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# A combination of Sinomenine and Methotrexate reduces joint damage of collagen induced arthritis in rats by modulating osteoclast-related cytokines

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ABSTRACT

*Objective:* To analyze the combination therapy of Sinomenine (SIN) and Methotrexate (MTX) in rheumatoid arthritis (RA), we herein demonstrated the combination effect of SIN and MTX on collagen-induced arthritis (CIA) in rats through their modulation on osteoclast-related cytokines.
*Methods:* CIA was induced by the immunization of type II collagen (CII) in SD rats. SIN and MTX were administrated alone or in combination after the onset of arthritis. Arthritis index and histological analysis were used to evaluate the effect of treatments. Effects of SIN and MTX on expression of receptor activator of NF-κB ligand (RANKL) and osteopontin (OPN) in synovial tissues were assayed by immunohistochemistry. RANKL, osteoprotegerin (OPG), IL-6, IL-17 and matrix metalloproteinases (MMPs) in rat serum were measured by ELISA. The expression of osteoclast-related cytokines in fibroblast-like synoviocytes (FLS) from RA patients was assayed by RT-PCR.
*Results:* SIN and MTX combination additively reduced the inflammatory symptoms and joint damage in CIA. Combination of SIN and MTX significantly repressed synovial RANKL and OPN production. SIN and MTX exhibited complementary and synergistic effect upon down-regulating RANKL, IL-6, IL-17 and MMPs in rat serum. SIN and MTX also modulated the expression of RANKL and OPG in RA-FLS.
*Conclusion:* SIN and MTX have additive effects, decreasing inflammation and joint damage in CIA rats by modulating osteoclast-related cytokines. These results are indicative of the combined effect of SIN and MTX for anti-arthritic treatment in RA.



## 1. Introduction

Rheumatoid arthritis (RA) is a systemic autoimmune disease characterized by polyarthric synovitis that frequently results in destruction of articular cartilage and bone [1]. Inflammation and joint damage are two closely linked processes. This joint-destructive feature of chronic inflammatory arthritis is a major cause of disability in RA patients.

Several studies in RA patients and animal models of arthritis support the potent role of osteoclasts in the pathogenesis of bone damage [2,3]. Osteoclasts are tissue-specific multinucleated cells that derive from monocyte/macrophage hematopoietic lineage. They are essential for the resorption of mineralized cartilage and subchondral bone, thus the enhanced activation of osteoclasts is responsible for joint damage in RA. Osteoclast formation, survival and activity can be regulated by a variety of cytokines produced by RA synovial tissues [4]. These osteoclast-related cytokines include receptor activator of NF-κB ligand (RANKL), osteoprotegerin (OPG), interleukin (IL)-17, IL-6, osteopontin (OPN) and macrophage colony stimulating factor (M-CSF) [5–7].

RANKL is an important factor for osteoclast differentiation by binding to the receptor activator of NF-κB (RANK) expressed on osteoclast precursors [8]. RANKL is mainly expressed by osteoblasts, stromal cells and activated T cells. OPG, also secreted by osteoblasts or stromal cells, prevents the binding of RANKL to RANK and thus inhibits osteoclast differentiation [9]. The expression levels of RANKL and OPG are instrumental in determining the degree of osteoclast-mediated bone resorption [10]. Within RA synovial tissues fibroblast-like synoviocytes (FLS) are the dominating resident stromal cells, and FLS are one of the major sources for osteoclast-related cytokines RANKL and OPG [11,12]. Furthermore, proinflammatory cytokines such as IL-6 and IL-17 promote osteoclastogenesis indirectly via up-regulating the RANKL/RANK system [13,14]. In addition, OPN, a secreted phosphoglycoprotein expressed by osteoclasts, synoviocytes, macrophages, and activated T cells, is important for osteoclast-mediated bone erosion. Previous studies indicate that OPN can promote osteoclasts recruitment and up-regulate RANKL/RANK system [15].

In the aspect of joint damage in RA, matrix metalloproteinases (MMPs) have also been demonstrated as important, because they can degrade components of the extracellular matrix. MMPs are amongst the key enzymes allowing osteoclasts to move and to initiate bone resorption. RA synovial tissues exhibit increased production of MMPs

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(including MMP-1, MMP-3 and MMP-13) [16]. MMP-1 and MMP-13 have predominant roles in collagen degradation, while MMP-3 degrades non-collagen matrix components of the joints.

For many years, attempts have been made to treat RA. In spite that biologic agents have been recently accepted clinically, treatment with a combination of Chinese herbal extracts and disease-modified anti-rheumatic drugs (DMARDs), is still often selected at present because of low cost and the less risk of side effects such as hypersensitivity, infections or oncogenesis. Among the DMARDs, Methotrexate (MTX) is generally accepted as the first-line drug for RA treatment. Sinomenine ($C_{19}H_{23}NO_4$, SIN), an alkaloid isolated from the stem of the Chinese medicinal plant, *Sinomenium acutum*, has been used to ameliorate the symptoms of rheumatic diseases [17]. Previous pharmacological studies have demonstrated that SIN has significant immunosuppressive, anti-inflammatory, analgesic and anti-arthritic properties [18,19]. SIN is capable of inhibiting the proliferation of FLS and the production of proinflammatory cytokines in RA [20,21]. Clinically, SIN can be used for long-term administration and may significantly alleviate the articular symptoms in RA patients. It was reported that the combination of SIN and MTX could reduce dosage of the drugs, decrease the adverse reactions and increase effective rate in RA patients [22]. However, to our knowledge, the effect of combined SIN and MTX on joint damage of RA has not yet been determined.

