Treatment with SiMiaoFang, an Anti-Arthritis Chinese Herbal Formula, Inhibits Cartilage Matrix Degradation in Osteoarthritis Rat Model

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Abstract

A Chinese herbal preparation, SiMiaoFang (SMF), has been used clinically for treating arthralgia by virtue of its anti-inflammatory and pain-relieving activities. However, no evidence base links SMF to anti-osteoarthritis (OA), particularly its link to inhibiting cartilage matrix degradation. In this study, we undertook a characterization of anti-OA activity of SMF using an *in vivo* rat model induced by anterior cruciate ligament transection and medial meniscus resection (ACLT + MMx) together with *in vitro* studies with chondrocytes for further molecular characterization. ACLT + MMx rats were treated with SMF at doses of 0.63, 1.25, and 2.5 grams/kg per day for 6 weeks. SMF treatments significantly inhibited cartilage matrix degradation, as indicated by increasing proteoglycan and collagen content, particularly type II collagen expression in articular cartilage, decreasing CTX-II (collagen type II degradation marker), and increasing CPII (collagen type II synthesis marker) in circulation. Moreover, SMF suppressed synovial inflammation and inhibited release of interleukin-1β (IL-1β) and tumor necrosis factor-α in serum. The levels of serum prostaglandin E_2 and nitric oxide productions were decreased via suppression of the production of cyclooxygenase-2 and inducible nitric oxide synthase, respectively. Importantly, SMF interfered with OA-augmented expression of matrix metalloproteinases (MMPs) -3 and -13 and aggrecanases (ADAMTS) -4 and -5, which are considered to be key enzymes in cartilage matrix degradation, and simultaneously augmented OA-reduced tissue inhibitors of metalloproteinases (TIMPs) -1 and -3 expression in the joints. The largest changes in these parameters were found at the highest dose. Meanwhile, SMF significantly decreased MMP-3 and -13 and increased TIMP-1 and -3 at mRNA and protein levels in IL-1β–induced chondrocytes. These findings provide the first evidence that SMF effectively treats OA by inhibiting cartilage matrix degradation.

Introduction

Osteoarthritis (OA), a musculoskeletal disorder of the bone and joint,[1] is the most common form of arthritis, affecting millions of people worldwide, and the major cause of disability in the elderly, affecting about 80% of individuals over the age of 75.[2] OA is characterized by progressive destruction of articular cartilage. An imbalance between the biosynthesis and degradation of matrix components leads to a progressive destruction of the tissue and finally causes complete damage of the articular surface. Cartilage matrix consists largely of proteoglycan and collagen; the loss of proteoglycan occurs early in the process of cartilage degeneration[3] and is followed by the catabolism of collagen fibrils, leading to the loss of cartilage structural integrity.[4] Interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α), the predominant cytokines involved in the initiation and processes of cartilage degradation, are potent inhibitors of cartilage matrix synthesis.[5] In addition, a relevant role as mediators of cartilage damage is carried out by nitric oxide (NO) and prostaglandins (PGE$_2$),[6,7] and the contribution of these molecules to cartilage degradation stems from their ability to enhance the production and activation of aggrecanases (ADAMTS) and matrix metalloproteinases (MMPs), which inhibit proteoglycan and collagen biosynthesis and induce chondrocyte apoptosis.[8,9] ADAMTS and MMPs are up-regulated in osteoarthritic-affected cartilage and act as downstream key players in the inflammatory

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signal cascade.[10,11] Additionally, MMP-induced cartilage degeneration is also regulated by inhibition of endogenous tissue inhibitors of metalloproteinase (TIMPs),[12] and the imbalance in the ratio of TIMPs and MMPs results in continued matrix destruction in OA.[13]

The current treatment strategies for OA are to decrease symptoms, improve function, and delay time to surgery. There are three kinds of therapeutic agents: Disease-modifying OA drugs (DMOADs) such as hyaluronic and glucosamine; nonsteroidal anti-inflammatory drugs (NSAIDs), such as loxoprofen and nabumetone; and steroid and biological response modifiers, which are all clinically used to relieve the severity of OA, slow disease progression, and prevent subsequent joint damage.[2] Although these drugs have good effects on OA, there were reports that long-term application of them causes side effects on the gastro-intestinal tract.[14]

SiMiaoFang (SMF) is a compound prescription comprised of four ingredients—Phellodendri Chines Cortex, Atractylodis rhizoma, Coicis Semen, and Achyranthis bidentatae Radix—in a specified ratio.[15] SMF has been used in traditional Chinese medicine since the Qing Dynasty (16th century CE) because of its function of clearing heat and removing dampness. Today, SMF is commonly used clinically for treatment of arthralgia due to its anti-inflammatory and pain-relieving activities,[16] as in gouty arthritis and rheumatoid arthritis.[17–19] But SMF's activity and mechanism of anti-OA remain uncharacterized, in particular its link to inhibiting cartilage matrix degradation. In this study we investigated the effects of SMF on cartilage matrix degradation, pro-inflammatory cytokines, and inflammatory mediators and mainly explored the further mechanism of MMPs/TIMPs using *in vivo* and *in vitro* assays, as part of an ongoing effort to identify novel and potent agents for the prevention and treatment of OA.

Materials and Methods

Herbal preparation

SMF was prepared as described in the Chinese Pharmacopeia of 2010. Briefly, the four ingredients—Phellodendri Chinensis Cortex (250 grams, beaked with salt), Coicis seuen (250 grams), Atractylodis Rhizoma (125 grams), and Achyranthis bidentatae radix (125 grams)—were pulverized to a fine powder, suspended in distilled water to a concentration of 0.25 gram/mL, and mixed well before administration. The samples for cell culture assays were prepared by extracting the powder with water (three times, for 1 h each). The combined extracts of SMF were concentrated *in vacuo* and dissolved in dimethylsulfoxide (DMSO; 1 g/mL). All of the samples were stored at 4°C.

The representative chemical compositions of berberine (0.7347%), glycerol trioleate (0.01%), atractylenolide (0.0028%), and β-ecdysone (0.0118%) in SMF were determined by high-performance liquid chromatography (HPLC) analysis.

OA Animals model and treatment

Ten-week-old male Sprague–Dawley rats (Animal Science Laboratory of Peking University Health Science department, PR China) were anesthetized with halothane.

After being shaved and disinfected, the right knee joint was exposed through a medial par patellar approach. The patella was dislocated laterally and the knee placed in full flexion, followed by anterior cruciate ligament transection and medial meniscus resection (ACLT+MMx) with microscissors. In sham-operated negative controls, the right knee joint was exposed and incisions were closed after subluxation of the patella and washing the joint surface with saline. The rats were randomly assigned to six groups: ACLT+MMx without treatment (Model, $n=10$), sham operated (Sham, $n=10$), ACLT+MMx rats treated with 0.16 gram/kg glucosamine (GS, $n=10$), and ACLT+MMx rats treated with SMF intra-gastrically at a daily dose of 0.63 gram/kg, 1.25 grams/kg, or 2.50 grams/kg ($n=10$ in per group) for 6 days per week for 6 weeks. Dose calculations followed guidelines correlating dose equivalents between humans and laboratory animals, on the basis of ratios of body surface area,[20] and 1.25 grams/kg is the dose equivalent based on a recommended dose of 0.1 gram/kg in human. Untreated control rats induced by ACLT+MMx and sham rats received distilled water only.

All animals were maintained on a 12-hr light/dark cycle under constant temperature (24±2°C) and humidity (55%±5%), and were allowed free access to food and water. All procedures for consideration of animal welfare were reviewed and approved by the ethical committee of China Academy of Traditional Chinese Medicine.

