Traditional Chinese Medicine Formula “Xiaofeng granules” suppressed gouty arthritis animal models and inhibited the proteoglycan degradation on chondrocytes induced by monosodium urate

Le Shi 1, Fangli Zhao 1, Fangfang Zhu 1, Yuqiong Liang, Fan Yang, Guangji Zhang, Li Xu *, Lian Yin *

College of Pharmacy, Nanjing University of Traditional Chinese Medicine, Nanjing 210023 PR China

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A B S T R A C T

Ethnopharmacological relevance: Xiaofeng Granules (XF) is a kind of granules prepared by the famous traditional Chinese medicine formula for its efficiency in treating gouty diseases.
Aim of the study: We investigated the relevance between XF that made from Modified simiaowan (MSW) as the anti-gouty arthritis drugs and protective mechanisms for cartilage matrix in order to provide the evidence for new drug application.
Materials and methods: In the present study, we evaluated the anti-gouty arthritis activity of XF in rats and rabbits models induced by MSU together with chondrocytes focusing on the link to proteoglycan degradation in vitro studies.
Results: The results demonstrated that XF significantly reduced the swelling rate and attenuated the pathological changes in joints. The XF-containing serum were used medicated serum in cellular experiments. The in vitro data were in accordance with the in vivo results, showing that the constituents in XF-containing serum had obvious inhibitory effects on the activation of pro-inflammatory mediators in chondrocytes. Moreover, XF-containing serum substantially inhibited MSU-induced expression of glycosaminoglycans (GAG) and hydroxyproline (Hyp), and up regulated proteoglycan, which might be associated with the regulation of the balance of MMP-3/TIMP-1 and ADAMTS-4/TIMP-3 in chondrocytes.
Conclusion: In conclusion, XF that made from MSW showed obvious effects on acute gouty arthritis, which also provided an effective protection on cartilage matrix degradation.

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1. Introduction

Gout is a common condition with an overall prevalence in adult men and has been an increasing cause of arthritis in women (Roddy and Doherty, 2010). It is also identified to be a type of inflammatory arthritis induced by deposition of monosodium urate (MSU) crystals in the joints and kidneys. The presence of MSU crystals has been considered as the gold standard for diagnosis of gout since it was identified in the synovial fluid analysis reported in 1961 (Pascual et al., 1999). MSU crystals in the joints stimulate synovial cells, monocytes-macrophages and neutrophils to produce different cytokines, including interleukin (IL)-1β, tumor necrosis factor-α (TNF-α) and inducible nitric oxide synthase (iNOS) (Hasselbacher et al., 1981; Lee et al., 2009; Tausche et al., 2004). Precisely, all these molecules play critical roles in developing acute inflammation in gout flares (Punzi et al., 2012). Nitric oxide (NO) generated by iNOS may react with superoxide anion causing oxidative/nitrosative stress resulting in joint damage by inhibiting chondrocyte proteoglycan synthesis via PGES induction of chondrocyte apoptosis (Blanco et al., 1995; Liu et al., 2004; Taskiran et al., 1994), and may influence synoviocyte survival through regulation of mitochondrial functionality (Cillero-Pastor et al., 2011). Inhibition of these pro-inflammatory mediators has been found to reduce the severity of the inflammatory reaction in gouty arthritis (Stow et al., 2009).

Abbreviations: AB, Achyranthes bidentata Bl; AC, Atractylodes chinensis (DC.) Koidz.; ADAMTS, a distintegrin-like and metal-loproteinase with thrombospondin type I motifs; COX-2, cyclooxygenase-2; CS, Coix laryma-jobi L var. mayuen (Roman); Statf; FBS, fetal bovine serum; GAG, glycosaminoglycans; Hyp, hydroxyproline; iNOS, inducible nitric oxide synthase; IL, Interleukin; Lj, Loniceraja japonica Thunb.; MSU, monosodium urate; MMPs, matrix metalloproteinases; MSW, Modified Simiaowan; NO, nitric oxide; NSAIDs, nonsteroidal anti-inflammatory drugs; PC, Phellodendron chinense Schneid.; SE, standard error; SG, Smilax glabra Roxb; TIMP, tissue inhibitor of metalloproteinase; TNF-α, tumor necrosis factor-α; XF, Xiaofeng

* Corresponding author.
E-mail addresses: xuliglp@126.com (L. Xu), yinlian162@163.com (L. Yin).

