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Original Article

Effect of methotrexate on collagen-induced arthritis in male Wistar rats

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Abstract

To evaluate the effect of methotrexate on collagen-induced arthritis, micro-computed tomography (micro-CT) and histopathological analyses were used in male Wistar rats. Rats were divided randomly into three groups. Group 1 was treated with 0.9% saline, and groups 2 and 3 were boosted with type Ⅱ collagen. From day 21 to 42, groups 1 and 2 were orally treated with 0.9% saline and group 3 was orally treated with 1.5 mg/kg methotrexate. All rats were sacrificed at day 42 after the first collagen treatment. Micro-CT analyses showed bony parameters, such as bone volume and trabecular number, were decreased in group 2 compared to group 1, and these parameters were recovered in group 3. Histopathological examination and pathological parameter scoring showed that the knee joints of rats in group 2 had severe joint destruction, showing cartilage and bone erosion, enlarged cavities with inflammatory cell infiltration and activation of synovial fibroblasts. By contrast, these changes were reduced in group 3. Taken together, methotrexate treatment showed therapeutic potential in male rat collagen-induced arthritis model, and micro-CT analysis and histopathological tools could be integrated to assess the quantification/qualification of arthritic lesions.

Keywords: arthritis, rat, collagen, micro-computed tomography, histopathology

Introduction

Arthritis is characterized by structural and biochemical changes in cartilage, bone and synovium[1–2]. Rheumatoid arthritis (RA) is manifested as joint destruction and chronic pain in association with serological evidence of immune reactivity[3]. In RA, potential therapeutics tend to achieve the reduction of joint inflammation and the recovery of erosive damage[4].

In RA researches, the collagen induced arthritis (CIA) model is usually used. CIA model is characterized by gradual degeneration of articular cartilage and destruction of the subchondral bone[5]. Pathogenic autoreactive antibodies were produced by immunization of rodents with type Ⅱ collagen, resulting in the development of severe arthritis[6], and involving macrophages[7], neutrophils, $CD4^+$ T cells and $CD8^+$ T cells[8–9]. Rat CIA model is useful in developing therapeutics, especially for arthritis of chronic stage, as it has many similarities with human RA[8]. Using the CIA model, it is possible to assess the recovery potential of therapeutic agents against cartilage degeneration and bone destruction. Methotrexate (MTX) is a disease-

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modifying antirheumatic drug to reduce synovitis and systemic inflammation and improve the joint function[3].

As histopathologic examination provides qualitative or semi-quantitative information, there have been some limitations for quantifying lesions. To assess arthritis in joint lesions, advanced bioimaging techniques with three-dimensional systems are used to provide more informative and disease-relevant platforms[10]. Furthermore, these may reduce the number of animals required per experiment[11]. As an advanced bioimaging technique, micro-computed tomography (micro-CT) can produce systematic and anatomical images of high resolution[12].

The purpose of this study was to examine the effect of MTX on rat CIA model using micro-CT analysis and conventional histopathological examination.

Materials and methods

Collagen induced arthritis

Fourteen male Wistar rats (8-week old) were obtained from Orient Bio (Kapyung, Korea) and kept in a temperature controlled environment [(22±3) °C], (55±5)% relative humidity with a 12-hour light/dark cycle. The rats were fed on a rodent diet and filtered water *ad libitum*.

Rats were divided separately into three groups randomly. The rats of group 1 (G1, $n = 3$) were treated with 0.9% saline, and those of group 2 (G2, $n = 5$) and group 3 (G3, $n = 6$) were boosted with type Ⅱ collagen at days 0, 7, 14 and 21 to induce arthritis. In brief, bovine type Ⅱ collagen (Chondrex, Redmond, WA, USA) was dissolved in 0.01 mol/L acetic acid overnight at 4 °C. This was emulsified in an equal volume of incomplete Freund's adjuvant (Chondrex). The rats of G2 and G3 were immunized intradermally at the base of tail with 0.1 mL of emulsion containing 100 μg of type Ⅱ collagen. From day 21 to 42, the rats of G1 and G2 were orally treated with 0.9% saline and those of G3 were orally treated with 1.5 mg/kg MTX. All rats were sacrificed at day 42 after the first collagen immunization and both hind knee joints were fixed in 10% formalin, and then micro-CT and histopathological analyses were carried out. This study was approved by the animal experiment committee of Namseoul University based on the Animal Protection Act.

