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Department of Veterinary Physiology, College of Veterinary Medicine and BK21 Program for Veterinary Science, Seoul National University, Seoul, South Korea

Suk-Yun Kang, Seo-Yeon Yoon, Dae-Hyun Roh, Hyoung-Sig Seo, Dong-Kyu Uh, Jang-Hern Lee

R&D Center, Daewoong Pharmaceutical Company, Yongin, South Korea

Mi-Jeong Jeon

Department of Pharmacology, Institute for Medical Science, Chonbuk National University Medical School, Jeonju, South Korea

Young-Bae Kwon

Department of Physiology, College of Medicine and Research Institute for Medical Sciences, Chungnam National University, Daejeon, South Korea

Hyun-Woo Kim

Biotherapy Human Resources Center, College of Veterinary Medicine, Chonnam National University, Gwang-ju, South Korea

Ho-Jae Han

Acupuncture and Meridian Science Research Center, Kyung Hee University, Seoul, South Korea

Hye-Jung Lee

Correspondence: J.-H. Lee, Department of Veterinary Physiology, College of Veterinary Medicine and BK21 Program for Veterinary Science, Seoul National University, Seoul, 151-742, South Korea.
E-mail: JHL1101@snu.ac.kr

Note: Suk-Yun Kang and Seo-Yeon Yoon contributed equally to this study.

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The anti-arthritic effect of ursolic acid on zymosan-induced acute inflammation and adjuvant-induced chronic arthritis models

Suk-Yun Kang, Seo-Yeon Yoon, Dae-Hyun Roh, Mi-Jeong Jeon, Hyoung-Sig Seo, Dong-Kyu Uh, Young-Bae Kwon, Hyun-Woo Kim, Ho-Jae Han, Hye-Jung Lee and Jang-Hern Lee

Abstract

Ursolic acid (UA) is pentacyclic triterpenoic acid that naturally occurs in many medicinal herbs and plants. In this study, we examined the possible suppressive effect of UA extracted from *Oldenlandia diffusa* on zymosan-induced acute inflammation in mice and complete Freund's adjuvant (CFA)-induced arthritis in rats. UA treatment (per oral) dose-dependently (25–200 mg kg^{-1}) suppressed zymosan-induced leucocyte migration and prostaglandin E2 (PGE_2) production in the air pouch exudates. Since the maximal effective dose of UA was 50 mg kg^{-1} in the zymosan experiment, we used this dose of UA in a subsequent study using an adjuvant-induced rheumatoid arthritis model. UA treatment (50 mg kg^{-1}, per oral, once a day for 10 days) was started from day 12 after adjuvant injection. UA dramatically inhibited paw swelling, plasma PGE_2 production and radiological changes in the joint caused by CFA injection. Moreover, UA significantly suppressed the arthritis-induced mechanical and thermal hyperalgesia as well as the spinal Fos expression, as determined by immunohistochemistry, which was increased by CFA injection. In addition, overall anti-arthritic potency of UA was comparable with ibuprofen (100 mg kg^{-1}, oral) while UA did not induce significant gastric lesions as compared with the ibuprofen treatment group. These findings strongly suggest that UA is a useful suppressive compound for rheumatoid arthritis treatment with low risk of gastric problems.

Introduction

Herbal medicines derived from plant extracts are utilized to treat a wide variety of clinical diseases. Of these, triterpenoids are compounds that have attracted considerable interest, based on their remarkable antioxidative, anti-inflammatory and anti-cancer biological functions. Ursolic acid (UA; 3β-hydroxy-12-urs-12-en-28-oic acid), a natural pentacyclic triterpenoid carboxylic acid, is abundant in a number of vegetarian foods and medicinal plants (Ahmad et al 2006). Recently, it has been suggested that some of the therapeutic effects of medical plants (i.e. Labrador tea, Ericaceae, Plantain, *Oldenlandia diffusa*, *Nepeta sibthorpill* Bentham) may, in part, originate from UA (Ikeda et al 2008).

Both in-vitro and in-vivo studies have demonstrated that UA has several biological and pharmacological effects, including anti-tumour, antioxidant and anti-inflammatory effects (Suh et al 1998; Ryu et al 2000; Banno et al 2004; Park et al 2004; Miceli et al 2005; Aggarwal & Shishodia 2006). Focusing on the anti-inflammatory effect, treatment with UA suppresses the phorbol 12-myristate 13-acetate (PMA)-induced expression of cyclooxygenase (COX)-2 protein and synthesis of prostaglandin E2 (PGE_2) in human mammary epithelial cells (Subbaramaiah et al 2000). Moreover, in animal models, single treatment with UA from *Nepeta sibthorpii* Bentham exhibited significant anti-inflammatory activity against carrageenan-induced rat paw oedema by scavenging free radicals (Miceli et al 2005). Moreover, chronic UA treatment suppressed the paw swelling (at dose levels of 80 and 160 mg kg^{-1}) in the complete Freund's adjuvant (CFA)-induced rheumatoid arthritis (RA) model (Ahmad et al 2006). Taken together, it

is suggested that UA has a suppressive role in inflammatory disease including RA. Therefore, this study is aimed to address precise therapeutic evidence (i.e. anti-inflammation, anti-nociception) of UA's effect in acute and chronic RA animal models.

UA was extracted from *Oldenlandia diffusa (Hedyotis diffusa* Willd, Rubiaceae), which is a Chinese herbal medicine and has long been used to treat many kinds of diseases, such as autoimmune diseases, rheumatism and arthritis.