Histological analysis

Animals were sacrificed after 6 weeks of treatment. The tibia and femur with synovium were fixed in 4% paraformaldehyde for 24 hr, de-calcified in 10% EDTA, and embedded in paraffin. Tissue sections (4 μm) were mounted on common slides for stained with Hematoxylin & Eosin (H&E), Toluidine Blue, and Masson's Trichrome as described.[21,22] Cartilage histopathological features were analyzed using the scoring system modified by Mankin et al. (score range 0–12, from normal to complete disorganization and hypocellularity).[23] Synovium histopathology was evaluated according to Yoshimi histological grading (score range 0–18, from normal to most severe reaction).[24] An Image-Pro Plus 6.0 System (IMS) image analysis system was used for quantitative analysis of Toluidine Blue and Masson staining. Six fields of view were randomly selected from each slice, and an index of positive staining was determined from the area of positive staining and the average optical density. The positive index was calculated as integrated optical density (IOD) (positive area×average OD). All sections were randomized and evaluated by a trained observer who was blinded to the treatment groups.

Serum radioimmunoassay and enzyme-linked immunosorbent assay analysis

Animal blood was collected from the abdominal aorta and serum was analyzed for IL-1β, TNF-α, PGE$_2$, and cyclooxygenase (COX) -1 and -2, by radioimmunoassay assay (RIA). NO and inducible nitric oxide synthase (iNOS) were detected by assay kits. Assays for serum CTX-II (collagen type II degradation marker) and CPII (collagen type II synthesis marker) levels were performed with serum Pre-Clinical CartiLaps ELISA kit (R & D System, USA) and

pro-collagen type II C-pro-peptide ELISA kit (R & D System, USA),[25] respectively.

Immunoblotting analysis

Paraffin sections (4 μm) of joint tissue were mounted on poly-L-lysine–coated slides. The paraffin sections were dewaxed by routine method and incubated for 10 min with 3% hydrogen peroxide (H_2O_2). Each section was incubated with blocking serum (Vectastain ABC Kit) at room temperature for 30 min and then with primary rabbit monoclonal antibody against type II collagen (dilution 1/30, Biosynthesis Biotechnology, China), rabbit polyclonal antibody against ADAMTS-4 (dilution 1/100, Abcam Biotechnology, UK), rabbit polyclonal antibody against ADAMTS-5 (dilution 1/20, Abcam Biotechnology, UK), rabbit polyclonal antibody against MMP-3 (dilution 1/50, Abcam Biotechnology, UK), rabbit polyclonal antibody against MMP-13 (dilution 1/50, Abcam Biotechnology, UK), rabbit monoclonal antibody against TIMP-1 (dilution 1/80, Abcam Biotechnology, UK) and rabbit monoclonal antibody against TIMP-3 (dilution 1/50, Abcam Biotechnology, UK), respectively overnight at 4°C, sections incubated in phosphate-buffered saline (PBS) without antibody served as negative controls. After incubation with biotinylated secondary antibody, sections were incubated with avidin–biotin complex reagent containing horseradish peroxidase for 30 min. The sections were then stained with 3,3'-diaminobenzidine (DAB) (Sigma). The Image-Pro Plus 6.0 System image analysis system was used for quantitative analysis.

Chondrocytes culture, cell viability, and samples collecting

Normal human chondrocytes were obtained commercially (ScienCell, USA). The chondrocytes were maintained in the special chondrocyte medium (ScienCell, USA) and 5% heat-inactivated fetal bovine serum (FBS; vol/vol), at 37°C in a humidified atmosphere of 95% air and 5% CO_2. At the third passage, cells were seeded at a density of 1×10^6 cells per dish or 5×10^3 cells per well in a 96-well plate and cultured to approximately 80% confluence, then made quiescent in serum-free medium for 24 hr. Chondrocytes were treated with SMF at 1.25, 3.125, and 7.8125 μg/mL for 1 hr, and then treated with IL-1β at 10 ng/mL for another 24 hr of incubation; DMSO and glucosamine (100 μg/mL) treatment were negative and positive controls. At the end of the incubation period, cell viability was determined by the MTT assay.[26] Chondrocytes in the dish were collected for further study using a western blot assay and real-time PCR assay, respectively.

Western blot

After treatment, all chondrocytes were harvested in cold PBS. The pellet was resuspended in lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 5 mM EDTA, 0.1% sodium dodecyl sulfate [SDS], 0.5% NP-40) containing 10 mM phenylmethylsulfonyl fluoride (PMSF) and 2 mg/mL aprotinin. Protein was obtained to detect the levels of MMPs and TIMPs in chondrocytes by western blotting. The western blot protocol and semi-quantitative analysis were carried out as described.[27] The following antibodies were used: Rabbit anti-MMP-3 polyclonal antibody (dilution 1/100, Abcam Biotechnology, UK), rabbit anti-MMP-13 monoclonal antibody (dilution 1/100, Abcam Biotechnology, UK), rabbit anti-TIMP-1 polyclonal antibody (dilution 1/50, Abcam Biotechnology, UK), rabbit anti-TIMP-3 polyclonal antibody (dilution 1/50, Abcam Biotechnology, UK), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody (internal control, rabbit polyclonal antibody, dilution 1:200, Santa Cruz Biotechnology, Inc., Santa Cruz, CA). All experiments were done in triplicate. The relative quantity of each antibody was measured by Alpha Ease FC (Fluorchem FC_2) software. The density ratio of protein to GAPDH was calculated from the band density.

Real-time quantitative PCR

After treatment, total RNA was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA) from the chondrocytes according to the manufacturer's instructions. The total RNA (2 μg) was reverse transcribed to complementary (c) DNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems Foster City, CA) according to the instruction manual. The specific transcripts were quantified by quantitative real-time PCR using a Quanti Tect SYBR Green PCR Kit (QIAGEN K.K., Tokyo, Japan) and analyzed with an ABI 7500 real-time PCR system (Applied Biosystems, USA). Gene-specific primers were used for MMP-3 (forward, TTTTGGCCATCT CTTCCTTCA; reverse, TGTGGATGCC TCTTGGGTATC), MMP-13 (forward, TGAGGATACA GGCAAGACTC T; reverse, CAATACGGTT ACTCCAG ATGC), TIMP-1 (forward, CTTCTGG CATCCTGTTGTT G; reverse, AGAAGGCCGTCTGTGGGT), TIMP-3 (forward, TACCGAG GCTTCACCAAGA TG; reverse, TCCCACC TCTCCACGAAGTTG), and GAPDH (forward, GAAGGT GAAGG TCGGAGTC; reverse, GAAGATGGTGATGGG ATTTC). The messenger (m) RNA levels of MMP-3 and -13 and TIMP-1 and -3 were normalized to the GAPDH mRNA level. PCR was performed as 95°C for 10 min, followed by 40 cycles of 95°C for 30 sec, and 60°C for 1 min. The quantification data was analyzed with ABI Prism analysis software. The relative mRNA expression was calculated with the comparative threshold cycle (C_T) method.[28]

Statistical analysis

The software of SPSS version 11.0 for Windows (SPSS Inc, Chicago, IL) and was used for statistical analysis. Continuous variables were expressed as means ± standard error of the mean (SEM). Content of collagen and proteoglycans were analyzed with non-parametric statistics (Kruskal–Wallis test). Other data were analyzed by one-way analysis of variance (ANOVA) followed by least significant difference (LSD) or Dunnett T3 test. Differences were considered statistically significant when p was less than 0.05.

