Anti-arthritic activity of Fu-Fang-Lu-Jiao-Shuang on collagen-induced arthritis in Balb/c mice and its underlying mechanisms

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Submitted: 21-04-2014 Revised: 09-06-2014 Published: 12-03-2015

ABSTRACT

Background: Rheumatoid arthritis (RA) is a common, autoimmune disorder characterized by progressive multiple joint destruction, deformity, disability and premature death in most patients. Fu-Fang-Lu-Jiao-Shuang (FFLJS) is an effective traditional Chinese medicine, which has long been used clinically to treat RA patients. **Objective:** The objective of this study is aimed to evaluate the anti-rheumatic effects of FFLJS on collagen induced arthritis (CIA) model, as well as the underlying mechanisms, which have not previously been explored. **Materials and Methods:** CIA was induced by immunization with type II collagen (CII) in male Balb/c mice. The mice in the onset of arthritis were treated daily with FFLJS (125 or 500 mg/kg) or 1% carboxymethyl cellulose-Na for 28 days. Paw thickness and arthritic score were evaluated to confirm the anti-arthritic effect of FFLJS on CIA in mice. Levels of anti-CII antibody, proinflammatory cytokines interleukin-1 (IL-1) β, IL-17, and tumor necrosis factor-α (TNF-α) as well as prostaglandin E-2 (PGE-2) in serum and histological changes in the ankle joint were also analyzed. In addition, expressions of matrix metalloproteinases-1 (MMP-1), MMP-3 and tissue inhibitors of matrix metalloproteases-1 (TIMP-1) in synovial tissue were also detected to further study the molecular mechanism of the anti-arthritic effects of FFLJS. **Results:** During therapeutic treatment, FFLJS significantly reduced paw thickness and arthritic score in CIA mice, decreased the amounts of TNF-α, IL-1 β, IL-17, PGE-2 and anti-CII antibody in serum. In addition, FFLJS treatment could prevent the bone destruction by reducing the expression of MMP-1 and MMP-3, increasing the expression of TIMP-1 in synovial tissue of CIA mice. **Conclusion:** These findings offer the convincing evidence for the first time that the anti-rheumatic effects of FFLJS might be related to down-regulation of TNF-α, IL-1 β, IL-17 and PGE-2 levels for acute arthritis, and regulation of MMP-1, MMP-3 and TIMP-1 protein expression for chronic arthritis.

Key words: Anti-arthritic activity, Fu-Fang-Lu-Jiao-Shuang, matrix metalloproteinases, traditional Chinese medicine

Access this article online

Website: www.phcog.com

DOI: 10.4103/0973-1296.153065

Quick Response Code:

INTRODUCTION

Rheumatoid arthritis (RA) is a kind of chronic and systemic autoimmune inflammatory disease, associated with synovial hyperplasia, cartilage destruction, and functional disability in most patients.[1] A multitude of different pathways participates in the pathogenesis of RA. Specifically, pro-inflammatory cytokines, including tumor necrosis factor-α (TNF-α), interleukin-1 β (IL-1 β) and IL-6 play key roles in RA.[2] These inflammatory cytokines induce the synthesis of matrix metalloproteinases (MMPs) which are a large group of matrix degrading enzymes that contribute to the joint destruction in RA by directly degrading the cartilage and bone and indirectly promoting angiogenesis.[3] High activities of diverse MMPs have been well-documented in tissues and synovial fluid from rheumatoid joints of human patients.[4] MMP-activity is controlled by four specific inhibitors termed tissue inhibitors of matrix metalloproteases (TIMPs). It is demonstrated that joint destruction in RA is partially due to a local imbalance between activated MMPs and TIMPs.[5]

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For the treatment of RA, immunosuppressants, steroidal agents and nonsteroidal anti-inflammatory drugs are usually used. However, these drugs are known to produce numerous side-effects including gastrointestinal disorders, immunodeficiency, and nephrotoxicity.[6] Therefore, growing interests and much effort have been put into screening new therapeutic agents from natural products that might not only prevent damage of arthritic joints, but also have fewer adverse effects and lower cost. Traditional Chinese medicine (TCM) is accepted as the most prevalent and effective treatment in the management of RA in China, Japan, and other Asian countries. Thus, studies exploring the traditional Chinese herbal formula may ultimately provide additional therapeutic agents for RA treatment.[7]