Firstly, we used a zymosan-induced inflammatory model to investigate the UA-induced acute anti-inflammatory effect and to determine the effective dose of UA by measuring the number of leucocytes migrating and the concentration of PGE_2 in the exudate fluid from the mouse air pouch (De Leon et al 2003). The local inflammatory response in the air pouch is considered a model of a synovial-like tissue inflammatory response resembling that seen in RA (Cabrera et al 2001). Subsequently, to evaluate the chronic anti-arthritic effect of UA treatment we utilized a CFA-induced arthritis rat model. Changes in paw volume (oedema), the concentration of PGE_2 and radiological changes were measured as a parameter of inflammation evoked by CFA injection. The potential anti-nociceptive effect of UA treatment on both thermal and mechanical hyperalgesia was also measured in both the ipsilateral and contralateral hind paws following induction of chronic arthritis. In addition, the suppressive effect of UA on CFA-induced spinal Fos protein (neuronal marker of nociception) expression (Hunt et al 1987; Catheline et al 1999) was determined by immunohistochemistry and was analysed using a computerized image analysis system as previously described (Abbadie & Besson 1994; Kwon et al 2001). Finally, we investigated whether long-term treatment with UA induces gastric lesions. In all steps, the results using UA were compared with those using ibuprofen, which has been used as a standard control for RA treatment.

Materials and Methods

Animals

Experiments were performed on male ICR mice (24–30 g) and Sprague–Dawley rats (130–150 g) obtained from the Laboratory Animal Center of Seoul National University. All of the experimental protocols for animal usage were reviewed and approved by the Animal Care and Use Committee at Seoul National University and conformed to NIH guidelines (NIH publication No. 86-23, revised 1985).

Preparation of UA

UA was provided by RNL Bio Co. (Seoul, Korea). Dried and powdered aerial parts (1 kg) of *Oldenlandia diffusa* were extracted by maceration with 80% methanol for 24 h at room temperature. The extract was concentrated by 20 fold under vacuum and partitioned with ethyl acetate. This ethyl acetate fraction was further concentrated and dissolved in the mixed solvent (ethyl actetate–acetone–water, 4.5:4.5:1 v/v) at 40–45°C. This resulting solution was stored at 4°C until precipitation occurred, and then centrifuged to collect UA. The purity of UA extracted from *Oldenlandia diffusa* was determined by high-performance liquid chromatography (HPLC) with UV detection using an Acme 9000 HPLC system (Younglin Instrument, Anyang, Korea). The detector was operated at 206 nm wavelength, which corresponded to the experimentally found absorption maximum of the UA standard (Sigma Chemical Co., MO, USA; Cat. No. U6753). A waters μBondapack C18 3.9 × 300 mm column was used. The solvent (80% methanol) flow rate was 1.0 mL min^{-1} and the mobile phase was acetonitrile–distilled water–phosphoric acid (8:1.98:0.02, v/v/v). The column temperature was maintained at 35°C by a column oven. For treatment, UA was dissolved in vehicle and it was mixed with 0.5% dimethyl sulfoxide, 10% polyethylene glycol 400 and 89.5% diluted water.

Mouse air pouch model

The air pouch was prepared in mice as previously described (Kwon et al 2003). Air (5 mL) was subcutaneously injected into the back of the mouse on day 0 and the pouch was reinforced with an additional 2.5 mL of air on days 2 and 5 to maintain the pouch cavity. Six days after the initial air injection, mice were injected with 0.5 mL of 1% zymosan (Sigma, St Louis, MO, USA) to induce local inflammation into the air pouch. Four hours after zymosan administration, the mice were anaesthetized with 3% isoflurane in a mixed N_2O/O_2 gas and the pouch exudate was collected with 2 mL of saline. The collected exudate was immediately diluted with Turk's solution at a ratio of 1:20. The total number of leucocytes in the exudate fluid was counted using a Neubauer hemacytometer counting chamber. To measure PGE_2 concentration, exudate samples were centrifuged for 15 min and were analysed by enzyme-linked immunosorbent assay (kit from Biosource International, CA, USA).

In this model, UA (12.5, 25, 50 and 200 mg kg^{-1}) and ibuprofen (100 mg kg^{-1}, Sigma Chemical Co.) (Singh et al 2007) was dissolved in vehicle and administered orally. Control mice received the same volume of vehicle. Each drug was given to mice 1 h before zymosan injection.

CFA-induced arthritis model

Arthritis was induced by a single subcutaneous injection (50 μL) into the plantar surface of the rat right hind-paw of heat-killed *Mycobacterium butyricium* (Difco Laboratory, Detroit, MI, USA) suspended in sterile mineral oil (20 mg mL^{-1}). Control rats were injected with sterile vehicle. In the rat arthritic model, UA was administered at a dose of 50 mg kg^{-1}, which was based on that producing maximum effect in the mouse air pouch model. The dose of ibuprofen was 100 mg kg^{-1} (Singh et al 2007). The drugs were given once daily by oral administration for 10 days beginning at day 12 after CFA injection. The number of rats was eight in each group.

Measurement of anti-inflammatory effect

Evaluation of paw volume

Paw volume was measured using a water displacement plethysmometer (Ugo Basil, Italy) every 3 days for 21 days after CFA injection and the mean values were recorded. Paw volume measured just before CFA injection was used as the control volume (day 0).

Evaluation of radiological change in the hind limb

At the end of experiment, rats were sacrificed with an overdose of ether. The hind limb was amputated and placed on a film carrier. All radiographs were taken with a Westinghouse Rivera Instrument set at 12.5 mA s^{-1}, 40 kV with Kodak Ektascan M Film. We evaluated two parameters (soft tissue swelling and new bone formation) in the lower part of the tibio-tarsal joint. Each parameter was analysed with a computer-assisted image analysis system (Metamorph, Universal Imaging, West Chester, PA, USA) using a modification of Esser's method (Esser et al 1995).

Evaluation of PGE_2 level in serum

At day 21 after CFA injection, blood samples were collected in sterile tubes by cardiac puncture and centrifuged for 15 min. The level of PGE_2 was measured by enzyme-linked immunosorbent assay (kit from Biosource International).

Measurement of anti-nociceptive effect

In the mechanical hyperalgesia test (Randall-Selitto test), a graded mechanical force was delivered through an analgesy meter (LETICA, LE7356) onto the convex surface of the paw. Rats withdrew their hind paw or vocalized when the applied force reached the nociceptive threshold. The test was duplicated at 5-min intervals in each hind paw.

