# Beneficial Effects of Rosmarinic Acid on Suppression of Collagen Induced Arthritis

JEEHEE YOUN, KYUNG-HEE LEE, JONGHWA WON, SUNG-JIN HUH, HYE-SUN YUN, WON-GIL CHO, and DOO-JIN PAIK

*ABSTRACT.* ***Objective.*** To assess the therapeutic potential of rosmarinic acid (RosA) in an inflammatory autoimmune arthritis model.

***Methods.*** Collagen induced arthritis is established in male DBA/1 mice. Mice were administered daily with 50 mg/kg/day of RosA for 15 days from Day 21 post-immunization and inspected daily to determine the progression of arthritis. After termination of injection, affected hindpaws were subjected to histopathological analyses and immunohistochemical assays for cyclooxygenase-2 (COX-2) expression.

***Results.*** Repeated administration of RosA dramatically reduced the arthritic index and number of affected paws. Histopathologic observations closely paralleled clinical data, showing that RosA treated mice retained nearly normal architecture of synovial tissues, whereas control mice exhibited severe synovitis. Synovial tissues from RosA treated mice exhibited remarkably reduced frequency of COX-2-expressing cells, compared to those from untreated mice.

***Conclusion.*** RosA suppressed synovitis in a murine collagen induced arthritis model; this effect may be beneficial for treatment of rheumatoid arthritis in clinical settings. (J Rheumatol 2003;30:1203–7)

*Key Indexing Terms:*
ROSMARINIC ACID
CYCLOOXYGENASE-2
COLLAGEN INDUCED ARTHRITIS
RHEUMATOID ARTHRITIS

Rosmarinic acid (RosA), α-[[3-(3,4-dihydroxyphenyl)-1-oxo-2-propenyl]-oxy]-3,4-dihydroxy-[R-(E)]-benzenepropanoic acid, is a polyphenolic metabolite widely distributed in Labiatae herbs[1]. RosA has diverse immunoregulatory functions including antimicrobial, antioxidant, and antiinflammatory activities[2-4]. The antiinflammatory activity of RosA is mainly attributed to its inhibition of cyclooxygenase (COX) and lipoxygenase (LOX) activities and complement activation[3,4]. Along with such activities, the capacity of RosA acting as a free radical scavenger may provide an additive effect to treat inflammatory diseases. However, *in vivo*, the role of RosA in the context of inflammatory autoimmune diseases remains to be assessed.

Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disease, characterized by the development of pathogenic T cells and subsequent inflammatory responses evident in multiple joints[5]. A murine model of collagen induced arthritis (CIA) resembles human RA in that both cellular and humoral responses are involved in the pathogenic process[6,7].

We investigated the therapeutic effects of RosA on the CIA model through clinical, histopathological, and immunological observations.

## MATERIALS AND METHODS

*Mice and arthritis induction.* Male DBA/1LacJ mice were immunized with 100 µg of bovine type II collagen (CII) (Chondrex, Seattle, WA, USA) emulsified in complete Freund's adjuvant (Chondrex) by intradermal injection at the base of the tail[8]. Two weeks later mice were subjected to booster immunization with 50 µg CII/incomplete Freund's adjuvant. RosA (Indofine Chemical Company, Somerville, NJ, USA) dissolved in absolute ethanol was mixed gently in olive oil at 5:95 (v:v) ratio and injected intraperitoneally daily from Days 21 to 35 at 50 mg/kg/day. Control mice were injected with ethanol mixed with olive oil (5:95, v:v). All *in vivo* studies complied with Korean legislation with policies by Hanyang University on the care and use of animals. Starting on Day 21 after primary immunization, mice were inspected for disease progression in a blinded manner. The clinical severity of disease was scored on a daily basis using a described scoring system[8]. Each limb was graded, resulting in a maximal clinical score of 16 per animal, expressed as the mean arthritic index on a given day. Mice were scored as arthritic after more than one paw was scored > 2.