Micro-CT analyses

Using a micro-CT system (Skyscan 1172, Bruker, Kontich, Belgium), quantitative analyses were performed. The specimens were scanned using micro-CT with X-ray source of 40 kV/250 μV, pixel size 23 μm and use aluminum 0.5 mm filter. After micro-CT scanning, cross-sectional slices were reconstructed and each scan result was reconstructed using the 0–0.14 threshold values to distinguish bone and air.

Using micro-CT analyzing software, CTAn software (Bruker), three-dimensional analysis was performed, including bone volume, percent bone volume, trabecular number, trabecular thickness, bone surface/ bone volume and trabecular separation.

Histopathological observation

The hind knee joints of male rats were fixed in 10% formalin for 24 hours and decalcified in 14% EDTA-glycerol for 14 days at room temperature. Samples were processed and embedded in paraffin, and 4 μm sections were stained with hematoxylin and eosin (HE) for histopathological examination. Furthermore, safranin O-fast green (SO) staining was conducted for cartilage staining.

Histopathological examination was scored as five categories: infiltration of inflammatory cell, cartilage degradation, bone destruction, synovial hyperplasia, degeneration/necrosis. Severity of lesions was classified into four grades: 0, no change; 1, slight; 2, moderate; 3, severe.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 6 (GraphPad Software, La Jolla, CA, USA). All data were analyzed using Dunnett's multiple comparison test following one-way analysis of variance and Student's *t*-test. *P*-values < 0.05 were considered statistically significant.

Results

X-ray, coronal and sagittal images of micro-CT showed that normal appearance of joints was presented in G1. However, joint destruction was observed in G2 and the destruction of bony surface was reduced in G3 (***Fig. 1***).

Micro-CT analysis showed that several bone parameters were altered (***Table 1***). In G2, bone volume was significantly decreased in tibia of animals compared to G1 ($P < 0.05$). However, bone volume in G3 had no significant difference compared to that of G1. Bone surface/bone volume ratio and trabecular thickness of G3 were significantly decreased compared to G1 ($P < 0.05$). Trabecular number of G2 was significantly decreased compared to G1 ($P < 0.01$), however, it showed no significant difference in G3 compared to G1.

H&E staining (***Fig. 2A***, ***B*** and ***C***) showed no inflammation or tissue destruction in G1. However,

