

The Antinociceptive and Anti-inflammatory Effect of Ethylacetate Extracts from Bang-Poong (*Radix ledebouriellae*) on the Freund's Adjuvant-Induced Arthritis in Rats

Hyun-Woo Kim¹, Young-Bae Kwon¹, Tae-Won Ham¹, Dae-Hyun Roh¹, Seo-Yeon Yoon¹, Ho-Jae Han², Sung-Keel Kang³, Hye-Jung Lee³, Woung-Chon Mar⁴, Il-Suk Yang¹, Alvin J. Beitz⁵ and Jang-Hern Lee^{1*}

¹Department of Veterinary Physiology, College of Veterinary Medicine and School of Agricultural Biotechnology, Seoul National University, Suwon, South Korea

²Hormone Research Center, College of Veterinary Medicine, Chonnam National University, Kwang-ju, South Korea

³Department of Acupuncture and Moxibustion, College of Oriental Medicine, Kyung-Hee University, Seoul, South Korea

⁴Natural Products Research Institute, Seoul National University, Seoul, South Korea

⁵Department of Veterinary Pathobiology, College of Veterinary Medicine, University of Minnesota, St Paul, MN, 55108, USA

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Abstract

In this study, we aimed to determine the antinociceptive and/or anti-inflammatory effect of Bang-Poong (BP, *Radix Ledebouriellae*) on Freund's adjuvant-induced arthritis in rats. Traditionally, BP has been used to treat several inflammatory diseases such as arthritis. Whole BP is extracted into two fractions that were ethylacetate and hexane-soluble fractions. Adult Sprague-Dawley rats (n=30, 130-150 g) were subcutaneously administered by the Freund's complete adjuvant (FCA) into the plantar surface of right hindpaw. Twelve days after the injection of FCA, the rats initially showed typical inflammatory edema and arthritis-related symptoms on the contralateral side (i.e. left hindpaw). Both antinociceptive (evaluation of mechanical, thermal pain threshold and analysis of spinal Fos expression) and anti-inflammatory (evaluation of paw edema, serum interleukin-6 level and x-ray analysis) effect of BP extracts were examined. The ethylacetate fraction of BP (BPE) significantly suppressed the FCA-induced paw edema as well as the serum level of interleukin-6 and it alleviated the radiological changes. Moreover, both mechanical and thermal hyperalgesia were attenuated by the treatment of BPE. In addition, spinal Fos expression that was increased by FCA-injection was suppressed in BPE group. Therefore, this study showed that BPE produced significant both antinociceptive and anti-inflammatory effects on FCA-

induced arthritis in rats, while hexane fraction of BP did not show these effects. In conclusion, it is suggested that the ethylacetate fraction of BP is recommended to alleviate the arthritis-related symptoms in human according to the results of this study.

Key words : Bang-Poong, *Radix Ledebouriellae*, antinociception, anti-inflammation, arthritis, rat.

Introduction

Clinically Bang-Poong (BP, *Radix Ledebouriellae*) has been widely used to treat several inflammatory diseases such as arthritis in oriental medicine. Although the anti-inflammatory effect of BP has not been critically examined, numerous studies have been performed to determine the anti-inflammatory effect of *Radix* herb (4, 12, 14, 15). It is reported that *Radix astragali* extract suppresses interleukin-6 elevation, tumor necrosis factor-alpha productions, prostaglandin E2 biosynthesis, and leukotrien C4 production from lipopolysaccharide-stimulated human amnion cells (14). Moreover, topically treated wogonin (5,7-dihydroxy-8-methoxyflavone), isolated from *Radix scutellaria*, inhibits cyclooxygenase 2 expression and prostaglandin E2 production induced by multiple treatments with 12-O-tetradecanoylphorbol-13-acetatein (TPA) in mouse skin (12). In addition, it is also reported that *Radix glycyrrhizae* produces suppressive effect on TPA-induced inflammation and TPA-induced tumor promotion in two-stage carcinogenesis in mouse skin (15). In oriental medicine, there are several acupunctural techniques such as manual acupuncture, acupressure, electroacupuncture, and moxibustion. One of acupunctural techniques, herbal acupuncture has been widely used to treat several diseases. Herbal acupuncture is acupoint stimulation by injection of medical herb extract into acupoint. Because of pharma-

