MASTER'S THESIS

Supercritical CO_2 extracts of Achyranthes japonica Nakai root inhibits arthritis pathogenesis in mice *in vitro* and *in vivo*

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A thesis submitted in partial fulfillment of the requirement for the degree of Master of Animal Biotechnology

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CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

Achyranthes japonica Nakai root (AJNR) is used to treat osteoarthritis (OA) and rheumatoid arthritis (RA) because of its anti-inflammatory and antioxidant effects. The present study investigated the inhibitory effects of AJNR on arthritis. AJNR was extracted using supercritical CO_2. The inhibitory effects of ANJR against arthritis were evaluated using primary cultures of articular chondrocytes and two *in vivo* arthritis models: destabilization of the medial meniscus (DMM) for the OA model and collagenase-induced arthritis (CIA) for the RA model. AJNR did not affect pro-inflammatory cytokine (IL-1β, TNF-α, and IL-6)-mediated cytotoxicity, but attenuated pro-inflammatory cytokine-mediated increases in catabolic factors and recovered pro-inflammatory cytokine-mediated decreases in anabolic factors in the *in vitro* model. The effect of AJNR is particularly specific for IL-6-mediated catabolic or anabolic alteration. In the DMM model, AJNR decreased cartilage erosion, subchondral plate thickness, osteophyte size, and osteophyte maturity. In the CIA model, AJNR effectively inhibited cartilage degeneration and synovium inflammation in either the ankle or knee, and reduced pannus formation in both the knee and ankle. Immunohistochemistry analysis revealed that AJNR mainly acted via the inhibitory effects of IL-6-mediated matrix metalloproteinase-3 and -13 in both arthritis models. AJNR is a potential therapeutic agent for relieving arthritis symptoms.

Keywords: *Achyranthes japonica* Nakai root; Osteoarthritis; Rheumatoid arthritis; Destabilization of the medial meniscus; Collagenase-induced arthritis

1. INTRODUCTION

Osteoarthritis (OA) is a severe chronic degenerative disease of the joints that is common in middle-aged and older people [1]. The main clinical manifestations of OA are degeneration of the articular cartilage and changes in the subchondral bone structure [2]. When joint cartilage is completely lost following disruption of cartilage homeostasis through the induction of catabolic factors as well as downregulation of anabolic factors, the bones and soft tissue structures around the joint are altered, resulting in joint pain, swelling, deformity, and disability [3,4]. Although several risk factors associated with OA have been proposed, including mechanical impairment, genetic factors, aging, obesity, sex, and metabolic diseases, the pathogenesis of OA is not fully understood. The current treatment of this disease is mainly aimed to relieve OA symptoms, such as pain relief, anti-inflammatory effects, and prevention of damage to the joint structure [5,6].

Chondrocytes produce various cytokines and chemokines and respond to them in a paracrine or autocrine manner in joint tissues or synovial fluid [7,8]. The relationship between elevated levels of catabolic enzymes and inflammatory mediators (e.g., prostaglandins and nitric oxide) in OA synovial fluid and joint tissue has been well studied [9-13]. IL-1β, IL-6, and TNF-α are well-known pro-inflammatory cytokines involved in arthritis pathogenesis [2]; however, the mechanisms that trigger the production of inflammatory mediators remain unclear [8].

OA clinical treatment is mainly performed using two methods: joint cavity injection and oral medication [14]. Glucocorticoid or sodium hyaluronate is commonly administered via injection, whereas amino grape sulfate, non-steroidal anti-inflammatory drugs (NSAIDs), and opioids have been administered orally. High doses of these drugs can cause many adverse reactions, such as gastrointestinal irritation, and apparent side effects, which can cause ulcers and perforations in severe cases [15]. Liver and kidney damages caused by arthritis medication also induce adverse effects such as skin rash, urticaria, headache,

dizziness, and drowsiness. Some patients even show symptoms such as hypertension and edema after arthritis medication [16]. Thus, it is important to find alternative effective medicines with low side effects.

As a traditional natural compound in many Asian countries, AJNR contains many types of compounds, including polysaccharides, saponins, sterones, flavonoids, polypeptides, organic acids, and various trace elements. These components have been used in OA treatment and have been shown to promote the proliferation of chondrocytes, reduce joint swelling, and inhibit synovial hyperplasia [17-20]. However, underline mechanisms of the inhibitory effects of AJNR on arthritis have not been elucidated.

The purpose of this study is to explore the remission effects of AJNR against OA and RA. In order to reach it, the AJNR compounds were extracted using supercritical CO_2. This extraction method is unique and does not require the use of toxic solvents. Next, we examined the ameliorative effect of AJNR on arthritis using the primary culture of articular chondrocytes and two *in vivo* model systems. The results indicate that AJNR is a potential therapeutic compound that can inhibit arthritis pathogenesis without any side effects. It provides an experimental and theoretic base for AJNR application in treating arthritis disease.

2. MATERIALS AND METHODS

2.1. Chemicals and laboratory ware

Unless otherwise stated, chemicals and laboratory wares were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and Falcon Labware (Becton-Dickinson, Franklin Lakes, NJ, USA). Dulbecco's modified Eagle medium (DMEM) and fetal bovine serum were purchased from Gibco (Grand Island, NY, USA).

2.2. Preparation of AJNR extracts by supercritical CO_2

AJNR extracts were prepared by extracting raw AJNR using a supercritical CO_2, which is widely applied to extract bioactive substances from natural materials [21,22]. The extraction was performed using a laboratory-scale supercritical CO_2 extraction device manufactured by Nantong Wisdom Supercritical Science & Technology Development Co., Ltd., Haian, China, which essentially consisted of a CO_2 source, a condenser, a CO_2 storage tank, a CO_2 pump, an extractor, and three separators, as shown in the schematic drawing in Figure 1. During extraction, 500 g of dried and powdered AJNR (approximately 40 meshes) mixed with 250 ml of absolute ethanol were loaded into the extractor, in which the air was replaced with CO_2 three times. The CO_2 was pumped into the extractor, and the extracts dissolved in CO_2 were transported out from the extractor and separated from CO_2 at the separators. The extraction pressure and temperature were set to 40 MPa and 60 °C, respectively. The pressure and temperature of the separators were set as 8 MPa, 50 °C (separators 6-Ⅰ), and 5 MPa, 40 °C (separators 6-II and 6-III). The CO_2 flow rate was controlled at 20 kg/h, and the extraction time was 2 h. After being separated from the extract, the CO_2 would return to the condenser and repeat the cycle. After the extraction was complete, the extracts were collected from the separators and further evaporated using a rotavapor to remove the ethanol. The final extract (approximately 10 g) was stored in a sealed airtight tube for subsequent tests.