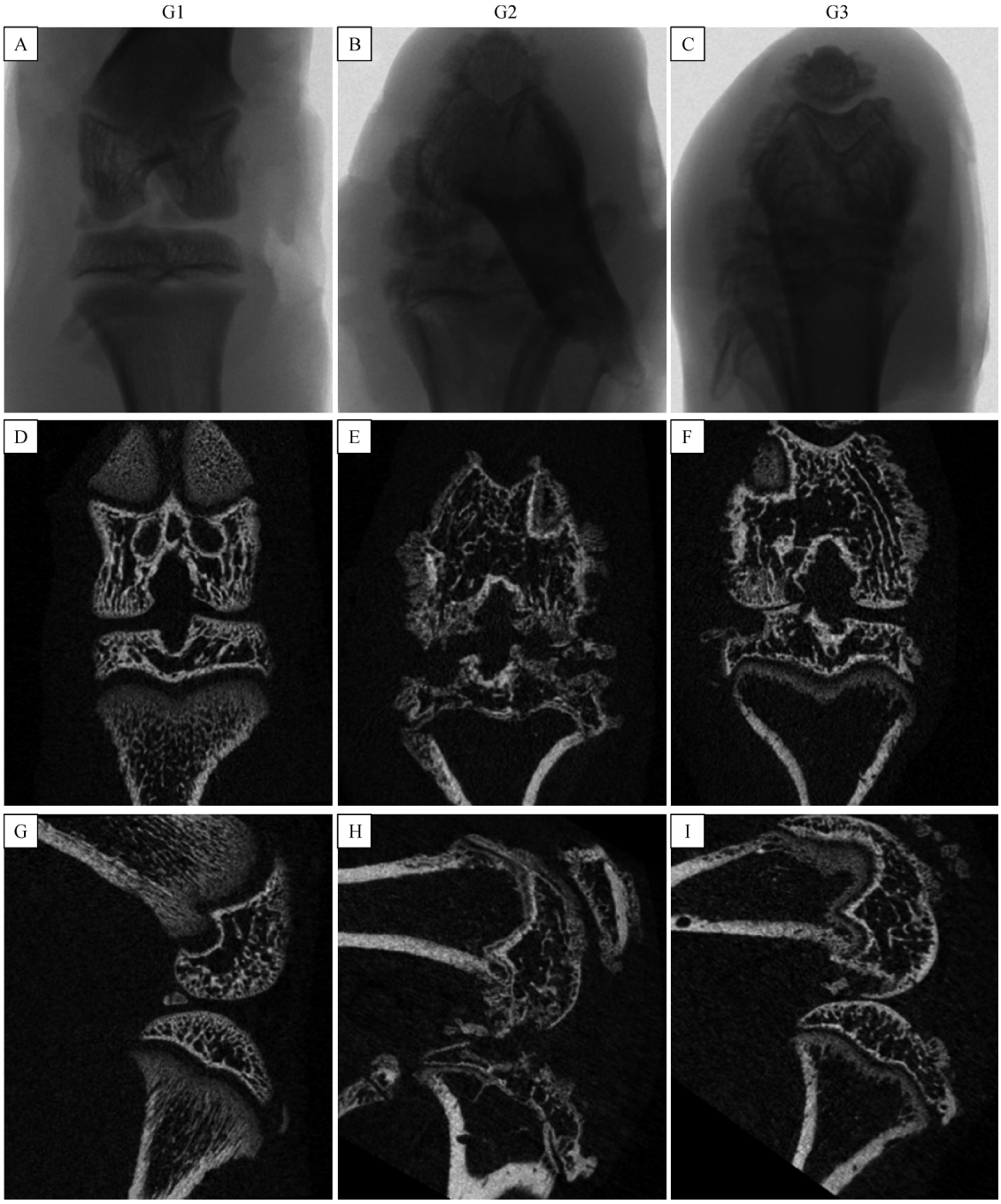

Fig. 1 **X-ray, coronal and sagittal micro-CT image of hind knee joint of rats treated with collagen.** Note the x-ray images (A–C), coronal images (D–F), sagittal images (G–I) for G1, 2, and 3, respectively. Note the normal appearance of joints for G1 and the destruction of bony surface for G2 and recovery of bony surface for G3 compared to G2.

Table 1 **Micro-CT analysis parameters of tibial trabecular bone in G1, G2 and G3**

Group	BV (mm^3)	BV/TV (%)	BS/BV (mm^{-1})	Tb.Th (mm)	Tb.N (mm^{-1})	Tb.Sp (mm)
G1	14.36±3.65	41.60±4.45	28.67±2.16	0.13±0.01	3.21±0.37	0.25±0.04
G2	10.46±4.22*	28.28±13.05	24.51±2.19	0.15±0.01	1.94±0.86**	0.55±0.41
G3	14.42±5.05	39.39±13.86	24.17±2.64*	0.15±0.02*	2.61±0.80	0.32±0.32

*,** Significantly different from G1 ($P<0.05$, $P<0.01$, respectively); Values are shown as mean±SD. BV: bone volume; BV/TV: percent bone volume; BS/BV: bone surface/volume ratio; Tb.Th: trabecular thickness; Tb.N: trabecular number; Tb.Sp: trabecular separation.

