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Anti-nociceptive effect of bee venom treament on chronic arthritic pain in rats

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Abstract : Bee venom (BV) has been traditionally applied to relieve pain and to cure inflammatory diseases such as rheumatoid arthritis (RA) and neuritis. While several investigators have evaluated the anti-inflammatory effect of BV treatment, the anti-nociceptive effect of BV treatment on inflammatory pain is not reported. Therefore, we decided to evaluate the analgesic effect of BV treatment using Freund's adjuvant induced chronic arthritis model. Freund's adjuvant-induced arthritis has been used as an experimental animal model for RA in humans to assess the efficacy of the anti-inflammatory/analgesic drugs. In this study, subcutaneous BV treatment (1 mg/kg/day) produced significantly reductions of symptoms related to arthritic pain (i.e. mechanical hyperalgesia and thermal hyperalgesia). The anti-nociceptive effect of BV was observed from at least 12 days after BV treatment. Furthermore, BV treatment significantly suppressed adjuvant induced Fos expression in lumbar spinal cord. We also found that local injection of BV into near the inflammatory site (especially Zusanli-acupoint) showed more potent analgesic effect on arthritic pain rather than distant injection of BV from inflammatory site (arbitrary side of back).

The present study demonstrates that BV treatment has anti-nociceptive effect on arthritis induced inflammatory pain. The analgesic effect of BV on RA is probably mediated by the effect of BV itself or possible other mechanism such as counter-irritation. Furthermore, it is possible that BV acupuncture is one of the promising candidates for long-term therapy of RA.

Key words : bee venom ; inflammatory pain ; anti-nociception ; rheumatoid arthritis ; Fos.

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Introduction

Rheumatoid arthritis (RA) is the inflammatory arthropathy associated with a major socio-economic impact. This chronic and unpredictable disease results in persistent joint pain and inflammation that increases joint damage and frequent extra-articular complication. Currently, nonsteroidal antiinflammatory drugs (NSAIDs) supplemented with steroid hormones remain the main strategy of its treatment[1]. While these drugs suppress inflammation and ameliorate symptoms, they do not improve the long-term disease outcome significantly[2]. Furthermore, NSAIDs have also serious side effect such as gastrointestinal ulcergenicity by long-term treatment[3]. Therefore, more effective and safe durgs have been required to cure RA.

Bee sting therapy or its deposition has been utilized to relieve pain and to cure inflammatory diseases such as RA, neuritis and fibromyositis in the oriental medicine. Whole bee venom (BV) and its disposition have been also reported to be effective in the treatment of human RA[4]. Hadjipetrou-Kourounakis and Yiangou[5] report that the induction of arthritis is successfully suppressed by long-term treatments of BV with low dose in rat. It has been reported that local injection of BV into inflamed site more potently inhibits the development of the Freund's adjuvant induced arthritis rather than the administration of BV into the back side[5,6]. Taken together, these observations implicate that the anti-inflammatory effects of BV on arthritis may be dependent on the site of administration in experimental animals. However, it has not been reported whether BV treatment has the anti-nociceptive effect of on RA, and whether its anti-nociceptive effect is dependent on the site of administration.

Complete Freund's adjuvant has been utilized to induce an arthritic immunopathological disease that resembles pathological features of human RA[7]. Unilateral injection of Freund's adjuvant develops "primary" inflammatory signs and hyperalgesia within hours at the paw of inoculation. Subsequently, "secondary" inflammation and pro-nociceptive sign appears between the 10th and 15th day after inoculation especially in the contralateral paw. The hyperalgesic sign may persist till about 8 weeks after inoculation[8]. Therefore, adjuvant induced arthritis model has been commonly used to analyze the anti-inflammatory/anti-nociceptive effect of newly developed drugs on chronic arthritis[7].

Therefore, we decided to evaluate the analgesic effect of BV treatment using this experimental animal model. We examined the anti-nociceptive effect of BV on thermal or mechanical hyperalgesia at both ipsilateral hind limb and contralateral hind limb after induction of chronic arthritis. In addition, the anti-nociceptive effect of BV is analyzed by Fos immunohistochemistry using computerized imaging analysis system as previously reported[9]. Finally, we investigated whether local injection of BV in the inflamed site has more potent analgesic effect on chronic arthritis rather than systemic injection.