Results

Effects of SMF on histopathology of articular cartilage and synovium in ACLT + MMx rats

To characterize the anti-OA activity of SMF, we treated ACLT + MMx rats with SMF and compared the activity with glucosamine. Histological analysis of sections revealed significant histopathological changes in the medial tibia
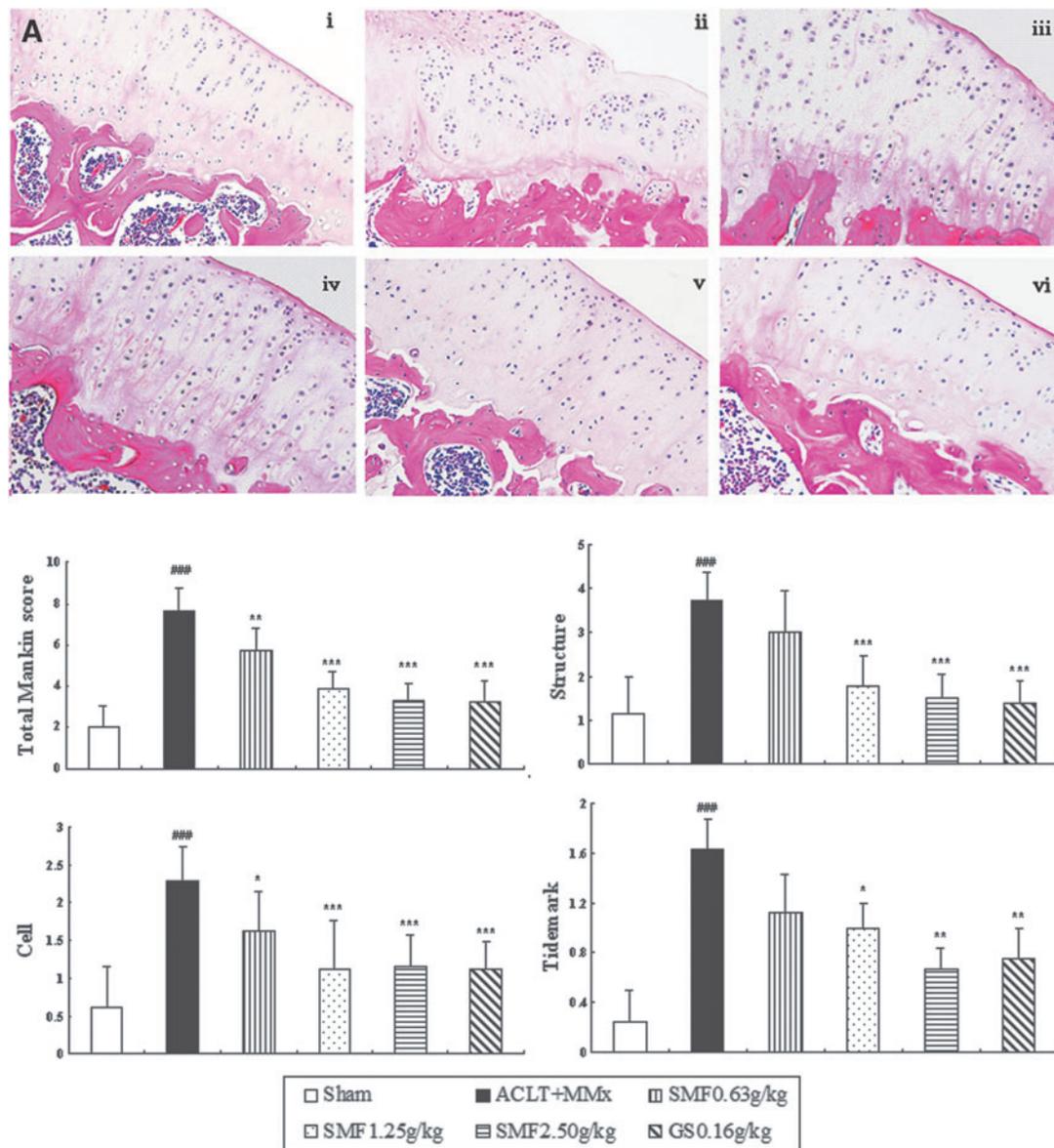
FIG. 1. Effects of SiMiaoFang (SMF) on histopathology of articular cartilage and synovium in anterior cruciate ligament transection and medial meniscus resection (ACLT+MMx) rats. The effect of SMF on morphology of articular cartilage (**A**) and synovium (**B**). Cartilage histology was observed in ACLT+MMx and sham-operated rats at the end of the 6-week treatment period. Photomicrographs taken at 200×magnification of representative cartilage sections from one animal per treatment group are shown: Sham (**i**), untreated ACLT+MMx rats (**ii**), ACLT+MMx rats treated with 0.625 gram/kg (**iii**), 1.25 grams/kg (**iv**), 2.50 grams/kg (**v**), ACLT+MMx rats treated with glucosamine 0.16 gram/kg (**vi**). Data are the mean±standard deviation (SD) of samples from 10 rats. p values are for the one-way analysis of variance (ANOVA) comparing treatment group with untreated ACLT+MMx rats. (*******) $p < 0.001$ and (******) $p < 0.01$, compared with ACLT+MMx; (**###**) $p < 0.001$, compared with sham group. (**A**) Significant histopathological changes were evident in the untreated ACLT+MMx rats, as indicated by surface irregularity and disorganization of articular cartilage with apparent cloning of chondrocytes in the transitional and radial zones (**i**). The cartilage histology of ACLT+MMx rats treated with SMF or glucosamine (**ii–iv, vi**), restored cartilage morphology (**Aii–iv**), as indicated by surface regularity of cartilage with normal cellularity in the transitional and radial zones. The overall Mankin scores of both the SMF and glucosamine groups were significantly decreased compared to untreated ACLT+MMx rats. (**B**) Untreated ACLT+MMX rats appeared more hypertrophic with infiltration of inflammatory cells in synovial tissue compared with sham rats (**Bi–ii**). SMF repressed massive cellular infiltration and dose-dependently inhibited the histological severity scores in ACLT+MMx rats. (Color images available online at www.liebertpub.com/rej)

plateau of un-treated ACLT+MMx rats (Fig.1A, ii), as indicated by surface irregularity, disorganization of articular cartilage with apparent cloning of chondrocytes in the transitional and radial zones, and disrupted tidemark. As expected, the overall Mankin score was significantly in-

creased by ACLT+MMx. Treatment of ACLT+MMx rats with SMF or glucosamine substantially restored cartilage morphology (Fig. 1A, iii–vi), as indicated by normal cartilage surfaces with normal cellularity in the transitional and radial zones and normal and intact. The overall modified
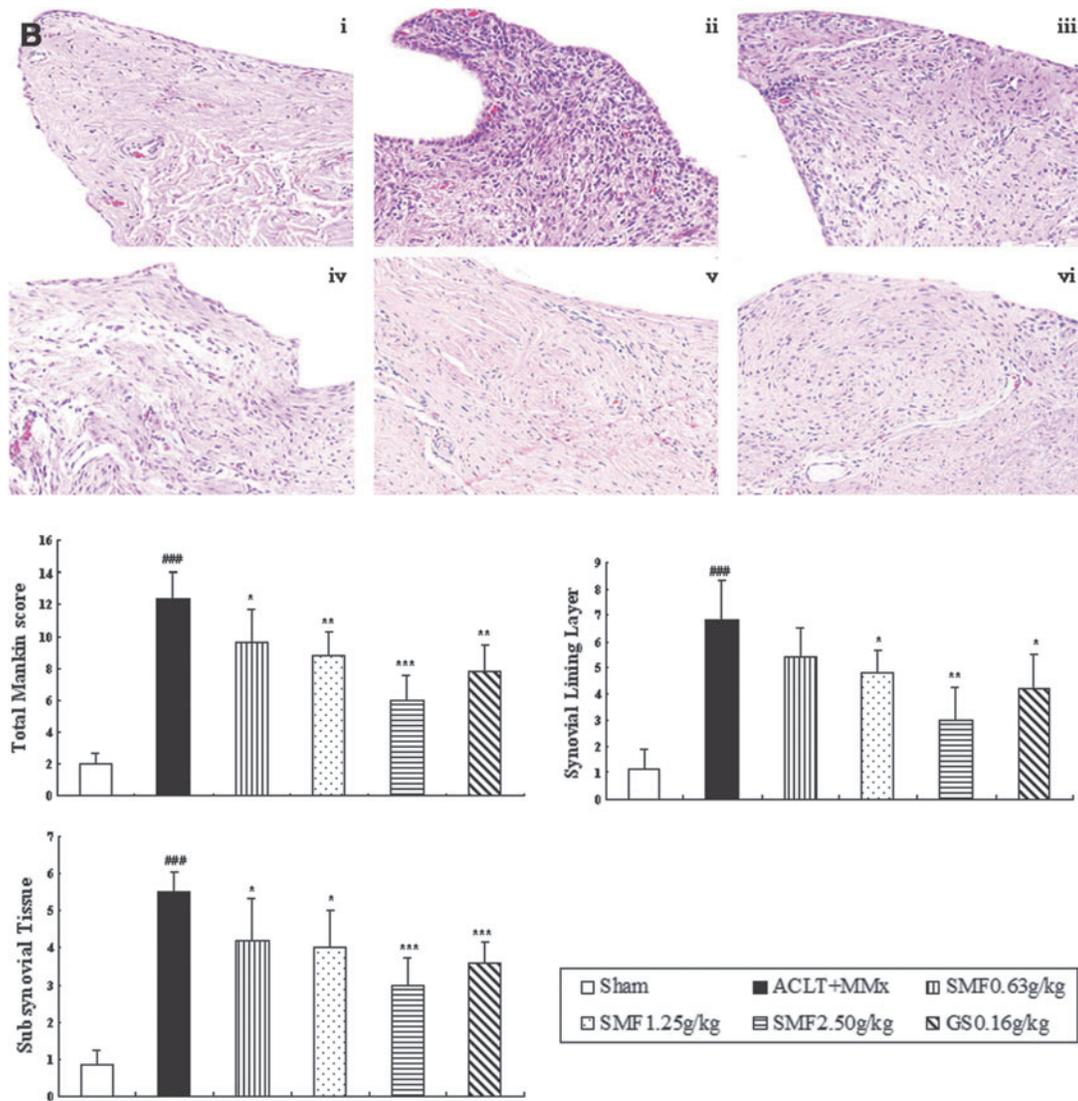
FIG. 1.   (*Continued*).