* Corresponding author: Jang-Hern Lee

Department of Veterinary Physiology, College of Veterinary Medicine, Seoul National University, Suwon 441-744, South Korea
Tel : +82-31-290-2732, Fax : +82-31-291-0536
E-mail : JHL1101@snu.ac.kr

cological effect of medical herb with traditional acupuncture effect, the application of herbal acupuncture has been gradually increased. BP is known to be one of effective medical herbs on human inflammatory diseases. In this study, we demonstrated that the anti-arthritic effect of herbal acupuncture with BP in rats.

Rheumatoid arthritis (RA) is a degenerative disease in human that is characterized by degenerative joint destruction, deformity and inflammatory pain in most cases. Currently non-steroidal anti-inflammatory drugs (NSAIDs) such as indomethacin are commonly used to cure the RA. Although the strong alleviative effect of NSAIDs on inflammatory diseases, those use to human is strictly limited because those produce severe adverse effect including gastric ulcer and dysfunction (2). For this reason, BP remedy is still performed because it produces strong curative effect with just a few adverse effects in human. To find effective component of BP on inflammation, ethylacetate fraction of BP (BPE) and hexane fraction of BP (BPH) were extracted from whole BP and antinociceptive and anti-inflammatory effect on experimentally evoked RA in rats was demonstrated.

Materials and Methods

Animals

Male Sprague-Dawley rats (the Laboratory Animal Research Center of Seoul National University, Seoul, Korea, n=30) weighing 130-150 g were used in this study. Animals were housed in colony cages with free access to food and water. The food was given on the wooden bed for animals under pathological state to take easily. They were maintained in temperature and light controlled rooms ($23 \pm 0.5^\circ\text{C}$, 12/12h light/dark cycle with lights on at 07:00). All of the methods used in the present study were approved by the Animal Care and Use Committee at SNU and conform to NIH guidelines (NIH publication No. 86-23, revised 1985). The ethical guidelines of the International Association for the Study of Pain (16) for investigating experimental pain in conscious animals was also followed.

The induction of arthritis

Arthritis was induced as previously used method by Kwon et al. (9). Animals were initially anesthetized with 3 % isoflurane in a mixed N₂O/O₂ gas. Then Freund's complete adjuvant (FCA) containing heat-killed *Mycobacterium butyricum* (Difco Laboratory, MI, USA) suspended in sterile mineral oil (20 mg/ml) was single injected subcutaneously into the plantar surface of right hindpaw at a volume of 50 μl .

BP treatments and experimental groups

Whole BP is extracted to two fractions such as BPE and BPH in Natural Products Research Institute of Seoul National University (Seoul, Korea). The air-dried roots of BP (500 g) were extracted with methanol during 3 days. The methanol extracts were evaporated using rotary evaporator,

suspended in water and fractionated successively with n-hexane and ethyl acetate respectively.

To investigate curative effect of BPE and BPH, we preliminarily examined the suppressive effect of these fractions in arthritic rats with various doses of BPE and BPH (data not shown). As a consequence of preliminary study, both BPE and BPH with a dose of 1mg/kg/day were showed the most significant effect on arthritis. Experimental groups were divided into 3 groups; (1) control group treated by saline/ethyl alcohol (9:1, vol/vol) (RA-vehicle, n=10), (2) BPE treatment group (RA-BPE, n=10), and (3) BPH treatment group (RA-BPH, n=10). BPE (1 mg/kg/day) and BPH (1 mg/kg/day) were dissolved in saline and ethyl alcohol solution with ratio of 9:1 (vol/vol), respectively and administered subcutaneously and bilaterally into lateral side of the knee that was adjacent site of inflammation. BP treatment was started the day after adjuvant injection and animals were injected daily for 3 weeks. All algometric assays were performed beginning 9 days after adjuvant injection at the time of induction of systemic arthritis (13).