1 These authors contributed equally to this work.

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Chondrocytes are vital cells in articular cartilage, which can synthesize matrix and fiber mainly including proteoglycans and type II collagen. Proteoglycan is composed of glycosaminoglycans (GAG) as side chains binding to the core protein, while collagen is the sole protein consists largely of hydroxyproline (Hyp). When the balance between the biosynthesis and degradation of matrix components is broken, a destruction of the tissue and eventually complete damage of the articular surface will be caused. The loss of proteoglycan occurs early in the process of cartilage degeneration and is followed by catabolism of collagen fibrils, leading to the loss of cartilage structural integrity (Jubb and Fell, 1980; Mankin and Lippiello, 1970). Namely, to ascertain the injury degree of articular cartilage, GAG and Hyp content regarded as the degradation indicators of proteoglycan and collagen can be examined.

Patients often suffer the rapid onset of severe pain, swelling, warmth, erythema, and decreased range of motion in the affected joint during acute phase (Cheng et al., 2004; Neogi, 2011; So et al., 2010), leading to joint destruction when become chronic. Generally, effective treatment of acute gouty arthritis must target both the pain and the underlying inflammation. Orally administered colchicines and non-steroidal anti-inflammatory drugs (NSAIDs) are primary agents for this phase (Neogi, 2010). The role of suppressing the inflammation for management of acute gouty arthritis has been well documented, but severe gastrointestinal reactions and toxicity remain unavoidable (Borstad et al., 2004; Sabina et al., 2011). Simiaowan is a compound prescription consisting of our individual herbs: Atractylodes chinensis (DC) Koidz. (AC), Phellodendron chinense Schneid. (PC), Coix lacryma-jobi L var. mayuen (Roman.) Stapf (CS), and Achyranthes bidentata Bl.(AB) (Shi et al., 2013), which is described as a famous traditional Chinese medicine (TCM) formula in a Chinese medicine book named Conventional Prescriptions for Easily Reading in Qing Dynasty, and now it has been recorded in the Chinese Pharmacopoeia for its efficacy, especially clearing heat and eliminating dampness retention (Pharmacopoeia Committee of PR China, 2010). According to Cheng-Fang-Bian-Du, Simiaowan is described to treat gout through eliminating dampness retention and strengthening the liver and kidney in terms of traditional Chinese medicine (Hu et al., 2010). And now simiaowan is commonly used in clinic for treatment of arthralgia due to its anti-inflammatory and pain-relieving activities, as in gouty arthritis and rheumatoid arthritis (Yin and Shi, 2004). It has been reported that simiaowan treatments significantly inhibit articular cartilage and synovial in the rats of OA (Xu et al., 2014), and ameliorates urate underexcretion and renal dysfunction in hyperuricemic mice (Hu et al., 2010).

According to 15 years clinical reports in treating acute gouty arthritis by traditional Chinese medicine, the combination of simiaowan and its additional drugs treated nearly 1500 cases of acute gout patients. The rate of total effective is 90%, uric acid returned to normal or significantly lower, and the recurrence rate were reduced. Statistics showed that the additional drugs of combination with clearing heat and promoting diuresis were more effective, and also pointed out that LJ and SG were most widely used in gouty treatment (Yin and Shi, 2004). Consequently, Modified simiaowan (MSW) is scientifically prepared with the classical formula Simiaowan with addition of Smilax glabra Roxb. (SG) and Lonicera japonica Thunb.(LJ). What is more, SG is reported to be effective in xeraxis, detoxification, and easing joint movement in traditional Chinese medical literature, which include the Compendium of Materia Medica and the State Pharmacopoeia of the People’s Republic of China, and has been described to possess hyperuricemia effects in vivo and XOD inhibitory activity in vitro (Xu et al., 2013). Pharmacological studies have shown that extracts of LJ flower buds have a broad spectrum of biological activity, including antibacterial, anti-inflammatory, antioxidiant, anti-angiogenic, antipyretic, antiviral, and hepatoprotective effects (Qian et al., 2007). It was also recorded that LJ extracts may be very effective against asthma and inflammation related diseases (Hong et al., 2013). MSW is a patented invention in Chinese (Yin et al., 2008), which developed based on TCM theory, clinical research, and pharmacological findings (Yin and Shi, 2006). It is able to invigorate vital energy, promote urination and detoxification (Hua et al., 2012; Hu et al., 2010), and diminish inflammation (Lower-Nedza et al., 2013). Previous studies in rodents have established its efficiency for treating all different symptoms, including hyperuricemia, inflammation and pain, which is more effective than indomethacin or colchicines (Shi et al., 2008).