Mankin scores of articular cartilage were significantly lower in SMF- and glucosamine-treated rats than in untreated ACLT + MMx rats ($p < 0.05$, 0.01, or 0.001) (Fig. 1A).

Un-treated ACLT + MMX rats appeared more hypertrophied, and there was infiltration of inflammatory cells in synovium tissue compared with sham rats (Fig. 1B i–ii). SMF repressed inflammatory cellular infiltration and dose-dependently inhibited the histological severity scores of synovium in ACLT + MMx rats (Fig. 1B).

Effects of SMF on cartilage matrix in the joints of ACLT + MMx rats

Proteoglycan and collagen are major constituents of articular cartilage matrix and were evaluated by Toluidine Blue and Masson Trichrome staining. In the untreated ACLT + MMx rats, proteoglycan and collagen were significantly reduced, as shown in Fig. 2A ii. Furthermore, immunohistochemistry revealed a significant decrease in typeII collagen, followed with a significant increase in CTX-II and a

decrease in CPII in the serum of untreated ACLT + MMx rats compared with the Sham group (Fig. 2B).

Treatment with SMF 1.25 and 2.5 grams/kg or glucosamine significantly inhibited the cartilage matrix degradation, as indicated by restoring the loss of proteoglycan and collagen (Fig. 2A), and up-regulated the expression of type II collage. Particularly at the dose of 2.5 grams/kg, there were around 1.8-fold, 2.1-fold, and 3.7-fold increase in proteoglycan ($p < 0.01$), collagen ($p < 0.001$) and type II collage ($p < 0.001$), respectively, as well as a significant difference with a 60% increase in CPII ($p < 0.001$) and a 24% decrease in CTX-II ($p < 0.01$) compared to those of untreated ACLT + MMx rats (Fig. 2B).

Effects of SMF on ADAMTS-4 and -5, MMP-3 and -13, and TIMP-1 and -3 expression in the joints of ACLT + MMx rats

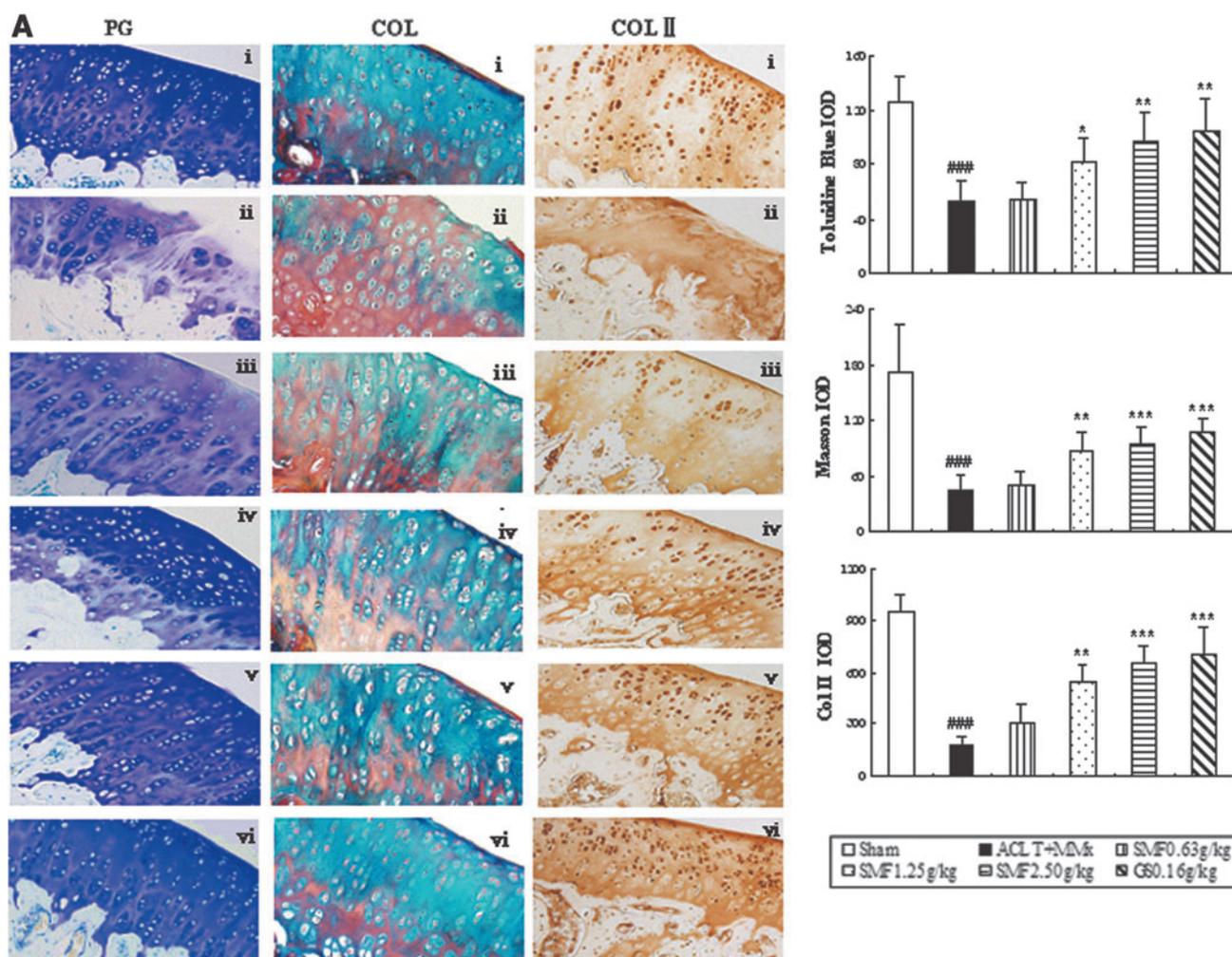The effects of SMF treatment on expression of ADAMTS, MMPs, and TIMPs in ACLT + MMx rats were assessed by

FIG. 2. The effect of SiMiaoFang (SMF) on cartilage matrix components in anterior cruciate ligament transection and medial meniscus resection (ACLT + MMx) rats. (**A**) The effect of SMF on proteoglycan (PG), collagen (COL), and type II collagen (COLII). Toluidine Blue-O, Masson staining, and immunohistochemistry were used to assess the proteoglycan (PG), collagen (COL), and type II collagen (COLII). Photomicrographs taken at 200× magnification of representative cartilage sections from one animal per group are shown on the left: Sham (**i**), untreated ACLT + MMx rats (**ii**), ACLT + MMx rats treated with 0.625 gram/kg (**iii**), 1.25 grams/kg (**iv**), 2.50 grams/kg (**v**), ACLT + MMx rats treated with glucosamine 0.16 gram/kg (**vi**). Data represents the mean ± SD of samples from 10 rats in each group. Comparing treatment group with untreated ACLT + MMx rats. (***) $p < 0.001$, (**) $p < 0.01$, and (*) $p < 0.05$, compared with ACLT + MMx; (###) $p < 0.001$, compared with the sham group. (**B**) The effects of SMF on CTXII and CPII in ACLT + MMx rats serum. (**B**) Data represent the mean ± SD of samples from 10 rats in each group. Comparing treatment group with untreated ACLT + MMx rats. (***) $p < 0.001$ and (**) $p < 0.01$, compared with ACLT + MMx; (###) $p < 0.001$, compared with the sham group.

