

## Inhibitory Effect of Whole Bee Venom in Adjuvant-induced Arthritis

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**Abstract.** *The aim of this study was to assess the inhibitory effect of whole bee venom (BV) on adjuvant-induced arthritis in the rat. Rats were divided into pre-apitherapy, post-apitherapy and control experimental groups. The pre-apitherapy group was subcutaneously stung with a honeybee (*Apis mellifera* L.) and the control group was subcutaneously injected with 0.1 ml of physiological saline solution one day prior to complete Freund's adjuvant (CFA) injection. The post-apitherapy group was subcutaneously stung with a honeybee on day 14 after CFA injection. When arthritis had developed in the rat, the post-apitherapy group was subcutaneously administered whole BV every other day for a further 14 days. Clinical signs, hematological values and radiological features were observed during the entire experimental period. In the pre-apitherapy group, the development of inflammatory edema and polyarthritis was inhibited. Significant differences in lameness score, hind paw edema volume and radiological features were observed between control and pre-apitherapy rats. White blood cell counts indicated that the degree of leucocytosis was significantly different between the pre-apitherapy and control groups ( $p < 0.01$ ). Inflammatory edema, polyarthritis and bone change into the right hind paw were effectively inhibited in pre-apitherapy rats during the two-week period post-CFA injection. In conclusion, whole BV was found to inhibit arthritic inflammation and bone changes in the rat. This may be an alternative treatment for arthritis in humans.*

Bee venom (BV) has been utilized as a traditional, alternative medicine for the relief of pain and treatment of

inflammatory diseases, such as rheumatoid arthritis (RA) in humans (1). Moreover, it contains several pharmaco-active substances that could account for its tocolytic activity in treating arthritis. Regulation of radical production (2), suppression of *alpha 1-acid glycoprotein* gene induction (3), and inhibition of phospholipase (PL) A<sub>2</sub> activity (4) have all been proposed as possible mechanisms of its anti-inflammatory effects. As in snake venom, PLs are major active components of BV. These chemical mediators are normally released from phagocytic lysosomes during the course of inflammation (5) and cleave phospholipids of the cell membrane to produce arachidonic acid, which is eventually converted into prostaglandins (PGs). Furthermore, a novel PLA<sub>2</sub> inhibitor has been demonstrated to block both acute and chronic inflammation (6).

Since PGs have been shown to have preventive and suppressive action against adjuvant arthritis in rats (7), it is possible that injections of honeybee venom in rats with arthritis might have a therapeutic effect similar to that of either PGs or anti-inflammatory drugs. In experimental animals, adjuvant-induced arthritis has previously been shown to be suppressed by long-term BV treatment (8, 9). BV and/or its constituents have also been reported to be effective in the treatment of RA in humans (10). Recently, we also demonstrated that whole BV produced anti-inflammatory effects in a complete Freund's adjuvant (CFA)-induced arthritis model (11).

This study was designed to investigate the inhibitory effects of pre- and post-treatment with whole BV in an animal model of CFA-induced arthritis. Its influence on both paw edema and radiological features produced by CFA injection into the hind paw were evaluated.

### Materials and Methods

**Animals.** Ninety male Sprague-Dawley rats (30 rats/group) were used. The animals were 6 weeks old and weighed  $180.5 \pm 14.7$  g at the time of study initiation. The rats were kept in a colony room with an ambient temperature of 22°C and a 12-h alternating light-

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dark cycle (09:00 onset). They were housed in cages and allowed water and food *ad libitum*.

**Treatments.** Arthritis was induced by CFA (Life Technologies, USA). A single injection of 1 mg *Mycobacterium butyricum* suspended in 0.1 ml paraffin oil was administered subcutaneously into the right hindpaw. The righting reflex was uniformly lost on day 14 after CFA injection, and this was considered to be the point of arthritis development (time of injection=day 0). The pre-apitherapy group was subcutaneously stung with a honeybee (*Apis mellifera* L.) and the control group was subcutaneously injected with 0.1 ml of physiological saline one day before CFA injection. When arthritis had developed, the post-apitherapy group was subcutaneously administered whole BV on alternate days for a further 14 days, beginning on day 15. The protocols employed in this study were approved by the Animal Care Committee at Chungbuk National University, Republic of Korea.