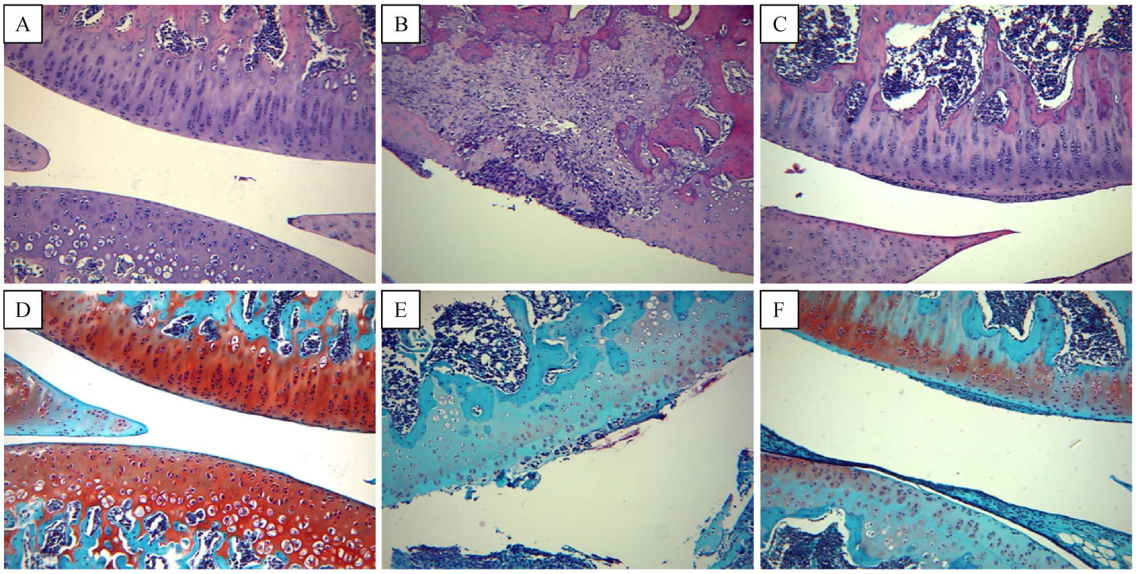

Fig. 2 **Hematoxin & eosin (H&E) and safranin O-fast green (SF) staining of hind knee joint of rats.** (A) G1; (B) G2; (C) G3 as H&E and (D) G1; (E) G2; (F) G3 as SF. Note the normal microscopic structure of the joint of control rat in G1. In G2, there are inflammatory cell infiltration, cartilage and bone erosion and activation of synovial fibroblasts. In G3, these are reduced. Magnification, ×200.

inflammatory cell infiltration, which was characterized by the collection of lymphocytes and macrophages, as well as cartilage and bone erosion and synovial fibroblast activation, was observed in G2. In contrast, these changes were reduced in G3. In SO staining (***Fig. 2D***, ***E*** and ***F***), cartilage structures in G1 were normal (appearing as red color). However, cartilage staining was remarkably reduced in G2. By comparison, in G3, there was moderate cartilage staining in joints of rats.

By scoring histopathological findings, infiltration of inflammatory cells was significantly increased in G2 compared to G1 ($P<0.01$), however, it showed no significant difference in G3 compared to G1. Cartilage degradation was significantly increased in G2 and G3, compared to G1 ($P<0.01$ and $P<0.05$, respectively). Bone destruction was significantly increased in G2 compared to G1 ($P<0.01$), however, it showed no significant difference in G3 compared to G1. Synovial hyperplasia was significantly increased in G2 and G3, compared to G1 ($P<0.001$). Degeneration/necrosis was significantly increased in G2 compared to G1 ($P<0.01$), however, it showed no significant difference in G3 compared to G1 (***Fig. 3***).

Discussion

In this study, micro-CT analysis provided several quantitative parameters for MTX evaluation and these results were in agreement with the conventional histopathology assessment.

In order to evaluate disease-specific regions, a consistent and reliable analysis was applied. Generally, micro-CT has advantage in bone detection. For quantification of arthritis progression, micro-CT was used in this study. It was reported that micro-CT could measure joint space narrowing and demonstrate trabecular bone structure and osteophyte formation[13]. In this study, bone volume and trabecular number were significantly decreased in G2 compared to G1 by micro-CT analysis. It represented that collagen treatment could decrease bone volume and trabecular number. However, these parameters showed no significant difference in G3 compared to G1, representing MTX treatment induced bone recovery in this study. Also MTX treatment restored bone volume to the control level. Compare to the previous report about the effect of methotrexate on female rats[14], MTX treatment in this study prevented bone loss induced by collagen treatment in male rats. Further studies will be warranted to clarify the difference of therapeutic potential between the two sexes.