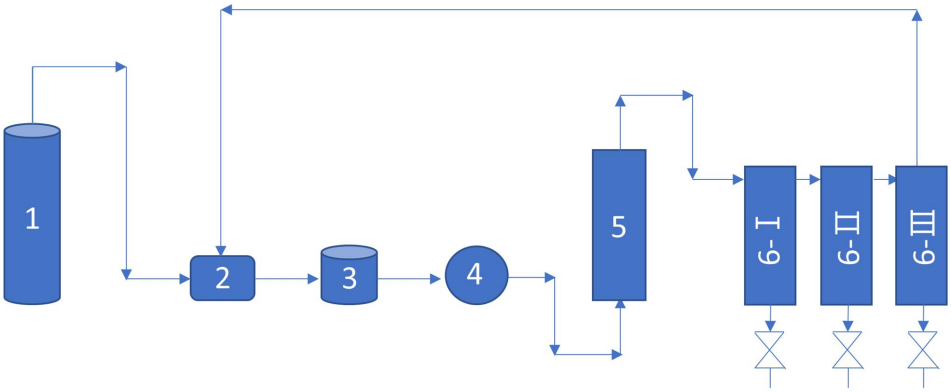

Figure 1. Schematic drawing of the supercritical CO_2 extraction device used in this study. CO_2 source (1), a condenser (2), a CO_2 storage tank (3), a CO_2 pump (4), an extractor (5), three separators (6)

2.3. Primary culture of mouse knee joint chondrocytes and treatment with AJNR

Chondrocytes were isolated from the femoral condyles and tibial plateaus of 4-day-old mice (n = 8) by digesting cartilage tissue with DMEM supplemented with 0.2 % collagenase (Sigma) [13,23,24]. The passage "0" (P0) primary chondrocytes (3×10^5/ 30 mm culture dish) were maintained as a monolayer in DMEM (Gibco, Waltham, MA, USA) supplemented with 10 % fetal bovine serum and antibiotics (100 units/ml penicillin G and 100 μg/ml streptomycin; Gibco, Waltham, MA, USA) for 24 h in a 5% CO_2 incubator at 37 °C. The chondrocytes were then exposed to various concentrations of AJNR (10, 20, and 50 μg/ml) in the absence or presence of IL-1β (1 ng/ml), IL-6 (100 ng/ml), and TNF-α (10 ng/ml).

2.4. MTT cell viability assay

Primary culture chondrocytes were exposed to various concentrations of AJNR (10, 20, and 50 μg/ml) in the absence or presence of IL-1β (1 ng/ml), IL-6 (100 ng/ml), and TNF-α (10 ng/ml). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed after 24 h of treatment. Briefly, the primary culture chondrocytes (5×10^4) were seeded in 96-well plates. After 24 h of incubation, AJNR (20, 50, 100 μg/mL) was added to the cells in the absence or presence of IL-1β (1 ng/ml), IL-6 (100 ng/ml), and TNF-α (10 ng/ml). MTT solution was added to each well for 4 h at the final incubation time. After adding 100 μl of dimethyl sulfoxide solution, optical density was recorded using a microplate reader (570 nm) [25,26].

2.5. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from primary cultured chondrocytes using TRIzol reagent (Molecular Research Center, Inc., Cincinnati, OH, USA). The quality and concentration of RNA were assessed using a NanoDrop™2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). The RNA was reverse transcribed, and the resulting cDNA was amplified by PCR or BIO-RAD Real-Time PCR system (CFX96™ Real-Time System,

Bio-Health Materials Core-Facility, Jeju National University) using SYBR premixed Extaq reagent (Takara Bio, Mountain View, CA, USA). The PCR primers and experimental conditions are summarized in Table 1. Glyceraldehyde-3-phosphate dehydrogenase (Gapdh) was used as an internal control [27,28].

Table 1. PCR primers and conditions

Genes	Strand	Primer Sequences	Size (bp)	AT (℃)	Origin
Gapdh	A AS	5′-TCACTGCCACCCAGAAGAC-3′ 5′-TGTAGGCCATGAGGTCCAC-3′	450	57.3	Mouse
Mmp2	A AS	5′-CCAACTACGATGATGAC-3′ 5′-ACCAGTGTCAGTATCAG-3′	233	60.0	Mouse
Mmp3	A AS	5′-AGGGATGATGATGCTGGTATGG-3′ 5′-CCATGTTCTCCAACTGCAAAGG-3′	434	58.0	Mouse
Mmp9	A AS	5′-TGCACTGGGCTTAGATCATTCC-3′ 5′-CCGTCCTTGAAGAAATGCAGAG-3′	450	58.4	Mouse
Mmp10	A AS	5′-AGAAATGGACACTTGCACCCTCAG-3′ 5′-CTGTCCGTGTTGTGAGCCTCATAG-3′	448	61.5	Mouse
Mmp12	A AS	5′-CCCAGAGGTCAAGATGGATG-3′ 5′-GGCTCCATAGAGGGACTGAA-3′	482	60.0	Mouse
Mmp13	A AS	5′-TGATGGACCTTCTGGTCTTCTGG-3′ 5′-CATCCACATGGTTGGGAAGTTCT-3′	474	58.2	Mouse
Mmp14	A AS	5′-GTGCCCTATGCCTACATCCG-3′ 5′-TTGGGTATCCATCCATCACT-3′	580	58.0	Mouse
Mmp15	A AS	5′-GAGAGATGTTTGTGTTCAAGGG-3′ 5′-TGTGTCAATGCGGTCATAGGG-3′	260	57.5	Mouse
Adamts 4	A AS	5′-CATCCGAAACCCTGTCAACTT-3′ 5′-GCCCATCATCTTCCACAATAGC-3′	287	58.4	Mouse
Adamts 5	A AS	5′-GCCATTGTAATAACCCTGCACC-3′ 5′-TCAGTCCCATCCGTAACCTTTG-3′	292	58.4	Mouse
Aggrecan	A AS	5′-GAAGACGACATCACCATCCAG-3′ 5′CTGTCTTTGTCACCCACACAT-3′	581	60.0	Mouse
Col2a1	A AS	5′-CACACTGGTAAGTGGGGCAAGACCG-3′ 5′-GGATTGTGTTGTTTCAGGGTTCGGG-3′	173	58.0	Mouse
Epas1	A AS	5′-CGAGAAGAACGACGTGGTGTTC-3′ 5′-GTGAAGGCTGGCAGGCTCC-3′	333	64.0	Mouse
Sox9	A AS	5′-ATGCTATCTTCAAGGCGCTG-3′ 5′-GACGTCGAAGGTCTCAATGT-3′	272	60.0	Mouse
Slc39a8	A AS	5′-GAACAATTGCCTGGATGATCACGC-3′ 5′-AAGCCGGTTAACATCCCTGCATTC-3′	430	63.0	Mouse

AT, annealing temperature; S, sense; AS, antisense

2.6. Animal model and AJNR treatment

All experiments were approved by the Jeju National University Animal Care and Use Committee. The DMM-mouse model was established using 12-week-old C57BL/6J mice (9 mice), as previously described [13,23,24]. The right knee of the medial meniscus was cut with surgical scissors in the patellar tendon in the middle and tendon of the tibial plateau. In the sham operation group, only arthrotomy was performed without resection of the tibial ligament of the medial meniscus [29,30]. The mice were intraperitoneal injection (IP) injected with AJNR (2 mg/kg) in 200 μl polyethylene glycol 400 (PEG-400) twice a week. The control group was injected with the same volume of PEG-400 reagent on the same schedule. The CIA mouse model was established using 7-week-old DBA/1 J (8 mice). RA was induced by type II collagen soluble in 0.05 M acetic acid at 4 °C. Emulsification was performed with an equal volume of Freund's complete adjuvant and boosted with Freund's incomplete adjuvant on day 21 [31]. Mice were IP injected with AJNR (2 mg/kg) with a newly formulated PEG-400 [32]. Control mice were injected with the same volume of PEG-400 reagent on the same schedule. The mice were examined every 3 days to measure the visual appearance of arthritis around the knee and ankle and to determine the corresponding severity and arthritis score [31,33].