Xiaofeng Granules (XF) is a kind of granules prepared for a novel agent that derived from the MSW. As clearing heat agents, PC, LJ and SG, the active drugs in XF, play the role of anti-inflammatory by targeting inflammatory cytokines, while AC, CS and SG of XF are more effective in promoting diuresis to eliminate dampness on gouty treatment. AB, a hemorheologic agent, is often used for promoting blood circulation. Diverse ingredients in six herbs of XF were well documented in treating acute gout. Caffeic acid, Luteoline and Loganin, the representative ingredients in LJ, show their potent antiinflammation and anti-inflammatory effects (Zhang et al., 2014). As active ingredients in SG, Quercetin, Astilbin and Resveratrol are rather effective for anti-inflammation, as well as benefit for lowering uric acid (Hu et al., 2010). Berberine, Jatrohizine, main active components in PC, are also useful for treating acute gout (Yin et al., 2004). As dampness-eliminating agents, many compounds in AC show their uric acid-lowing abilities on gouty treatment except anti-inflammation, such as Vanillic acid, Palmitic acid (Wang et al., 2002). Besides, Coixol, Oleic acid, the representative compounds in CS, have also been shown their ability to reduce serum uric acid levels (Zhang et al., 2015).

It has been well documented that the joints in patients with gouty arthritis may be injured severely by uric acid crystals, which leads to repeated attack. But little is currently known about alteration of cartilage matrix by anti-gouty arthritis drugs. We here explored the anti-gouty arthritis activity of XF and its mechanism for cartilage matrix degradation. In this study, the therapeutic effects of XF on gouty arthritis models and inflammatory mediators, and cartilage matrix degradation in chondrocytes were examined. We further investigated the mechanisms of XF focusing on proteoglycan degradation using MSU-stimulated chondrocytes in vitro, which could mimic the clinical pathological characteristics of gouty arthritis.

2. Materials and methods

2.1. Reagents and materials

Microcrystalline urate was purchased from Sigma (St. Louis, MO, USA). ELISA Kits for human IL-1β and IL-6 were provided by Shanghai Ex Cell Biology Inc (PR China). NO test kit, NOS test kitand Hyp test kit were products of Nanjing Jioncheng Bioengineering Institute (PR China). MMP-3 andTIMP-1 ELISA kits and BCA Protein Assay Kit were supplied by Wuhan Boster Biotechnology Co., Ltd. (PR China). Antibodies were purchased as follows: β-actin monoclonal antibody, Nanjing SunShine Biotechnology Co., LTD.(PR China); Goat anti-Rabbit IgG (H+L)-TRITC, Bioworld(PR China); ADAMTS4 polyclonal antibody, Biorbyt(Shanghai, PR China);TIMP-3 polyclonal antibody, R&D SYSTEMS(USA).

2.2. Animals

Male Sprague Dawley rats (200 ± 20 g) and male New Zealand rabbits (2.0–2.5 kg) were supplied by Comparative Medicine Centre of Yangzhou University (No. SYXK(Su)2007-0001). The animals...
were housed at room temperature (22 ± 2 °C) with relative humidity (55 ± 5%) and were given standard chow and water ad libitum for the duration of the study. The Provision and General Recommendation of Chinese Experimental Animals Administration Legislation Guidance were approved by the Science and Technology Department of Jiangsu Province and also by the Animal Care and Use Committee of Nanjing University of Chinese Medicine.

2.3. Preparation of XF extractive

All herbs used in the study were all commercially available dry matter, which were supplied by the Department of Chinese Medicine Chemistry, Nanjing University of Chinese Medicine. The mixtures, including Atractyloides chinesis (DC.) Koidz., Phellodendron chinense Schneid., Coix lacryma-jobi L. var. mayuen (Roman.) Stapf., Achyranthes bidentata Bl., Smilax glabra Roxb. and Lonicerajaponica Thunb., were soaked in distilled water for 30 min and then boiled in 10 volumes of water (v/w) for 1 h and extracted twice. The suspension was then centrifuged (3000 rpm, 20 min) and the supernatant was decompressed. And we selected 10% of dextrin as adhesive, mixed well and granulated. The XF were turned into powder form and extracted with the 80% ethanol for 10 times (2 times × 0.5 h). The extractive solutions were filtered, concentrated under vacuum and dried, obtaining XF extracts. The yield of the XF extractive was 20.05% (w/w). XF were stored at 4 °C.

2.4. MSU crystal-induced inflammation

The animals (rats and rabbits) were randomly divided into five groups (Control, Colchicine, XF at three doses). Each group was orally administered with Colchicine or XF once daily for 5 consecutive days. One hour after the final administration, animals were injected intrarticularly under anesthesia with normal saline and MSU crystal suspension at the medial side of the right ankle joint of the hind limb for control group and all other groups respectively.