In the thermal hyperalgesia test (Hargreaves's method), to assess nociceptive responses to thermal stimuli, paw withdrawal latency was tested using the procedure previously described by Hargreaves (Hargreaves et al 1988). Briefly, rats were placed in a plastic chamber with a glass floor and a radiant heat source was positioned under the glass floor beneath the hind paw to be tested. The withdrawal latency of both hind paws was measured to the nearest 0.1 s using a photoelectric cell connected to a digital clock. The test was duplicated at 5-min intervals in each hind paw.

Fos immunohistochemistry

At the end of the experiments (day 21), the animals were deeply anaesthetized with 5% isoflurane, perfused transcardially with calcium-free Tyrode's solution, followed by a fixative containing 4% paraformaldehyde and 0.2% picric acid in 0.1 M phosphate buffer (pH 6.9). The spinal cord was then removed immediately after perfusion, post-fixed in the same fixative for 4 hours and then cryoprotected in 30% sucrose in phosphate buffered saline (PBS, pH 7.4) for 48 hours. Frozen serial frontal sections (40 μm) were cut through the lumbar L3–L5 spinal cord using a cryostat (Microm, Germany). After elimination of endogenous peroxidase activity with 0.3% hydrogen peroxide in PBS and pre-blocking with 1% normal goat serum and 0.3% Triton X-100 in PBS, the free-floating sections were incubated in polyclonal rabbit anti-Fos antibody (Calbiochem, 1:10,000) at 4°C overnight. The sections were subsequently processed using the avidin–biotin–peroxidase procedure previously described (Lee & Beitz 1993; Kwon et al 1999). Fos-like immunoreactive (FLI) neurons were visualized using a 3-3 diamino-benzidine reaction intensified with 0.2% nickel chloride.

Imaging analysis of Fos-like immunoreactive neurons

Tissue sections were examined using darkfield microscopy (Zeiss Axioscope, Germany) to determine the segmental level as well as the gray matter landmarks to define individual spinal cord laminae (Abbadie & Besson 1994). Individual sections were digitized with 4096 gray levels using a cooled CCD camera (Micromax Kodak 1317; Princeton Instruments, Tucson, AZ, USA) connected to a computer-assisted image analysis system (Metamorph, Universal Imaging). To assess the effect of UA treatment on spinal cord Fos expression, the following four gray matter regions were selected for analysis based on cytoarchitectonic criteria: superficial dorsal horn (SDH, laminae I and II); nucleus proprius (NP, laminae III and IV); neck (NECK, laminae V and VI); and the ventral horn (VENT, laminae VII ± IX).

Gastric damage scoring

Rats were sacrificed on day 21 after arthritis induction for blind assessment of gastric damage, and gastric damage was scored as the lengths (in mm) of all haemorrhagic lesions. The scoring of damage involved measuring the length of each lesion with digital calipers, then summing these data for each stomach to give a gastric damage score. The assessment of damage was carried out by an individual unaware of the treatment the rats had received.

Statistics

Data values were expressed as mean ± s.e.m. The level of statistical significance was determined by unpaired Student's *t*-test for comparisons between two means, and by analysis of variance followed by Student–Newman–Keuls test for multiple comparisons. Mann–Whitney test was used for comparison of gastric damage score data between each group.

Results and Discussion

The purity of UA extracted from *Oldenlandia diffusa*

Both the standard UA (Sigma, purity: 97%) and the extracted UA (purity: 89.5%) showed a highly identified peak at similar retention time (18.85 min vs 18.73 min) (Figure 1). In this study, the range of purity of extracted UA was 89~91%. The species *Oldenlandia diffusa* (Rubiaceae) is an annual herb widely distributed in northern Asian countries. In traditional Chinese medicine, it has been used frequently for the treatment of inflammatory diseases such as rheumatism, arthritis and appendicitis. Therefore, we proposed that UA is one of the active anti-inflammatory compounds of *Oldenlandia diffusa*.

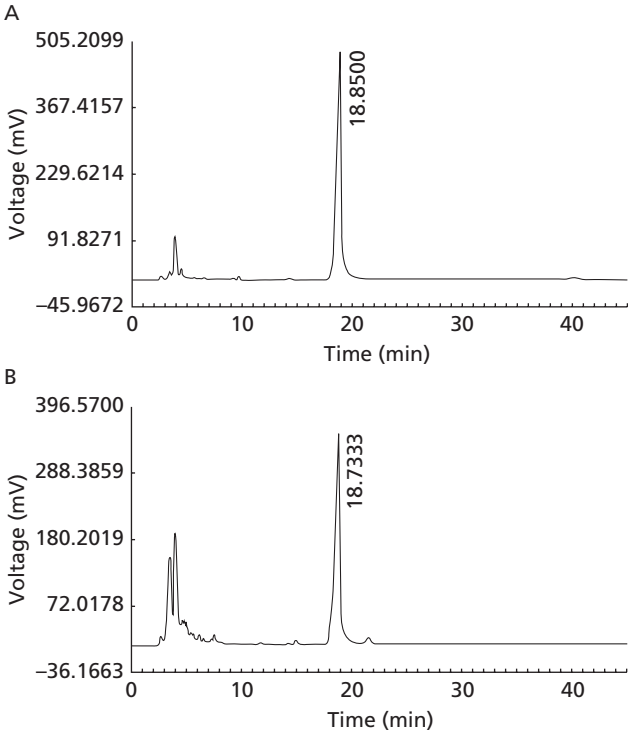

Figure 1 The HPLC chromatogram of standard commercial UA (A) and extracted UA from *Oldenlandia diffusa* (B) under the same condition. Both the standard and the extracted UA showed a highly identified peak at similar retention time (18.85 min vs 18.73 min). Purity of standard and extracted UA was 97% and 89.5%, respectively.