immunohistochemistry. Representative joint sections from SMF 2.5 gram/kg or glucosamine treatment groups and quantitative analysis are shown in Fig. 3. ACLT + MMx induced increased expression of ADAMT-4 and -5 and MMP-3 and -13 and decreased expression of TIMP-1 and -3 in the joints. Compared with un-treated ACLT + MMx rats, treatment with SMF 2.5 grams/kg increased positive expression by 42% in ADAMT-4 ($p < 0.001$) and 38% in ADAMT-5 ($p < 0.01$) (Fig. 3A). Meanwhile, SMF 2.5 grams/kg also induced a clear down-regulation of MMP-3 and -13 ($p < 0.001$ and $p < 0.01$, respectively) and marked up-regulation of TIMP-1 and -3 ($p < 0.001$ and $p < 0.05$, respectively) compared with untreated ACLT + MMx rats (Fig. 3B). ADAMTS-4 and -5, MMP-3 and -13, and TIMP-1 and -3 were expressed in the chondrocytes of the knee joint of all groups, and the positive

staining was predominantly cytoplasmic or cytosolic in the chondrocytes. These results showed that SMF exerted anti-OA activity *in vivo* through ADAMTS and MMPs/TIMPs system.

Effects of SMF on the production and gene expression of MMP-3 and -13 and TIMP-1 and -3 in human chondrocytes

It is well known that MMPs/TIMPs play a key role in OA progression according to *in vivo* results. We carried out *in vitro* studies to elucidate further effects on MMPs/TIMPs by western blotting. The expression of MMP-3 and -13 was significantly down-regulated when incubated with SMF at 3.125 or 7.8125 μg/mL or after incubation with glucosamine. The down-regulating effects were augmented as the dose of SMF
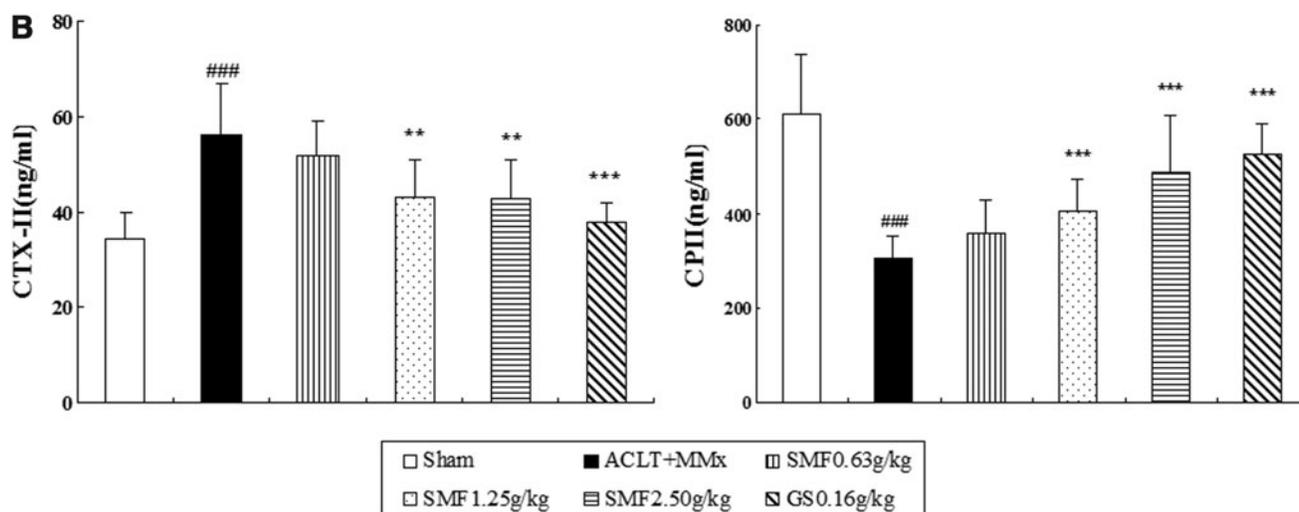
FIG. 2. (*Continued*).

increased, and SMF at 7.8125 µg/mL induced a 40% decrease in MMP-3 ($p < 0.01$) and a 42% decrease in MMP-13 ($p < 0.001$) compared to untreated IL-1β–induced chondrocytes. The down-regulated effect of SMF on MMP-13 expression is weaker than that of glucosamine. Meanwhile, the expression of TIMP-1 and -3 was remarkably up-regulated when incubated with SMF at all three concentrations or with glucosamine. The largest increases in the two parameters were found at SMF 3.125 µg/mL (both $p < 0.01$) and with 2.2-fold up-regulation, respectively. The expression of TIMP-1 and -3 was up-regulated
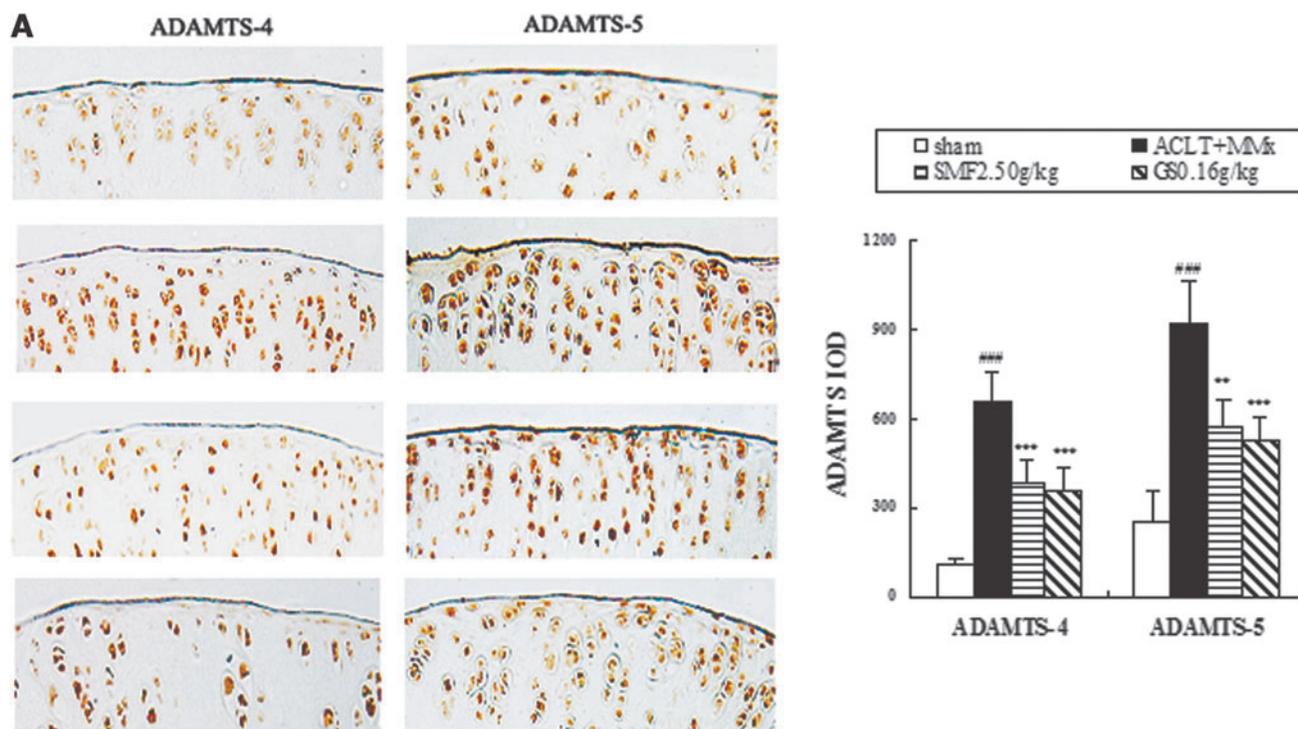