All the procedures were conducted according to the methods described before (Codorex and Wall, 1987) with few modifications. The inflammation was quantified by measuring the volume (or perimeter) of the paw with foot volumometer at 1, 3, and 5 h after MSU crystal injections. Paw swelling ratio was calculated using the following formula:

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\text{Paw swelling ratio (\%)} = \left( \frac{\text{Paw volume (or perimeter) after MSU crystal injection}}{\text{Paw volume (or perimeter) before MSU crystal injection}} \right) \times 100\%.
\]

2.5. Histological analysis

Rabbits were sacrificed and whole knee joints were removed and fixed in 4% formaldehyde for 7 days before decalcification in 5% formic acid and processing for paraffin embedding. Tissue sections were stained with haematoxylin/eosin. Scoring was performed on decoded slides by two separate observers, using the following parameters: the amount of cells infiltrating the synovial lining and the joint cavity was scored from 0 to 3 (Abdollahi-Roodsaz et al., 2008; Joosten et al., 2008; Joosten et al., 2009).

2.6. Preparation of XF serum

Male Sprague Dawley rats were randomly divided into two groups (Control and XF) with three animals per group. Each group were administered intragastrically daily once for five days. One hour after the last administration, blood samples from the rats were obtained and germ-free sera which contained drug metabolites were prepared under sterile condition.

The blood samples were placed in a water bath for 10 min at 37 °C, centrifuged at 4000 rpm for 10 min to obtain the sera (supernatant). Then the sera was used for HPLC analysis. Methanol (6 mL) was added to the serum (2 mL), and the solution was vortexed for 1 min. The suspension was centrifuged at 10,000 rpm for 10 min to obtain the supernatant and then dried using nitrogen gas at 30 °C. The residues were redissolved in 200 μL of methanol and centrifuged at 10,000 rpm for 10 min. The supernatant was subjected to HPLC analysis.

A total of eleven compounds including Sweroside, Loganin, Logatanin sulfate, aglycone of Loganic acid, Caffeic acid glucuronide, methyl Iridin, Sinapaldehyde glucuronide from LJ and Luteolin glucuronide, Resveratrol glucuronide, Quercetin glucuronide, Luteolin glucoside from SG are main components of XF-treated rat serum, and they were absorbed into blood and distributed into tissues after oral administration by our previous HPLC-MS analysis.

2.7. Primary chondrocyte culture

Thin cartilage slices were digested overnight in 0.25% Trypsin at 37 °C and 5% CO2. The cell in suspension was filtered and washed before the first expansion, and cultured in high glucose DMEM with 10% fetal bovine serum (FBS). The cells were transferred into 12-well plates while the confluence reached. Only first and second passage cells were used in this study.

2.8. Cell viability assay

Survival rate of cells in each group was analyzed by the conventional MTT assay. Cells were properly cultured in 96-well plates at 1 × 104 cells/well overnight. After incubation with the germ-free sera (5% XF-serum + MSU, 10% XF-serum + MSU, 20% XF-serum + MSU) for the indicated time period, 20 μL MTT (5 g/L MTT in PBS) was added to each well and incubated at 37 °C for 4 h and the supernatant was then removed. DMSO (200 μL/well) was added to dissolve the cell pellets. Absorbance was measured at a wavelength of 570 nm with enzyme-linked immunity implement.

2.9. Staining with Alcian blue 8GX and the determination of glycosaminoglycan (GAGs)

Chondrocytes were immersed in 0.025 M sodium acetate buffer (pH 5.8), containing 0.05% Alcian blue 8GX (Sigma, Poole, U.K.) for 10 min. Color development was monitored with an ELISA plate reader at 480 nm, and using a chondroitin sulfate standard curve to obtain GAGs content.

2.10. Western blot analyses

Western blot analyses were performed as follows. Incubation of cells with blank serum, MSU (1000 μM) and MSU + XF (5%, 10%, 20% serum) were performed for 48 h. Thereafter, cells were collected and lysed with 100 μL RIPA buffer containing a protease inhibitor cocktail. After incubation for 10 min, lysates were collected and centrifuged at 15,000g for 20 min at 4 °C. The total cell lysates (20 μg protein) obtained were subjected to 10–12% SDS-PAGE and transferred to polyvinylidene fluoride membranes. Proteins of interest were identified by reaction with specific primary and secondary antibodies linked to horseradish peroxidase and detected by chemiluminescence. The relative protein levels were determined by the formula: relative protein level =
expression of target protein / expression of internal control protein × 100%. Each experiment was performed independently in triplicate.