*Histopathologic assessment.* Hindpaws were removed post mortem on Day 37, fixed in 10% (w/v) phosphate buffered formalin, and decalcified in 5.5% EDTA in phospahate buffered formalin. Decalcified paws were embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Arthritic changes in the ankle and foot were scored as described[9] with modifications, where 0 = normal, 1 = weak leukocyte infiltration but no

*From the Department of Anatomy and Cell Biology and Institute of Biomedical Science, College of Medicine, Hanyang University, Seoul; and the Signal Transduction Laboratory, Mogam Biotechnology Research Institute, Yonginsi, Korea.*

*Supported by grants from the Korean Ministry of Science and Technology (M1-9808-00-0036) and the Korean Green Cross Company.*

*J. Yun, PhD, Assistant Professor; K-H. Lee, MS; S-J. Huh, MS; H-S. Yoon, MS; W-G. Cho, PhD; D-J. Paik, MD, PhD, Professor, Hanyang University; J. Won, PhD, Senior Scientist, Mogam Biotechnology Research Institute.*

*Address reprint requests to Dr. D-J. Paik, Department of Anatomy and Cell Biology, College of Medicine, Hanyang University, 17 Haengdang-dong, Sungdong-gu, Seoul 133-791, Korea. E-mail: paikdj@hanyang.ac.kr*

*Submitted July 29, 2002; revision accepted November 28, 2002.*

erosion, 2 = modest infiltration and weak erosion, 3 = severe infiltration and invasion to bones, and 4 = loss of bone integrity.

*Immunohistochemical assays*. After deparaffinization and endogenous peroxidase quenching, the sections were permeabilized and blocked with 2% normal rabbit serum. The sections were incubated with goat anti-COX-2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 1:50 dilution, washed, and incubated with biotinylated anti-goat IgG antibody (Vector Laboratories, Burlingame, CA, USA). Specific labeling was detected using avidin-biotin-peroxidase complex (Vector) and DAB. The sections were counterstained with 1% methylene blue solution.

## RESULTS

*Suppressive effect of RosA on progression of CIA*. To determine the suppressive effect of RosA on inflammatory autoimmune arthritis, CIA mice were intraperitoneally injected with 50 mg/kg/day of RosA for 15 days from the early phase of disease initiation. Administration of RosA markedly reduced clinical manifestations of established CIA, as indicated by the mean arthritis index and number of affected paws (Figure 1). The reduction of the arthritis index became evident from Day 25, when the 4th injection of RosA was made, and was sustained with gradual decrease of the arthritis index with additional RosA injections until the day of termination.

The hindpaws were subjected to histologic inspections at termination of the injection protocol. The histopathologic index of each hindpaw, which was scored according to the severity of leukocyte infiltration and bone invasion, was closely correlated with the clinical data from individual mice (Figure 2). The mean histopathologic index of vehicle-treated control mice was $3.31 \pm 0.25$, as manifested by severe leukocyte infiltration and loss of bone integrity found in most of the mice. In contrast, the joints of RosA injected mice were essentially normal, with some instances of early arthritis in some mice, as shown by much lower mean histopathologic index of $0.60 \pm 0.21$ ($p < 0.0001$, Student's t test).

*Reduced COX-2 expression in joints of RosA-treated mice*. After RosA treatment, the concentration and distribution of COX-2 in affected joint tissues were assessed using immunohistochemical methods. The level of COX-2 positivity closely paralleled clinical and histopathologic data from individual mice. Joints from CIA control mice exhibited intensive staining with anti-COX-2 antibody, notably in leukocyte infiltrates and fibroblast-like stromal cells (Figure 3, panels C–F). However, the number of COX-2 positive cells was drastically reduced in the joint tissue from RosA-treated mice, comparable to that from normal mice (Figure 3, G–H).

## DISCUSSION

The results indicate an *in vivo* therapeutic effect of RosA in a murine experimental model of RA. Repeated administration of RosA during the early phase of the disease was sufficient to achieve a dramatic arrest in overall disease progression, judged by significantly reduced clinical and histopathological manifestations of arthritis. We also observed that RosA administration resulted in depletion of COX-2 positive cells in the affected joint tissues.