2.7. Histological analysis

The CIA (knee and ankle) or DMM (knee) samples were fixed with 4 % paraffin formaldehyde for 24 h and then decalcified in 0.5 M EDTA solution at 4 °C for 4 weeks. Next, the samples were dehydrated using gradient ethanol and embedded in paraffin. Finally, the sample was sliced at a thickness of 5 mm and stained with Safranin-O/Fast Green for evaluation [34,35]. Cartilage destruction and severity of synovitis were evaluated by experienced histological researchers who were blinded to the study groups. The

Osteoarthritis Research Society International (OARSI) scoring system was used to evaluate cartilage degeneration, as previously described [36-39].

2.8. Immunohistochemistry

The dehydrated slides were incubated with hydroperoxide (DACO LSAB 2 SYSTEM, HRP KIT; DAKO real^{TM}), and then incubated for 45 min with trypsin at 37 °C. The slides were blocked with 1 % bovine serum albumin for 60 min at room temperature. The sections were incubated with rabbit polyclonal antibody (4 μg ml^{-1}, ab52915; Abcam, Cambridge, United Kingdom) against Mmp3, rabbit polyclonal antibody (1:100 dilution, ab51072; Abcam) against Mmp13, and rabbit monoclonal antibody (1:200 dilution, #12912; Cell Signaling, Danvers, MA, USA) against IL-6 at 4 °C overnight. After incubation with anti-mouse or anti-rabbit secondary antibodies for 60 min, the slides were stained (Dako Real Envison^{TM}, Santa Clara, CA, USA).

2.9. Statistical analysis

All statistical analyses were performed using IBM SPSS Statistics 24 (IBM Corp., Armonk, NY, USA). Experimental data were analyzed using the non-parametric Mann–Whitney U test and two-tailed Student's *t*-tests with unequal sample sizes. Significance was accepted at a level of probability of 0.05 ($p < 0.05$) [40]. The results are shown as the mean ± standard error of the mean (S.E.M).

3. RESULTS

3.1. Effects of AJNR on cell viability

The effects of AJNR on cell viability were evaluated by the MTT assay (Figure 2A). Treatment with AJNR (0-100 μg/ml) for 48 h did not result in a significant cytotoxic effect. Moreover, following treatment with AJNR (10, 20, 50 μg/ml) in the absence or presence of the pro-inflammatory cytokines IL-1β (1 ng/ml), IL-6(100 ng/ml), and TNF-α (10 ng/ml), the cell viability of primary chondrocytes was not recovered (Figure 2B,C,D). These results suggest that AJNR does not affect cell viability, either as a single treatment or in the presence of pro-inflammatory cytokines.

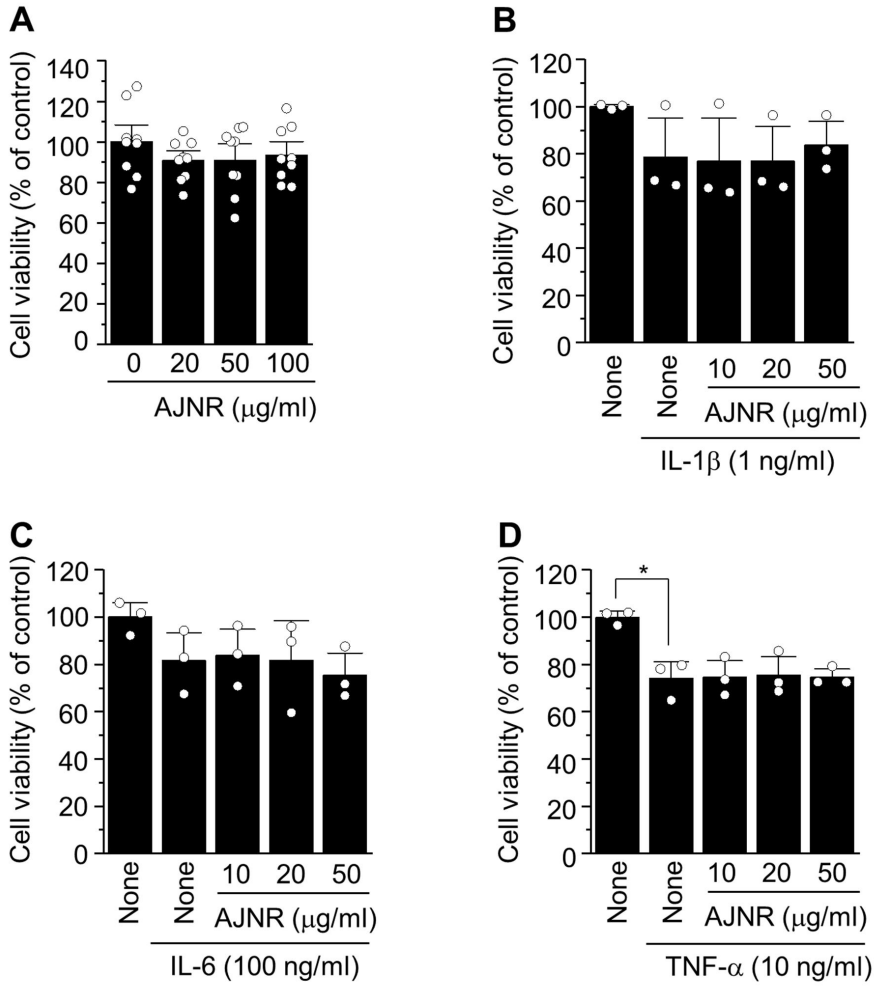

Figure 2. The effects of AJNR on the viability of primary cultured articular chondrocytes. (A) Primary cultured mouse chondrocytes were exposed to increasing concentration (0-100 μg/ml) of AJNR for 24 h and then subjected to MTT assay. The other groups were treated with AJNR (10, 20, 50 μg/ml) in the absence or presence of IL-1β (1 ng/ml) (B), IL-6 (100 ng/ml) (C), or TNF-α (10 ng/ml) (D), and then the cell viability of primary chondrocytes were evaluated by MTT assay. The results are shown as the mean ± S.E.M of three separate experiments. * $p < 0.05$.

3.2. Effects of AJNR on anabolic or catabolic factors in primary cultured articular chondrocytes

We analyzed the effects of AJNR on anabolic or catabolic factors in primary cultured articular chondrocytes. RT-PCR analyses revealed that AJNR (0-50 μg/ml) did not affect the expression of key OA inducer genes, such as endothelial PAS domain protein 1 (Epas1) [2], solute carrier family 39 member 8 (Slc39a8) [41], estrogen-related receptor gamma (Esrrg) [23], nicotinamide phosphoribosyltransferase (Nampt) [42], and metal regulatory transcription factor 1 (Mtf1) (Figure 3A) [43]. In addition, AJNR had almost no effect on the expression of Mmp2, -3, -8, -9, -10, -12, -13, -14, and -15 (Figure 3B). Moreover, the expression of the anabolic factors collagen type II alpha 1 chain (Col2a1), Aggrecan, and SRY-box transcription factor 9 (Sox9), as well as the catabolic factors ADAM metallopeptidase with thrombospondin type 1 motif (Adamts)-4 and -5, was not affected by AJNR (Figure 3C).