2.11. Statistical analysis

Graph data were presented as mean ± standard error (SE). All analyses were performed by using a two-way ANOVA and values in the treated samples were compared to the corresponding controls. p < 0.05 was considered statistically. All analyses were performed by using a two-way ANOVA and values in the treated samples were compared to the corresponding controls. p < 0.05 was considered statistically significant and GraphpadPrism6 was used to analyze the data.

3. Results

3.1. Effects of XF on acute gouty arthritis in rats and rabbits

Injection with MSU crystals caused a significant increase in joint thickness compared to negative control (paw volume or perimeter before MSU crystal injection). Paw swelling was found

![Fig. 1. Effects of XF on acute gouty arthritis model. (a) The swelling rate of rat joint. (b) The swelling rate of rabbit joint. (c) White blood count in rabbits' synovial fluid. (d) IL-6 in rabbit synovial fluid. (e) IL-1β in rabbit synovial fluid. (f) Histological examination of rabbit joints. (g) Articular lesions severity score. Values are means ± SEM (n=8). Significance: **p < 0.01, *p < 0.05 compared with the group of model.](image-url)
to be remarkably reduced in both rats and rabbits treated with XF at high dose from 1 to 5 h (Fig. 1a and b). XF at medium dose was also able to reduce the paw swelling induced by MSU crystal injection. However, XF at the low dose showed weak anti-inflammatory activity in this study. White blood count, and levels of IL-1β and IL-6 in rabbits' synovial fluid were determined by counting plate and ELISA analysis. The results showed that XF exhibited inhibitory effects on them in a dose-dependent manner (Fig. 1c-e). Histopathological analysis provided the proof for the anti-inflammatory effects of XF (Fig. 1f-g). Histology confirmed the joint inflammation after local injection with MSU crystals. Intra-articular injection with MSU crystals led to enhanced influx of granulocytes. Compared to model group, synovial specimens of XF groups, especially the group at high dose showed marked reduction in edema and less leukocyte infiltration due to the anti-inflammatory effect of XF. Fig. 1g demonstrated the corresponding articular lesions severity score of each group. All these facts demonstrated that XF reduced the leukocyte infiltration. Consequently, we postulated that XF might perform its action by inhibiting the proinflammatory cytokines.

3.2. XF enhanced the viability of chondrocytes stimulated by MSU

The viability of chondrocytes was observed under an inverted microscope and by MTT assay. Chondrocytes were incubated with MSU at different concentrations for different periods (24, 48, and 72 h), and cell viability was assessed (Fig. 2a). MSU at 2000 μM inhibited the proliferation of chondrocytes. Massive dead cells and severe broken cytoplasm were detected under the microscope. The cell viability of MSU group (1000 μM) was lower than 500 or 250 μM of MSU, but the cytoplasm of the majority of cells had integrity. Based on the above results, MSU at 1000 μM was chosen as the suitable concentration for the following study of XF effects on chondrocytes. We identified that treatment with XF sera showed no effect on chondrocytes compared with the blank serum (Fig. 2b). What is more, XF was well established to have the capability of protecting the chondrocytes treated with MSU at 1000 μM (Fig. 2c). Therefore, concentration of 1000 μM of MSU and time point of 48 h were chosen for subsequent research.

3.3. XF suppressed the growth of cytokines in vitro

As compared with the control group, production of IL-1β and TNF-α were elevated considerably in chondrocytes treated with MSU (Fig. 3a-b). In contrast, chondrocytes were incubated with different concentrations of XF-serum for 48 h, and we found that TNF-α levels were decreased dose-dependently. At the same time, IL-1β was also declined when subjected to XF sera (10%, 20% XF-serum). It has been established that IL-1β plays a major role in gout, and that TNF-α plays a critical role in the development of gout arthritis (Di Giovinle et al., 1991). Simultaneously, inducible nitric oxide synthase (iNOS) can be induced in chondrocytes, and therefore, NO production can be seen following stimulation with either IL-1β or TNF-α. Previous studies have confirmed that NO reduced the survival of osteoarthritis synoviocytes by regulating mitochondrial functionality, as well as the proteins controlling the cell cycle (Cillero-Pastor et al., 2011). As shown in Fig. 3c-d, XF significantly reduced the content of iNOS and NO. These findings indicated the suppression of the acute inflammatory pathway treated with XF.

3.4. XF protected chondrocytes by inhibiting degradation of cartilage matrix induced by MSU

The balance of degradation and synthesis of cartilage matrix plays a key role in the pathophysiology of articular cartilage. In the absence of MSU, incubation with blank serum slightly stimulated GAGs or Hyp release from chondrocytes (Fig. 4a-b). However, significant induction of GAGs and Hyp release induced by MSU was observed, while proteoglycan was definitely decreased simultaneously (Fig. 4c). Treatment with different XF-serum concentrations significantly inhibited the cartilage matrix degradation, and upregulated the expression of proteoglycan, particularly at the dose of 20% XF-serum group.