COX-2 is induced by various proinflammatory agents and plays a predominant role in inflammatory responses. Accordingly, COX-2 inhibitors exert their therapeutic effects as nonsteroidal antiinflammatory drugs[10]. Our result showing a lack of COX-2-expressing cells in joint tissues from RosA-treated mice may simply reflect RosA activity serving as a COX-2 inhibitor, since prostaglandin $E_2$ has been shown to enhance COX-2 expression[11,12]. The possibility that RosA may directly regulate the expression of COX-2 gene remains to be determined. Alternatively, RosA may interfere with the activation and/or recruitment of COX-2-expressing leukocytes to affected joints. Recently,

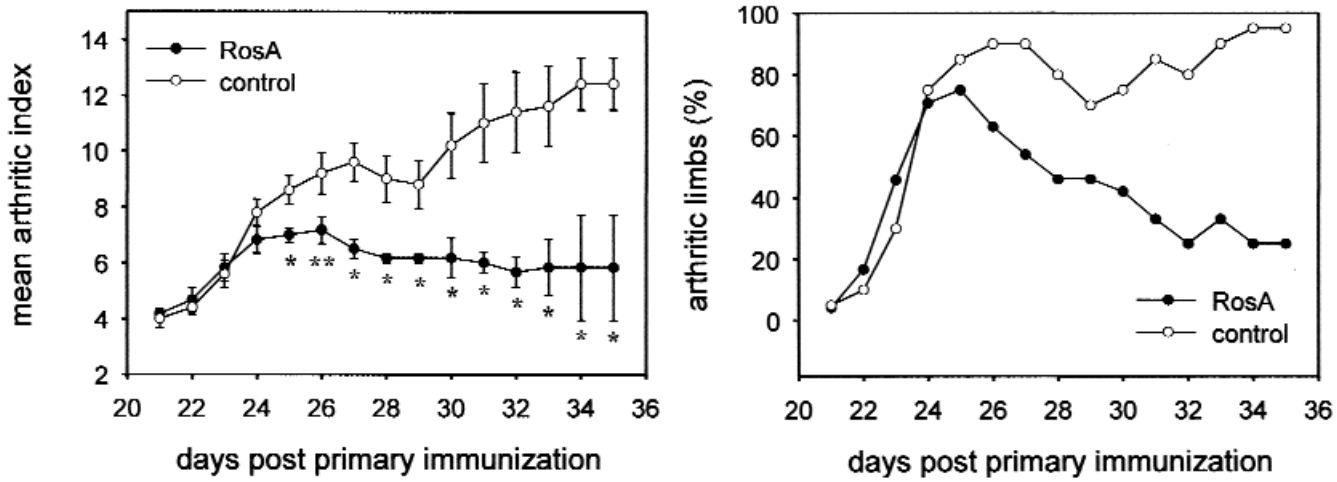


*Figure 1*. Suppression of clinical manifestations of CIA by RosA. DBA/1LacJ mice were immunized with CII emulsion with complete Freund's adjuvant at Day 0, followed by booster immunization with CII/incomplete Freund's adjuvant emulsion at Day 14. From Days 21 to 35, mice (5–6 per group) were injected intraperitoneally with 50 mg/kg/day of RosA or vehicle. These data are representative of 3 independent experiments. *$p < 0.02$, **$p < 0.05$ versus controls, Student's t test.