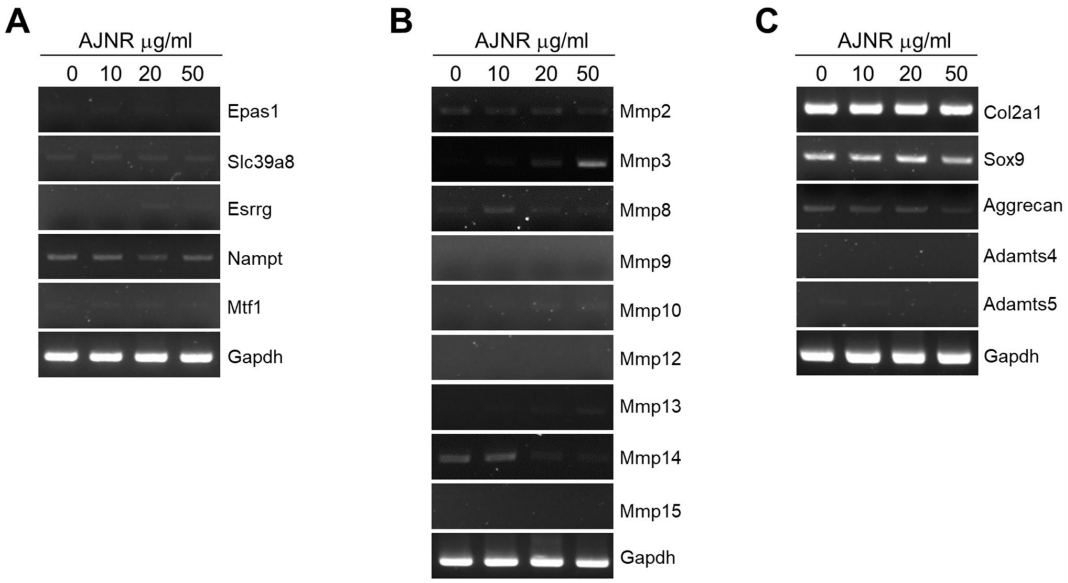

Figure 3. Effects of AJNR on anabolic or catabolic factors in primary cultured articular chondrocytes. Primary cultured mouse chondrocytes were exposed to AJNR (0-50 μg/ml) for 24 h. Next, the levels of osteoarthritis inducer genes (A), matrix metalloproteinases (B), and anabolic/catabolic factors (C) were measured by RT-PCR. The results are representative of three independent experiments from different mouse pups. Glyceraldehyde-3-phosphate dehydrogenase (Gapdh) was used as an internal marker. Epas1, endothelial PAS domain protein 1; Slc39a8, solute carrier family 39 member 8; Esrrg, estrogen-related receptor gamma; Nampt, nicotinamide phosphoribosyltransferase; Mtf1, metal regulatory transcription factor 1; Mmp2, -3, -8, -9, -10, -12, -13, -14, and -15, matrix metalloproteinase-2, -3, -8, -9, -10, -12, -13, -14, and -15; Col2a1, collagen type II alpha 1 chain; Sox9, SRY-Box transcription factor 9; Adamts-4 and -5, ADAM metallopeptidase with thrombospondin type 1 motif-4 and -5.

3.3. Effects of AJNR on chondrocytes exposed to pro-inflammatory cytokines

Treatment with pro-inflammatory cytokines, namely IL-1β (0, 0.1, 0.5, 1 ng/ml), TNF-α (0, 1, 5, 10 ng/ml), and IL-6 (0, 10, 50, 100 ng/ml), increased catabolic factors (Mmp3, Mmp10, Mmp13, and Adamts5) and decreased anabolic factors (Col2a1, Sox9, and Aggrecan) remarkably in mouse primary cultured chondrocytes in a dose-dependent manner (Figure 4A, B, C). However, AJNR attenuated the increases in catabolic factors induced by IL-1β, TNF-α, and IL-6. AJNR also recovered IL-1β-, TNF-α-, and IL-6-induced decreases in anabolic factors (Figure 4D, E, F). Interestingly, AJNR showed a specific effect on IL-6-mediated anabolic and catabolic alterations (Figure 4F).