In the context of TCM, RA belongs to the category Bi Zheng, which is defined as a syndrome marked by arthralgia and dyskinesia of the joints and limbs due to attack of the meridians of the limbs by wind, dampness, and heat or cold pathogens.[8] Fu-Fang-Lu-Jiao-Shuang (FFLJS) is an anti-arthritic Chinese formula, which has been used as a complementary and alternative medicine for the treatment of RA in Yichang Center People's Hospital (Hubei, China) for over 60 years, which can reduce and ultimate replace the use of steroidal agents. FFLJS is formulated according to cold-heat theory of TCM especially for the treatment of Bi Zheng, functioning as draining dampness, dispelling wind, freeing the flow of network vessels and relieving pains.[9] It consists of a mixture of six TCMs [Table 1] and each of crude drugs have been used for hundreds of years to treat various bone disorders in China, and their safety and efficacy are documented through a long history of human use.[10] LJS is the residue of *cornu Cervi* extracted by water and primarily described as a famous TCM of Yang and Qi acting through the kidney meridian in a Chinese medicine book named Ben Cao Meng Quan in Ming Dynasty.[11] Due to the unique efficacy and high yield, it has been included in many traditional Chinese formulas and also has been confirmed to show potential positive effects on modern ailments associated with aging, infection, and immune dysfunction during long-term clinical application, such as RA, acute gouty arthritis and ankylosing spondylitis.[12,13] As the "principal drug" of this formula, LJS is chiefly improving the liver and kidney, releasing humidity and heat as well as strengthening sinews and bone based on TCM theory.[14] In LJS there are kinds of chemical composition: 25% of soluble collagen, calcium carbonate, calcium phosphate and some kinds of amino acids, polysaccharides and endocrine, according to the literature date, the mechanism of actions is probably because of the mild hormone-like effect for expelling wind-damp.[15,16] Moreover, the dried whole plant of *Pyrola decorata* H. Andres has been used as tonics, sedatives, hemostatics, anti-inflammatory, and analgesics against RA in China since the antiquity.[17] *Cinnamomum cassia* Presl is used as an analgesic and anti-pyretic against influenza and rheumatic pain.[18] *Lonmicera japonica* Thunb and *Morus alba* L were also investigated in various inflammatory models.[19,20]

Our previous work have indicated that FFLJS could decrease the levels of IL-18, TNF-α and transforming growth factor-β in serum as well as improve joint and bone destruction in complete Freund's adjuvant induced arthritis rats with a dose-depended manner. The preliminary clinical study also showed that FFLJS had the analgesia and anti-arthritic effect on the RA patents.[21] However, the underlying mechanism of FFLJS is still unknown. Therefore, the current study was designed to confirm the anti-arthritic effect and explore the potential mechanism of FFLJS on collagen induced arthritis (CIA) in mice, which is a favorable animal model of RA.[22]

MATERIALS AND METHODS

Preparation of Fu-Fang-Lu-Jiao-Shuang extracts

The composition of FFLJS was listed in Table 1. All these drugs were purchased from Fu Chun Tang Herbal Pharmaceutical Union Company in China, and carefully authenticated by Prof. Jianping Wang, according to the Chinese pharmacopoeia, 2010. The voucher specimens have been deposited in Hubei Key Laboratory of Natural Medicinal Chemistry and Resource Evaluation, School of Pharmacy, Tongji Medical College, Huazhong University of Science and Technology.

Table 1: Composition of FFLJS

Chinese name	Botanical name	English name	Voucher numbers	Amount (g)
Lu-Jiao-Shuang	*Cervus nippon* Temminck	Cornu cervi degelatinatum	FFLJS01-120306	15.0
Lu-Xian-Cao	*Pyrola decorata* H. Andres	Pyrola Herb	FFLJS02-120306	15.0
Ren-Dong-Teng	*Lonmicera japonica* Thunb.	Honeysuckle stem	FFLJS03-120306	15.0
Sang-Zhi	*Morus alba L.*	Mulberry twig	FFLJS04-120306	9.0
Gui-Zhi	*Cinnamomum cassia* Presl	Cassia twig	FFLJS05-120306	9.0
Feng-Fang	*Polistes japonicus* Saussure	Honeycomb	FFLJS06-120306	5.0
	Total amount			68.0

FFLJS: Fu-Fang-Lu-Jiao-Shuang

Aqueous extract of FFLJS were prepared according to the following procedure: Extract amounts of component drugs were weighed according to the classic percentage and mixed well. The mixture (5 kg) was soaked in distilled water for 1 h and then boiled in 12 volumes of water (v/w) for 3 h and extracted twice, after extraction, the solvent was filtered and evaporated till dryness under reduced pressure (rate of yield 14.6%). The obtained extract was stored in a refrigerator at 4°C until time of use.