The evaluation of antinociceptive effect of BPE and BPH

Mechanical hyperalgesia test

The analgesy meter (LETICA, LE7356) was used to evaluate mechanical hyperalgesia in arthritic animals (Randall-Selitto method). A graded mechanical force (g) was delivered onto the convex surface of the left paw. Mechanical threshold was determined by these two indices; (1) withdrawal behavior or (2) vocalization. The mechanical threshold in normal animal was ranged from 140 to 160 g. The test was duplicated at 5 min intervals and mean value was applied to analyze.

Thermal hyperalgesia test

To determine the thermal hyperalgesia of arthritic animals, Hargreaves' method was used as previously described (5). Rats were acclimated in a plastic chamber with a glass floor for 5 min. Then a radiant heat was focused under glass floor beneath the hind paw. The withdrawal latency (sec) was measured using photosensitive cell connected to a digital clock. The intensity of light source was calibrated to produce withdrawal within 9-10 sec in normal animals. The test was duplicated at 5 min intervals and mean value was applied to analyze.

Fos immunohistochemistry and image analysis

Spinal Fos expression was performed to analyze the antinociceptive effect of BP as previously described (9). Briefly, animals were deeply anesthetized with isoflurane, perfused transcardially with calcium-free tyrode's solution, followed by a fixative containing 4 % paraformaldehyde and 0.2 % picric acid in 0.1M phosphate buffer (pH 6.9). Then spinal cord was removed immediately, post-fixed in same fixative and then cryoprotected in 30 % sucrose in phosphate buffered saline (pH 7.4). Frozen serial frontal sections (40

μm) were cut through the lumbar L3-L5 spinal cord using a cryostat (Microm, Germany). After quenching with 0.3 % hydrogen peroxide and preblocking with 1 % normal goat serum (0.3 % triton X-100/ 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 (11). Fos-like immunoreactive (FLI) neurons were visualized using a 3-3 diaminobenzidine reaction intensified with 0.2 % nickel chloride.

Tissue sections were examined using dark field microscopy (Zeiss Axioscope, Germany) to determine the segmental level according to Abbadie and Besson (1) as well as the gray matter landmarks to define individual spinal cord laminae. Individual sections were digitized with 4096 gray levels using a cooled CCD camera (Micromax Kodak 1317, Princeton Instruments, AZ, USA) connected to a computer-assisted image analysis system (Metamorph, Universal Imaging, PA, USA). Image analysis of spinal Fos expression was followed by the method of Kwon et al. (9), as previously described. The following four gray matter regions were selected for analysis based on cytoarchitectonic criteria: (1) superficial dorsal horn (SDH, laminae I and II); (2) nucleus proprius (NP, laminae III and IV); (3) neck (NECK, laminae V and VI); and (4) the ventral horn (VENT, laminae VII-IX).

The evaluation of anti-inflammatory effect of BPE and BPH

Paw volume

Paw volume of left hind paw was measured by a water displacement plethysmometer (UGO BASIL, Italy) every 3 day during 3 weeks after adjuvant injection. Paw volume was measured by blind experimenter and performed twice and mean value was recorded for analysis. Paw volume measured just before the adjuvant injection was used as the control volume (day 0).

X-ray analysis

All hind paws in each group were exposed to x-ray film (10 mA sec, 40kV). Then these images were analyzed using image analysis system (Metamorph, Universal Imaging Corporation, PA, USA) by two categories such as soft tissue swelling and bone proliferation. Each value was calculated by these equations;

- (1) Area of soft tissue = [whole area of paw] - [bone area]
- (2) Area of soft tissue swelling = [area of inflamed soft tissue] - [area of normal soft tissue]
- (3) Area of bone proliferation = [area of proliferated bone] - [area of normal bone]

Serum concentration of interleukin-6

At the end of whole experiment, rats (n=10 each group) were sacrificed and blood was collected by cardiac syringe puncture. Collected blood samples were placed in temperature-regulated chamber (37 °C) for 1 hour. Then, this blood

samples were centrifuged (15,000 rpm) in 4°C during 15 min and supernatant serum was collected. The enzyme-linked immunosorbent assay kit (cytoscreen, Biosource International Inc., CA, USA) was used to determine the serum concentration of interleukin-6 (IL-6). Blood samples of sham animals (n=10) were simultaneously tested to compare the normal and arthritic serum level of IL-6 in this experiment.