Anti-inflammatory effect of UA in zymosan-induced mouse air pouch model

Zymosan, a polysaccharide composed primarily of glucan and mannan residues, activates the complement system through the alternative complement pathway. Consequently, it induces the leucocyte migration and the production of cytokines and various inflammatory mediators such as PGE_2, eicosanoids, nitric oxide and enzymes such as phospholipase A_2 (PLA_2) (Cabrera et al 2001; Du et al 2006). In this study, zymosan injection into the air pouch caused a rapid influx of leucocytes at 4 h post-injection ($19.7 \pm 1.3 \times 10^6$ cells/mL), whereas saline injection showed negligible leucocyte infiltration ($2.9 \pm 0.4 \times 10^6$ cells/mL). Oral treatment with UA (12.5, 25, 50 and 200 mg kg^{-1}) significantly suppressed leucocyte migration in a dose-dependent manner in comparison with the group pretreated with vehicle (Figure 2). Especially, the 50 mg kg^{-1} dose of UA produced maximal anti-inflammatory effect, which was similar to that produced by ibuprofen (100 mg kg^{-1}) (Figure 2). Furthermore, the 50 mg kg^{-1} dose of UA (9.94 ± 1.63 ng mL^{-1}) and ibuprofen (4.47 ± 1.38 ng mL^{-1}) significantly suppressed PGE_2 production in the exudates as compared with the vehicle group (24.65 ± 6.21 ng mL^{-1}). The basal level of PGE_2 in the exudates was very low (0.23 ± 0.05 ng mL^{-1}). Based on these anti-inflammatory effects of UA in the acute inflammatory air pouch model, subsequent experiments in chronic adjuvant-induced arthritis were performed using a 50 mg kg^{-1} dose of UA.

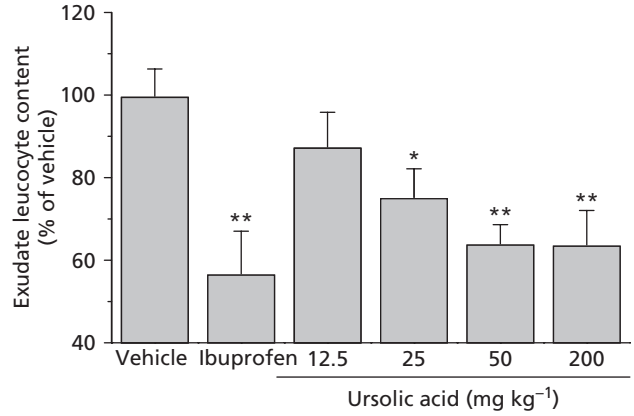

Figure 2 Anti-inflammatory effect of ursolic acid (UA) and ibuprofen in the zymosan-injected air pouch model in mice. Pretreatment with UA and ibuprofen significantly reduced leucocyte migration into the air pouch exudate. Data are expressed as a percentage of the vehicle value ($19.7 \pm 1.3 \times 10^6$ cells/mL) and are presented as the mean ± s.e.m., n = 7 mice per group. $^*P < 0.05$, $^{**}P < 0.01$, compared with vehicle group.

Anti-inflammatory effect of UA in CFA-induced rat arthritis model

Unilateral CFA injection into the right paw was found to evoke paw oedema initially in the ipsilateral limb and subsequently in the non-injected contralateral limb and tail (Philippe et al 1997). Injection of CFA into the right hind paw produced a primary swelling of the injected paw that was first measured at day 3 after CFA injection (Table 1). A secondary response, characterized by swelling of the non-injected contralateral hind paw and tail, was first evident at day 12 post-injection (Table 1). One of the goals of this study was to determine whether UA was responsible for the anti-inflammatory effect on this arthritis-induced primary and secondary inflammatory response. In the paw oedema study, UA (50 mg kg^{-1}) treatment significantly inhibited paw oedema in both CFA-injected ipsilateral and non-injected contralateral paw when compared with the vehicle group. Moreover, UA showed the anti-inflammatory effect on radiological changes induced by CFA injection. A number of changes in bone and peri-articular soft tissue has been reported to occur during the course of CFA-induced arthritis (Esser et al 1995). Using image analysis of radiographs, we measured new bone proliferation and soft tissue swelling in the tibio-tarsal joint at day 21 after arthritis induction. In this study, CFA-induced arthritis produced severe radiological changes in both soft tissue and bone in the vehicle group (Figure 3A, B, C). UA treatment significantly suppressed the soft-tissue swelling in right limb and also reduced the bone proliferation in both limbs as compared with the vehicle group. The effect of ibuprofen treatment was similar to that of UA treatment. The results obtained using this method clearly demonstrated that UA treatment inhibited the radiological changes (i.e. new bone proliferation and soft tissue swelling) induced by systemic arthritis.

Additionally, we measured effects of UA (50 mg kg^{-1}) and ibuprofen (100 mg kg^{-1}) on the increase in plasma PGE_2 concentration (normal basal level: 0.09 ± 0.03 ng mL^{-1}).

Table 1 Effects of ursolic acid (UA, 50 mg kg^{-1}) and ibuprofen (100 mg kg^{-1}) on CFA-induced increase of paw volume and on both mechanical and thermal hyperalgesia in right and left paw in rats