Materials and Methods

Experimental animals : Experiments were performed on 60 male Sprague-Dawley rats (the Laboratory Animal Center of Seoul National University, South Korea) Weighing 130-150g at the beginning of the experiment. They were kept in a 12 : 12 light-dark cycle (light on 7 : 00 AM to 7 : 00 PM) in a temperature controlled room (20±0.5℃). Food and water were available *ad libitum*. The food was directly available on the sawdust in the cage to minimize the need for animals to make potentially painful movements to obtain food. All of the methods used in the present study were approved by the Animal Care and Use Committee at Seoul National University and conform to NIH guidlines (NIH publication No. 86-23, revised 1985). The ethical guidelines of the International Association for the Study of Pain (IASP) for investingating experimental pain in conscious animals were also followed out[10].

The inductions of arthritis : Experimental animals were briefly anesthetized with 3% of isoflurane in a mixed N_2O/O_2 gas. Polyarthritis was induced by a single subcutaneous injection (50μl) of heat-killed *Mycobacterium butyricium* (Difco Laboratory, Detroit, MI) suspended in sterile mineral oil (20mg/ml) into the right hind paw as previously described[9]. Sham animals were injected with sterile vehicle.

Experimental groups and drug treatments : Experimental animals were divided into four groups; 1) non-arthritic animals (Sham, n = 10), 2) saline treated arthritis group (RA-Sal, n = 10), 3) BV administrated into hind limb (RA-BV/Z, n = 10) or 4) back (RA-BV/B, n = 10) in arthritic animal. BV (Sigma, Cat #: V3125, 1 mg/kg) was subcutaneously administrated into the either hind limbs (especially Zusanli point, RA-BV/Z) or arbitrary site of back (RA-BV/B) by dissolving in saline (0.5mg/ml). Zusanli point (S36) was located at 5mm lower and lateral to the anterior tubercle of the tibia. Sham control and RA-Sal animals were injected into both hind limbs with same volume of saline. BV treatment was started from the next day of adjuvant injection and lasted three weeks. All algesiometric assays were made from 9 days after the injection of adjuvant to end of experiment. All algesiometric assays were conducted between 10 : 00 AM and 4 : 00 PM. The BV was injected between 5 : 00 PM and 6 : 00 PM to minimize the stress of BV injection in nociceptive sensitivity[11].

Ankle flexion test : Arthritis induced hyperalgesia was quantitatively evaluated by the total number of vocalization. After rat comfortably hold, gentle flexion and extension evoked the vocalization at both hind limbs. Five stimuli were repeated at 5 sec intervals and a rating 0 or 1 was given if the animal emitted a vocalization or not, respectively. Thus each animal the rating ranged from 0 to 10 at each hind limb.

Thermal hyperalgesia test (Hargreaves's Method) : To assess nociceptive response to thermal stimuli, paw withdrawal latency was tested as previously described[12]. Briefly, rats were placed in a plastic chamber with a glass floor and allowed to acclimate to their environment for 5 min before testing. Radiant heat source positioned under glass floor beneath the hindpaw. The withdrawal latency of both hind paws was measured to the nearest 0.1 sec with electrical clock and photoelectric cell. The intensity of light source was calibrated 9-10 sec in sham animal, and test was duplicated at 5 min time intervals in both hind paw.

The mechanical hyperalgesia test : Evaluation of mechanical hyperalgesia in arthritis animal was made using the Randall-Sellito test. In this experiment, a graded mechanical force (g) was delivered through the analgesy meter (LETICA, LE7356) onto the convex surface of paw. A rat withdrew its hindpaw or vocalized when the applied force reached its pain threshold. Test was duplicated at 5 min time intervals in both hind paw, threshold force in sham animals was ranged from 140 to 160g.