FIG. 3. The effects of SiMaioFang (SMF) on aggrecanases-1 and -2 (ADAMTS-4 and -5), matrix metalloproteinases-3 and -13 (MMP-3 and -13), and tissue inhibitors of metalloproteinases-1 and -3 (TIMP-1 and -3) in the knee joints. (**A**) The effects of SMF on expression of ADAMTS-4 and -5 in the knee joints. (**B**) The effects of SMF on expression of MMP-3 and -13 and TIMP-1 and -3 in the knee joints. These enzymes expressions were assessed by quantitative immunohistochemistry. Photomicrographs taken at 200× magnification of representative cartilage sections from one animal per group are shown: Sham (**i**), anterior cruciate ligament transection + medial meniscus resection ACLT + MMx (**ii**), ACLT + MMx + SMF at 2.50 grams/kg (**iii**), ACLT + MMx + glucosamine 0.16 gram/kg (**iv**). Quantitative immunohistochemistry analyses of the expression of these enzymes in the tibia are shown, and data represent the mean ± standard deviation (SD) of samples from 10 rats in each group. Comparing treatment group with untreated ACLT + MMx rats. (***) $p < 0.001$, (**) $p < 0.01$, and (*) $p < 0.05$, compared with ACLT + MMx; (###) $p < 0.001$, (##) $p < 0.01$, and (#) $p < 0.05$, compared with the sham group. (Color images available online at www.liebertpub.com/rej)

slightly more by glucosamine than by SMF (Fig. 4A). Meanwhile, SMF at doses of 1.25, 3.125, and 7.8125 μg/mL did not affect IL-1β–induced chondrocyte viability (Fig. 4B).

To further validate the above mechanisms, we also assessed the effects of SMF on mRNA expression of MMP-3 and-13 and TIMP-1 and -3 in IL-1β–induced chondrocytes by real-time quantitative PCR. SMF significantly inhibited the IL-1β–induced gene expression of MMP-3 and-13 in a dose-dependent manner and SMF at the highest concentration produced with a 31% decrease in MMP-3 mRNA ($p < 0.01$) and 52% decrease in MMP-13 mRNA ($p < 0.001$) compared with the model group. Interestingly, the effects of SMF and glucosamine on gene expression of TIMP-1 and -3 were similar to those in protein levels. SMF at 3.125 μg/mL induced 1.5-fold and 2.0-fold up-regulation in TIMP-1 and -3 mRNA compared with the model group, respectively (both $p < 0.01$) (Fig. 5).

Effects of SMF on pro-inflammatory cytokines and inflammatory mediators in ACLT + MMx rats

Because the high levels of serum IL-β in mono-iodoacetate–induced OA rats and serum TNF-α as well as IL-6 in OA patients were observed, respectively.[29,30] We detected pro-inflammatory cytokines and inflammatory mediators in the serum of ACLT + MMx rats, and the results are indicated in Fig. 6. Treatment of ACLT + MMx rats with SMF at 1.25 or 2.5 grams/kg, or with glucosamine, resulted in a significant difference in IL-1β and with a 15% decrease at the highest dose of 2.5 grams/kg compared with untreated ACLT + MMx rats ($p < 0.01$). SMF at any of three doses significantly decreased levels of circulating TNF-α by approximately 20% compared to those of untreated ACLT + MMx rats (all $p < 0.01$).

There was a trend of decreasing in serum PGE$_2$ with an increasing dose of SMF; SMF at 2.5 grams/kg resulted in a 30% decrease compared with untreated ACLT + MMx rats ($p < 0.001$). SMF did not cause any effect on serum COX-1 levels. However, at any of three doses of SMF, there were significant differences in COX-2 levels compared with untreated ACLT + MMx rats, and the highest dose resulted in a 33% decrease in COX-2 ($p < 0.001$), which was comparable to the decrease induced by glucosamine treatment.

SMF treatment at all three doses significantly decreased levels of NO and iNOS in serum, and SMF at 2.5 grams/kg or glucosamine significantly decreased contents of NO and
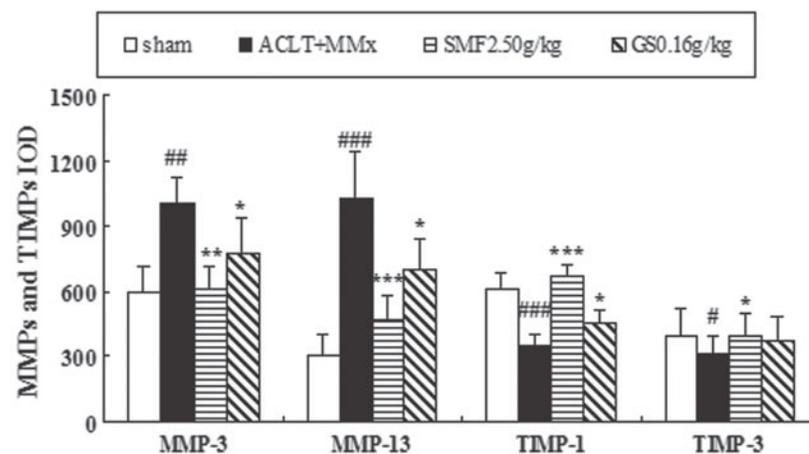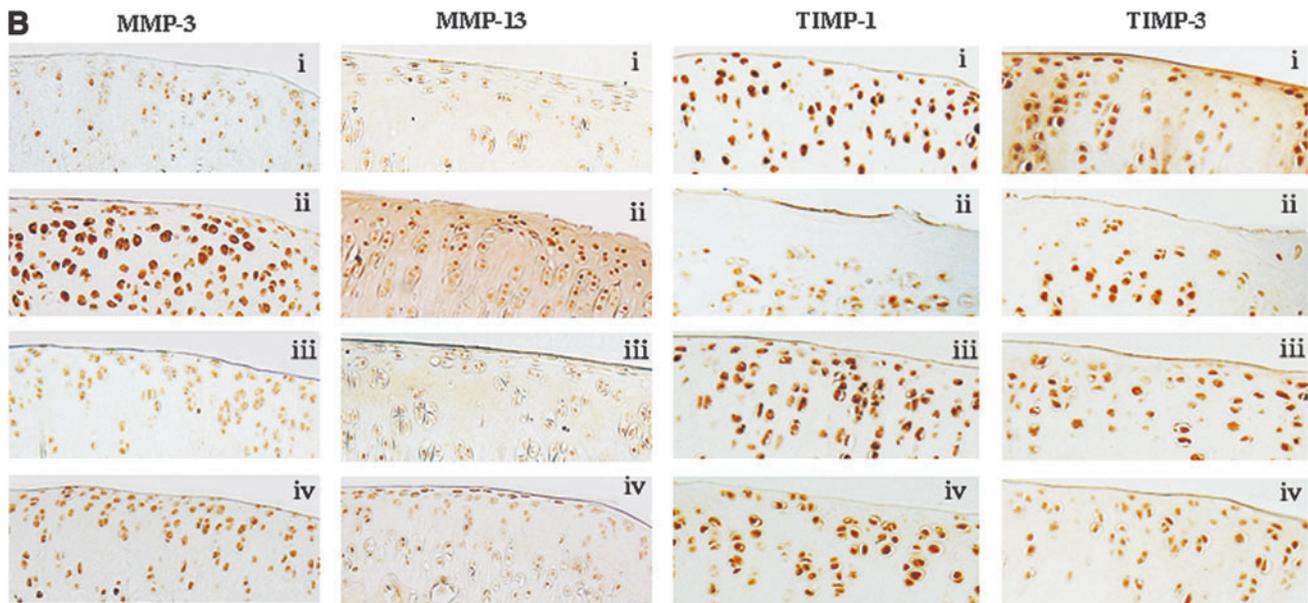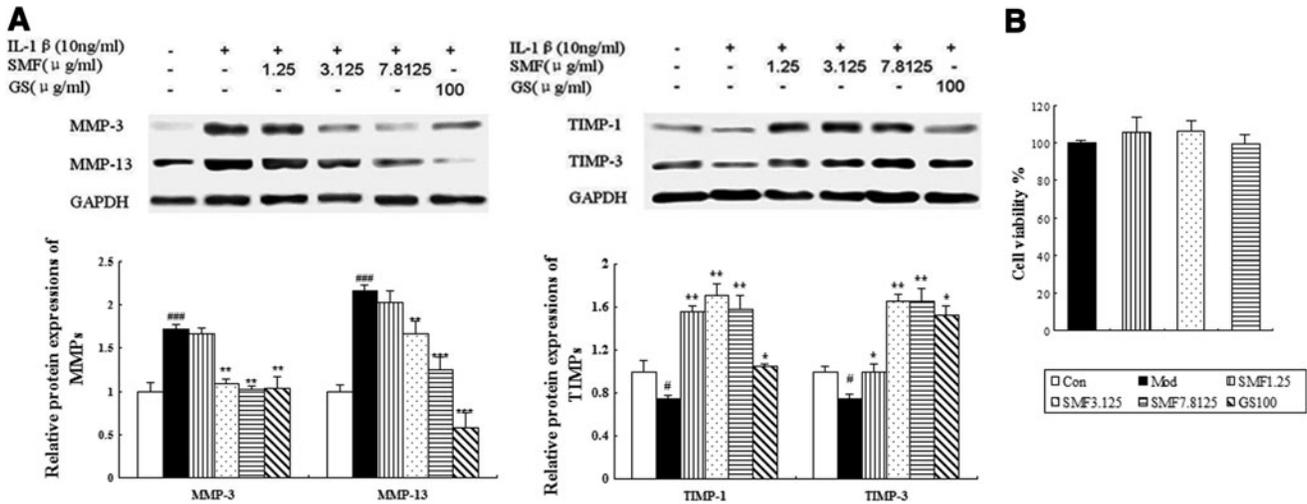