	Paw	Treatment	Days after athritis induction							
			0	3	6	9	12	15	18	21
Paw volume (mL)	Right	Vehicle	1.48 ± 0.03	2.29 ± 0.15	2.29 ± 0.14	2.40 ± 0.17	3.06 ± 0.12	3.31 ± 0.15	3.25 ± 0.10	3.28 ± 0.12
		Ibuprofen	1.48 ± 0.03	2.42 ± 0.14	2.44 ± 0.12	2.56 ± 0.11	2.87 ± 0.14	2.63 ± 0.08*	2.46 ± 0.05**	2.75 ± 0.14*
		UA	1.51 ± 0.02	2.33 ± 0.14	2.26 ± 0.13	2.40 ± 0.16	2.74 ± 0.23	2.76 ± 0.21*	2.66 ± 0.20**	2.73 ± 0.16*
	Left	Vehicle	1.44 ± 0.02	1.52 ± 0.03	1.57 ± 0.02	1.60 ± 0.07	2.33 ± 0.11	2.46 ± 0.09	2.34 ± 0.14	2.54 ± 0.10
		Ibuprofen	1.42 ± 0.02	1.49 ± 0.06	1.65 ± 0.05	1.63 ± 0.03	2.02 ± 0.11	2.02 ± 0.09**	2.05 ± 0.08	2.17 ± 0.08*
		UA	1.47 ± 0.01	1.52 ± 0.03	1.60 ± 0.02	1.59 ± 0.05	1.95 ± 0.13	2.11 ± 0.10*	2.04 ± 0.10	2.20 ± 0.11*
Mechanical threshold (g)	Right	Vehicle	111.6 ± 2.8	71.6 ± 6.4	73.8 ± 8.5	64.2 ± 6.2	76.0 ± 4.1	87.9 ± 11.2	79.0 ± 10.8	79.2 ± 10.7
		Ibuprofen	114.4 ± 5.2	82.7 ± 7.7	84.8 ± 7.2	75.9 ± 9.1	80.0 ± 5.6	84.1 ± 10.4	91.1 ± 5.1	100.3 ± 9.1*
		UA	112.9 ± 3.5	82.9 ± 6.5	73.3 ± 5.4	73.0 ± 11.2	77.2 ± 5.2	81.3 ± 5.5	101.0 ± 7.2	121.6 ± 3.1**
	Left	Vehicle	110.4 ± 3.9	112.3 ± 3.9	97.9 ± 5.7	94.2 ± 5.8	94.0 ± 7.1	83.8 ± 10.0	82.8 ± 13.1	84.6 ± 8.6
		Ibuprofen	107.9 ± 5.9	114.3 ± 6.2	100.4 ± 4.1	100.3 ± 3.8	99.4 ± 4.8	79.2 ± 11.8	81.6 ± 7.3	108.6 ± 5.6*
		UA	107.3 ± 2.8	115.0 ± 4.5	109.4 ± 6.6	97.4 ± 3.6	100.1 ± 3.6	86.4 ± 7.2	87.5 ± 7.2	110.6 ± 6.2*
Thermal withdrawal latency (s)	Right	Vehicle	10.17 ± 0.48	6.21 ± 0.35	5.75 ± 0.49	5.23 ± 0.64	5.80 ± 0.72	5.57 ± 0.52	5.22 ± 0.38	4.88 ± 0.29
		Ibuprofen	10.56 ± 0.43	6.45 ± 0.34	5.32 ± 0.42	5.35 ± 0.22	6.39 ± 0.63	8.95 ± 0.99*	9.23 ± 0.57**	7.14 ± 0.58**
		UA	10.90 ± 0.36	6.02 ± 0.58	5.77 ± 0.54	5.51 ± 0.46	7.62 ± 1.33	7.16 ± 0.91	7.82 ± 0.52**	6.55 ± 0.50*
	Left	Vehicle	10.37 ± 0.41	9.89 ± 0.59	9.39 ± 0.41	10.34 ± 0.69	6.28 ± 0.76	7.17 ± 0.63	6.75 ± 0.35	6.92 ± 0.37
		Ibuprofen	10.11 ± 0.43	9.54 ± 0.40	8.84 ± 0.34	9.26 ± 0.53	8.85 ± 0.37**	9.91 ± 0.33*	12.25 ± 0.52**	10.17 ± 0.64**
		UA	10.67 ± 0.17	9.07 ± 0.36	9.36 ± 0.56	9.89 ± 0.50	9.47 ± 0.84*	9.17 ± 0.93*	9.93 ± 0.57**	8.74 ± 0.24**

UA or ibuprofen treatment (per oral, once a day for 10 days) was started from day 12 after adjuvant injection. Each value represents the mean ± s.e.m. *$P < 0.05$, **$P < 0.01$, compared with the vehicle group.