Fos immunohistochemistry : In another experimental group, animals were left without any pain test through all the experiment periods (n = 5/group). At the end of experiments (21 days), the animals were deeply anesthetized with 5% isoflurane. And then subjected to a transcardial perfusion 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). The spinal cord was then removed immediately after perfusion, post-fixed in the same fixative for 4h and then cryoprotected in 30% sucrose in PBS (pH 7.4).

Frozen serial frontal sections (40μm) of the lumbar L3-L5 segments were cut with cryostat microtome (Microm, Germany). After elimination of endogenous peroxidase activity with 3% hydrogen peroxide in PBS and preblocking with 1% normal goat serum and 0.3% triton X-100 in PBS, immunohistochemistry of the free-floating sections was incubated in polyclonal rabbit anti-Fos antibody (Calbiochem, diluted 1 : 10000). The avidin-biotin-peroxidase procedure was sequentially processed as previously described[13]. Finally, Fos-like immunoreactive (FLI) neurons were visualized by 3-3 diamino-benzidine reaction intensifying with 0.2% nickel chloride.

Imaging analysis of Fos like immunoreactive neurons : Tissue sections were examined using darkfield microscopy to determine the segmental level according to Abbadie and Besson[9] as well as the gray matter landmarks using microscopy (zeiss axioscope, Germany). The sections were then examined under a lightfield microscopy at X100 to localize FLI neurons. For quantitative analysis of segmental and regional FLI neurons, lumbar spinal cord sections were scanned and the five sections with the greatest number of labeled cells at the L3-5 level were selected from each animal. And then, each segments were digitized with 4096 gray levels using a cooled CCD (Micromax Kodak

1317, Princeton instrument, USA) equipped computer-assisted imaging analysis system (Metamorph, Universal Imaging Co, USA). In order to keep constant threshold of each image considering subtle variablility of staining, we only counted labeled nuclei having less 70% intensity than the average gray level of each image (shown as regional statistics menu in software) after background subtraction and shading correction were done. All illuminations and data acquisition settings were fixed. And all procedures described above were performed blindly to the experimental condition of each animal.

To assess the effect of bee venom pretreatment on suppressing spinal FLI neurons, four regions were divided according to cytoarchitectonic criteria: superficial laminar (SDH, laminar I-II), nucleus proprius (NP, laminar III-IV) and neck (NECK, laminar V-VI) of dorsal horn and, in addition, the ventral horn (VENT, laminar VII-X).

Statistical analysis : Data were expressed as the mean± SEM. Repeated measures ANOVAs were performed to determine to overall effect. And then paired *t*-test was used to determine probability values when repeated measures ANOVAs indicated the significant drug effect. We considered the critical value of $p < 0.05$ as a statistically significant level.

Results

Induction of arthritis ; Injection of the adjuvant in the right hind paw produced a "primary" swelling of the injected paw from 3 days after adjuvant injection. Swelling of the non-injected contralateral hind paw and tail manifested the "secondary" response, which occurred from 12 days after injection. The incidence ratio of arthritis at contralateral paw was 100% in RA-Sal group. In addition, the arthritic symptoms were persisted till the end of experiments.

Ankle flexion test : Gentle flexion and extension of the inflamed ankle elicited vocalizations that were recorded a measure of hyperalgesia. In RA-Sal group, the vocalization by flexion/extension were significantly increased at ipsilateral hind limb (Fig 1a). The hyperalgesic sign of ipsilateral hind limb was suppressed in RA-BV/Z, whereas the suppressive effect was not shown in RA-BV/B (Fig 1a). In the contralateral limb of RA-Sal group, pain related vocalization was gradually increased from 12 days after adjuvant injection (Fig 1b). BV treatments significantly decreased vocalization of ipsilateral limb in both RA-BV/Z and RA/BV/B group ($p < 0.05$, Fig 1b).