FIG. 3. (*Continued*).

FIG. 4. The effects of SiMiaoFang (SMF) on matrix metalloproteinases-3 and -13 (MMP-3 and-13) and tissue inhibitors of metalloproteinases-1 and -3 (TIMP-1 and -3) expression in IL-1β–induced chondrocytes. (**A**) Western blot analysis of MMP-3 and -13 and TIMP-1 and -3 expression in human chondrocytes were carried out as described in Materials and Methods. Representative blots are shown above, and quantitative analysis and cell viability are shown below. Values given are the mean ± standard deviation (SD) of three independent experiments. (***) $p < 0.001$, (**) $p < 0.01$, and (*) $p < 0.05$, compared with Model (Mod); (###) $p < 0.001$ and (#) $p < 0.05$, compared with the Control (Con) group. (**B**) The effect of SMF on viability of IL-1β–induced chondrocytes. GS, glucosamine.

iNOS by 50% and 42%, respectively, compared to those of untreated ACLT + MMx rats ($p < 0.001$, respectively).

Discussion

OA is a degenerative joint disease with multiple underlying pathogenic mechanisms caused by various risk factors. The balance of degradation and synthesis of cartilage matrix plays a key role in inhibiting the destruction of articular cartilage. Therefore, inhibiting degradation and promoting synthesis of cartilage matrix is an important issue in the

treatment of OA. In the present study, we evaluated the anti-OA activity of SMF *in vivo* using a ACLT + MMx rat model. The results showed SMF could clearly inhibit the degradation of cartilage matrix, as indicated by increasing proteoglycan and collagen content, particularly type II collagen expression in articular cartilage, and decreasing CTX-II and increasing CPII in circulation, showing a significant anti-joint degeneration.

Cartilage matrix is composed of proteoglycan and collagen. Type II collagen is the most important biomarker for OA because of a 90–95% content in total collagen.[31] It has been
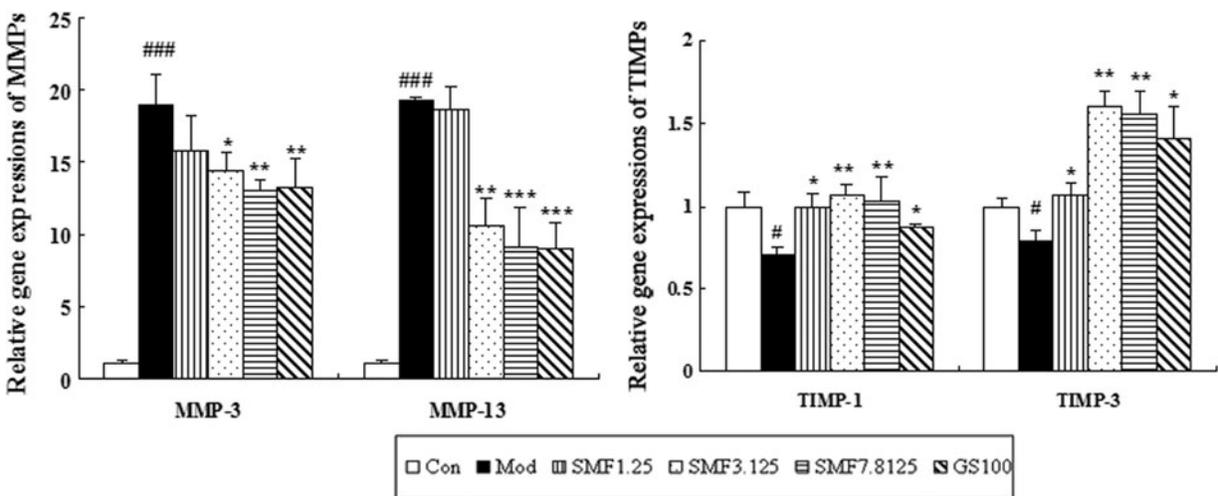