Statistical analysis

Thermal and mechanical hyperalgesia data were expressed as percent change and compared to that of the sham group at each time point. Data were expressed as the mean SEM. Repeated measures ANOVA were performed to determine the overall effect. Paired *t*-tests were then used to determine probability values when repeated measures ANOVAs indicated a significant drug effect. Throughout, $P < 0.05$ was considered to be statistically significant.

Results

The antinociceptive effect of BPE and BPH

In RA-vehicle group, the mechanical threshold in left hind paw was significantly decreased about 50 % as compared with that of normal animal from 12 days after adjuvant injection (Fig. 1). BPE treatment dramatically increased the mechanical pain threshold and there was a statistical significance between vehicle and BPE treated groups. In contrast, BPH did not produce an increase of mechanical pain threshold in this test. The paw withdrawal latency (PWL) of animals in RA-vehicle group significantly decreased in thermal hyperalgesia test and the PWL of RA-vehicle group was about 60% as compared with that of normal animal (Fig. 2). Likely to mechanical hyperalgesia test, BPE treatment strongly increased the PWL from 12 days after adjuvant injection to the end of this study. However, BPH produced a similar level of PWL with that of RA-vehicle group. Analysis result of spinal Fos expression was represented in Fig. 3. In RA-vehicle group, Fos expression was dramatically increased in the spinal cord such as 28.65 ± 3.40 in SDH, 14.20 ± 1.70 in NP, 14.95 ± 1.72 in NECK, and 3.30 ± 0.50 in VENT. BPE treatment significantly decreased the number of Fos-positive neurons in SDH (18.86 ± 1.93) and NP (7.86 ± 0.86) as compared to that of RA-vehicle group. However, BPH treatment did not suppress the number of Fos expression in every region of spinal cord as compared with that of RA-vehicle group. The pattern of spinal Fos expression was represented in Fig. 7.

The anti-inflammatory effect of BPE and BPH

Intraplantar injection of FCA rapidly produced typical inflammatory swelling and redness in the injected right hind paw. As time goes on, arthritis is transferred into contralateral left side, fore paws and tail. In the left hind paw, paw volume was initially increased 12 days after adjuvant injection in systemic arthritic phase (Fig. 4). The

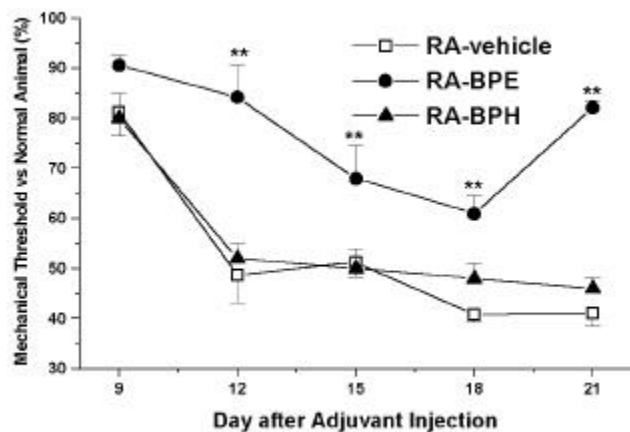


Fig. 1. Effect of BPE and BPH on adjuvant-induced mechanical hyperalgesia. Animals of RA-vehicle group showed the significantly decreased level of mechanical pain threshold. In contrast to this BPH did not increase the mechanical pain threshold as compared with that of RA-vehicle group. (Abbreviations) **RA**: rheumatoid arthritis, **BPE**: ethylacetate fraction of Bang-Poong, **BPH**: hexane fraction of Bang-Poong. ** $p<0.01$, significantly different from that of RA-vehicle group.