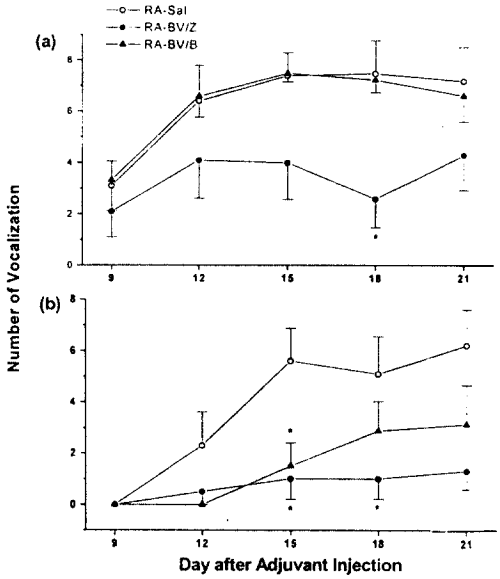

Fig 1. Effect of bee venom (BV) on ankle flexion/extension score in saline-treated arthritis group (RA-Sal) and BV treated arthritis group. BV (1 mg/kg) was administrated into either Zusanli (RA-BV/Z) or back (RA-BV/B). (a): score in ipsilateral right hind limb. (b): score in contralateral left hind limb. *$p < 0.05$: a significantly different from RA-Sal group.

Thermal hyperalgesia test : In ipsilateral hind paw of the arthritis-induced groups, there was a significant decrease of paw withdrawal latencies (PWL) from 9 days after adjuvant injection as compared to Sham group(Fig 2a). However, the PWL of RA-BV/Z group showed significantly higher thermal threshold as compared with those of RA-Sal and RA-BV/B group in the ipsilateral paw ($p < 0.05$, Fig 2a). In contralateral paw, RA-BV/B group gradually increased in the reduction of PWL to the level of Sham group whereas RA-Sal group showed continuously lower PWL as compared with Sham group (Fig 2b). Interestingly, the PWL of RA-BV/Z group were similar with the level of Sham group in contralateral paw (Fig 2b).

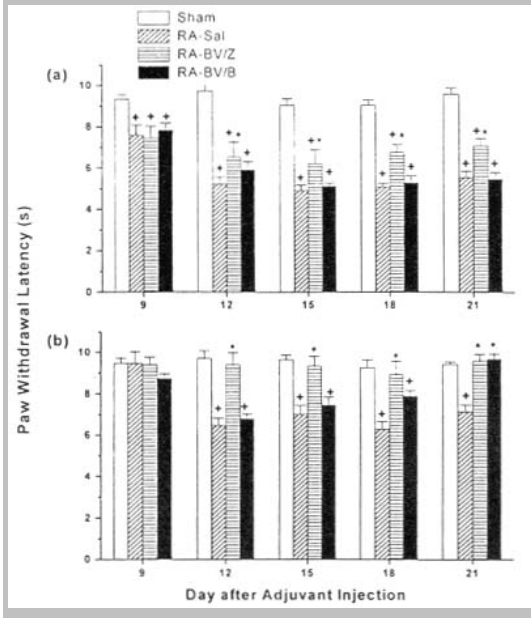

Fig 2. Effect of bee venom (BV) on paw withdrawal latency (PWL) by noxious heat stimuli in non-arthritic animal (Sham), saline-treated arthritis group(RA-Sal) and BV treated arthritis group. BV (1 mg/kg) was administrated into either Zusanli (RA-BV/Z) or back (RA-BV/B). (a) : PWL in ipsilateral right hind paw. (b) : PWL in contralateral left hind paw. +p 〈 0.05 : a significantly different from Sham group. *p 〈 0.05 : a significantly different from RA-Sal group.