High-performance liquid chromatography analysis

High-performance liquid chromatography (HPLC) analysis was performed on a Dionex UltiMate 3000 system (Dionex, USA) equipped with Dionex VWD-3100 ultraviolet (UV) detector (Dionex, USA). Chromatographic separation was carried out on a Dionex C18 column (4.6 mm × 250 mm, 5 μM) at room temperature with an injection volume of 10 μl using a gradient elution of solvent A (methanol) and B (water containing 0.3% formic acid) at a flow rate of 1 mL/min as follows: (a) A progressively raised from 5% to 40% at 30 min; (b) A then raised to 60% at 50 min. Peaks were detected at 236 nm for UV detection.

In order to control the quality of FFLJS, seven marker compositions from of FFLJS were detected by comparison of the retention times and on-line UV spectra. One organic acid (Chlorogenic acid) and two iridoid glycoside (Sweroside and Loganin) were attributed to *L. japonica* Thunb. Three aromatic compounds (Cinnamic acid, caffeic acid and ferulic acid) were owed to *C. cassia* Presl, and a flavonoid glycoside was ascribed to *P. decorata* H. Andres. The qualitative HPLC analysis was preliminary elucidated the main chemical composition of FFLJS.

Animals

Male Balb/c mice[18-22] were bought from the Center for Disease Prevention and Control in Hubei province, China (Reg. No. SCXK [Hubei] 2008–0005). The animals were kept in the room maintained at 22°C ± 2°C with alternating 12-h light-dark cycle and given free access to both food and water. They were allowed to adapt to the environment for 1 week before the experiments. All experiments were carried out in accordance with the Animals in Research: Reporting *in vivo* experiments guidelines[23] and approved by the Committee on the Ethics of Animal Experiments of Huazhong University of Science and Technology. All care was taken to minimize the suffering of the animals.

Induction of arthritis and drug administration

Collagen-induced arthritis was induced as previously described, with minor modification.[24] Briefly, bovine type II collagen (CII) (CII; Sigma, St Louis, MO, USA) was dissolved overnight at 4°C in 0.1 mol/l acetic acid to 2 mg/ml. Balb/c mice were given an intradermal injection of CII emulsified in complete Freund's adjuvant into the base of the tail. Three weeks later, the mice were given a booster intra-peritoneal injection of equal volume of CII emulsified in incomplete Freund's adjuvant. After onset of CIA, mice were divided into five groups randomly. The CIA mice were treated with FFLJS, at low (125 mg/kg) and high (500 mg/kg) oral administration does, respectively. Dexamethasone (DEX, 3 mg/kg) was used as a reference drug by intragastric administration. The animals in the normal control group and CIA model ones were administered with the same volume of saline. The administration was conducted after onset of CIA every day and lasted for 4 weeks.

Assessment of arthritis severity in collagen induced arthritis mice

Arthritis severity was assessed by measuring the thickness of the affected hind paws with the use of Vernier Caliper and clinical arthritis was graded in all four paws of the mice by a triple blind test. The results were assessed according to a previously described method.[25] Briefly, the severity was scored as follows: (0) Normal; (1) mild, apparent swelling limited to individual digits; (2) moderate, redness and swelling of the ankle; (3) redness and swelling in the paw as well as in the digits; and 4, maximally inflamed leg with involvement of multiple joints. The arthritis score for each mouse was the sum of arthritis severity in all four paws, with the highest score being 16.