Matrix metalloproteinases (MMPs), collectively called matrixins, play a vital role in the progression of extracellular matrix degradation (Sternlicht and Werb, 2001). TIMPs are key regulators of the metalloproteinases to degrade the extracellular matrix. MMPs/TIMPs have been demonstrated to play a key role in osteoarthritis. However, little is known about their roles in gout. Aggrecan and type II collagen are the major constituents of articular cartilage. Degradation of cartilage aggrecan has mainly been attributed to the action of glutamyl endopeptidases, a family of metalloproteinases with thrombospondin motifs (ADAMTs), termed “aggrecanases” (Vankemmelbecke et al., 2003). TIMP-3 is a powerful inhibitor of ADAMTS, and ADAMTS/ TIMPs also have As compared with the control group, production of IL-1β and TNF-α were elevated considerably in chondrocytes treated with MSU (Fig. 4a-b). In contrast, chondrocytes were incubated with different concentrations of XF-serum for 48 h, and we found that TNF-α levels were decreased dose-dependently. At the same time, IL-1β was also declined when subjected to XF sera (10%, 20% XF-serum). It has been established that IL-1β plays a major role in gout, and that TNF-α plays a critical role in the development of gout arthritis (Di Giovinle et al., 1991). Simultaneously, inducible nitric oxide synthase (iNOS) can be induced in chondrocytes, and therefore, NO production can be seen following stimulation with
either IL-1β or TNF-α. Previous studies have confirmed that NO reduced the survival of osteoarthritis synoviocytes by regulating mitochondrial functionality, as well as the proteins controlling the cell cycle (Cilleró-Pastor et al., 2011). As shown in Fig. 4c-d, XF significantly reduced the content of iNOS and NO. These findings indicated the suppression of the acute inflammatory pathway treated with XF. Similar effects on proteoglycan as MMPs/TIMPs. Further effects on MMPs/TIMPs and ADAMTS/TIMPs were carried out in our in vitro studies (Fig. 4d-e). The levels of MMP-3 and ADAMT-4 were markedly increased when exposed to MSU and reduced when incubated with XF-serum of 10% or 20%. Meanwhile, XF-serum at different doses down-regulated the ratio of MMP-3/TIMP-1 dose-dependently and decreased the ratio of ADAMTS-4/TIMP-3 obviously at dose of 10% or 20%. These findings reinforced the results of proteoglycan and GAGs which had been shown above. Taken together, inhibiting degradation and promoting synthesis of cartilage matrix are promising strategies for the treatment of gout.

4. Discussion

Gout is an inflammatory arthritis as a result of precipitation of serum urate into crystallized deposits of MSU in and around the joints. XF is a Chinese compound preparation for the treatment of acute gouty arthritis, which had completed its preclinical study and was being proceeded the new drug application procedure. Previous animal experiments have confirmed these effects of XF including decreasing serum uric acid, anti-inflammatory and analgesic effects. The contribution of cartilage matrix degradation to gouty arthritis mechanism remained elusive, but was especially important for elucidating drug effect. In the present study, we carried out the animal experiments in vivo with acute gouty arthritis to evaluate the anti-gouty arthritis effect of XF. At the same time, chondrocytes stimulated by MSU were designed to demonstrate the generation of inflammatory cytokines and degradation of cartilage matrix in vitro. To further investigate whether treatment with XF could inhibit the destruction to joint, we studied the effects of XF-containing serum on proteoglycan, MMPs and ADAMTs.