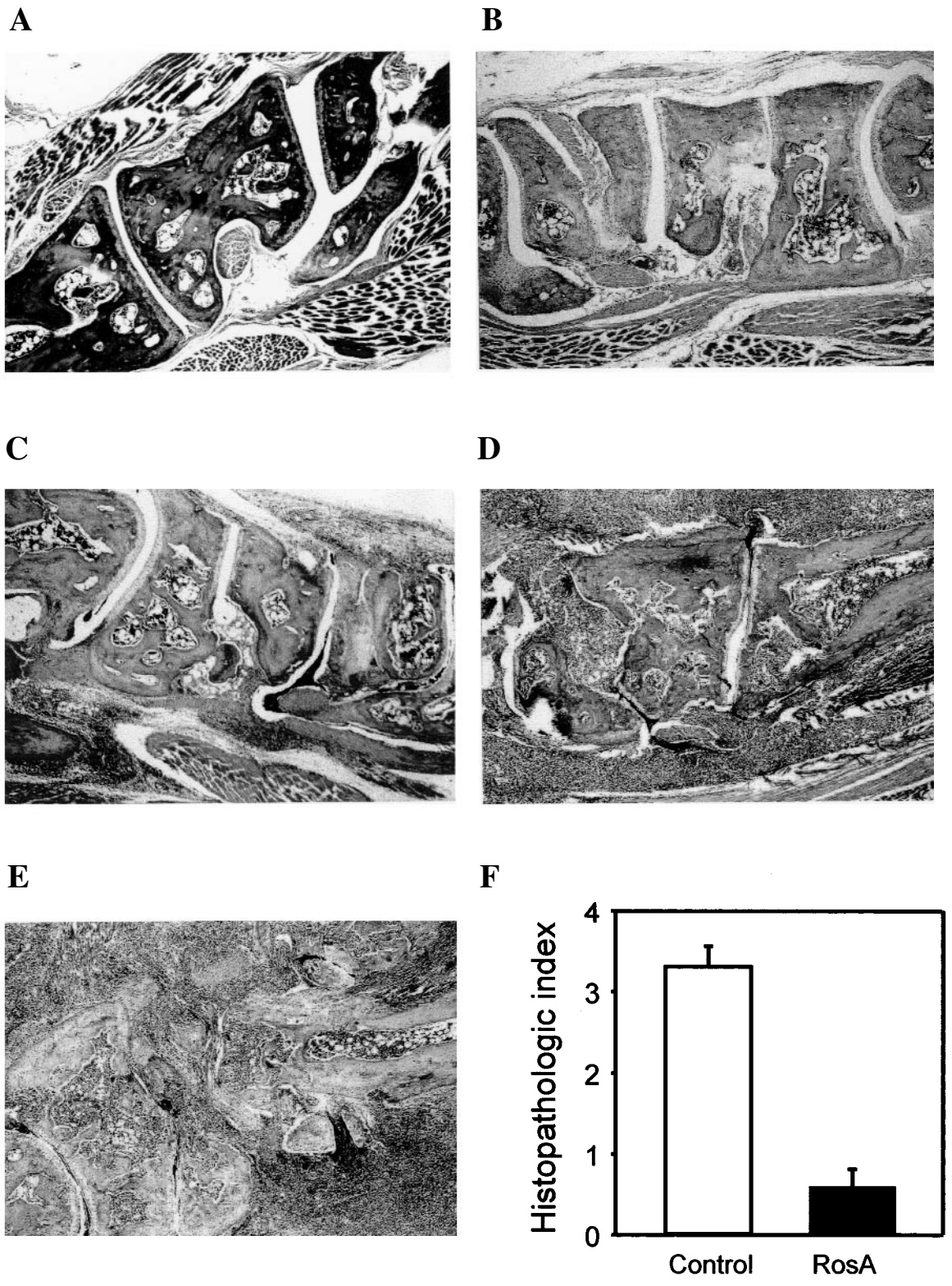


*Figure 2*. Histopathologic assessment of effect of RosA on CIA. On Day 37 post-immunization, mice were sacrificed and hindpaws were fixed, decalcified, embedded in paraffin, sectioned, and stained (H&E). Figures are representative of each score (A, normal mice, score 0; B, RosA-treated, score 1; C, RosA-treated, score 2; D, vehicle-treated, score 3; E, vehicle-treated, score 4; original magnification ×40). The mean histopathologic scores of each group are plotted as a histopathologic index (F). This result was reproducible in 2 independent experiments.