**Honeybees for apitherapy.** Natural honeybees (*Apis mellifera* L.) used for stinging in this study were raised on the farm of the Agricultural College of Chungbuk National University. Bees of about 15 days old (after metamorphosis) were used since they are known to have approximately 0.1 mg BV in their poison sac – a strong bee sting – and are easy to use for acupuncture.

**Clinical assessment of arthritis.** Lameness score, body weight, edema volume, hematological values and bone changes were observed clinically during the entire experimental period. The criterion of lameness score was based on severity with a range of 0 to 3: 0-plain, 1-mild, 2-moderate and 3-severe.

Edema volumes were assessed with a plethysmometer (Ugo Basile, Italy) as the change of water volume. The magnitude of the initial inflammatory response was evaluated by measuring the volume of both hind paws at day 14. Edema was calculated as the mean increase in paw volume.

**Measurement of leucocyte counts.** Peripheral blood samples were obtained at 0, 7, 14, 21 and 28 days after CFA treatment. Total peripheral blood cells were counted with a blood cell counter (Celltac MEK-4000, Nihon Kohden Co., Japan).

**Radiographic assessment of bone changes.** Hind limb radiographs were obtained using an X-ray unit (BLD-15RK, Dong-A X-ray Co., Korea) on 14 x 14-inch diagnostic film (medium speed, Kodak Co., USA) exposed at 51 kVp and 7.5 mAs. Rats were anesthetized with xylazine HCl (0.1 mg/kg, *i.m.*) and ketamine HCl (0.2 mg/kg, *i.m.*) prior to X-ray. Whole bodies were X-rayed using a 90-degree projection from the dorsal-ventral aspect.

The severity of bone changes was assessed blindly from the radiographs by grading osteoporosis, erosions and joint space narrowing in the distal tibia and calcaneus. A grade of 0 to 3 was assigned to each bone area on the basis of the following scoring system (12): 0=no change, 1=dimming of bone surface and minimal exostosis in a few areas by lyses of the compact substance and periosteal new bone formation and/or mild osteoporosis in some areas with little change in bone area size, 2=dimming of bone surface in all areas, exostosis by new bone formation, joint space narrowing and moderate osteoporosis, and 3=physiological structures no longer detectable due to the irregular proliferation of spongiosa, entire loss of joint spaces and severe osteoporosis.

Each paw was graded and the 4 scores were added to obtain a maximum possible score of 12.

**Statistical analysis.** All data are expressed as the mean±SD and were analyzed using a one-way analysis of variance (ANOVA). Significant differences between groups were detected with a paired Student's *t*-test. The criterion for significance was  $p<0.05$ .

## Results

Subcutaneous administration of CFA at a site on the right hindpaw induced inflammatory edema and polyarthritis during the two-week study period. The effects of whole BV on CFA-induced arthritis in the rat are presented in Figures 1 and 2. The inhibitory effect of whole BV, compared with control, on the lameness score is illustrated in Figure 1. For both end-points, significant differences between control and pre-apitherapy groups were observed throughout the experimental period. When body weight was analyzed, it was evident that rats in the control group lost weight over the experimental period, which was probably a reaction to the stressful pain, while both groups of rats treated with BV gained weight (Figure 2).

While rats injected with saline had severe symptoms of inflammatory edema, this effect was ameliorated in those pre-treated with BV (Tables I and II). Furthermore, while significant leucocytosis developed in the control group ( $p<0.01$ ), rats pre-treated with whole BV did not display any marked change in white blood cell count over the study period (Table III). On the contrary, the red blood cell count, hematocrit and hemoglobin did not differ among the three groups (data not shown).

