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Original Article

Effects of Herbal Acupuncture of *Clematis Mandshurica* Maxim. at *Sinsu-hyul* (BL 23) on Adjuvant Arthritis in Rats.

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Objective : Based on the immunological dysfunction in RA and the immunological feature of *Clematis mandshurica* Maxim. herbal acupuncture solution (CM-HAS), this study examined whether subcutaneous (s.c.) administration of CM-HAS has anti-inflammatory effects on adjuvant arthritis (AA) in the rat.

Methods : Complete Freund's adjuvant was used to induce AA in rats. Secondary paw swelling of AA rats was measured, and polyarthritis index was scored. The administration of CM-HAS (2, 5, 10 mg/kg) to the *Sinsu-hyul* (BL 23) acupuncture point subcutaneously (s.c.) inhibited the inflammatory response and restored the weight of body and immune organs of AA rats. In this study, inhibitory effect of CM-HAS on cyclooxygenase-2 (COX-2) was evaluated. The plant material selected for this study has been used in Korean medicine for the treatment of various diseases that are considered as inflammatory in nature, e.g. asthma, arthritis, rheumatism, fever, edema, infections, snakebite and related inflammatory diseases.

Results : There were significant secondary inflammatory reactions in AA rats, accompanied with the simultaneous decrease of body and immune organs weight. The administration of CM-HAS (2, 5, 10 mg/kg, s.c.) improved the above changes significantly. CM-HAS showed inhibitory activity against COX-2, which supports their traditional uses. In this study, aspirin and indomethacin were used to contrast with CM-HAS as the COX-2 inhibitors.

Conclusion: The findings of this study may explain at least in part why CM-HAS has been traditionally used for the treatment of inflammatory conditions in traditional Korean and Chinese medicine.

Key Words : herbal acupuncture, *Clematis mandshurica* Maxim., *Sinsu-hyul* (BL 23)

Introduction

Rheumatoid arthritis (RA) is a chronic auto-immune disease characterized by joint swelling, synovial membrane inflammation and cartilage destruction.

Clematis mandshurica Maxim. herbal acupuncture solution (CM-HAS) has been used as a potent anti-inflammatory agent for arthritis and inflammation for hundreds of years in Asian countries, including Korea and China[1-6]. For scientific study, Choi et al. (2002) and Jung et al. (2001) have recently reported that natural herbal product extracted from three kinds of *Clematis mandshurica* is protective on articular cartilage for osteoarthritis (OA) of *in vivo* models and OA-like degeneration of the articular cartilage and synovial tissue[5,6]. Based on the immunological dysfunction in RA and the immunological feature of CM-HAS, we examined whether subcutaneous (s.c.) administration of CM-HAS has anti-inflammatory effects in rat adjuvant arthritis (AA).

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Despite progress in medical research during recent decades, the treatment of many serious diseases remains problematic[6]. Chronic inflammatory diseases remain among the world's major health problems[7]. Currently, both steroidal anti-inflammatory drugs and non-steroidal anti-inflammatory drugs (NSAIDs) are used in the relief of inflammation. Steroids have an obvious role in the treatment of inflammatory diseases, but due to their toxicity, can only be used over short periods except in very serious cases where the risks are acceptable. Prolonged use of NSAIDs is also associated with severe side effects, notably gastrointestinal hemorrhage[8,9]. Even the newer cyclooxygenase-2 (COX-2) selective drugs do not seem to be free of risk[10]. Consequently, there is a need to develop new anti-inflammatory agents with minimum side effects[11].

A number of plant species used in Australian aboriginal medicine and traditional Korean medicine (TKM) have been claimed to possess anti-inflammatory activities. As part of a cross-cultural medicinal plant research project, Korean plants that have been used to treat inflammatory disorders such as asthma, arthritis, rheumatism, fever, joint swelling, edema, infections and snakebite were developed. We were interested in CM-HAS, because it has been used in the treatment of similar pathological conditions by peoples of totally different cultural backgrounds, thus would provide a strong probability for the development of potential anti-inflammatory agents. Earlier studies on some of these plants including ethnobotany, phytochemistry and the evaluation of anti-tumor and antidiarrhoeal activity were reported; however, very little pharmacological investigation has been reported on their claimed anti-inflammatory effects.

The aim of this study was to evaluate the anti-inflammatory activity of CM-HAS extracts using a method based on the inhibitory effect on the biosynthesis of prostaglandin E2 (PGE2) and prostaglandin D2 (PGD2) from arachidonic acid catalyzed by COX-2.

Materials and Method

1. Animals

Male Sprague-Dawley (SD) rats weighing 180-220 g were obtained from the Biochemical Animal Department, Dongguk Oriental Medical Experimental Animal Center in Korea. All animals were maintained at a controlled temperature (22±2℃), and a regular light/dark cycle (7:00 to 19:00 h, light), and all animals had free access to food and water. CM-HAS was kindly provided by the Department of Acupuncture & Moxibustion, Oriental Medical College, Dongguk University.