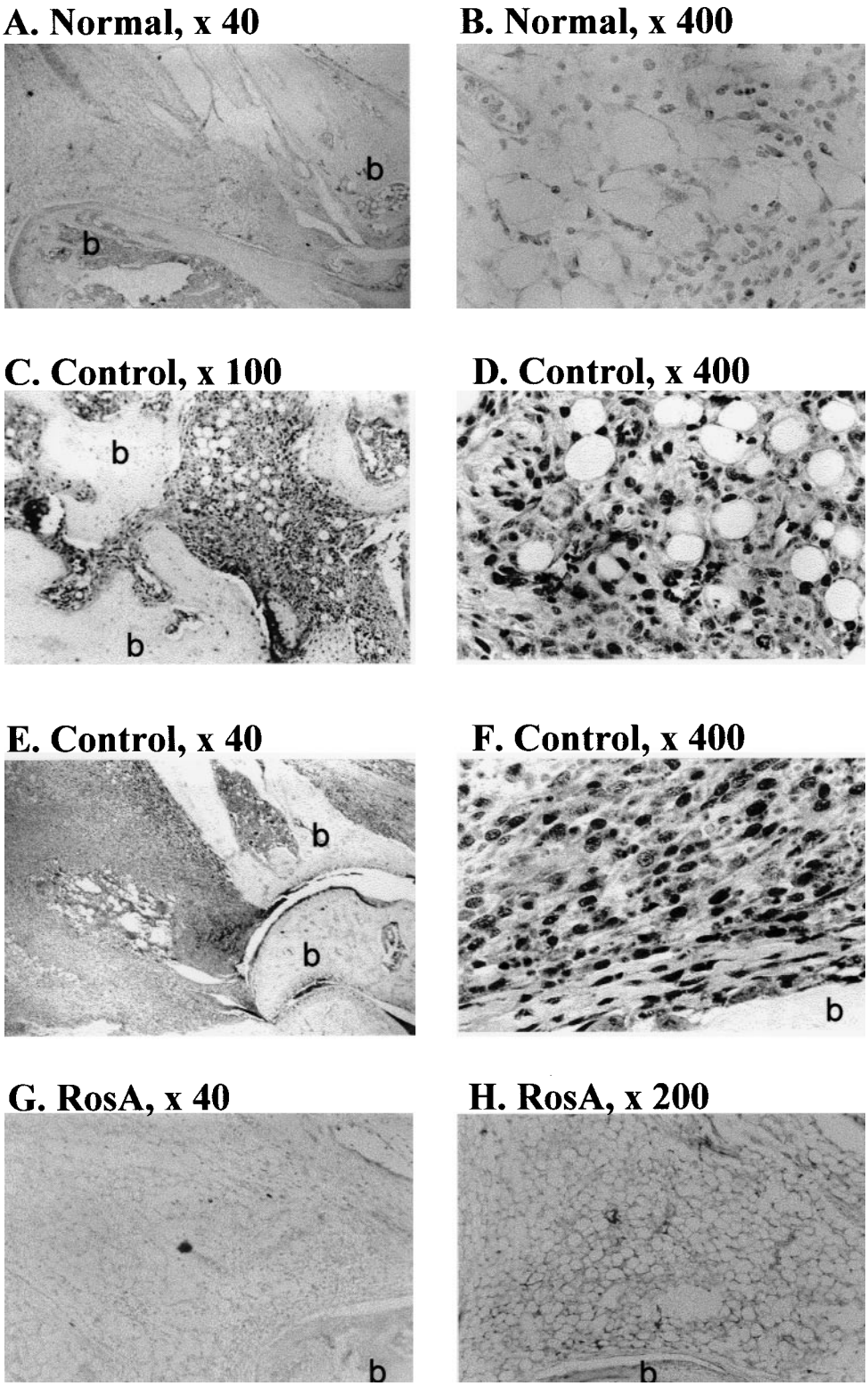


*Figure 3*. Deficiency of COX-2-producing cells in joints of RosA-treated mice. Hindpaw sections were stained with anti-COX-2 antibody, followed by standard immunohistochemical investigation. Figures are representative of each group. A, B, normal joint; C to F, vehicle-treated; G, H, RosA-treated; b: bone (original magnification: A, E, G, ×40; C, ×100; H, ×200; B, D, F, ×400).

we observed that RosA inhibits T cell receptor-mediated T cell activation and proliferation, implying an immunosuppressive potential of RosA in T cell-mediated immune disorders (unpublished observations). In accord with this observation, T cells obtained from RosA-treated CIA mice exhibited a decrease in the proliferative response upon *in vitro* stimulation with CII, as compared with those from vehicle-treated mice (data not shown). By inhibiting both COX and LOX, it is anticipated that RosA may have reduced side effects, such as gastrointestinal toxicity, that result from the compensatory increase of leukotriene production induced by selectively blocking the COX-dependent pathway[13]. Indeed, several other dual inhibitors of COX and LOX have been reported to have reduced irritant effects on the stomach, relative to nonselective Cox inhibitors[13-15]. Further investigations including the molecular mechanisms of RosA activity and its side effects are required.