To further study the influence of whole BV on arthritis, radiological features were investigated (Figure 3). Bone changes indicative of CFA-induced arthritis became apparent radiographically on day 14. The control group had widespread hind paw edema, polyarthritis and large areas of erosion with persistent, severe peri-articular soft tissue swelling emerging from day 21. Radiological bone changes in the tibia were effectively inhibited in the pre-apitherapy group. X-ray scores of the control group were significantly higher than those of the pre- and post-apitherapy groups from days 21 to 28 ( $p<0.01$ ). Pre-apitherapy suppressed bone changes in the hindpaw over the entire experimental period. Saline did not inhibit bone changes induced by CFA.

## Discussion

The rat CFA model has been used to induce an arthritic immunopathological disease that displays many pathological features of human RA (13). Unilateral injection of CFA into the paw induces 'primary' inflammatory signs and hyperalgesia within hours at the site of inoculation. Subsequently, 'secondary' inflammation and pro-nociceptive

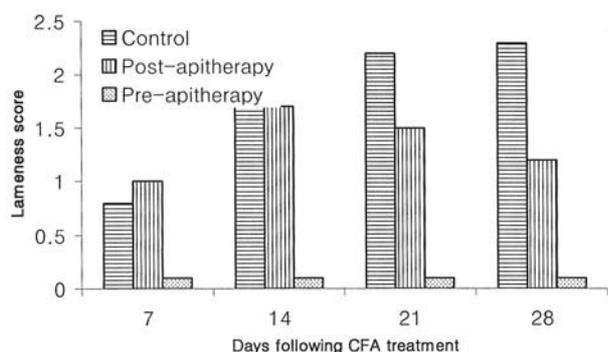


Figure 1. Lameness scores in rats with adjuvant arthritis. The pre-apitherapy group was subcutaneously stung with a honeybee and controls were subcutaneously injected with 0.1 ml of physiological saline solution one day before CFA injection. The post-apitherapy group was subcutaneously stung with a honeybee on day 14 after CFA injection. Lameness score: 0-plain, 1-mild, 2-moderate, 3-severe.

signs appear between days 10 to 15 post-inoculation, especially in the contralateral paw. The hyperalgesia associated with CFA may persist for 8 weeks after injection (14). This CFA-induced arthritis model has been used extensively to analyze the anti-inflammatory/anti-nociceptive effects of new drugs with potential therapeutic application to chronic arthritis (15).

BV has traditionally been used in oriental medicine to relieve pain and to treat chronic inflammatory diseases such as RA (1, 8, 9). It has also been widely used by scientists and medical personnel. Whole BV is a complex mixture of substances such as PLA, hyaluronidase, melittin, apamin, peptide 401 and a myriad of other, as yet unidentified, factors (15). The known constituents of BV have been demonstrated to produce a variety of physiological and pharmacological changes in experimental animals. For example, Vick and Shipman (16) have shown that of the different fractions of crude BV separated using a column, PLA was identified as being the most powerful component since it caused a precipitous fall in arterial blood pressure and heart rate in the dog. Furthermore, a pharmacological PLA inhibitor was found to suppress BV-derived enzymes *in vitro* (6). Moreover, peptide 401 was found to be effective in edema (17), whereas the other two basic peptides, melittin and apamin, produced sharp elevations in plasma cortisol. The purported involvement of the adenohypophysis in anti-inflammatory actions, through adrenal cortex-derived corticosterone production (16, 18, 19), has been disputed by Hanson and colleagues (20) who found that adrenalectomized animals were also sensitive to anti-inflammatory effects.