Biochemical assays

Blood samples were collected from all mice via cardiac puncture prior to sacrificing the mice on day 28 after the secondly immunization. Blood was centrifuged at 6000 rps for 10 min to obtain serum. Serum levels of anti-CII antibody, TNF-α, IL-1 β, IL-17 and prostaglandin E-2 (PGE-2) were determined using commercially available Enzyme Linked Immune Sorbant Assay (ELISA) kits according to the manufacturer's recommendations.

Histological assessment

The mice were euthanized and joints were removed, immediately fixed in 10% buffered formalin, and decalcified in a decalcifying solution for 4 days. The decalcified paws were then dehydrated in a gradient ethanol series (70–100%), washed twice with xylene for 3 min, and then embedded in paraffin. Five-micrometer tissue sections were prepared and stained with hematoxylin and eosin using standard methods. Histopathological changes were observed under a light microscope and read blindly by a pathologist.

Western blot analysis

Protein of synovial tissue from CIA mice was used in western blot analysis for MMP-1, MMP-3 and TIMP-1. Total protein was extracted with a radio immunoprecipitation assay buffer solution at −20°C overnight. We used bovine serum albumin as a protein standard to calculate equal total cellular protein amounts. Same amounts of protein were resolved by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel electrophoresis under 4°C, the protein was transferred onto microporous polyvinylidene fluoride membranes in running buffer with 20% methanol. After nonspecific sites were blocking with 5% nonfat dried milk-tris-buffered saline with tween (TBST) buffer (10 mM Tris–HCl, pH 7.4, 100 mM NaCl and 1% [v/v] Tween 20), membranes were incubated overnight at 4°C with 1:1000 dilution of antibodies for MMP-1, MMP-3 and TIMP-1, equal lane loading was assessed using β-actin. The blots were rinsed four times with TBST buffer for 5 min each. Washed blots were incubated with 1:10,000 dilution of the horseradish peroxidase conjugated-secondary goat anti-mouse or goat anti-rabbit antibodies for 1 h at room temperature and washed 4 times with TBST buffer. The bands of interest were detected using an enhanced chemiluminescent technique. Densities of bands were measured by an image analyzer.

Statistical analysis

All results were expressed as means ± standard error of the mean statistical significance was determined using One-way analysis of variance test, followed by Dunnet's *t*-test or Student–Newman–Kauls test. The analysis was performed using Statistical Package for the Social Sciences software, version 18.0 (SPSS Inc, Chicago). Statistical significance was considered for $P < 0.05$.

RESULTS

High-performance liquid chromatography analysis

To establish the fingerprint chromatogram for the quality control of FFLJS, chlorogenic acid, caffeic acid, sweroside, loganin, ferulic acid, hyperoside and cinnamic acid were used as markers. HPLC chromatograms showed seven marker components present in FFLJS [Figure 1].

Effects of Fu-Fang-Lu-Jiao-Shuang on disease progression of collagen induced arthritis

Treatment with FFLJS showed a progressive ($P < 0.05$) decrease in the incidence and severity of CIA compared with the untreated group, as assessed by the arthritic score and paw swelling [Figure 2]. From 21th to 48th (after the primary immunization), dose-dependent amelioration of the paw swelling and polyarthritis index when compared to the CIA model was observed after the administration of FFLJS. The DEX also significantly reduced arthritis scores and decreased the severity of arthritis, but there were no significant ($P > 0.05$) differences in paw swelling and arthritis index between the FFLJS groups. It was showed that FFLJS significantly blocked development of CIA.

Effects of Fu-Fang-Lu-Jiao-Shuang on histopathological changes

To further evaluate the inhibitory effects of FFLJS on synovial inflammation in CIA mice, histological assessment of the ankle joints was carried out. As shown in Figure 3, histopathological evaluation indicated that the ankle joints of CIA mice exhibited notable synovial hyperplasia, partial bone destruction, and inflammatory cell infiltration into the joint capacity [Figure 3b]. In contrast, CIA mice treated with 125 mg/kg or 500 mg/kg of FFLJS showed a normal joint space and well-preserved articular cartilage, indicating that the administration of FFLJS directly correlated with a reduction in disease severity compared with control CIA mice [Figure 3c and d]. These data also showed that oral administration of DEX resulted in a significant reduction in synovial hyperplasia, inflammatory cell infiltration [Figure 3e].