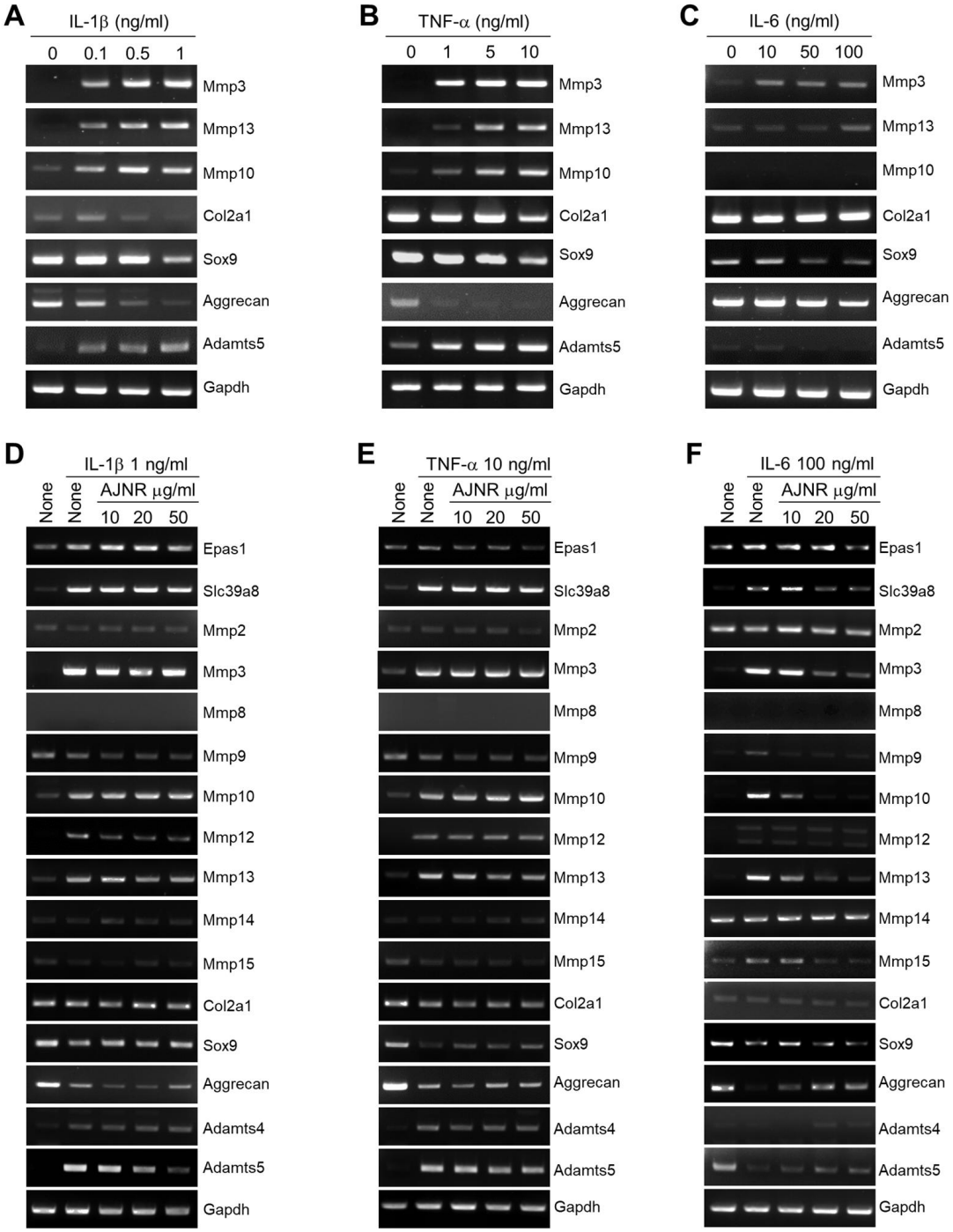

Figure 4. The inhibitory effects of AJNR on pro-inflammatory cytokine-induced catabolic expression in primary cultured chondrocytes. (A)(B)(C) Anabolic or catabolic factors in primary cultured mouse chondrocytes were analyzed following treatment with IL-1β, TNF-α, and IL-6. Chondrocytes were pre-incubated with the indicated concentrations of AJNR for 1 h and then stimulated with IL-1β (D), TNF-α (E), and IL-6 (F) for 24 h. RT-PCR

analysis revealed the levels of anabolic and catabolic factors. The results are representative of three independent experiments from different pups. Gapdh was used as an internal marker. AJNR, Achyranthes japonica Nakai Root); IL-1β, interleukin-1β; IL-6, interleukin 6; TNF-α, tumor necrosis factor-α; Epas1, endothelial PAS domain protein 1; Mmp2, -3, -8, -9, -10, -12, -13, -14, and -15, matrix metalloproteinase-2, -3, -8, -9, -10, -12, -13, -14, and -15; Col2a1, collagen type II alpha 1 chain; Sox9, SRY-Box transcription factor 9; Adamts-4 and-5, ADAM metallopeptidase with thrombospondin type 1 motif-4 and -5; Gapdh, glyceraldehyde 3-phosphate dehydrogenase.

3.4. AJNR attenuated DMM-induced OA pathogenesis

We further investigated the effect of supercritical CO_2 extract of AJNR on OA pathogenesis in a DMM model. AJNR was *intraperitoneal injection (IP)* injected twice a week for 8 weeks after DMM surgery (Figure 5A). The structural integrity of the articular cartilage was evaluated via staining with Safranin-O/Fast Green (Figure 5B). The results showed that AJNR attenuated DMM-induced cartilage degeneration and erosion. The other OA parameters, such as OARSI ($p < 0.0001$), sclerosis ($p = 0.005$), and osteophytes ($p < 0.0001$), were completely blocked by AJNR in the DMM model (Figure 5C, D, E, F). In addition, DMM-induced synovial inflammation (as indicated by increased articular cavity inflammatory cells, thickened synovial membrane, and synovial tissue edema) was attenuated by AJNR ($p < 0.0001$) (Figure 5G, H). In both sham groups, the articular cartilage surface was smooth, and the synovial tissue was not hyperplastic.

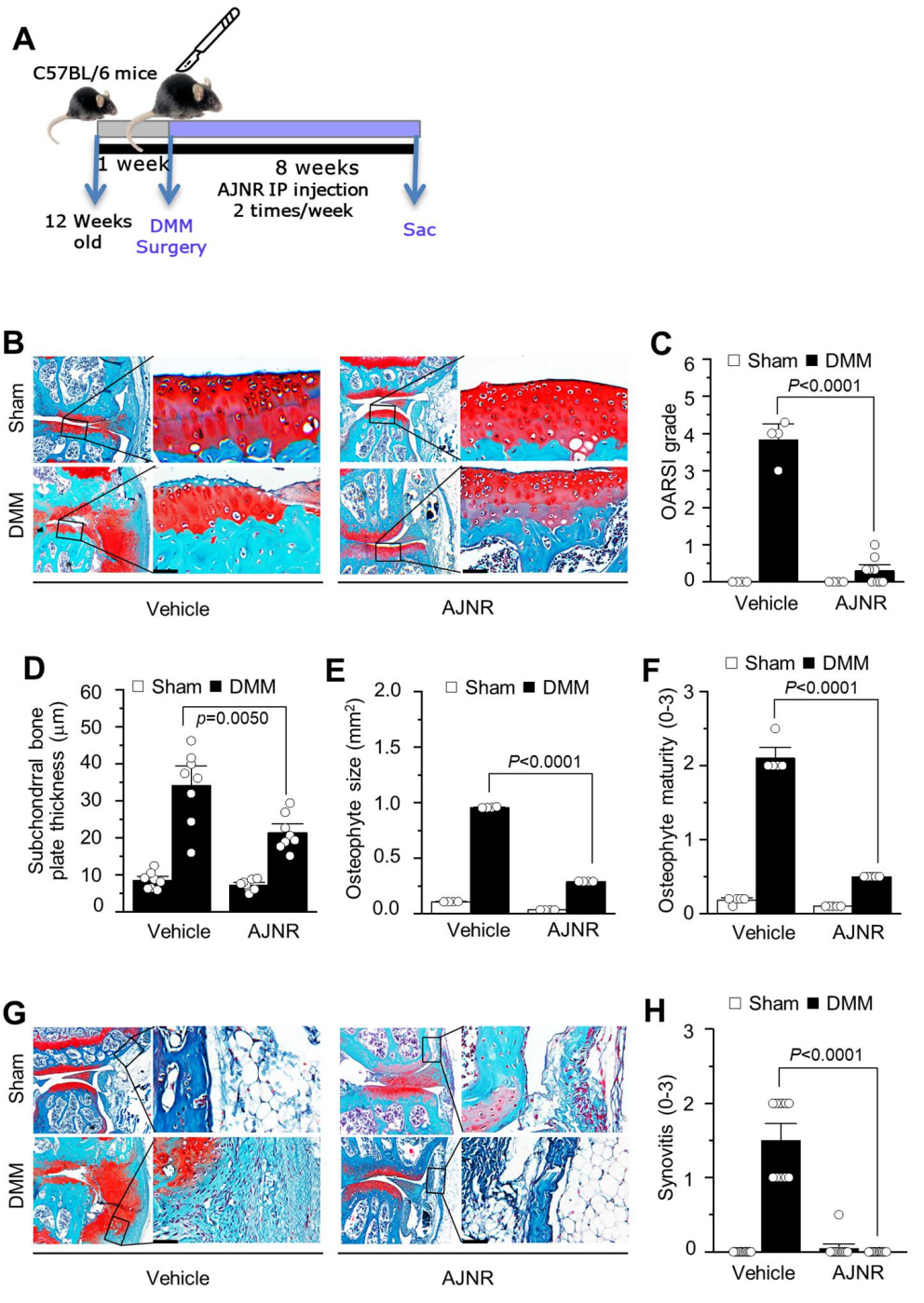

Figure 5. The inhibitory effects of AJNR in DMM-induced osteoarthritis mouse model. First, 12-week-old C57BL/6 mice were subjected to sham operation or DMM surgery. Next, sham-or DMM-operated mice were intraperitoneal injection (IP) injected with AJNR (2 mg/kg) (2 times per week for 8 weeks). (A) The experiment scheme of AJNR in mouse DMM model. (B) Representative Safranin-O staining images showing the whole joint (40x),