2. Adjuvant arthritis induction and clinical evaluation[12,13,14]

Briefly, rats were immunized on day 0 by intradermal injection of Freund's complete adjuvant (FCA), containing 10 mg heat-inactive BCG (Bacillus Calmette-Gue?ri) in 1 ㎖ paraffin oil, into the left hind paw in 0.1 ㎖ for each rat. As a sham control, the same volume of paraffin oil alone was given into the left hind paw. Right hind paw volume was determined with MK-550 volume meter (Muromachi Kikai, Japan) before immunization (basic value, day 0) and repeated on days 14, 17 and 21. Right hind paw swelling was expressed as increase in hind paw volume in milliliters calculated by subtracting the basal value from the hind paw volume measured at all times considered.

The polyarthritis severity was graded on a scale of 0-4[12]: grade 0, no swelling; grade 1, isolated phalanx joint involvement; grade 2,

involvement of phalanx joint and digits; grade 3, involvement of the entire region down to the ankle; and grade 4, involvement of the entire paw, including ankle. The maximum joint score was 12, excluding the evaluation of the left hind paw for each rat.

3. Drug treatment

CM-HAS in 0.5% methylcellulose at concentrations of 2, 5 or 10 mg/kg/0.2 ml was administered by *Sinsu-hyul* (BL 23) injection of 0.2㎖ aliquots once daily from the day when the adjuvant was given to day 21 of the experiment. As a positive control, leflunomide (20 mg/kg) was intragastrically (i.g.) administrated[15].

4. Body weight and index of organ

In the course of the experiment, the body weight of rats was measured every 7 days. At day 21 after immunization, the animals were killed, and the thymus and spleen were promptly removed and weighed. The index of thymus and spleen were expressed as the percentage (%) of thymus and spleen wet weight versus body weight, respectively.

5. Cyclooxygenase-2 assay

Experiments were performed according to the methods described previously by White and Glassman (1974)[16], with the modification made by Noreen et al. (1998)[17]. A total of 0.01㎖ of ovine COX-2 (0.35 g protein) was activated on ice for 5 min with 0.06 ㎖ of co-factor solution in Tris-HCl buffer (pH 8.0), which included epinephrine (1.0 mM), reduced glutathione (1.0 mM), and hematin (0.001mM) in the reaction mixture. A total of 0.01 ㎖ of plant extract or vehicle was added to 0.07 ㎖ of the enzyme solution and pre-incubated at room temperature for 10 min. The reaction was started by adding 0.02 ㎖ of [1-14C] arachidonic acid (50 nCi). The samples were incubated at 37 ℃ for 15 min. The enzymatic reaction was terminated by adding 0.04 ㎖ of 4 M formic acid. Arachidonic acid metabolites, PGE2 and PGD2, were extracted with 0.2 ㎖ of chloroform (CHCl3) and separated from the CHCl3 extract by thin layer chromatography (TLC). The TLC plates were developed in hexane: diethyl ether:glacial acetic acid (70:30:1 v/v/v). After drying, the plates were further developed in the top layer of ethyl acetate:methanol:water mixture (80:20:50 v/v/v). The plates were placed in a chamber saturated with iodine vapor for 1 h to visualize the bands of PGE2 and PGD2. The bands of PGE2 and PGD2 were scraped off and their radioactivity (cpm) was measured on a Packard Tri-Carb 400 liquid scintillation counter. Percentage inhibition of COX-2 was determined. The negative control experiments were performed with boiled enzymes and the positive control experiments were conducted with aspirin and indomethacin.

The corresponding amounts of ethanol were used as solvent control. Each experiment was performed in three or five replicates.

6. Statistical analysis

Unless stated otherwise, data are expressed as mean ± S.D. and evaluated using an ANOVA followed by Dunnett's test. $P<0.05$ was considered statistically significant.

Results

1. Effects of CM-HAS on secondary arthritis in adjuvant arthritis (AA) rats

Inflammatory polyarthritis was induced in all immunized rats. The peak incidence occurred on d 14 after immunization. Treatment with CM-HAS (2, 5 and 10 mg/kg, s.c.) and leflunomide (20

Table 1. Effects of CM-HAS on Hind Paw Swelling in Rats with Adjuvant Arthritis (AA)

Group Dose (mg/k)g)	Hind paw swelling (ml) Day 14	Day 17	Day 21
Sham	0.06±0.03	0.10±0.03	0.11±0.03
AA	0.65±0.13**	0.89±0.27**	0.98±0.15**
CM-HAS 2	0.46±0.13#	0.53±0.23##	0.70±0.24
CM-HAS 5	0.40±0.14##	0.50±0.21##	0.59±0.31##
CM-HAS 10	0.40±0.21##	0.47±0.13##	0.43±0.23##
Leflunomide 20	0.33±0.11##	0.49±0.21##	0.55±0.33##

n=9. Mean ± SD. # P<0.05. ## P<0.01 compared with AA.
** P<0.01 compared with sham

