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The antinociceptive and anti-inflammatory effect of water-soluble fraction of bee venom on rheumatoid arthritis in rats

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ABSTRACT

We recently demonstrated that bee venom (BV) injection into acupoint (i.e. Zusanli) produced more potent anti-inflammatory and antinociceptive effect in Freunds adjuvant induced rheumatoid arthritis (RA) model as compared with that of non-acupoint injection (i.e back). However, the precise components underlying BV-induced antinociceptive and/or anti-inflammatory effects have not been fully understood. Therefore, we further investigated the anti-arthritic effect of BV after extracting the whole BV according to solubility (water soluble: BVA, ethylacetate soluble: BVE).

Subcutaneous BVA treatment (0.9 mg/kg/day) into Zusanli acupoint was found to dramatically inhibit paw edema and radiological change (i.e. new bone proliferation and soft tissue swelling) caused by Freunds adjuvant injection. In addition, the increase of serum interleukin-6 by RA induction was normalized by the BVA treatment as similar with that of non-arthritic animals. On the other hand, BVA therapy significantly reduced

arthritis induced nociceptive behaviors (i.e., nociceptive score for mechanical hyperalgesia and thermal hyperalgesia). Furthermore, BVA treatment significantly suppressed adjuvant induced Fos expression in the lumbar spinal cord at 3 weeks post-adjuvant injection. However, BVE treatment (0.05 mg/kg/day) has not any anti-inflammatory and anti-nociceptive effect on RA.

Based on the present results, we demonstrated that BVA might be a effective fraction in whole BV for long-term treatment of RA-induced pain and inflammation. However, it is clear necessary that further fraction study about BVA was required for elucidating an effective component of BVA.

Key words: Bee venom; Fraction: Anti-nociception; Anti-inflammation; Arthritis; Acupuncture

1. Introduction

Bee venom (BV) therapy has been utilized to relieve pain and to cure inflammatory diseases such as rhumatoid arthritis (RA) in humans (Billingham et al., 1973) and experimental animals (Eiseman et al., 1982; Hadjipetrou -Kourounakis and Yiangou, 1984).

The BV contains a variety of different peptides including melittin, apamin, adolapin and mast cell degranulating (MCD) peptide (Billingham et al., 1973). In addition, it also contained enzyme (i.e. phospholipase A2) and active amine (i.e. histamine) and non-peptide components such as lipid, carbohydrate and amino acid (Lariviere and Melzack, 1996). Until now, the individual components of BV have been tested to verify the anti -inflammatory and/or antinociceptive effect of BV.

In early study, it has been reported that adolapin and purified MCD peptide have anti-inflammatory and/or antinociceptive activity (Martin and Hartter, 1980; Koburova et al., 1985). However, it is notable that these substances are present in very small quantities (1-2%) in the dried whole BV. As we know about the pain evoked by bees sting, whole BV injection produced tonic pain response and developed hyperalgesia to mechanical and heat stimuli (Lariviere, et al., 1996; Chen, 1999). Furthermore, the major components of BV such as melittin (50%

in whole BV) and phospholipase A2 (10% in whole BV) are responsible for local inflammation and induce nociceptive responses (Hartman et al., 1991; Koyama et al., 2000; Landucci et al., 2000).

Therefore, it is clear at this point in time that further investigation is required to clarify the major components underlying the anti-arthritic effect of whole BV in RA.

In experimental animals, the induction of arthritis is successfully suppressed by long-term BV treatment (Eiseman et al., 1982; Hadjipetrou-Kourounakis and Yiangou, 1984).

In our recent study, we further demonstrated that injection of whole BV into the Zusanli acupoint produced a significantly greater anti-arthritic effect as compared to BV injection into non-acupoint on back in Freund adjuvant induced RA model (Kwon et al., in press). The modification of anti-arthritic effect by injection site suggested that BV induced anti-arthritic effect may be produced by specific point stimulation causing by BV injection rather than the systemic anti-arthritic effect induced by the analgesic and/or anti-inflammatory substance among whole BV components. However, it is not reasonable that individual stimulating component of BV (i.e. melittin) was only responsible for this site-specific anti-arthritic effect of BV. Therefore, we hypothesized that the complex stimulation by individual component of BV produced the anti-arthritic effect.

Base on this hypothesis, we grossly extracted whole BV according to solubility to evaluate more potent anti-arthritic BV complex.