In the present study, we aimed to investigate the anti-arthritic effects of SIN and MTX, used alone or in combination, in a rat model of collagen induced arthritis (CIA) which is a representative animal model used in studies of RA. The underlying mechanisms were focused on the effect of these treatments upon osteoclast-related cytokines in vivo. We also analyzed the effect of SIN and MTX on human RA-FLS, one of the important sources of osteoclast-related cytokines.

## 2. Material and methods

### 2.1. Drugs and reagents

Bovine type II collagen (CII), complete Freund's adjuvant (CFA) and incomplete Freund's adjuvant (IFA) were purchased from Sigma. Sinomenine (SIN) of 98% purity verified by HPLC was purchased from Zhengqing Pharmaceutical Group (Hunan, China). Methotrexate (MTX) of 99% purity verified by HPLC was purchased from Xinyi Pharmaceutical Group (Shanghai, China). Antibodies used for immunocytochemistry were purchased from the Santa Cruz. Dulbecco's modified eagle's medium (DMEM) and fetal calf serum (FCS) were purchased from GIBCO. TRIzol and primers used for RT-PCR were obtained from Invitrogen. Restriction enzymes and Taq polymerase were purchased from Promega.

### 2.2. Animals

Female Sprague–Dawley rats (7–8 weeks old) were obtained from Experimental Animal Center of Drum Tower Hospital (Nanjing, China). Animals were housed in a temperature-controlled room (21 to 26 °C) with a 12-hour alternating light/dark cycle. Animals were given rat chow and water *ad libitum* before and throughout the experiments. Animals were acclimated to their surroundings over one week to eliminate the effect of stress prior to initiation of the experiments. All animal care and treatment procedures were approved by the Committee of Experimental Animal Administration of Nanjing University.

### 2.3. Induction of collagen-induced arthritis

Arthritis was induced by immunizing with CII as described previously [23]. Dissolved CII (4 mg/ml in 0.05 M acetic acid) was emulsified with CFA or IFA (ratio of 1:1). The final concentration of CII was 2 mg/ml. CII emulsified in CFA was used for the first immunization on day 0, while CII emulsified in IFA was used for the second immunization on day 14. Rats were immunized intradermally (i.d.) in the tail with 150 μl of emulsion.

### 2.4. Experimental protocol

Treatment was initiated on day 21. 28 rats were assigned to four groups of 7 rats each and treated with SIN, MTX, SIN plus MTX or placebo (treated with physiological saline) respectively. MTX dissolved in physiological saline at a dose of 1 mg/kg/week, was administered intraperitoneally (i.p.). SIN dissolved in physiological saline at a dose of 120 mg/kg/d, was administered intragastrically (i.g.). All the treatments were used at doses previously described in the literature as being effective [24,25]. Arthritis was not induced in 7 rats, acting as normal controls.

### 2.5. Assessment of arthritis activity

Disease progression was monitored from the secondary immunization (day 14). Arthritis activity was evaluated by using arthritis index characterized by edema and/or erythema in the paws as described previously [25]. Arthritis index was measured every 3 to 4 days by inflammation of paws (0 = normal, 1 = mild swelling and erythema of digits or ankles, 2 = moderate swelling and erythema of digits or ankles, 3 = marked swelling of paws including digits, 4 = severe swelling and erythema with limited motion in many joints). This system yielded a total score between 0 and 16 for each rat. Rats were sacrificed on day 58, and both ankles were removed for histologic examination to assess joint damage.

### 2.6. Histologic analysis

Formalin-fixed limbs were decalcified and paraffin-embedded using standard histologic techniques. Serial sections were cut and stained with hematoxylin and eosin to examine morphologic features and to assess the histological score. Sections were analyzed microscopically for the degree of inflammation and for cartilage and bone destruction according to the method reported previously [26]. The histological scores were determined as follows: score 0, no signs of inflammation; 1, mild inflammation with hyperplasia of the synovial lining without cartilage destruction; 2 through 4, increasing degrees of inflammatory cell infiltration and cartilage/bone destruction.

### 2.7. Immunohistochemical analysis

Rat synovial tissue specimens (also collected on day 58) were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS). Using immunohistochemistry, sections were incubated with anti-RANKL antibody or anti-OPN antibody according to the manufacturer's instructions. Primary antibody staining was followed by treatment with biotinylated secondary antibody. The stained samples were examined by microscopy, and representative sections were photographed. Immunostained sections were analyzed semi-quantitatively using image-pro plus 6.0.

### 2.8. Measurements of cytokine and protease levels

Levels of IL-6, IL-17, MMP1, MMP3, MMP13, RANKL and OPG in rat serum collected on day 58 were measured using commercially available ELISA kits (Sigma) according to the manufacturers' instructions.

### 2.9. Isolation and culture of FLS from RA patients

Synovial tissues were obtained from 3 patients with RA at time of knee replacement surgery. All participants gave informed consent to the study, which was approved by the Ethics Committee of the Affiliated Drum Tower Hospital of Nanjing University Medical School. All RA