It is well documented in recent studies that MSU crystals are among the most potent pro-inflammatory stimuli and intimately involved in the pathology of gouty arthritis (Popa-Nita and Naccache, 2010). The deposition of MSU crystals into the joint cavity promotes an acute inflammation, and thus MSU crystal is most frequently employed to develop an animal model of gouty arthritis (Martin et al., 2009; Torres et al., 2009). In this article, we established the models to examine the effects of XF on anti-gouty arthritis. XF at different doses severely ameliorated the joint swelling degree, and reduced the increased white blood count and inflammatory cytokines of articular tissue in MSU crystals-treated rabbits. It was also shown in the histological assessment of joint section from rabbit’s ankle that XF reduced the infiltration of inflammatory cells in the synovium, and diminished the erosive damage in the cartilage. Evidence in animal experimental model of gouty arthritis indicated that MSU crystals stimulated the synthesis and release of IL-1β and IL-6, which resulted in an acute inflammation (Di Giovine et al., 1987). This phenomenon has been well documented and both in vivo and in vitro methods have shown the production of large numbers of pro-inflammatory mediators, such as IL-1β and IL-6 in the mediation of MSU crystal-
Fig. 4. XF protected chondrocytes by restraining degradation of cartilage matrix induced by MSU. (a) The expression of GAG was assessed by Alcian blue stain. (b) ELISA of Hyp secreted from the cells. Values are means ± SEM (n=6). Significance: **p < 0.01,*p < 0.05 compared with the group of model. (c) The expression of proteoglycan was assessed by Toluidine blue stain. A showed nucleus, B showed cytoplasm, C showed proteoglycan. (d) ELISA of MMP-3 and TIMP-1 secreted from the cells. Values are means ± SEM (n=4). Significance: **p < 0.01,*p < 0.05 compared with the group of model. (e) Immunoblot of ADAMTS-4 and TIMP-3. Values are means ± SEM (n=3). Significance: **p < 0.01,*p < 0.05 compared with the group of model. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
induced inflammatory responses. According to the anti-inflammatory effect evaluated in the MSU crystals-treated animal and chondrocytes, XF did show obvious inhibitory effects on the activation of pro-inflammatory mediators.

Then XF-containing serum was selected to replace the drug for the research in vitro followed by animal experiments. Totally, 11 compounds were finally identified in the XF-treated rat serum. Most of them or their parent compounds could be classified into phenolic acids, such as caffeic acid hexoside glucuronide; flavonoids, such as luteolin glucuronide and quercetin glucuronide, and iridoids, such as sweroside and loganin, and many of them were derived from SG and LJ in XF. As we all know, LJ and SG, as anti-inflammatory agents, were commonly effective in clearing heat on anti-inflammation in clinical treatment (Chinese Pharmacopoeia). It has also been reported that loganin and sweroside showed anti-inflammatory and analgesic activities, which appeared to be the mechanism underlying anti-inflammatory efficacy by inhibiting cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) activities (Oku et al., 2011; Ryu et al., 2010). As the metabolite of loganin aglycon, its sulfate may also induce inflammatory cytokines production. Resveratrol, a natural phytoalexin with low toxicity and anti-inflammatory properties, was found to inhibit the mRNA expression of all pro-inflammatory markers, including interleukin-8 (IL-8), IL-6 and tumor necrosis factor-α (TNF-α), which were the key markers of inflammation (Cullberg et al., 2013). Besides, it markedly decreased iNOS and COX-2 protein expression (Chung et al., 2015). Inflammatory cytokines, such as IL-1β, IL-6, IL-8, and TNF-α, which play vitally important roles in inflammation and skin damage, could be inhibited by quercetin. NF-κB pathway, which plays crucial roles in the regulation of gene expression involved in immune and inflammatory responses (Karim and Greten, 2005), could be regulated by quercetin through inhibiting NF-κB DNA-binding activity. As the metabolites of quercetin and resveratrol, we supposed that quercetin glucuronide and resveratrol glucuronide may all have a certain amount of impact on inflammatory response. Report well demonstrated that luteolin exerted anti-inflammatory activities in RAW264.7 macrophages by inducing heme oxygenase-1 expression and inhibiting the secretion of inflammatory cytokines such as IL-1β and TNF-α (Sung and Lee, 2015). Thus, luteolin glucuronide, as its metabolite, was also identified as a reference to the comprehensive understanding of inflammatory information of luteolin (Jeon et al., 2014). What is more, luteolin glucoside could also inhibit lipopolysaccharide-induced inflammatory responses through modulation of NF-κ B/AP-1/P38-Akt signaling cascades, but less potently ameliorated LPS-induced inflammation than luteolin (Park and Song, 2013). Caffeic acid was reported to have anti-inflammatory activities in both acute and chronic contact dermatitis models via blockade of the mRNA and protein synthesis of these cytokines, such as TNF-α, IL-6 and IL-1β, and neutrophil-mediated myeloperoxidase activity, and it can target inflammatory mediators especially in the keratinocytes (Zhang et al., 2014). Furthermore, the present results suggest that the metabolite of caffeic acid and caffeic acid glucuronide might be therapeutic agents against inflammatory response.