Effect of Fu-Fang-Lu-Jiao-Shuang on serum level of anti-type II collagen antibody

Anti-CII antibody plays an important role in the pathogenesis of CIA.[26] The level of serum anti-CII antibody in model group mice was significantly higher than that in normal group, and this elevation was attenuated by treatment with FFLJS at dose of 125 mg/kg and 500 mg/kg ($P < 0.05$) [Figure 4]. This suggested that the reduced incidence and severity of arthritis in FFLJS mice could be due to the modulation of the humoral immune response to CII antigen.

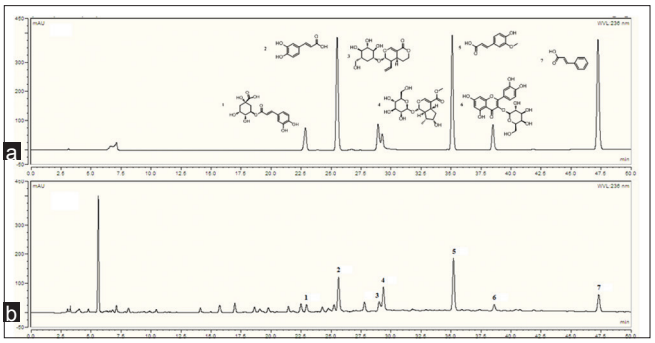

Figure 1: High-performance liquid chromatography (HPLC) fingerprint analysis of the Fu-Fang-Lu-Jiao-Shuang (FFLJS). (a) HPLC Chromatograms of seven marker compositions (1: Chlorogenic acid; 2: Caffeic acid; 3: Sweroside; 4: Loganin; 5: Ferulic Acid; 6: Hyperoside; 7: Cinnamic acid); (b) HPLC chromatograms of FFLJS. Peaks were detected at 236 nm

Effects of Fu-Fang-Lu-Jiao-Shuang on serum levels of tumor necrosis factor-α, interleukin-1 β, interleukin-17 and prostaglandin E-2

Proinflammatory cytokines TNF-α, IL-1 β and IL-17 as well as PGE-2 have central roles in the maintenance of chronic inflammation and tissue damage during the progression of RA, thus, the regulation of these inflammatory mediators may be important in the pathogenesis and therapy of RA. Therefore, levels of TNF-α, IL-1 β, IL-17 and PGE-2 in serum were detected by ELISA. As shown in Figure 5, serum levels of TNF-α, IL-1 β, IL-17 and PGE-2 in the nontreated CIA mice were systemically increased, which suggested that model group had obvious response to inflammation. Meanwhile, a marked and dose-dependent decrease was observed in the serum of FFLJS-treated groups. The overall results suggested that FFLJS could reduce the levels of pro-inflammatory cytokines and finally relieve the inflammatory response in CIA mice. As a result, the joint damage was gradually alleviated.

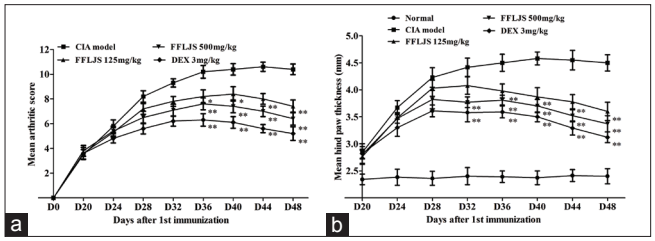

Figure 2: Effects of Fu-Fang-Lu-Jiao-Shuang (FFLJS) on articular swelling. (a) Arthritic score were shown for 7 weeks after the first collagen injection. Mice treated with higher dose of FFLJS revealed a remarkable amelioration of arthritic score. (b) The paw swelling of FFLJS groups were significantly decreased compared with that of collagen induced arthritis group. Data are expressed as means ± standard error of the mean; $n = 10$ in each group, the symbols $^{*}P < 0.05$, $^{**}P < 0.01$ denote the significance level when compared to the control group