In the present study, pre-treatment with whole BV was used to evaluate its effectiveness as a curative agent for arthritis. Whole BV was infused subcutaneously by stinging

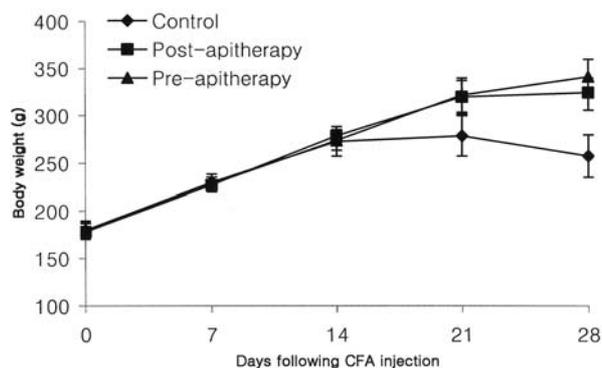


Figure 2. Changes in body weight in rats with arthritis. Data are expressed as mean  $\pm$  SD ( $n=10$ ). The pre-apitherapy group was subcutaneously stung with a honeybee and controls were subcutaneously injected with 0.1 ml of physiological saline solution one day before CFA injection. The post-apitherapy group was subcutaneously stung with a honeybee on day 14 after CFA injection. \* $p<0.05$  significantly different compared to the control group.

on alternate days for 14 days once the righting reflex was uniformly lost. The amount of venom from each live bee sting was presumed to be approximately 0.1 mg, based on previous reports (16). The appearance of edema followed a time-course similar to that previously described in other studies (8, 14). The development of arthritis-associated thermal and mechanical hyperalgesia, following the induction of systemic arthritis, begins at approximately 9 days after CFA injection, as previously reported by Philippe *et al.* (14). Here, in agreement with other studies, whole BV administration effectively inhibited the development of edema, a characteristic sign of inflammation, in both hind paws in arthritic rats (8, 21). Numerous inflammatory mediators, including histamine, prostaglandins, leukotrienes and bradykinins, are released at sites of inflammation (22). Both pre- (23) and post-treatment (24) with dry BV significantly inhibited lameness, and this effect is most probably mediated through anti-inflammatory and counter-irritant actions. As described above, BV constitutes a variety of different peptides, including melittin, apamin, adolapin and mast cell degranulating (MCD) peptide (17). Although adolapin and purified MCD peptide are known to have anti-inflammatory activity (25), these substances are present in very small quantities (1-2%) in whole BV. It is possible, however, that these peptides play a minor role in the anti-inflammatory effect of BV at the dose used in the present study. Melittin is a major component of BV (50% of dry weight) and binds to secretory PLA<sub>2</sub> and inhibits its enzymatic activity (4). Because PLA<sub>2</sub> is a major inflammatory trigger whose activity is enhanced in rheumatoid arthritis, it is possible that the formation of a melittin-PLA<sub>2</sub> complex by BV injection effectively suppresses some of the symptoms associated with the development of arthritis (23). However, paradoxically,

Table I. Changes in right hind paw volume in arthritic rats. (Unit: ml)

Group	Days after CFA treatment				
	0	7	14	21	28
Control	2.56±0.04	3.19±0.11	3.60±0.64	3.83±0.93	4.48±0.66
Post-apitherapy	2.57±0.05	3.23±0.10	3.58±0.14	3.45±1.09	3.39±0.56*
Pre-apitherapy	2.66±0.04	2.78±0.28	3.12±0.43	3.23±0.37	3.26±0.48*

Data are expressed as mean±SD (n=10). Significant differences as compared with control: \**p*<0.05. Controls were subcutaneously injected with 0.1 ml of physiological saline solution and the pre-apitherapy group was subcutaneously stung with a honeybee one day before CFA injection. The post-apitherapy group was subcutaneously stung with a honeybee on day 14 after CFA injection.