The adjuvant induced chronic RA model is widely used for studies of the evaluation of potential new therapeutic agents. In general, the therapeutic potency of arthritic disease was evaluated from both anti-inflammatory effect and anti-nociceptive effect. The change of paw volume classically used for evaluating anti-inflammation for RA (Eiseman et al., 1982). Plasma concentration of inflammatory cytokine (i.e. interleukin-6) was also a valuable clinical index in adjuvant induced RA model in rats (Philippe et al., 1997). It has been demonstrated that the measurement of bone changes that occur during course of experimental arthritis by quantitative image analysis applied for evaluating the anti-inflammatory effect on RA (Esser et al., 1995).

For evaluating the potential anti-nociceptive effect on RA, it has been used that both thermal and mechanical hyperalgesia was measured following induction of chronic arthritis. In addition, the anti-nociceptive effect on adjuvant induced spinal cord Fos expression was analyzed using a computerized image analysis system.

2. Materials and Methods

2.1. Experimental animals

Experiments were performed on 60 male Sprague-Dawley rats (obtained from the Laboratory Animal Center of Seoul National University, South Korea) weighing 130-150 g at the beginning of the experiment. Animals were kept in a 12:12 light-dark cycle (7:00 AM onset) in a temperature controlled room (23 0.5C). Food and water were available *ad libitum*. The food was placed 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 guidelines (NIH publication No. 86-23, revised 1985). All algesiometric assays were conducted under the ethical guidelines set forth by the International Association for the Study of Pain (IASP) (Zimmermann, 1983).

2.2. The induction of arthritis

Experimental animals were briefly anesthetized with 3% isoflurane in a mixed N_2O/O_2 gas. Arthritis was induced by a single subcutaneous injection (50 l) into the plantar surface of the right hind paw of heat-killed *Mycobacterium butyricium* (Difco Laboratory, Detroit, MI, USA) suspended in sterile mineral oil (20 mg/ml). Control animals were similarly injected with sterile vehicle.

2.3. Extract preparation and experimental groups

The whole BV (*Apis mellifera*) was purchased from Sigma. The BV was dissolved with water and partitioned with hexane (1:1 vol/vol). The hexane fraction was evaporated to dry and the resulting water layer partitioned with ethylacetate fraction and water fraction. Each fraction was completely dried and stored at refrigerator. The BV contains 90% water-soluble (BVA), 5% ethylacetate

-soluble (BVE) and 5% hexane-soluble substances. In this study, we only evaluated the BVA and BVE extract because hexane soluble extract was generally known as toxic substance. Therefore, experimental animals were divided into four groups: 1) saline treated non-arthritic animals (Sham, n=10), 2) saline treated arthritic group (RA-Sal, n=10), 3) BVA treated arthritic group (RA-BVA, n=10) or 4) BVE treated arthritic group (RA-BVE, n=10). Each extract was dissolved in appropriate vehicle [BVA: 0.9% saline, BVE: ethanol and saline (1:10 vol/vol)] for experiment.

In previous study, we observed that whole BV had antinociceptive effect in the concentration of 1 mg/kg (Kwon et al., in press). The dose of each extract was determined by considering the partial ratio in whole BV (1 mg/kg). Thus, BVA (0.9 mg/kg) and BVE (0.05 mg/kg) were used for the evaluating whether each extract was responsible for the anti-arthritic effect of whole BV.

Each extract was administrated subcutaneously and bilaterally into the Zusanli point as previously described (Kwon et al., in press). Sham and RA-Sal animals were injected bilaterally into the Zusanli point with an equal volume of saline. Treatment was started the day after adjuvant injection and animals were injected daily for a period of three weeks. All algesiometric assays were performed beginning 9 days after adjuvant injection at the time of induction of systemic arthritis.

2.4. Evaluation of anti-inflammatory effect

2.4.1. Evaluation of paw volume

Paw volume of contralateral hind paws was duplicately measured using a water displacement plethysmometer (UGO BASIL, Italy) every three days for 21 days after adjuvant injection and the mean values were recorded. Paw volume measured just prior to adjuvant injection was used as the control volume (day 0). Data were plotted as the change of paw volume versus control volume at each time point. Measurement of paw volume and all behavioral tests were performed blindly.

2.4.2. Evaluation of radiological change in the hind limb

At the end of experiment, rats were sacrificed with ether. Left hind limb was amputated and placed on film carriers

with the medial aspect of the limb down (lateromedial view). All radiographs were taken with a Westinghous Rivera Instrument set 12.5 mA/s, 40 kV with Kodak Ektascan M Film. The film-to-source distance was 40 inch.

Radiographs were digitized with Hewlett-Packard scanner (in a 200 % magnification of the original image and save as TIFF files for later measurements.