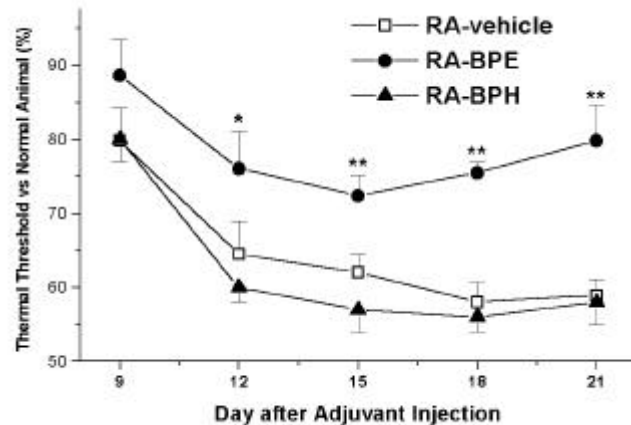


Fig. 2. This represented that the effect of BPE and BPH on PWL in arthritic rats. BPE treatment significantly increased the PWL, however, BPH did not produce this effect. (Abbreviations) **PWL**: paw withdrawal latency. * $p<0.05$ and ** $p<0.01$, significantly different from that of RA-vehicle group, respectively.

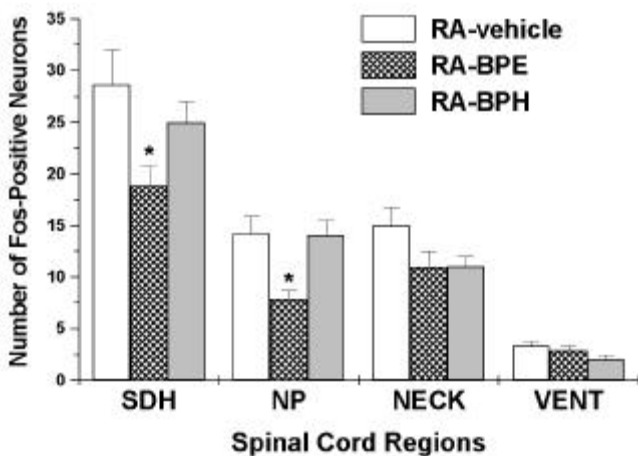


Fig. 3. Effects of BPE and BPH on spinal Fos expression. BPE treatment significantly reduced the number of Fos-positive neurons in SDH (18.861.93) and NP (7.860.86) of the spinal cord, respectively (* $p<0.05$). However, BPH did not reduce the number of spinal Fos-positive neurons in this study. (Abbreviations) **SDH**: spinal dorsal horn, **NP**: nucleus of proprius. * $p<0.05$, significantly different from that of RA-vehicle group.