Table II. Changes in left hind paw volume in arthritic rats. (Unit: ml)

Group	Days after CFA treatment				
	0	7	14	21	28
Control	2.57±0.05	2.65±0.08	2.76±0.05	2.92±0.08	3.22±0.06
Post-apitherapy	2.56±0.03	2.68±0.09	2.75±0.06	2.88±0.30	2.84±0.04*
Pre-apitherapy	2.66±0.04	2.66±0.06	2.67±0.09	2.70±0.07	2.71±0.08*

Data are expressed as mean±SD (n=10). Significant differences as compared with control: \**p*<0.05. Controls were subcutaneously injected with 0.1 ml of physiological saline solution and the pre-apitherapy group was subcutaneously stung with a honeybee one day before CFA injection. The post-apitherapy group was subcutaneously stung with a honeybee on day 14 after CFA injection.

Table III. Changes in white blood cell counts in arthritic rats. (Unit: x10<sup>3</sup>/μl)

Group	Days after CFA treatment				
	0	7	14	21	28
Control	9.40±2.26	14.51±2.19	15.40±2.57	16.80±0.70	22.13±2.20
Post-apitherapy	10.42±0.93	16.26±1.95	15.40±1.44	14.60±0.47	14.46±1.74**
Pre-apitherapy	10.11±1.07	14.46±1.16	11.76±1.67*	13.60±2.98	13.60±2.55**

Data are expressed as mean±SD (n=10). Significant differences as compared with control: \**p*<0.05, \*\**p*<0.01. Controls were subcutaneously injected with 0.1 ml of physiological saline solution and the pre-apitherapy group was subcutaneously stung with a honeybee one day before CFA injection. Post-apitherapy was subcutaneously stung with a honeybee on day 14 after CFA injection.

melittin injection into mouse paws elicits paw edema at 60 minutes after injection (26).

While the rats in the control group showed a trend of decreasing body weight due to pain, both pre- and post-BV-treated rats gained weight over the study period. These changes in body weight were very similar to the observations of Zurier *et al.* (5). Furthermore, rats treated with BV did not develop leucocytosis or anemia, both characteristics of the

disease, also consistent with the former study (5). The control group did exhibit significant leucocytosis as compared to the other groups. Previously, pre-treatment with whole BV has been demonstrated to significantly reduce peripheral edema (23), and an anti-inflammatory effect of BV has been reported in a CFA-induced chronic arthritis model (8, 9).

Thus, to further assess the influence of BV in this rat model, inflammatory edema, polyarthritis, periarticular

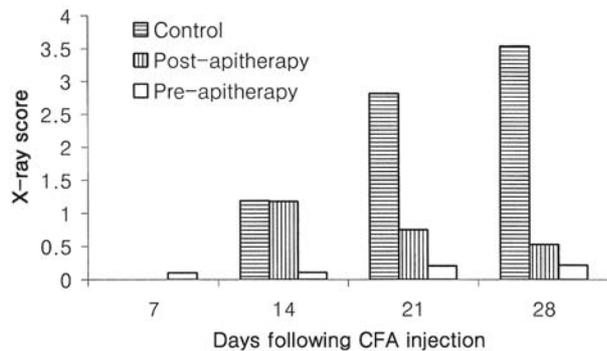


Figure 3. Total X-ray scores in rats with adjuvant arthritis following complete Freund's adjuvant administration.

tissue, erosions and joint space in the distal tibia were monitored. Pre-apitherapy protected the joint space by inhibiting inflammatory edema, leucocytosis and erosion of articular cartilage. Radiological scores of the pre-apitherapy group were significantly lower than those of the control group during the entire experimental period. Pre-apitherapy inhibited the development of poly-arthritis in CFA-induced arthritic rats during our study period. The major radiological changes in control rats appeared in the injected hind paw on day 14 after CFA injection.

In summary, whole BV ameliorated inflammatory edema, lameness and polyarthritis of both hind paws in rats. This treatment also decreased the infiltration of leucocytes and the erosion of articular cartilage into joints and inhibited the development of leucocytosis. These observations may explain the reported preventive and therapeutic applications of BV.

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