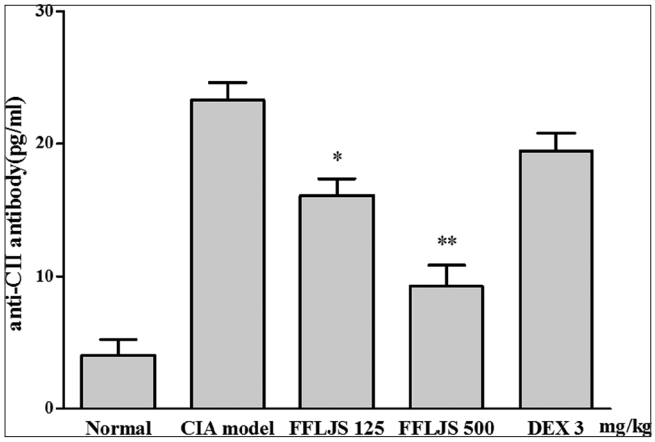

Figure 4: Effects of Fu-Fang-Lu-Jiao-Shuang (FFLJS) on the expression of anti-type II collagen (CII) antibody in serum. The concentration of anti-CII antibody in serum of collagen induced arthritis (CIA) mice 7 weeks after the first collagen injection was obviously increased. Treatment with higher dose of FFLJS for 4 weeks reduced significantly the concentration of anti-CII antibody in serum. Data are expressed as means ± standard error of the mean; $^{\#\#}P < 0.01$ compared with sample of normal group, $^{*}P < 0.05$, $^{**}P < 0.01$ compared with the CIA group ($n = 10$ in each group)

Effects of Fu-Fang-Lu-Jiao-Shuang on matrix metalloproteinases-1, matrix metalloproteinases-3 and tissue inhibitors of matrix metalloproteases-1 expressions in synovial tissue

After the experimental period of 28 days, the expression levels of MMP-1, MMP-3 and TIMP-1 in the synovial tissue of CIA mice were measured [Figure 6]. The release of MMP-1 and MMP-3 were significantly higher in the model group, compared to the normal group, in the group

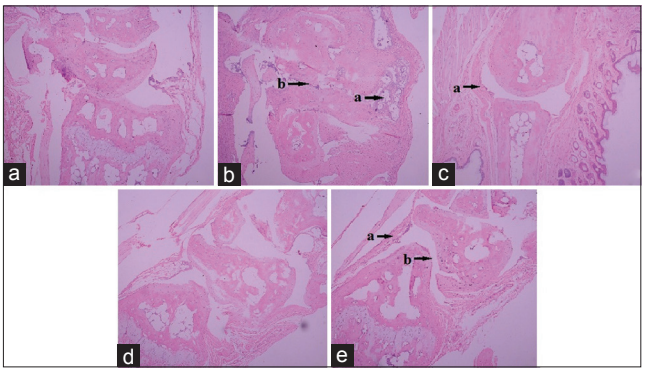

Figure 3: Influence of Fu-Fang-Lu-Jiao-Shuang (FFLJS) on the histopathological change of ankle joint in collagen induced arthritis (CIA) mice. Fu-Fang-Lu-Jiao-Shuang inhibits the histological changes in the ankle joints of CIA mice. Paraffin sections of ankle joints were stained with H and E. (a) Nonimmunized mice showed normal articular cartilage, absence of damage in the synovium and open joint space. (b) CIA mice showed marked infiltration of inflammatory cells, narrow joint space with synovia hyperplasia. (c and d) CIA mice treated with FFLJS (125, 500 mg/kg) and (e) dexamethasone (3 mg/kg) showed less inflammatory cells infiltration, well preserved joint spaces and minimal synovia hyperplasia. (a) Infiltration of inflammatory cells; (b) cartilage hyperplasia and close articular cavity