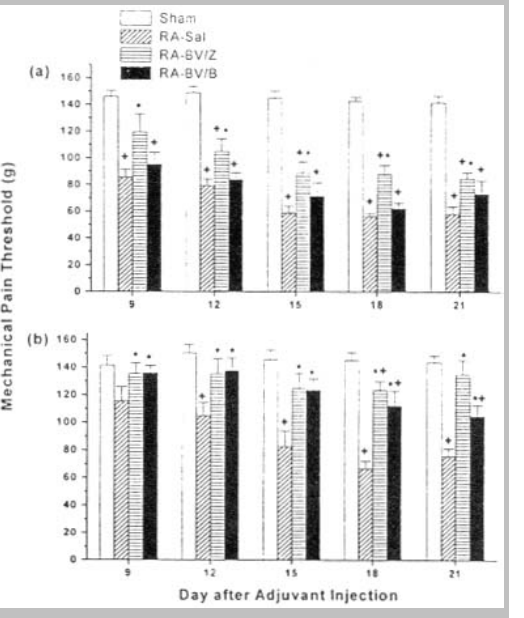

Fig 3. Effect of bee venom (BV) on mechanical threshold (Randall-Selitto) in non-arthritic animal (Sham), saline-treated arthritis group(RA-Sal) and BV treated arthritis group. BV (1 mg/kg) was administrated into either Zusanli (RA-BV/Z) or back (RA-BV/B). (a) : The mechanical threshold in ipsilateral right hind paw. (b) : mechanical threshold in contralateral left hind paw. +p 〈 0.05 : a significantly different from Sham group. *p 〈 0.05 : a significantly different from RA-Sal group.

The mechanical hyperalgesia test : There was a significant decrease in the mechanical pain threshold in RA-Sal group as compared with Sham group at both hind paws ($p < 0.05$, Fig 3a,b). In ipsilateral paw, the pain threhold of RA-BV/B group was not statistically different from that of RA-Sal group (Fig 3a). In contrast, it is observed that pain threshold of RA-BV/Z group was significantly increased as compared with that of RA-Sal group in ipsilateral paw ($p < 0.05$, Fig 3a). Furthermore, the pain threshold at contralateral paw in RA-BV/Z and RA-BV/B group was similar with the level of Sham group (Fig 3b).

Fos immunohistochemistry : Very few FLI neurons were observed in the lumbar spinal cord of the Sham group (Fig 4a, 4b). In contrast, RA-Sal group was observed significantly higher number of FLI neurons at both ipsilateral and contralateral side of lumbar spinal cord (especially L3-L5) after unilateral Freund's adjuvant injection. FLI neurons were mainly distributed in the NECK (about 50%) of dorsal horn, whereas small FLI neurons observed in SDH, NP and VENT regions of spinal cord (Fig 4a and b). While ipsilateral side of spinal cord in RA-BV group is not significantly different from that of RA-Sal (Fig 4a), the number of FLI neurons was significantly reduced in all regions of contralateral spinal cord as compared with RA-Sal ($p < 0.05$, Fig 4b). Interestingly, RA-BV/Z group was observed significantly small number of FLI neurons in both side of the spinal cord as compared with that of RA-Sal group ($p < 0.05$, Fig 4a, b).

Discussion

Unilateral Freund's adjuvant injection into right paw induces RA from ipsilateral limb to contralateral limb with a time course as previously described[6,8]. In this study, we ob-