Table 2. Effects of CM-HAS on Polyarthritis Index in Rats with Adjuvant Arthritis (AA)

Group Dose (mg/k)g)	Polyarthritis index Day 14	Day 17	Day 21
AA	7.8±1.8	8.9±2.6	9.0±3.2
CM-HAS 2	6.3±3.2	6.9±1.8	7.6±1.8
CM-HAS 5	5.0±2.3#	6.0±2.1#	6.4±2.4#
CM-HAS 10	3.3±1.5##	4.3±2.2##	4.8±2.5##
Leflunomide 20	4.4±2.1##	5.3±2.4##	5.7±2.3##

n=9. Mean ± S.D. CM-HAS # P<0.05. ## P<0.01 compared with AA

mg/kg, i.g.) attenuated the right hind paw swelling and polyarthritic symptoms from day 14 to day 21 after immunization (Table 1 and Table 2).

2. Effects of CM-HAS on body weight and the weight of immune organs of AA rats

The increase of body weight between immunized and nonimmunized rats was compared. Beginning on day 7, the body weigh increase of immunized

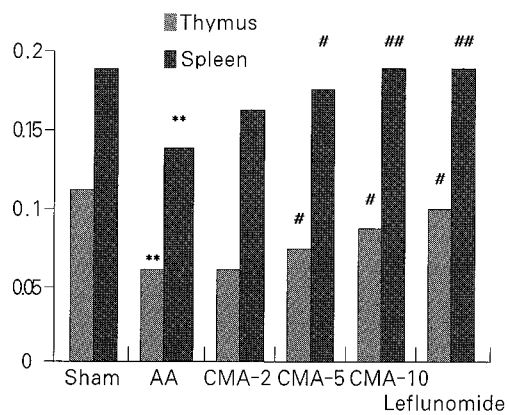

Fig. 1. Effect of CM-HAS on Index of Thymus and Spleen in AA Rats.
CM-HAS (5, 10mg/kg, sc) increased the weight of thymus and spleen of AA rats.
n=9, mean ± S.D.;
**P<0.01 compared with sham; #P<0.05, ##P<0.01 compared with AA.

Table 3. Effect of CM-HAS on Body Weight Increase in Adjuvant Arthritis (AA) Rats

Group Dose (mg/k)g)	Body weight increase (g)		
	Day 7	Day 14	Day 21
Sham	10.3±2.3	14.7±1.6	27.5±4.3
AA	6.8±2.2**	8.4±2.1**	14.3±3.2**
CM-HAS 2	7.2±2.1	10.5±2.3	17.3±1.5
CM-HAS 5	8.0±2.3	12.3±2.6##	18.3±3.2##
CM-HAS 10	8.7±2.4#	13.8±1.7##	20.4±2.6##
Leflunomide 20	8.5±2.3	14.1±1.5#	21.2±1.5##

n=9. Mean ± S.D.
** P<0.01 compared with sham. ## P<0.01 compared with AA. # P<0.05.

Table 4. Inhibition of COX-2 by CM-HAS

Drugs treatment	Inhibition of PGE2 and PGD2 (%)b
CM-HAS 1.5 mg/ml	23.3
CM-HAS 3.0 mg/ml	90.2

The concentration of extracts was 1.5 and 3.0mg/ml. In this experiment, aspirin (0.3mg/ml) and indomethacin (0.01mg/ml) used as positive controls produced 55 and 74% inhibition on COX-2, respectively.

rats was significantly less than the sham group, and this trend continued throughout the experiment. After administration of CM-HAS, the AA rats exhibited a significant weight gain (Table 3). The index of thymus and spleen of AA rats were determined at day 21 after immunization. It was found that there was a decrease of thymus and spleen in AA rats. The administration of CM-HAS (5, 10 mg/kg, s.c.) evidently increased the weight of thymus and spleen of AA rats, the same result as leflunomide (20 mg/kg, i.g.; Fig. 1).