subchondral bone sclerosis, and cartilage destruction (400x). Osteoarthritis Research Society International (OARSI) grade (for cartilage destruction) (C), subchondral bone plate thickness (for subchondral bone sclerosis) (D), osteophyte size (E), and osteophyte maturity (F) were quantified (n ≥ 4 mice per group). Two-tailed *t*-test and Mann–Whitney U test. Scale bar: 50 μm.

3.5. AJNR attenuated CIA-induced RA

We determined whether AJNR has inhibitory effects on RA in a CIA model. IP injection of AJNR (2 mg/kg) was performed during CIA induction (Figure 6A). Histological assessments were conducted on mouse ankle, knee, and toe joints. There were no pathological symptoms of arthritis in the non-immunized (NI) group. However, severe synovitis with synovial hyperplasia, erosion of the bone and cartilage, and infiltration of inflammatory cells were observed in the CIA group (Figure 6B, E). CIA-induced cartilage degeneration and synovitis were dramatically reduced in the AJNR-treated group (Figure 6B, E). The histological scores of synovitis and OARSI were also significantly attenuated in the ankle, knee, and toe of mice treated with AJNR (Figure 6C, D, F, G, H, I). These results suggest that AJNR has beneficial effects against inflammation and cartilage degeneration in RA.

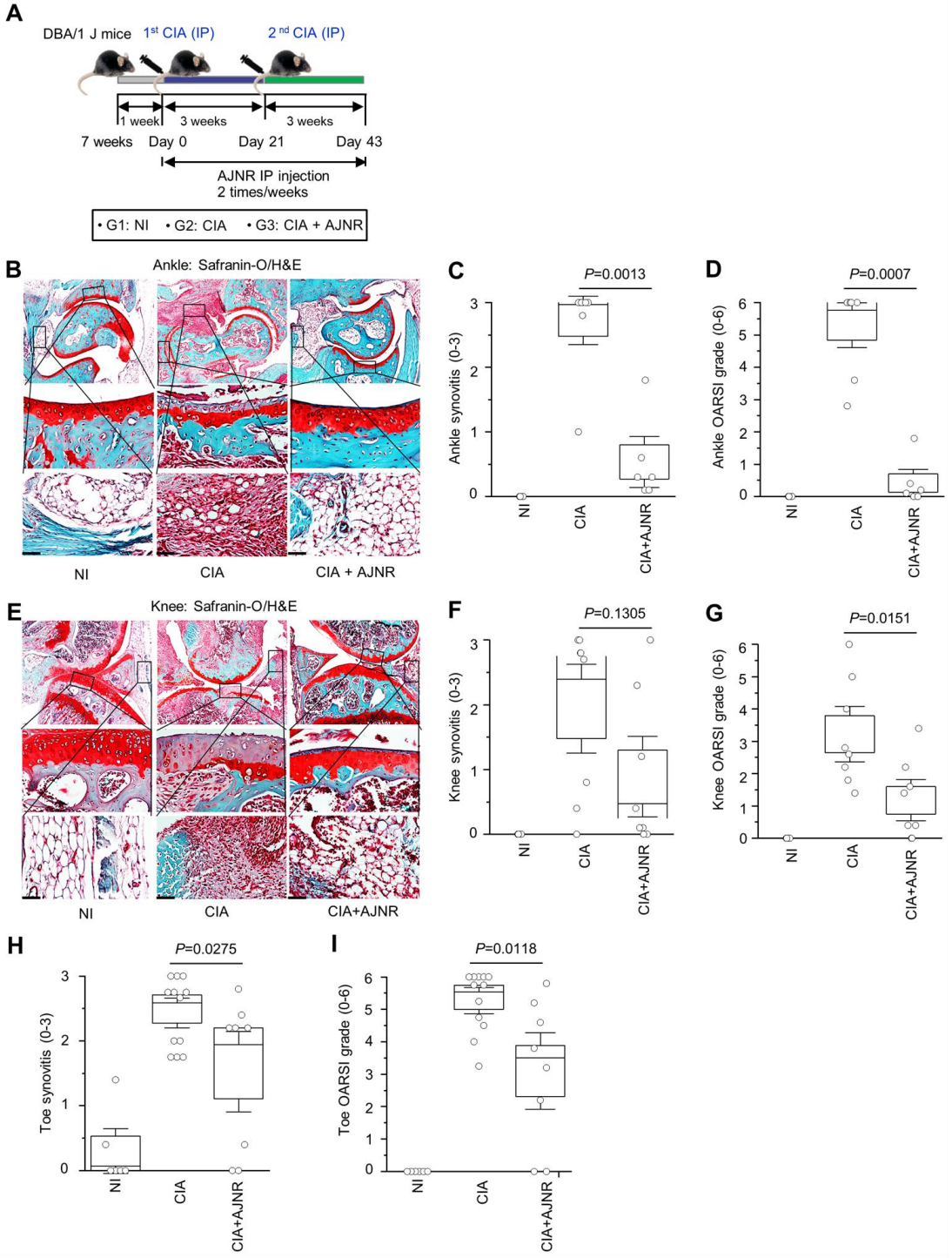

Figure 6. The inhibitory effects of AJNR against collagenase-induced arthritis (CIA)-induced cartilage degeneration and synovitis. First, CIA models were established using 10-week-old DBA/1 J mice. During arthritis induction, AJNR intraperitoneal injection (IP) injections were performed (2 times per week for 6 weeks). (A) The scheme of AJNR treatment in the CIA mouse model. (B) Representative Safranin-O and hematoxylin and

eosin stain (H&E) staining images of ankle cartilage degeneration and synovial inflammation, respectively, in the non-immunized (NI), CIA, and AJNR-treated CIA mice. Scores of synovial inflammation (C) and Osteoarthritis Research Society International (OARSI) (D) in the ankle are presented (n ≥ 4 mice per group). (E) Representative Safranin-O and H&E staining images of knee cartilage degeneration and synovial inflammation, respectively, in non-immunized (NI), CIA, and AJNR-treated CIA mice. Scores of synovial inflammation (F) and OARSI (G) in the knee are presented (n ≥ 4 mice per group). Scores of toe synovitis (H) and OARSI (I) are also presented (n ≥ 6 mice per group). Two-tailed *t*-test and Mann–Whitney U test. Scale bar: 50 μm.

3.6. AJNR attenuated CIA-induced pannus formation in ankle and knee

Pannus formation is an essential indicator of severe arthritis. Therefore, we evaluated CIA-induced pannus formation in mouse ankles and knees. The results showed that pannus formation in the ankle and knee was not observed in the NI group; however, CIA-induced pannus formation was significantly reduced by AJNR in both the ankles ($p=0.0007$) and knee ($p=0.0293$) (Figure 7).