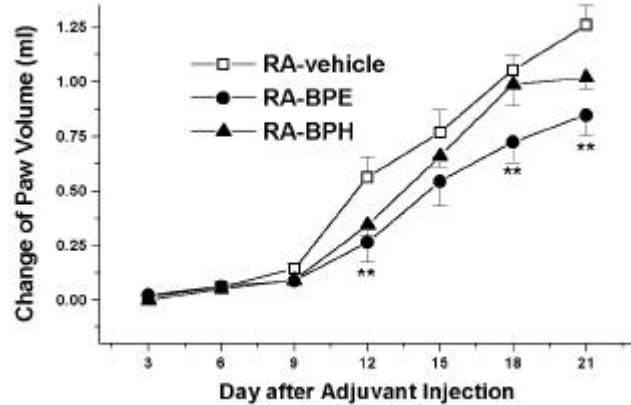
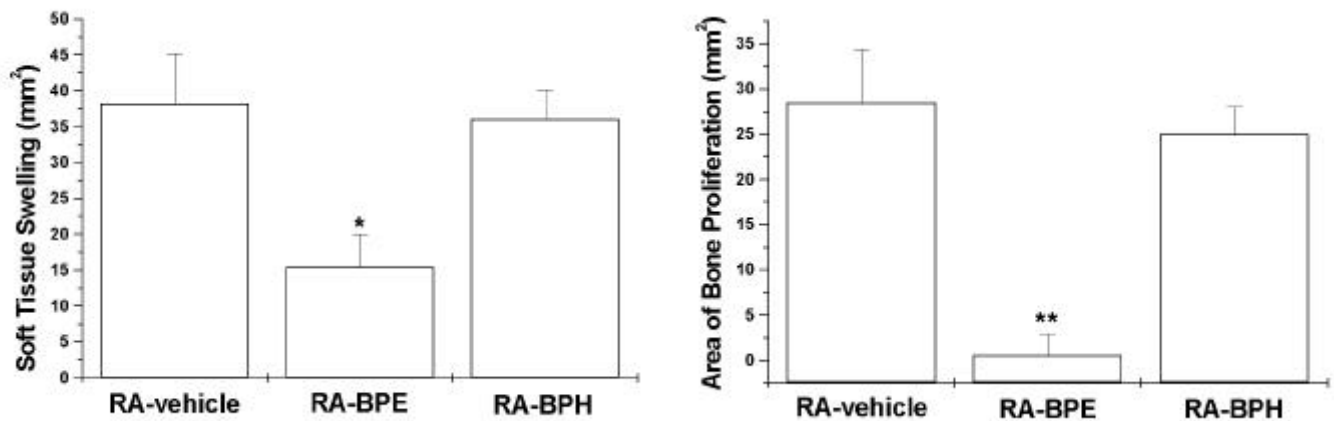


Fig. 4. This represents that the suppressive effect of BP on adjuvant-induced paw edema of the left hind paw. BPE significantly reduced the paw edema from 12 days after the FCA injection except 15 day as compared with that of vehicle control group. However, BPH did not suppress the paw edema in this study. ** $p<0.01$, significantly different from that of RA-vehicle group.



suppressed both soft tissue swelling and bone proliferation. In contrast to this, BPH failed to reduce these inflammation-related symptoms as compared with those of RA-vehicle group. * $p < 0.05$ and ** $p < 0.01$, significantly different from that of RA-vehicle group, respectively.

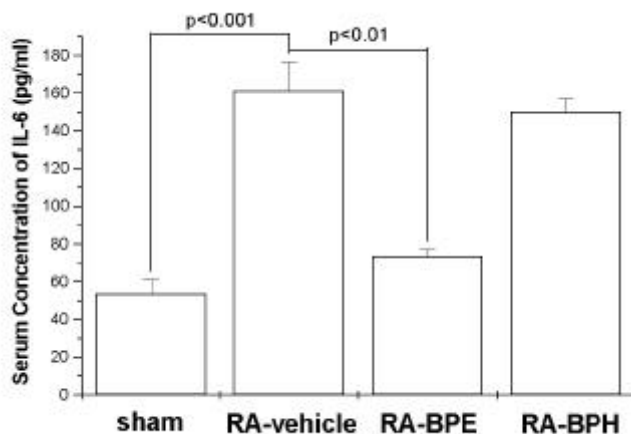


Fig. 6. Effects of BPE and BPH treatment on adjuvant-elevated serum IL-6 level. Vehicle treated arthritic rats significantly increased the serum IL-6 level as compared with sham animal ($p < 0.001$). BPE treatment significantly reduced the level of IL-6 ($p < 0.001$). However BPH did not affect the adjuvant-elevated serum IL-6 level. (Abbreviations) **IL-6:** interleukin-6