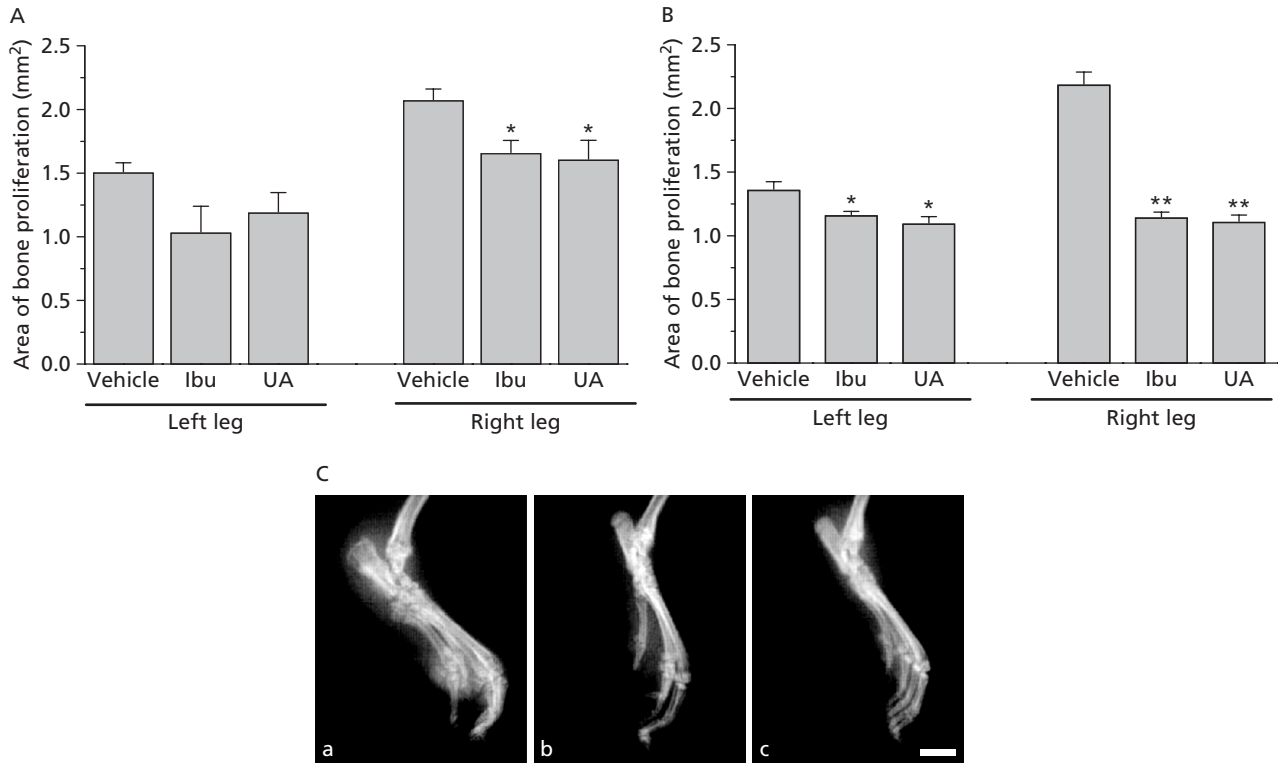

Figure 3 Effect of ursolic acid (UA, 50 mg kg^{-1}) and ibuprofen (Ibu, 100 mg kg^{-1}) on soft-tissue swelling (A) and bone proliferation (B) and X-ray images of each right limb in the vehicle group (C-a), Ibu group (C-b) and UA group (C-c) at day 21 after arthritis induction in rats. CFA-induced arthritis produced severe soft-tissue swelling and new bone proliferation in the vehicle group. UA significantly inhibited the soft-tissue swelling in right limb and also reduced the bone proliferation in both limbs. This effect in the UA group was similar to that in the Ibu group. $*P < 0.05$, $**P < 0.01$, compared with the vehicle group. Scale bar, 1 cm.

UA significantly suppressed PGE_2 concentration in the serum (1.04 ± 0.12 ng mL^{-1}) as compared with the vehicle group (1.56 ± 0.12 ng mL^{-1}) at day 21 post-arthritis induction. Furthermore, ibuprofen also suppressed PGE_2 production (1.11 ± 0.06 ng mL^{-1}). These results were consistent with the results of zymosan-induced air pouch inflammation. PGE_2 is one of the critical mediators produced at inflammatory sites by COX and is considered to be of great importance in the pathogenesis of RA (Anderson et al 1996; Darshan & Doreswamy 2004). In this regard, it has been reported that UA produced a significant COX-2 inhibitory effect, directly on the enzyme activity (Ringbom et al 1998). Moreover, (Subbaramaiah et al 2000) have demonstrated that UA suppresses the expression of COX-2 protein in human mammary epithelial cells. Taken together, it is possible that the UA-induced suppression of PGE_2 biosynthesis in this study results from the inhibition of COX-2 enzyme or COX-2 protein expression. However, it is unclear how UA modulates the COX-2 enzyme activity directly or indirectly in the RA model. Further study is necessary to evaluate this mechanism.

Anti-nociceptive effect of UA in CFA-induced rat arthritis model

Several investigators have previously reported that hyperalgesia was found to persist up to at least 3 weeks after CFA injection (Abbadie & Besson 1992; Philippe et al 1997). In this study, there was a bilateral decrease in the hind-paw mechanical pain threshold of rats in all groups (Table 1). UA increased the mechanical threshold in both hind paws as compared with the vehicle at the end of the experiment (day 21). Additionally, in the thermal hyperalgesia test, the paw withdrawal latency (PWL) in the ipsilateral paw of the vehicle group decreased from day 3 after CFA injection and in the contralateral paw also decreased from day 9. The UA group had a significantly increased PWL in the ipsilateral hind paw from day 18 after injection of CFA. In the contralateral hind paw, UA also increased the PWL from day 12 after injection of CFA as compared with the vehicle group (Table 1). Moreover, ibuprofen treatment also increased the ipsilateral PWL from day 15 and contralateral PWL from day 12 to the end of this study. In addition, we examined changes in spinal cord Fos expression following UA treatment. It has been previously reported that arthritis-induced nociception significantly increases Fos expression in the lumbar spinal cord 21 days after CFA injection (Abbadie & Besson 1992; Kwon et al 2001). In this study, the vehicle group also showed a significantly higher number of Fos-like immunoreactive (FLI) neurons in the ipsilateral and contralateral sides of the L3–L5 lumbar spinal cord segments on day 21 after CFA injection. Approximately 50% of FLI neurons were distributed in the NECK region of the dorsal horn at this time point, whereas smaller numbers of FLI neurons were observed in the SDH, NP and VENT regions of the spinal cord injection