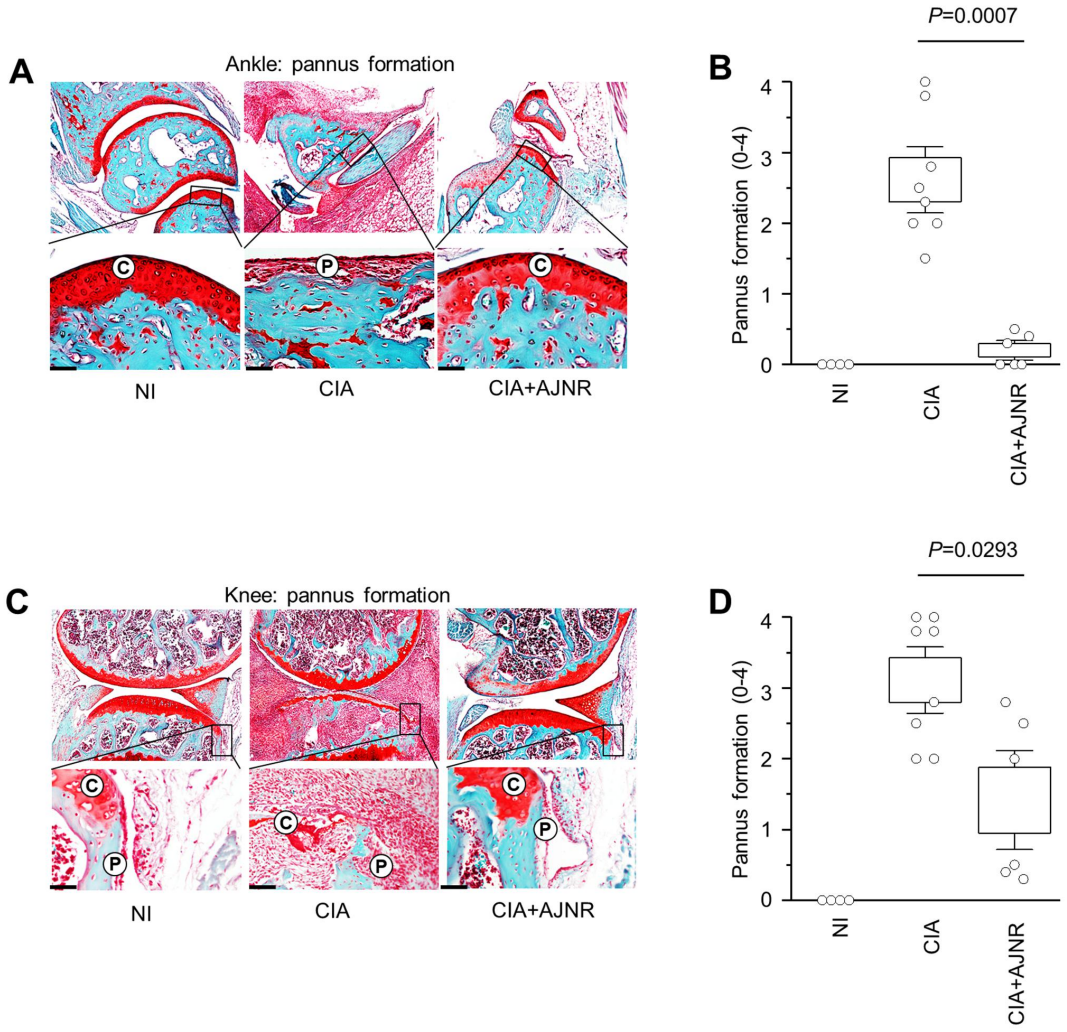

Figure 7. The inhibitory effects of AJNR in CIA-induced pannus formation. Collagenase-induced arthritis (CIA) models were established using 7-week-old DBA/1 J mice. During arthritis induction, AJNR IP injections were performed (2 times per week for 6 weeks). (B) Representative Safranin-O and H&E staining images of the ankle pannus of non-immunized (NI), CIA, and AJNR-treated CIA mice. Score of ankle pannus formation is presented in B (n ⩾ 4 mice per group). (C) Representative Safranin-O and H&E staining images of the knee pannus of NI, CIA, and AJNR-treated CIA mice. Score of knee pannus formation is presented in D (n ⩾ 4 mice per group). Two-tailed *t*-test and Mann–Whitney U test. Scale bar: 50 μm.

3.7. AJNR inhibited IL-6 mediated Mmp3 and Mmp13 expression in DMM-induced OA model

Our *in vitro* mechanistic studies revealed that the inhibitory effects of AJNR were specific against IL-6. To further verify the inhibitory effects of AJNR on IL-6-mediated OA pathogenesis, we performed immunohistochemical staining of samples from the DMM model. As shown in Figure 8, IL-6, Mmp3, and Mmp13 expression was elevated in both the knee cartilage and knee synovium of the DMM model, compared with that in the sham group; however, the elevated expression of these proteins was remarkably attenuated in the AJNR-treated group (Figure 8).

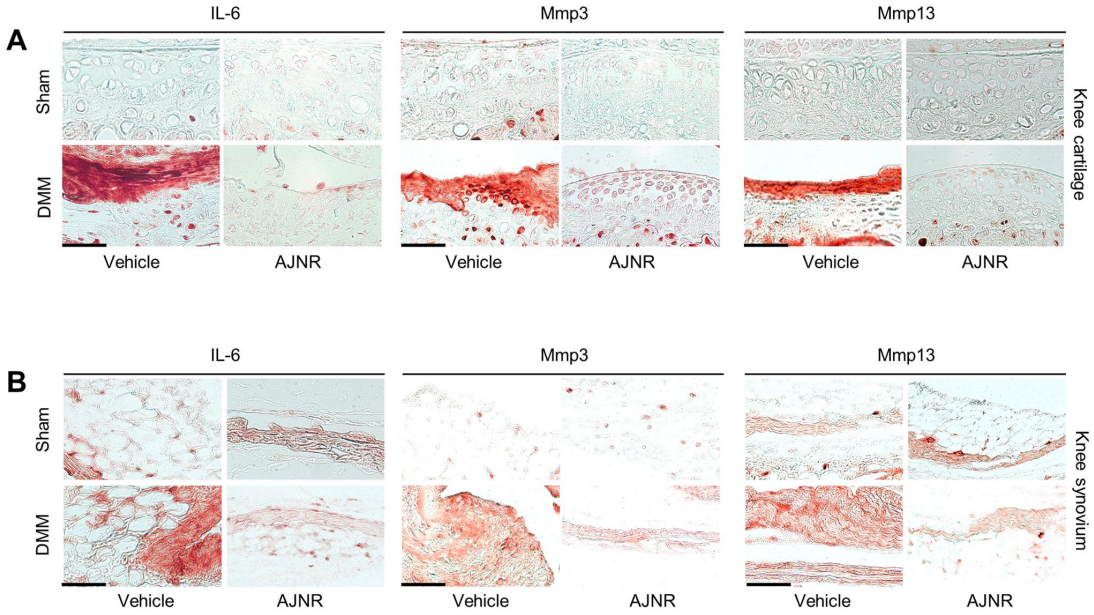

Figure 8. AJNR inhibits IL-6-mediated Mmp3 and Mmp13 expression in destabilization of the medial meniscus (DMM) surgery-induced osteoarthritis. First, 12-week-old C57BL/6 mice were subjected to sham operation or DMM surgery. Sham-or DMM-operated mice were intraperitoneal injection (IP) injected with AJNR (2 mg/kg) (2 times per week for 8 weeks). (A) Representative images of IL-6, Mmp3, and Mmp13 immunostaining in the knee cartilage tissue sections of sham, DMM, and AJNR-treated DMM mice (n = 4 mice per group). (B) Representative images of IL-6, Mmp3, and Mmp13 immunostaining in the synovial tissue sections of sham, DMM, and AJNR-treated DMM mice (n = 4 mice per group). Scale bar: 50 μm.