morbidity of arthritis in the left hind paw was 100% ($n=10$) in vehicle control group. BPE treatment significantly suppressed the volume of paw swelling as compared with that of vehicle control group except day 15. However, BPH failed to suppress paw swelling during 21 days. This result was consistent with the data of x-ray image analysis. The soft tissue swelling was significantly inhibited by the treatment only with BPE while BPH treatment did not produced this suppressive effect (Fig. 5a, Fig. 7). In addition, adjuvant injection-increased area of bone proliferation was decreased by the treatment with BPE as compared with vehicle control group (Fig. 5b). Likely to paw volume and soft tissue swelling results, BPH failed to inhibit

arthritis-increased area of bone proliferation. Serum level of IL-6 was 53.54 ± 7.79 pg/ml in sham group (Fig. 6). Adjuvant injection and vehicle treated animals showed the significantly increased the serum IL-6 at a level of 161.44 ± 15.32 pg/ml. BPE treatment strongly suppressed the serum level of IL-6 (73.44 ± 3.87 pg/ml) as compared with vehicle control group. However, BPH produced the similar high level of serum IL-6 with that of RA-vehicle group.

Discussion

Freund's complete adjuvant (*Mycobacterium butyricum*) is generally used to induce arthritis in animal models (8, 13). In this study, intraplantar injection of adjuvant into right hind paw rapidly induced paw swelling and redness and from 12 days after the secondary arthritic symptoms were observed in the contralateral left hind paw. Paw volume of left hind paw was increased significantly in RA-vehicle group and other inflammatory signs such as radiological changes and serum IL-6 levels. In addition, both mechanical and thermal pain threshold of RA-vehicle group was strongly decreased (Fig. 1, 2). Fos expression was also increased in both ipsi- and contra-lateral side of the spinal cord in RA-vehicle group. Fos expression was usually applied as a neuronal activity marker (6). Hunt and his co-workers reported that the peripheral noxious stimulation increases the spinal Fos expression and it is reported that morphine, one of potent analgesics, reduces the spinal Fos expression that was elevated by noxious stimulation (3). These results indicate that the Fos protein can be used as a neuronal marker of nociception. Therefore reduced spinal Fos expression by the treatment of BP in this study suggested that the antinociceptive effect of BP on adjuvant-induced arthritis in rats.

To investigate the anti-inflammatory effect of genus *Radix* medical herb, numerous studies has been performed.