FIG. 5. The effects of SiMiaoFang (SMF) on matrix metalloproteinases-3 and -13 (MMP-3 and -13) and tissue inhibitors of metalloproteinases-1 and -3 (TIMP-1 and -3) mRNA expression in IL-1β–induced chondrocytes. Real-time PCR analyses of MMP-3 and -13 and TIMP-1 and -3 gene expression in human chondrocytes were carried out as described in Materials and Methods. Quantitative analysis is shown and values given are the mean ± standard deviation (SD) of three independent experiments. (***) $p < 0.001$, (**) $p < 0.01$, and (*) $p < 0.05$, compared with Model (Mod); (###) $p < 0.001$ compared with the Control (Con) group.
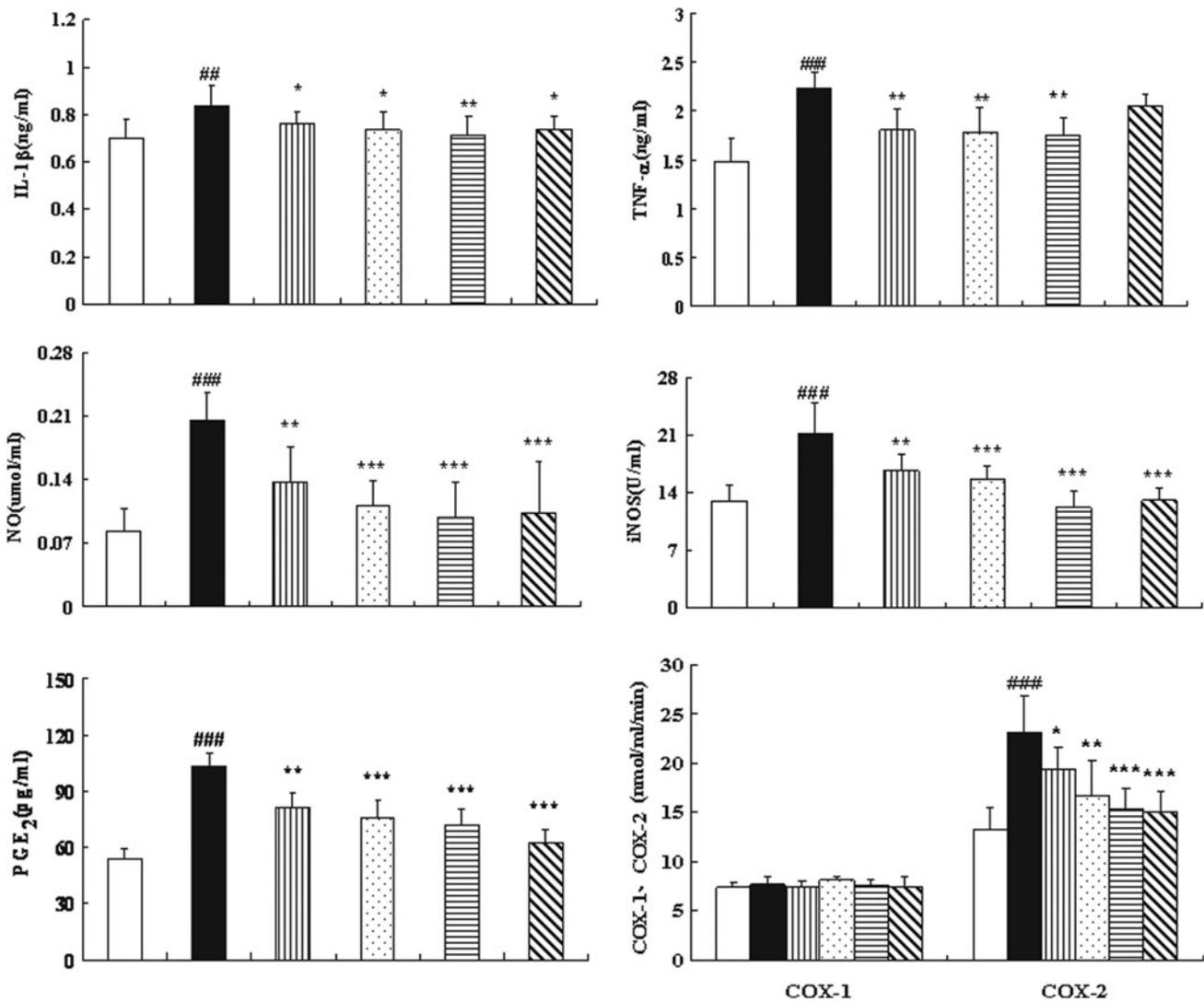
FIG. 6. The effects of SiMiaoFang (SMF) on interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), nitric oxide (NO), inducible nitric oxide synthase (iNOS), prostaglandin E (PGE$_2$), cyclooxygenase-1 (Cox-1), and Cox-2 in serum of cruciate ligament transection+medial meniscus resection (ACLT+MMx) rats. Blood was obtained from the abdominal aorta at 6 weeks of treatment after the ACLT+MMx operation and sham-operated, and serum was used for the assays of IL-1β, TNF-α, NO, iNOS, PGE$_2$, COX-1, and COX-2. Data represent the mean±standard deviation (SD) of samples from 10 rats in each group compared the treatment group with untreated ACLT+MMx rats. (***) $p < 0.001$, (**) $p < 0.01$, and (*) $p < 0.05$, compared with ACLT+MMx; (###) $p < 0.001$, (##) $p < 0.01$, compared with the sham group.

recognized that MMP-3 and -13 play important roles in the degradation of cartilage matrix during the development of OA. MMP-3 can degrade proteoglycan and type II collagen.[32] MMP-13 can degrade type II collagen and is considered the primary collagenase in OA.[33] The expression and production of MMPs are regulated at the mRNA and protein levels by various factors; the most important effect is from the TIMP family.[34] The enzymatic activities of MMP-3 and −13 are inhibited by TIMP-1, and TIMP-3 has been recognized as a potent inhibitor of MMP-3.[35,36] Under normal conditions, TIMPs bind to active MMPs in a ratio of 1:1 to make an inactive complex. Therefore, an imbalance in the ratio of TIMPs to MMPs causes continued matrix destruction in OA.[13,15]

In the present study, we demonstrated that SMF significantly interfered with the OA-induced expression of

MMP-13 and -3, while augmenting that of TIMP-1 and -3 in the joints of ACLT+MMx rats. These results are supported by our *in vitro* study, where IL-1–augmented expression of MMP-3 and -13 was inhibited and expression of TIMP-1 and -3 was up-regulated in protein and gene levels by SMF in IL-1β–induced chondrocytes. A recent study has also showed that the primary component berberine inhibited the induction of MMP-13 and -3 and increased TIMP-1 expression in IL-1β– chondrocytes.[37,38] Thus, SMF can block cartilage matrix degradation by mediating the balance of MMPs/ TIMPs.

Moreover, ADAMTS-4 and -5 are considered to be the most efficient aggrecanases.[39,40] Recent papers have reported that deleting ADAMTS-4 and -5 in mice significantly protected against proteoglycan degradation and decreased the severity of murine OA.[41] As the most likely

candidates to play a role in the pathogenesis of OA, ADAMTS-4 and -5 are inhibited by TIMP-3.[42] Our study shows that SMF markedly decreased ADAMTS-4 and -5 expression and increased TIMP-3 expression in ACLT + MMx rat joints, which may be one of mechanisms of SMF anti-OA activity.

The progression of OA is now believed to be relevant to inflammation in the early stages of the disease. Among the pro-inflammatory cytokines involved in OA, IL-1β and TNF-α are considered the major players. IL-1β and TNF-α produced by activated synoviocytes, mononuclear cells, or chondrocytes can directly induce degradation of cartilage matrix[43,44] and promote the production of PGE$_2$ and NO as well as up-regulation of MMP-3 and -13, which together induce the progression of OA.[45–48] Among the numerous pro-inflammatory enzymes, inducible COX-2 is a key enzyme for production of PGE$_2$, and iNOS regulates the generation of NO. Our results showed that SMF significantly decreased the secretion of IL-1β and TNF-α in serum. Additionally, SMF markedly inhibited the release of PGE$_2$ and NO in circulation through suppression of COX-2 and iNOS production, respectively. SMF was able to suppress the inflammatory response of arthritis. Wang et al. reported that SMF effectively down-regulated overexpression of IL-1β, TNF-α, and IL-6 mRNA in adjuvant arthritis (AA) rats.[49] The dose of SMF used in AA rats is similar to OA rats in our study. Moreover, all SMF four ingredients contain the anti-inflammatory substances, such as berberine and atractylodine, which were confirmed to have anti-inflammatory activity.[50–53] Thus, we deduce that SMF inhibits inflammation response in OA by interfering with secretion of pro-inflammatory cytokines and inflammatory mediators.

Because glucosamine is effective not only in prevention of cartilage matrix degradation but also against inflammatory symptoms in OA animal experiments,[54–56] we used it as the control drug, and its activity was also proved in our study. Compared with glucosamine at the clinically equivalent dose, SMF at 2.5 grams/kg had a similar ability to suppress cartilage matrix components mass loss and anti-inflammation. Moreover, both SMF and glucosamine were able to regulate the balance of the MMPs/TIMPs system, which has been confirmed by in vitro results. Meanwhile, SMF exerted anti-OA activity with no evidence of adverse effect on viscera (heart, liver, spleen, lung, and kidney), body weight, skin, hair, and activities of ACLT + MMx rats. It is worth mentioning that the cost of SMF is far lower than glucosamine in the marketplace. Thus, SMF could be a potential novel agent that could effectively prevent cartilage matrix degradation in OA.

In conclusion, these results demonstrated that SMF effectively inhibited cartilage matrix degradation and interfered with the secretions of pro-inflammatory cytokines and inflammatory mediators in ACLT + MMx rats. The effects of preventing cartilage matrix were mainly from the direct suppressing MMPs expression, simultaneous up-regulation of TIMPs production, and then maintaining the balance of MMPs to TIMPs in the joints and chondrocytes. Moreover, down-regulation of the expression of ADMATS-4 and -5 was highly effective in ACLT + MMx rats. Accordingly, this study provides the first evidence that SMF can effectively treat OA.

Author Disclosure Statement

Ying Xu and Qian Liu contributed equally to this paper and all authors have no conflicts of interest to disclose.

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