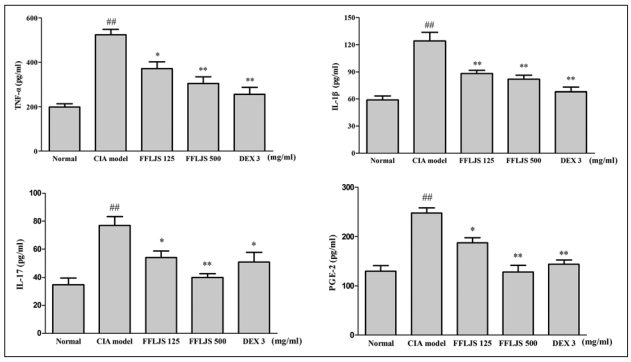

Figure 5: Effects of Fu-Fang-Lu-Jiao-Shuang (FFLJS) on the production of tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1) β, IL-17 and prostaglandin E-2 (PGE-2) in serum. The amounts of TNF-α, IL-1 β, IL-17 and PGE-2 in serum of collagen induced arthritis (CIA) mice 7 weeks after the first collagen injection were obviously increased. Treatment with 125 or 500 mg/kg of FFLJS for 4 weeks decreased significantly the levels of TNF-α, IL-1 β, IL-17 and PGE-2. Data are expressed as means ± standard error of the mean; $^{\#\#}P < 0.01$ compared with sample of normal group, $^{*}P < 0.05$, $^{**}P < 0.01$ compared with the CIA group ($n = 10$ in each group)

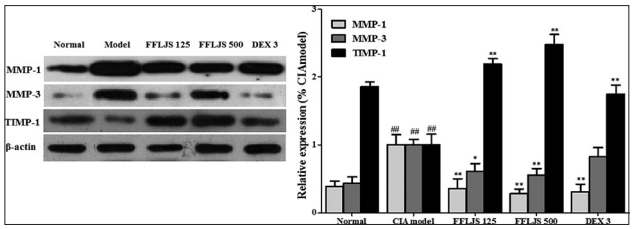

Figure 6: Effects of Fu-Fang-Lu-Jiao-Shuang on the levels of matrix metalloproteinases-1 (MMP-1), MMP-3 and tissue inhibitors of matrix metalloproteases-1 (TIMP-1) protein expression. Representative images of agarose gel electrophoresis from western blotting analyses of MMP-1, MMP-3 and TIMP-1 in synovial tissue of the collagen induced arthritis (CIA) mice after 4 weeks of treatment, β-actin was used as an internal control. $^{\#\#}P < 0.01$ compared with sample of normal group, $^{*}P < 0.05$, $^{**}P < 0.01$ compared with the CIA group

treated with FFLJS or DEX, the release of MMP-1 and MMP-3 were lower when compared to the model group. Meanwhile, the expression of TIMP-1 was much lower in the model group and both FFLJS and DEX can increase the TIMP-1expression in cartilage of CIA mice.

DISCUSSION AND CONCLUSION

Rheumatoid arthritis is a progressive, disabling, chronic multisystem disorder and characterized by pain, swelling and stiffness of the synovial joints. Current therapies for RA are often based on the concept of "polypharmacy" with the hope that each drug will be targeted at interrupting one or another of the pathways involving acute and chronic inflammation, the immune response, and metalloproteinase biochemistry.[27] Unfortunately, the current agents, which can generally control the pain and inflammation, are hard to prevent the progressive joint destruction and have multiple side-effects in the long term. In this regard, an effective anti-rheumatic formula, comprising multiple active components with broad ranging pharmacological activities, will be a more effective strategy for RA treatment.

Fu-Fang-Lu-Jiao-Shuang is derived from a traditional Chinese formula "Fu Fang Lu Jiao Shuang", which has been long used in the treatment for RA. It is also used in Yichang Center Peeople's Hospital (Hubei, China) as a hospital preparation for many years. This formula is chemically quite complex, with hundreds, if not thousands, of components. Although the exact chemical nature and the interaction of all of these components have not been clearly defined, certain bioactive chemicals have been identified, monotropein in *P. decorata*;[28] chlorogenic acid, loganin and sweroside in *L. japonica*;[29,30] mulberrin in *M. alba*;[31] cinnamaldehyde and cinnamic acid in *C. cassia*.[32]

Type II CIA is an autoimmune model that manifests common immunological and pathological features associated with human RA, and serves as an animal model for testing novel therapeutics to treat anti-inflammatory or anti-rheumatic drugs.[33] The present study demonstrated that FFLJS induced a significant reduction in paw swelling and arthritis score. Microscope examination further indicated the role of FFLJS in attenuating the major pathological characters of RA, including synovial cell hyperplasia, inflammatory cell infiltration and cartilage erosion, which indicated that the FFLJS had anti-inflammatory and anti-rheumatic activities on RA.