By scoring the histopathological findings, infiltration of inflammatory cells was significantly increased in G2 compared to G1, however, it was not significantly different in G3 compared to G1. This indicates that MTX treatment can slightly reduce inflammatory cell infiltration, however, there could exist individual variation. In G2 and G1, synovial hyperplasia was significantly increased compared to G1, representing MTX treatment did not reduce synovial hyperplasia in knee joints. In early inflammatory arthritis, hypertrophy of the synovial lining appeared and in the later stage, a

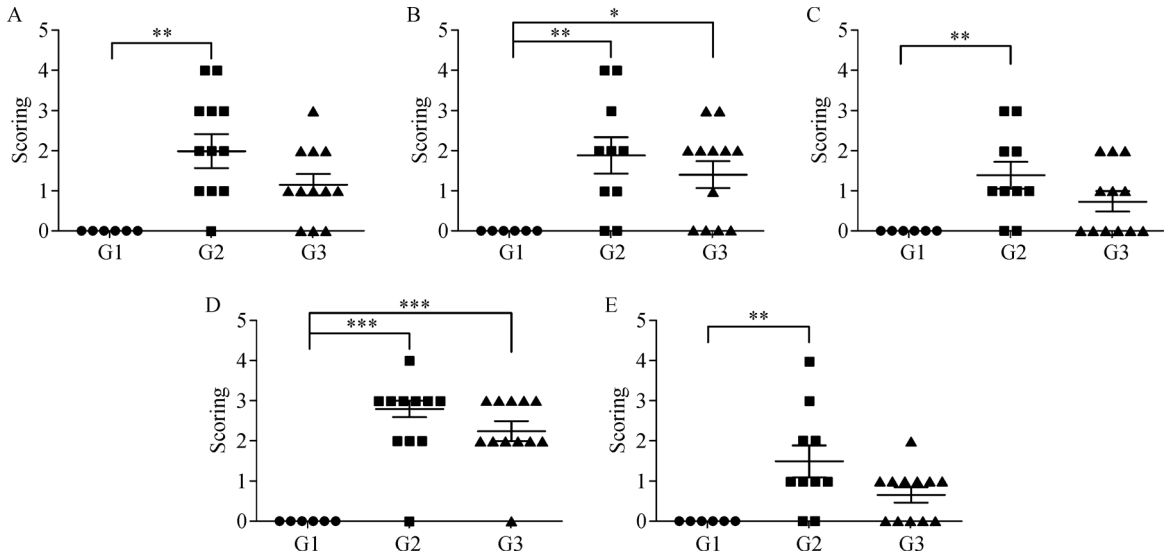

Fig. 3 **Histopathological scores in both hind knee joints of male rats.** A: infiltration of inflammatory cells; B: cartilage degradation; C: bone destruction; D: synovial hyperplasia; E: degeneration/necrosis. Values are shown as mean±SD.

fibrovascular pannus was formed[15]. In this study, MTX treatment induced the decrease of inflammatory cell infiltration, not synovial hyperplasia. There were significantly increased bone destruction and degeneration/ necrosis in G2 compared to G1, however, no significant difference was found between G3 and G1. It revealed that MTX treatment could slightly reduce bone destruction and degeneration/ necrosis.

As micro-CT analysis has a limitation for the detection of cartilage, safranin O-fast green staining was used. There were normal cartilage structures in the rats' joints (appearing as red color) in G1. However, cartilage staining was remarkably reduced in the joints of rats in G2. In G3, there was slight cartilage staining in joints of rats in G3. By scoring histopathological findings, cartilage degradation was significantly increased in G2 and G3 compared to G1 ($P<0.01$ and $P<0.05$, respectively). These results indicated that MTX treatment showed slight reduction of cartilage destruction.

Even though histopathological scoring paradigm has been found to be sensitive for evaluating therapeutics[16], using histopathological scores in this study may have a limitation in obtaining quantitative information. For quantification of lesions, magnetic resonance imaging (MRI) was applied to assess cartilage degeneration[17]. As matrix metalloproteinases or gelatinases were involved in joint destruction in arthritis[18], quantitative analysis of the breakdown products of the joint matrix components was carried out[2]. A near-infrared fluorescence dye[19] or a bone specific polymeric probe[20] was used to visualize arthritis progression and produce even quantitative readouts. As some bioimaging techniques have a strong point for quantification, and histopathologic tools have a strong point for qualification of lesions, an integration of these techniques can ensure a better assessment of the lesions. Further studies will be warranted to monitor the animals *in vivo* after treatment using these tools.

Taken together, MTX treatment showed therapeutic potentials in male rat CIA model; integrating micro-CT analysis and histopathological tools could make it possible to assess the quantification/qualification of arthritic lesions.

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