3. Effects of CM-HAS on histopathology of AA rats

Histologically, AA rats had severe infiltration of inflammatory cells, with disruption and loss

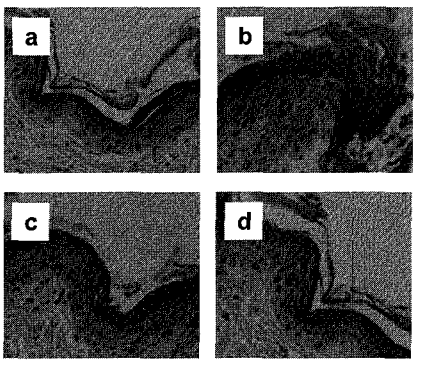

Fig. 2. Changes of the histological morphology of Rat Knee Joints by HE staining. × 100. (A) Sham rats showed normal articular cartilage and absence of infiltration in the synovium. (B) Rats with AA showed marked infiltration of inflammatory cells, with disruption and loss of articular cartilage. (C or D) AA rats treated with CM-HAS (10 mg/kg) or leflunomide showed less articular cartilage damage and inflammatory cells infiltration.

of articular cartilage. In the rats treated with CM-HAS, those histological finding were markedly improved (Fig. 2).

4. Inhibition of cyclooxygenase-2 by CM-HAS

The inhibitory effects of CM-HAS on arachidonic acid metabolites, PGE2 and PGD2, were measured. Aspirin showed 55% inhibition of COX-2 at 0.306 mg/㎖ and indomethacin showed 74% inhibition at 10 ug/ml. The negative controls showed no inhibitory activity. Table 4 shows the percentage inhibition of PGE2 and PGD2 conversion from arachidonic acid by CM-HAS. CM-HAS inhibited COX-2 from 23.3 to 90.2% at concentrations of 1.5 and 3. mg/㎖.

Discussion

Plants used in folk medicine have been accepted as one of the main sources of drug discovery and development. Natural products of plant origin are still a major part of traditional medicinal systems in developing countries. There is also a resurgence of interest in herbal medicines in western countries as an alternative source of drugs often for intractable diseases such as rheumatoid arthritis[21]. In Korea, there is a rich treasury of ethnobotanical knowledge and over the past decade we have been widely engaged in research on this subject. During our field studies, we have coincided several Oriental and herbal remedies used in the treatment of rheumatism-related inflammatory diseases.

The aim of this study was to investigate the effects and mechanisms of *Clematis mandshurica* Maxim. herbal acupuncture solution (CM-HAS) on rat adjuvant arthritis (AA). Complete Freund's adjuvant was used to induce AA in rats. CM-HAS was investigated by using an in vitro screening method based on the inhibitory effects on cyclooxygenase & COX-2 production and histopathology.

Although previous studies showed that CM-HAS could prevent the onset and progression of AA and collagen-induced arthritis[4,5], the mechanisms of CM-HAS on arthritis are not fully understood. Because AA was often used as an animal model of RA in the evaluation of anti-rheumatic drugs[18], the present study was to elucidate the effects and mechanisms of CM-HAS on AA. The results demonstrated that CM-HAS markedly inhibited joint swelling and the index of polyarthritis, and significantly reduced the histological degrees of joint injury. Increase of body weight was also apparently improved in the rats treated with CM-HAS. The above results suggested that CM-HAS would be effective in rat AA. Inflammatory paw edema by CM-HAS stimulated at *Sinsu-hyul* (BL 23) locus at low and high doses (2~10mg/kg) were significantly reduced in this animal model during the experimental duration (14th-21st day).

The increase of body weight between immunized and nonimmunized rats was compared. Beginning on day 7, the body weigh increase of immunized rats was significantly less than the sham group, and this trend continued throughout the experiment. After administration of CM-HAS, the AA rats exhibited a significant weight gain. The index of thymus and spleen of AA rats were determined at day 21 after immunization. It was found that there was a decrease of thymus and spleen in AA rats. Also, AA rats had severe infiltration of inflammatory cells, with disruption and loss of articular cartilage. In the rats treated with CM-HAS, those histological findings were markedly improved.

I investigated the inhibitory effects of CM-HAS

on arachidonic acid metabolites, so PGE2 and PGD2 were measured. Aspirin showed 55% inhibition of COX-2 at 0.306 mg/㎖ and indomethacin showed 74% inhibition at 10 ug㎖ l. The negative controls showed no inhibitory activity. Table 4 shows the percentage inhibition of PGE2 and PGD2 conversion from arachidonic acid by CM-HAS. CM-HAS inhibited COX-2 from 23.3 to 90.2% at concentrations of 1.5 and 3. mg/㎖. Therefore, I found that CM-HAS could be potent to regulate the inflammation at the chronic phase of the disease of rodent adjuvant-induced arthritis.

In summary, these results strongly suggested that CM-HAS could be useful for treating & inhibiting joint swelling and production of COX-2 associated with poly arthritis. Our results suggest that the effect of CM-HAS in the inhibition of inflammatory diseases may be partially associated with the down-regulation of COX-2. CM-HAS has great potential as an alternative treatment, and has no adverse effects. CM-HAS can be given on *Sinsu-hyul* (BL 23), and it inhibits disease progression by both controlling inflammatory proteins and protecting cartilage. CM-HAS warrants further investigation, including preclinical and clinical studies. We are now in progress to isolate active molecules.

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