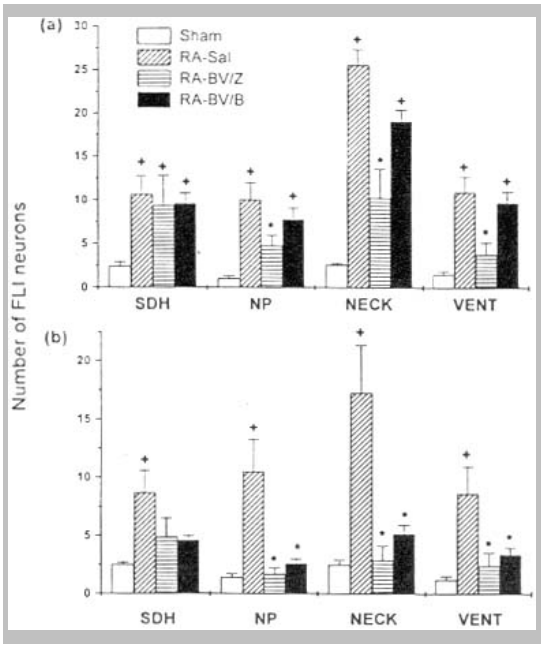

Fig 4. Histograms showing the number of Fos-like immunoreactive (FLI) neurons in non arthritic animal (Sham, n = 5), saline-treated arthritis group (RA-Sal, n = 5) and bee venom (BV) treated arthritis group at 3 weeks after adjuvant injection. BV(1 mg/kg) was administrated into either Zusanli (RA-BV/Z, n = 5) or back (RA-BV/B, n = 5). (a) : The number of FLI neurons in ipsilater side (right) of spinal cord. (b) : The number of FLI neurons in contralateral side (left) of spinal cord. + : a significantly higher numbers of FLI neurons as compared to Sham group ($p < 0.05$). * : a significantly different from the number of FLI nuerons of RA-Sal group($p < 0.05$). Abbreviation : SDH; superficial dorsal horn (laminar I-II), NP ; nucleus proprius (laminar III-IV), NECK; neck of dorsal horn (laminar V-VI), VENT; ventral horn (laminar VII-X).

served the same pattern of progressive arthritic induction (data not shown). Furthermore, we also demonstrated that the thermal and mechanical hyperalgesia were gradually increased after adjuvant injection according to the development of arthritis. In addition, the inflammatory hyperalgesia is persisted at least 8 weeks. Therefore, this animal model was utilized to evaluate the anti-nociceptive effect of BV treatment in this study.

It has been shown that the number of FLI neurons in lumbar spinal cord is increased and peaks at 3 weeks after the inoculation that corresponds to the maximal stage of hyperalgesia[14]. In this study, we demonstrated that the number of FLI neurons increased in parallel with the clinical and behavioral signs of RA at 3 weeks after adjuvant injection. When chronic treatment of NSAIDs (i.e. aspiriin and acetaminophen) are applied during the development of RA, the number of FLI neurons and behavioral sign of RA are significantly decreased in drug treatment group as compared to vehicle-treated groups[9]. We also observed that the treatment of BV was effectively suppressed the number of FLI-neurons and pain related symptoms. Thus, the reduction of FLI neuron in the spinal cord indicates that the arthritis related pain is suppressed by BV treatment.

Surprisingly, arthritic pain of secondary inflammation site (contralateral limb) was dramatically suppressed by BV therapy up to the normal level, while that of primary inflammation site (ipsilateral limb) did not completely abolished (Fig 1, 2, 3). Furthermore, the suppression of FLI neurons showed the same manner as the behaviroal data (Fig 4). Eiseman and his co-workers also report that decrease of contralateral paw edema was more potently than that of ipsilateral paw edema according to long-term BV treatment[6]. Until now, it is unclear why anti-arthritic drug shows different therapeutic efficacy between the primary inflammation site and secondary inflammation site in unilateral adjuvant induced arthritis model

Although many investigators have reported that BV is a useful therapeutics to manage arthritis, it still remains controversial in effective dose, injected site or route in experimental animal model. The induction of arthritis is successfully suppressed by intramuscular BV injection into both hind limbs at the dose of 0.5mg/kg[5]. However, the administration of BV (2mg/kg) into the back did not completely abolish the primary and secondary inflammatory responses induced by chronic arthritis [6]. In this study, subcutaneous BV treatment (1 mg/kg) produced significant reductions of arthritis induced pains (i.e. mechanical hyperalgesia and thermal hyperalgesia).

Until now, the mechanism of anti-arthritic action of whole BV remains uncertain. However, several investigations suggest the possible mechanisms of BV therapy for RA using individual its components. BV consists of different peptides such as melittin, apamin, adolapin and mast cell degranulat-