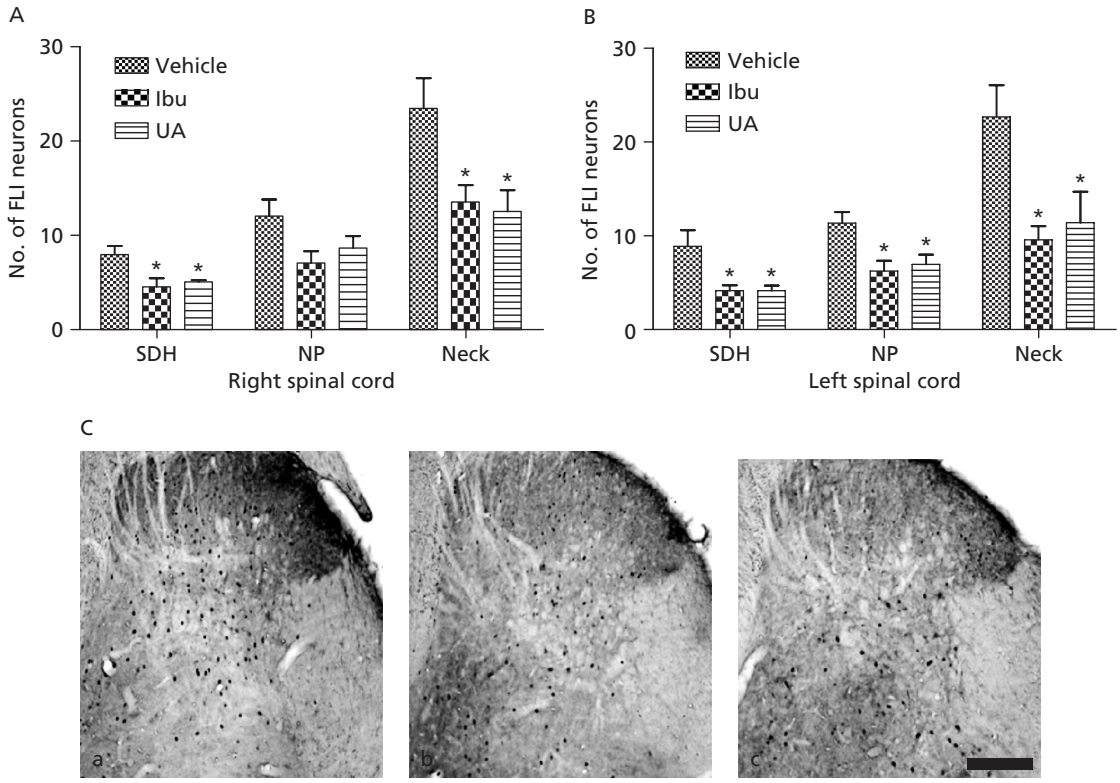

Figure 4 The number of Fos-like immunoreactive (FLI) neurons in the vehicle group (Vehicle), UA group (UA) and ibuprofen group (Ibu) at the right paw (A) and left paw (B) of rats and photomicrographs of Vehicle group (C-a), Ibu group (C-b) and UA group (C-c) on the Fos expression in ipsilateral lumbar spinal cord. The number of FLI cells was analysed in three regions (SDH, NP, NECK) as described in Materials and Methods. UA significantly reduced FLI neurons in both right and left regions of the spinal cord. $*P < 0.05$, compared with the vehicle-treated group. Scale bar, 200 μm.

(Kwon et al 2001). The UA and ibuprofen groups demonstrated significantly decreased numbers of FLI neurons as compared with the vehicle group (Figure 4A, B, C). Interestingly, the number of FLI neurons was reduced in the NP regions of the contralateral spinal cord of rats as compared with those of the ipsilateral group. Together with these findings, it is suggested that UA potentially suppressed the arthritic nociceptive input to the spinal cord. In this regard, the decrease in the number of FLI neurons induced by UA treatment paralleled the increase in anti-nociception indicated by the behavioural data. Collectively these data suggest that UA treatment has a potent anti-nociceptive effect on CFA-induced arthritic pain.

Evaluation of gastric damage by UA

The major side effect of non-selective COX inhibitors, such as ibuprofen, is their induction of gastrointestinal lesions (Whittle 2003; Peng & Duggan 2005). In this study, the number of rats with gastric lesions and the gastric damage score of the ibuprofen group was significantly higher than in the vehicle group (Table 2). However the UA group was not statistically different from the vehicle group and showed gastric lesions in less than half the ibuprofen group. In this regard, as previously described, UA selectively inhibited COX-2 activity (Ringbom et al 1998; Ikeda et al 2008). Moreover, it was reported that derivatives from UA, such as dihemisuccinate sodium salt and uvaol dihemiphthalate sodium salt analogues, show potent anti-ulcer activity. Taken together, these results demonstrate that long-term oral treatment with UA is safer than ibuprofen in RA with respect to gastric damage.

Table 2 The severity of gastric mucosal injury in rats produced by vehicle, ibuprofen and UA

Treatment	Gastric damage total scoring	Rats with gastric legion/total rats
Vehicle	0.5 ± 0.5	1/8
Ibuprofen (100 mg kg^{-1}) (positive control)	10.7 ± 5.0*	6/8*
UA (50 mg kg^{-1})	2.1 ± 1.2	3/8

A total lesion score for each rat was calculated as the total lengths of lesions multiplied by the respective severity scores at day 21 post-arthritis induction. While gastric mucosal injury was produced in the ibuprofen group, the UA group showed a relatively minor degree of gastric mucosal damage. $*P < 0.05$, compared with the vehicle group.

Conclusions

In this study, UA treatment exerted an anti-inflammatory effect in zymosan-induced acute inflammation in mice. Moreover, in the CFA-induced chronic arthritis model in rats, long-term UA treatment significantly reduces arthritic symptoms (pain, inflammation, radiological changes).

This anti-arthritic effect of UA was similar to that of the synthetic drug ibuprofen. Additionally, while ibuprofen caused severe gastric damage, UA did not. Based on these results, we propose that UA may be a safe and useful suppressive compound for the disease progression of RA.

References

Abbadie, C., Besson, J. M. (1992) c-fos expression in rat lumbar spinal cord during the development of adjuvant-induced arthritis. *Neuroscience* **48**: 985–993

Abbadie, C., Besson, J. M. (1994) Chronic treatments with aspirin or acetaminophen reduce both the development of polyarthritis and Fos-like immunoreactivity in rat lumbar spinal cord. *Pain* **57**: 45–54

Aggarwal, B. B., Shishodia, S. (2006) Molecular targets of dietary agents for prevention and therapy of cancer. *Biochem. Pharmacol.* **71**: 1397–1421

Ahmad, S. F., Khan, B., Bani, S., Suri, K. A., Satti, N. K., Qazi, G. N. (2006) Amelioration of adjuvant-induced arthritis by ursolic acid through altered Th1/Th2 cytokine production. *Pharmacol. Res.* **53**: 233–240

Anderson, G. D., Hauser, S. D., McGarity, K. L., Bremer, M. E., Isakson, P. C., Gregory, S. A. (1996) Selective inhibition of cyclooxygenase (COX)-2 reverses inflammation and expression of COX-2 and interleukin 6 in rat adjuvant arthritis. *J. Clin. Invest.* **97**: 2672–2679

Banno, N., Akihisa, T., Tokuda, H., Yasukawa, K., Higashihara, H., Ukiya, M., Watanabe, K., Kimura, Y., Hasegawa, J., Nishino, H. (2004) Triterpene acids from the leaves of Perilla frutescens and their anti-inflammatory and antitumor-promoting effects. *Biosci. Biotechnol. Biochem.* **68**: 85–90