In RA, the formation of anti-CII antibody from B cells plays an important role in initiating autoimmune responses and maintaining synovial inflammation by activating the complement system, recognizing the surface of cartilage and initiating the inflammatory responses.[34] In this study, the administration of FFLJS significantly reduced the anti-CII antibody level in serum from CIA mice. This result suggests that FFLJS could alleviate arthritis by relieving autoimmune response of the animals. The reduction in anti-CII antibody level suppresses the formation of autoimmunity complex, thus restoring inflammatory injure in synovium and cartilage of joint.

To further study the molecular mechanism of the therapeutic effects of FFLJS on CIA in mice, levels of some pro-inflammatory cytokines in blood were detected. Studies have demonstrated that pro-inflammatory cytokines TNF-α, IL-1 β and IL-17 play central roles during the progression of RA, and the efficacy of their blockade has been proven by their specific inhibitors.[35] TNF-α and IL-1 β promote induction of adhesion molecule and expression of proteinase gene. IL-17 binding to an IL-17 receptor expressed on epithelial, endothelial, and fibroblastic stromal cells triggers the activation of transcription factor Nuclear factor-κB and mitogen-activated protein kinase (p-38), which in turn results in the secretion of IL-1, TNF-α, IL-6, IL-8, PGE-2, in addition, IL-17 exerts additive or even synergistic effects with IL-1 and TNF-α in inducing cytokine expression and joint damage.[36] PGE-2 is an inflammatory mediator produced by PG endoperoxide synthase at inflammatory sites, which can cause inflammatory reactions, such as local congestion, edema, and pain in RA.[37] It is, therefore, not surprising that therapies for RA have targeted these inflammatory mediators. In the present studies, the protective effect of FFLJS appeared to result from its regulation of key cytokines in RA pathogenesis, such as decreasing the levels of TNF-α, IL-1 β, IL-17 and PGE-2.

Accumulating evidences implicated MMPs are cytokine-modulated enzymes that play an important role in the pathogenesis of RA by inducing bone resorption and cartilage destruction.[38] Among the various MMPs, MMP-1 and MMP-3 are key enzymes that degrade articular

extracellular matrix (ECM) constituents and subsequent articular destruction.[39] The enzymatic activities of MMPs are specifically controlled by TIMPs.[40] TIMP-1 is capable of inhibiting the activities of all known MMPs and as such play a key role in maintaining the balance between ECM deposition and degradation in different physiological processes, accelerated breakdown of ECM occurs in various pathological processes, including inflammation, chronic degenerative diseases.[41] In healthy tissue, there is a homeostasis between MMPs and TIMPs, which is disturbed in RA. Joint destruction in RA is probably due to a local imbalance between activated MMPs and TIMPs. Therefore, down-regulation of MMPs would be reasonable therapeutic targets for the treatment of arthritis. Indeed, collagen and proteoglycan released from cartilage can be prevented by treatment with inhibitors of MMP transcription, synthetic MMPs inhibitors, or agents that up-regulate TIMPs.[42] In the present study, after 4 weeks of treatment, the levels of MMP-1 and-3 proteins in joint were dramatically suppressed, meanwhile, the expression of TIMP-1 was increased, suggesting that the ameliorative effect of FFLJS on articular destruction in CIA probably was achieved through regulating the expression of MMP-1, MMP-3 and TIMP-1.

In this study, we confirmed the therapeutic effects of FFLJS using CIA mouse model, and found the effects were related to down-regulation of TNF-α, IL-1 β, IL-17 and PGE-2 levels for acute arthritis, and regulation of MMP-1, MMP-3 and TIMP-1 protein expression for chronic arthritis. It was also associated with suppression of the abnormal humoral immune responses. Efficacious treatment with FFLJS on CIA in the present study may explain why FFLJS can improve RA on a long-term therapeutic basis. Further studies on the mechanism of action and the active principles of this formula may contribute to developing novel drugs for controlling RA in the future.

ACKNOWLEDGMENTS

This work was financially supported by Program for New Century Excellent Talents in University (No. NCET-2008-0224) and National science and technology major project (No. 2011ZX09102-006-05).

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Cite this article as: Wang Y, Sun W, Chen L, Xu X, Wu Y, Zhang J, *et al.* Anti-arthritic activity of Fu-Fang-Lu-Jiao-Shuang on collagen-induced arthritis in Balb/c mice and its underlying mechanisms. Phcog Mag 2015;11:242-9.

Source of Support: Nil, **Conflict of Interest:** None declared.