To determine whether XF-containing serum can take the place of the compound and has a protective effect on chondrocytes damaged by MSU crystals, cell viability of the growth of cytokines was assessed first. Results of the experiments confirmed that chondrocytes were protected against MSU by at XF-containing serum at concentrations ranging from 5% to 20%. The cell viability of MSU crystal-induced chondrocytes treated with XF-serum was enhanced significantly. IL-1β and TNF-α can activate inducible NOS, which is induced in response to inflammatory-like stimuli and is capable of sustained production of high levels of NO that predominate during inflammation (Clancy et al., 1998). The excessive or inappropriate production of NO can damage tissue through the superoxide anion (Yaren et al., 2007). The content of IL-1β, TNF-α, iNOS and NO decreased significantly in XF-serum-treated groups. These data were consistent with the results of rabbit experiment in vivo. Therefore, we think the constituents in XF-containing serum can basically represent the effect of the prescription.

Reduction of pro-inflammatory mediators is efficient for anti-gouty arthritis drugs because they can suppress the acute progression of the disease. However, most evidence suggests that treatment with drugs which could protect the joint from uric acid crystals might reduce the repeated attack. But little is currently known about cartilage matrix regulated by anti-gouty arthritis drugs. Thus, it warrants further investigation of drug mechanism on cartilage matrix such as GAG and Hyp.

Proteoglycans and collagen are the major structural components of the extracellular matrix of cartilage. Collagen provides the tensile strength and the proteoglycans are largely responsible for the compressive stiffness of the tissue. The chondrocytes experiment was mainly designed to assess the proteoglycans and Hyp by means of Alcian blue stain and Toluidine blue stain. Our current data demonstrated a significant production of GAG and Hyp from cultured chondrocytes stimulated with MSU, compared with the control group. Therefore, as the degradation product, the increase of GAG naturally led to down regulation of proteoglycan. These results were consistent with clinical pathological characteristics of gouty arthritis in patients and indicated that the model in vitro was reliable and accurate. XF-containing serum substantially interfered with the MSU-induced expression of GAG and Hyp, while upregulated proteoglycan. Thus, it can protect the chondrocytes by blocking cartilage matrix degradation.

Increasing evidence has implicated that matrix metalloproteinases and aggrecanases are important for regulation of cartilage matrix degradation. The families of MMPs are regarded as major factors involved in the pathophysiology of osteoarthritis. Among the MMPs, stromelysin-1, also termed MMP-3 or proteoglycanase, secreted from chondrocytes, is capable of degrading the protein core of proteoglycans, resulting in the release of soluble glycosaminoglycans (Hall et al., 1999). Aggrecan degradation products resulting from aggrecanase action have been found incultures of cartilage treated with pro-inflammatory cytokines as well as in synovial fluid of arthritis patients (Arner et al., 1998; Illic et al., 1998; Lohmander et al., 1993). ADAMTS and MMPs are up-regulated in osteoarthritic-affected cartilage and act as downstream key players in the inflammatory signal cascade. ADAMTS-4, also called aggrecanase-1, is considered to play a key role in aggrecan degradation in human osteoarthritic cartilage. It can cleave other members of the large aggregating proteoglycan family such as versican and brevican, but information about gout of aggrecanase activity remains limited (Matthews et al., 2000; Nakamura et al., 2000; Sandy et al., 2001).

The ELISA results and Western blot assays showed that MSU considerably induced MMP-3 and ADAMTS-4 expression compared with the control group, while treatment with XF-containing serum indicated that it could suppress the production of MMP-3 and ADAMTS-4 in a dose dependent fashion. TIMPs were originally known as specific inhibitors of MMPs, but their range of activities has now been found to be broader as it includes the inhibition of several of the disintegrin-metalloproteinases, ADAMs and ADAMTs. It has been reported that TIMP-1 inhibited not only collagensases, but also gelatinases and MMP-3, while TIMP-3 has the additional ability to inhibit the aggrecanases ADAMTS-4 (Cawston et al., 1981; Gendron et al., 2003; Kashiwagi et al., 2001). According to our data, TIMP-1 and TIMP-3 did not differ significantly between the groups, but the ratio of MMP-3/TIMP-1 and ADAMTS-4/TIMP-3 were decreased by XF-containing serum dose-
dependently, which may be one of mechanisms of XF’s anti-gout arthritis activity.

With the knowledge of XF, which could suppress the body’s defense on inflammation and cartilage matrix components mass loss. Moreover, XF could regulate the balance of MMP-3/TIMP-1 and ADAMTS-4/TIMP-3 in chondrocytes, which has been confirmed by the in vitro results.

5. Conclusion

In the present study, we performed in vivo studies using experimental gouty arthritis models and in vitro experiments in chondrocytes induced by MSU. Our data collectively suggest that XF that made from MSW showed the obvious effects on acute gouty arthritis and protective mechanisms for cartilage matrix, which might provide evidence for new drug application.

Conflict of interest

The authors declare that they have no conflict of interest.

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