Cabrera, P. V., Blanco, G., Gravisaco, M. J., Alvarez, E., Hajos, S. (2001) Zymosan modulates CD44 isoform expression in a murine model of inflammation resembling rheumatoid arthritis synovitis. *J. Rheumatol.* **28**: 943–949

Catheline, G., Le Guen, S., Besson, J. M. (1999) Effects of opioid receptor antagonists on the effects of i.v. morphine on carrageenin evoked c-Fos expression in the superficial dorsal horn of the rat spinal cord. *Brain. Res.* **824**: 105–111

Darshan, S., Doreswamy, R. (2004) Patented antiinflammatory plant drug development from traditional medicine. *Phytother. Res.* **18**: 343–357

De Leon, E. J., Alcaraz, M. J., Dominguez, J. N., Charris, J., Terencio, M. C. (2003) A new chloroquinolinyl chalcone derivative as inhibitor of inflammatory and immune response in mice and rats. *J. Pharm. Pharmacol.* **55**: 1313–1321

Du, Z., Kelly, E., Mecklenbrauker, I., Agle, L., Herrero, C., Paik, P., Ivashkiv, L. B. (2006) Selective regulation of IL-10 signaling and function by zymosan. *J. Immunol.* **176**: 4785–4792

Esser, R. E., Hildebrand, A. R., Angelo, R. A., Watts, L. M., Murphey, M. D., Baugh, L. E. (1995) Measurement of radiographic changes in adjuvant-induced arthritis in rats by quantitative image analysis. *Arthritis Rheum.* **38**: 129–138

Hargreaves, K., Dubner, R., Brown, F., Flores, C., Joris, J. (1988) A new and sensitive method for measuring thermal nociception in cutaneous hyperalgesia. *Pain* **32**: 77–88

Hunt, S. P., Pini, A., Evan, G. (1987) Induction of c-fos-like protein in spinal cord neurons following sensory stimulation. *Nature* **328**: 632–634

Ikeda, Y., Murakami, A., Ohigashi, H. (2008) Ursolic acid: an anti- and pro-inflammatory triterpenoid. *Mol. Nutr. Food Res.* **52**: 26–42

Kwon, Y. B., Yoon, Y. S., Han, H. J., Lee, J. H. (1999) Effect of 7-nitroindazole, a selective neuronal nitric oxide synthase inhibitor, on parvalbumin immunoreactivity after cerebral ischaemia in the hippocampus of the Mongolian gerbil. *Anat. Histol. Embryol.* **28**: 325–329

Kwon, Y. B., Lee, J. D., Lee, H. J., Han, H. J., Mar, W. C., Kang, S. K., Beitz, A. J., Lee, J. H. (2001) Bee venom injection into an acupuncture point reduces arthritis associated edema and nociceptive responses. *Pain* **90**: 271–280

Kwon, Y. B., Kim, H. W., Ham, T. W., Yoon, S. Y., Roh, D. H., Han, H. J., Beitz, A. J., Yang, I. S., Lee, J. H. (2003) The anti-inflammatory effect of bee venom stimulation in a mouse air pouch model is mediated by adrenal medullary activity. *J. Neuroendocrinol.* **15**: 93–96

Lee, J. H., Beitz, A. J. (1993) The distribution of brain-stem and spinal cord nuclei associated with different frequencies of electroacupuncture analgesia. *Pain* **52**: 11–28

Miceli, N., Taviano, M. F., Giuffrida, D., Trovato, A., Tzakou, O., Galati, E. M. (2005) Anti-inflammatory activity of extract and fractions from Nepeta sibthorpii Bentham. *J. Ethnopharmacol.* **97**: 261–266

Park, J. H., Son, K. H., Kim, S. W., Chang, H. W., Bae, K., Kang, S. S., Kim, H. P. (2004) Antiinflammatory activity of Synurus deltoides. *Phytother. Res.* **18**: 930–933

Peng, S., Duggan, A. (2005) Gastrointestinal adverse effects of non-steroidal anti-inflammatory drugs. *Expert Opin. Drug Saf.* **4**: 157–169

Philippe, L., Gegout-Pottie, P., Guingamp, C., Bordji, K., Terlain, B., Netter, P., Gillet, P. (1997) Relations between functional, inflammatory, and degenerative parameters during adjuvant arthritis in rats. *Am. J. Physiol.* **273**: 1550–1556

Ringbom, T., Segura, L., Noreen, Y., Perera, P., Bohlin, L. (1998) Ursolic acid from Plantago major, a selective inhibitor of cyclooxygenase-2 catalyzed prostaglandin biosynthesis. *J. Nat. Prod.* **61**: 1212–1215

Ryu, S. Y., Oak, M. H., Yoon, S. K., Cho, D. I., Yoo, G. S., Kim, T. S., Kim, K. M. (2000) Anti-allergic and anti-inflammatory triterpenes from the herb of Prunella vulgaris. *Planta Med.* **66**: 358–360

Singh, S., Khajuria, A., Taneja, S. C., Khajuria, R. K., Singh, J., Qazi, G. N. (2007) Boswellic acids and glucosamine show synergistic effect in preclinical anti-inflammatory study in rats. *Bioorg. Med. Chem. Lett.* **17**: 3706–3711

Subbaramaiah, K., Michaluart, P., Sporn, M. B., Dannenberg, A. J. (2000) Ursolic acid inhibits cyclooxygenase-2 transcription in human mammary epithelial cells. *Cancer Res.* **60**: 2399–2404

Suh, N., Honda, T., Finlay, H. J., Barchowsky, A., Williams, C., Benoit, N. E., Xie, Q. W., Nathan, C., Gribble, G. W., Sporn, M. B. (1998) Novel triterpenoids suppress inducible nitric oxide synthase (iNOS) and inducible cyclooxygenase (COX-2) in mouse macrophages. *Cancer Res.* **58**: 717–723

Whittle, B. J. (2003) Gastrointestinal effects of nonsteroidal anti-inflammatory drugs. *Fundam. Clin. Pharmacol.* **17**: 301–313