We observed that RosA ameliorated CIA, as manifested by reduction of synovitis and depletion of COX-2 positive cells in affected joints. Thus, administration of RosA may provide a therapeutic effect to treat RA in clinical settings.

## ACKNOWLEDGMENT

We gratefully acknowledge technical assistance from Chulshik Lim and a critical reading of the manuscript by Dr. Andrew H. Kang, University of Tennessee, and Dr. Sungsoo Jung, Hanyang University.

## REFERENCES

1. Al-Sereiti MR, Abu-Amer KM, Sen P. Pharmacology of rosemary (Rosmarinus officinalis Linn.) and its therapeutic potentials. Indian J Exp Biol 1999;37:124-30.
2. Van Kessel KP, Kalter ES, Verhoef J. Rosmarinic acid inhibits external oxidative effects of human polymorphonuclear granulocytes. Agents Actions 1986;17:375-6.
3. Kelm MA, Nair MG, Strasburg GM, DeWitt DL. Antioxidant and cyclooxygenase inhibitory phenolic compounds from Ocimum sanctum Linn. Phytomedicine 2000;7:7-13.
4. Sahu A, Rawal N, Pangburn MK. Inhibition of complement by covalent attachment of rosmarinic acid to activated C3b. Biochem Pharmacol 1999; 57: 1439-46.
5. Feldmann M, Brennan FM, Maini RN. Rheumatoid arthritis. Cell 1996;85:307-10.
6. Durie FH, Fava RA, Noelle RJ. Collagen-induced arthritis as a model of rheumatoid arthritis. Clin Immunol Immunopathol 1994;73:11-8.
7. Seki N, Sudo Y, Yoshioka T, et al. Type II collagen-induced murine arthritis. I. Induction and perpetuation of arthritis require synergy between humoral and cell-mediated immunity. J Immunol 1988;140:1477-84.
8. Rosloniec EF, Cremer M, Kang A, Myers LK. Collagen induced arthritis. In: Coligan JE, Kruisbeek AK, Margulies DH, Shevach EM, Strober W, editors. Current protocols in immunology. New York: John Wiley & Sons; 1996:15.5.1-15.5.24.
9. Neurath MF, Hildner K, Becker C, et al. Methotrexate specifically modulates cytokine production by T cells and macrophages in murine collagen-induced arthritis: a mechanism for methotrexate-mediated immunosuppression. Clin Exp Immunol 1999;115:42-55.
10. Hawkey CJ. Cox-2 inhibitors. Lancet 1999;353:307-14.
11. Faour WH, He Y, He QW, et al. Prostaglandin $E_2$ regulates the level and stability of cyclooxygenase-2 mRNA through activation of p38 mitogen-activated protein kinase in interleukin-1 beta-treated human synovial fibroblasts. J Biol Chem 2001;276:31720-31.
12. Tjandrawinata RR, Hughes-Fulford M. Up-regulation of cyclooxygenase-2 by product-prostaglandin $E_2$. Adv Exp Med Biol 1997;407:163-70.
13. Gay RE, Neidhart M, Pataky F, Tries S, Laufer S, Gay S. Dual inhibition of 5-lipoxygenase and cyclooxygenases 1 and 2 by ML3000 reduces joint destruction in adjuvant arthritis. J Rheumatol 2001;28:2060-5.
14. Asako H, Kubes P, Wallace J, Gaginella T, Wolf RE, Granger N. Indomethacin-induced leukocyte adhesion in mesenteric venules: role of lipoxygenase products. Am J Physiol 1992;262:G903-8.
15. Wallace JL, Whittle BJ. Role of prostanoids in the protective actions of BW755C on the gastric mucosa. Eur J Pharmacol 1985;115:45-8.