3.8. AJNR inhibited IL-6-mediated Mmp3 and Mmp13 expression in CIA-induced RA model

To further verify the mechanism of IL-6-mediated Mmp3 and Mmp13 expression, we examined the expression of IL-6, Mmp3, and Mmp13 in the CIA model. The expression of IL-6, Mmp3, and Mmp13 was dramatically enhanced in the ankle cartilage and synovium of CIA-induced RA mice (Figure 9). Similar to that in the DMM model, the elevated expression of IL-6, Mmp3, and Mmp13 was reduced in the AJNR-treated CIA model (Figure 9). These results indicate that the inhibitory effects of AJNR are specific for IL-6-mediated Mmp3 and Mmp13 expression.

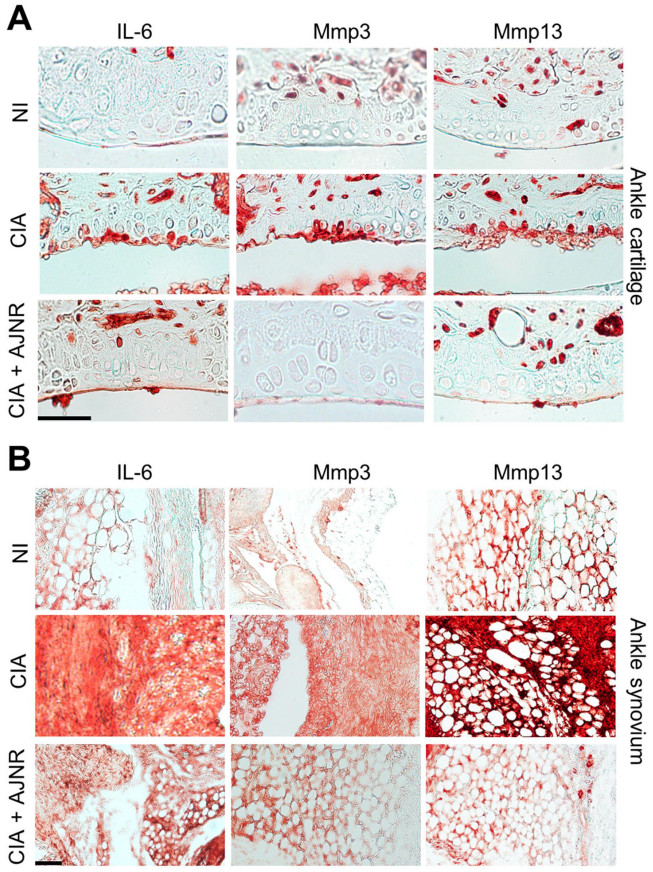

Figure 9. AJNR inhibits IL-6-mediated Mmp3 and Mmp13 expression in CIA-induced rheumatoid arthritis. CIA models were established using 7-week-old DBA/1 J mice. During arthritis induction, AJNR intraperitoneal injection (IP) injections were performed (2 times per week for 6 weeks). (A) Representative IL-6, Mmp3, and Mmp13 immunostaining in the ankle cartilage tissue sections of non-immunized (NI), CIA, and AJNR-treated CIA mice (n = 4 mice per group). (B) Representative images of IL-6, Mmp3, and Mmp13 immunostaining in the synovial tissue sections of NI, CIA, and AJNR-treated CIA mice (n = 4 mice per group). Scale bar: 50 μm.

4. DISCUSSION

In this study, we investigated the effect of supercritical CO_2 extracts of AJNR against OA and RA. IP injection of AJNR effectively protected and slowed down the pathogenesis of post-traumatic OA in the mouse DMM and CIA models of RA. Compared with those in the arthritis group, cartilage erosion and proteoglycan loss, synovitis, and subchondral plate thickness were reduced in the AJNR treatment group. These phenomena are explicitly related to IL-6-mediated Mmp3 and Mmp13 expression. Taken together, our findings showed that the potential of AJNR for joint protection and arthritis treatment was significant.

In accordance with the OA concept put forward by Garrod in 1890, the pathological characteristics of OA in the inflammatory lesions of joints were evaluated [44]. The main pathological changes in OA are loss of articular cartilage and collagen fiber degeneration [36], which can lead to bone hyperplasia and osteophyte formation when the original mechanical balance of the knee joint is disrupted [45]. Therefore, these pathological changes can reflect the clinical characteristics of this degenerative joint disease [46,47]. Mmps participate in the degradation of many matrix components, and the activities of Mmps are regulated by hormones and cytokines [41,48]. Mmps play an essential role in synthesizing and decomposing the matrix and in the intervention of many physiological and pathological processes, such as arthritis and tissue remodeling [48]. ADAMTS4 and ADAMTS5 are the main proteases responsible for the degradation of proteoglycans in the articular cartilage of OA [49-52].

There has been no evidence of the complete curation of OA. The main clinical treatments for this disease are oral drugs, such as NSAIDs, which can cause many adverse side effects [53-55]. In addition, it is important to protect the joints of patients with the disease. The inhibitory effect of AJNR against OA symptoms indicates its potential as a

therapeutic agent against OA; however, further validation in other animal models and clinical trials is required [56].

The saponins and sterones in AJNR, which have anti-tumor or anti-inflammatory effect, are believed to be the main medicinal substances of AJNR [57,58]. The flavonoids, alkaloids, allantoin, succinic acid, and β-sitosterol of AJNR have also been studied [58]. As a traditional plant medicine, AJNR has been used in the clinical treatment of OA for many years [58-60]. In addition to the therapeutic effects of AJNR against OA, our data support the therapeutic effects of AJNR against RA. Our results suggest that AJNR is specific for IL-6 mediated anabolic or catabolic alterations in arthritis pathogenesis.

5. CONCLUSIONS

We have shown that AJNR extracts inhibits arthritis pathogenesis by reducing the expression of catabolic factors and enhancing the expression of anabolic factors without altering cell viability. Interestingly, AJNR has therapeutic effects in both OA and RA animal models. In particular, AJNR has specific inhibitory effects against IL-6-mediated anabolic and catabolic imbalances. Our results indicate that AJNR has vast potential for the treatment of arthritis pathogenesis, which can lead to drug development.

6. REFERENCES

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