ing (MCD) peptide[4]. Although purified MCD peptide (1 mg/kg) and adolapin (20μg/kg) has anti-inflammatory activity[15,16], these substances are comprised in only low part of whole BV to the nearest 1-2%. Therefore, these peptides play a minor role in the anti-arthritic effect of BV at the dose of this study. Recently, Saini and his co-workers[17] report that melittin as a major component of BV (50% of dry wt) binds to secretory phospholipase A_2 (PLA_2) and inhibits its enzymatic activity. Because PLA_2 is a major inflammatory trigger (i.e. release arachidonic acid) and is enhanced its activity in RA, it possible that the formation of melittin-PLA_2 complex by BV injection is able to suppress the symptom of RA. However, other observation shows inconsistent data that melittin injection into mouse paw elicits paw edema at 60 min after injection[18]. Furthermore, whole BV injected into hindpaw also produced local inflammation for more than 48 hours[11]. Therefore, further studies are required to understand the mechanisms for the anti-inflammatory effect of BV in RA.

We found that BV injection into both hind limbs (especially Zusanli acupoint) showed more potent analgesic effect on arthritic pain rather than arbitrary injection into back (non-acupoint). It is questionable why long-term BV treatment into near inflammation site produces higher anti-nociceptive effect in RA rather than distant inflammation site. As mentioned above, the anti-nociceptive effect can be partially induced by the analgesic component (i.e. adolapin) of BV. The other possibility of anti-nociceptive effect of BV may be produced by counter-irritation ; when noxious stimuli are applied to widespread body, these stimuli increase the pain thresholds and reduce pain rating. For centuries, pain is relieved by counter-irritation such as moxibustion on an arthritic limb or cauterizing above the hip for sciatica[19]. In recent clinical practice, counter-irritation such as electric stimulation and some types of accupuncture has been used to induced analgesia in local and widespread area of the body[20,21]. After electroacupuncture was applied at Zusanli acupoint, the tail flick latencies are significantly prolonged[22]. Furthermore, the analgesic effect of electroacupuncture is associated with a significantly increased number of FLI neurons in the dorsal horn of spinal cord[23]. In addition, capsaicin, extracts of the red pepper, produces itching, pricking and burning sensations caused by the excitation of nociceptors. Since repeated application of capsaicin is followed by a prolonged period of hypalgesia that is usually referred to as desensitization, or nociceptor inactivation, it traditionally used as a remedy for some types of pain[24,25]. Bee sting also evokes local and brief pain symptoms as capsaicin[11]. Furthermore, the number of FLI neurons is prolonging increased at lumbar spinal cord following intraplantar BV injection as that of electroacupuncture[26]. Therefore, it is possible that the anti-nociceptive effect of BV is partially mediated by counter-irritation. Because the anti-arthritic action of BV is more potently produced by the application into Zusanli acupoint and BV acupuncture can be more easily applicable than electroacupuncture, BV therapy may be a promising candidate for alternative medicine.

Current strategies for arthritis are limited by NSAIDs and steroid hormone[1]. However, these drugs not only inadequately treat the arthritis, but also result in serious side effect[2]. These side effects significantly limit their usefulness in the long-term therapy. In this study, we observed that anti-arthritic effect of BV was produced by long-term treatment for more than 12 days without side effects. It has been reported that allergy to BV has an incidence of about 3% in the general population[27]. However, it has been also demonstrated that long-term BV treatment induces T-cell hyporesponseness to allergen and modulation of cytokine secretion in human[28]. Consistently, long-term BV treatment for 18 days reduces the population of intereukin-1 and intereukin-2 in the splenocyte of normal and arthritic rat[29]. Thus, hyposensitization induced by BV is currently recognized by the successful long-term therapy for this allergy in human[30]. Interestingly, the whole BV but not the individual component produces early and persistent increase in corticosterone concentration[31]. Since RA is evoked by an autoimmune reaction against self-cartilage antigen, modification of the level of cytokine and corticosteroid by BV therapy may inhibit the immune cell from the attack of synovial cartilage. However, further investigations are necessary to analyze the precious effect of BV on immune system or other hormonal change.

The present study demonstrates that BV treatment has anti-nociceptive effect on arthritis induced inflammatory pain. The analgesic effect of BV on RA is probable mediated by effect of BV itself or possible other mechanism such as counter-irritation. Furthermore, it is possible that BV acupuncture is one of the promising candidates for long-term therapy of RA.

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