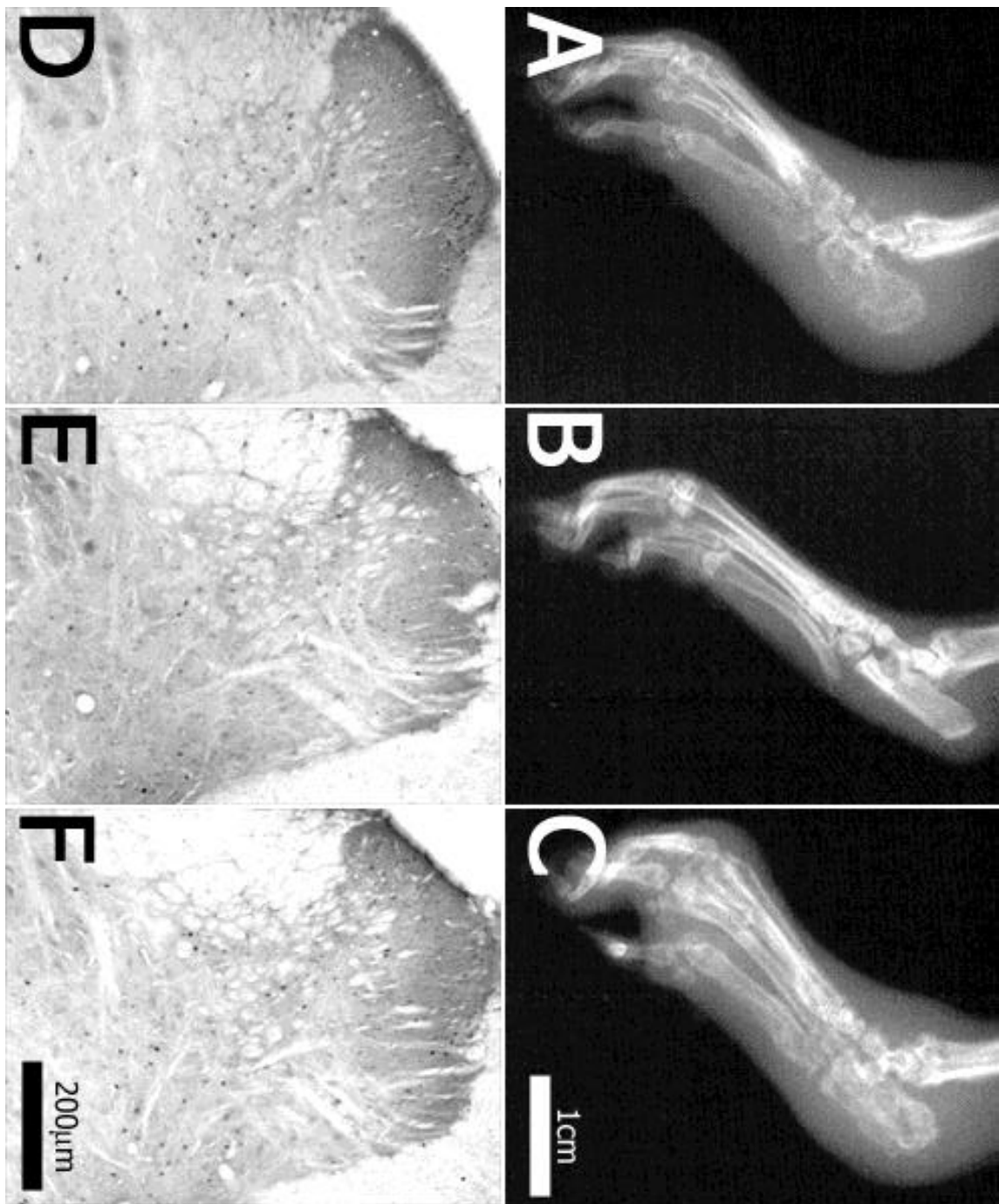


Fig. 7. Effects of BPE and BPH treatment on radiological changes and spinal Fos expression. In vehicle control group, soft tissue was significantly swelled and newly proliferated bone was observed (A) and spinal Fos expression was increased (D). Treatment with BPE significantly suppressed adjuvant-induced radiological changes (i.e. soft tissue swelling and bone proliferation) (B) and spinal Fos expression in SDH and NP (E). In contrast to this, BPH did not inhibit these arthritis-related changes (C and F).

It was reported that several extracts of oriental herbal medicines including radix of *Aralia continentalis* inhibits interleukin-8 induction in lipopolysaccharide-activated rat macrophages (10). In addition Radix ginseng is well known to produce curative effect on several disease states (10). In this study, daily treatment of BPE at a dose of 1 mg/kg/day significantly suppressed arthritis-related symptoms. BPE suppressed the adjuvant-induced paw edema during 3 weeks in contralateral left hind paw and it also inhibited soft tissue swelling. In RA-vehicle group, proliferated bone area and deformed joint were observed, whereas BPE treated animals dramatically reduced both soft tissue swelling and radiological changes as compared with that of RA-vehicle group. Moreover serological inflammatory marker, IL-6 level of serum was also inhibited by treatment of BPE. In nociceptive tests, BPE also produced a suppressive effect on mechanical and thermal hyperalgesia. Moreover, spinal Fos expression evoked by adjuvant injection also decreased by BPE treatment. However, BPH treatment failed to reproduce these suppressive and curative effects on adjuvant-induced arthritis in this study. Although the significant suppressive effect of BPE on arthritis in rats was demonstrated in this study, the most effective component of whole BP is still unknown. To find the most effective component of BPE and suppressive mechanism of BP on arthritis, further study is required. In conclusion, it is suggested that BPE had a anti-inflammatory effect on FCA-induced arthritis in rats. However, effective component of BP and its anti-inflammatory mechanism could not be elucidated in this study.

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