We evaluated two parameters including the new bone formation and soft tissue swelling in the lower part of tibio-tasal joint. Each parameter was analyzed by the modified Essers method (Esser et al., 1995) using computer-assisted image analysis system (Metamorph, Universal Imaging Co., West Chester, PA, USA).

Briefly, the mean area of calcaneus was determined in the normal animal (Sham) which is indicated the standard bone area (SBA). New bone proliferation was measured by substracting the value of SBA from the bone area of testing animal.

Soft tissue swelling was also calculated by following equation: [soft tissue area (whole paw area - bone area) in test animal -mean soft tissue area in sham animals].

2.4.3. Evaluation of interleukin 6 level in serum

Blood samples were collected in sterile tubes by cardiac puncture and centrifuged, and the serum was stored at -20C. The level of IL-6 was measured by enzyme-linked immunosorbent assay for rat IL-6 (kit from Biosource international Inc., CA, USA)

2.5. Evaluation of antinociceptive effect

2.5.1. Thermal hyperalgesia test (Hargreavess Method)

Rats were placed in a plastic chamber with a glass floor and allowed to acclimate to their environment for 5 min before testing. A radiant heat source was positioned under the glass floor beneath the hindpaw to be tested.

The withdrawal latency of both hind paws was measured to the nearest 0.1 sec using a photoelectric cell connected to a digital clock. The intensity of the light source was calibrated to produce withdrawal in 9-10 sec in normal animals. The test was duplicated at 5 min intervals in each hind paw.

2.5.2. The mechanical hyperalgesia test

A graded mechanical force (g) 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. The threshold force in normal animals ranged from 160 to 180 g.

2.5.3. Fos expression in lumbar spinal cord

One group of adjuvant injected rats was not subjected to any of the above nociceptive behavioral tests (n=5/group), but at the end of the experiments (21 days) were used for Fos immunohistochemistry.

The animals were deeply anesthetized with 5% isoflurane, perfused transcardially with calcium-free Tyrodes solution, followed by a fixative containing 4% paraformaldehyde and 0.2% picric acid in 0.1 M phosphate buffer (pH 6.9). All perfusions were performed between 9:00 AM and 12:00 AM. The spinal cord was then removed immediately after perfusion, post-fixed in the same fixative for 4 h and then cryoprotected in 30% sucrose in phosphate buffered saline (PBS, pH 7.4) for 48 h.

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 preblocking 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 4C overnight.

Fos-like immunoreactive (FLI) neurons were visualized using a 3-3 diamino -benzidine reaction intensified with 0.2% nickel chloride.

For quantitative analysis of FLI neurons, lumbar spinal cord sections were scanned and the five with the greatest number of labeled cells at the L3-5 level were selected from each animal. 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 Co., West Chester, PA, USA) as previously described (Abbadie and Besson, 1994; Kwon et al., in press).

To assess the effect of BV extract on spinal cord Fos expression, 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).

2.6. 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 ANOVAs 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.

3. Results

3.1. Evaluation of paw volume

Latent systemic arthritic response that characterized by swelling of the non-injected contralateral hind paw and tail was first evident at 12 days post-adjuvant injection into right hind paw (Fig. 1). Therefore, we measured the paw volume of left hind paw for evaluating anti-inflammatory effect of BV fraction on RA.

In the RA-BVA group, arthritis induced paw edema was significantly decreased from 12 days post RA induction as compared to that of the RA-Sal group (Fig. 1). However, the anti-inflammatory effect in RA-BAE group was first evident at 21 days post-RA induction.

In addition, the RA-BVA group showed a significant decrease in paw volume in comparison to that of the RA-BVE group (Fig. 1).

3.2. Evaluation of radiological change

Radiographs of the left hindpaw taken at 3 weeks after RA induction were evaluated by image analysis. Adjuvant induced arthritis induced severe soft tissue swelling and new bone proliferation in RA-Sal group (Fig 2b, Fig 3a and b) as compared with Sham group (Fig 2a). BVA treatment had significantly inhibited these pathological changes (Fig 2c, Fig 3a and b) whereas it is not different from that of RA-Sal in RA-BVE group (Fig 2d, Fig 3a and b). This data indicated that BVA treatment successfully suppressed RA induction.

Fig. 1. The change of paw volume in saline-treated arthritic animals (RA-Sal, n=10), the aqua fraction of BV treated arthritic animals (RA-BVA, n=10) and the ethylacetate fraction of BV treated arthritic animals (RA-BVE, n=10). Vehicle or fractions of BV was administrated into the Zusanli acupoint for 3 week after RA induction. Graph depicts the change in the paw volume of the contralateral left hind limb. **p<0.01: significantly different from RA-Sal group.

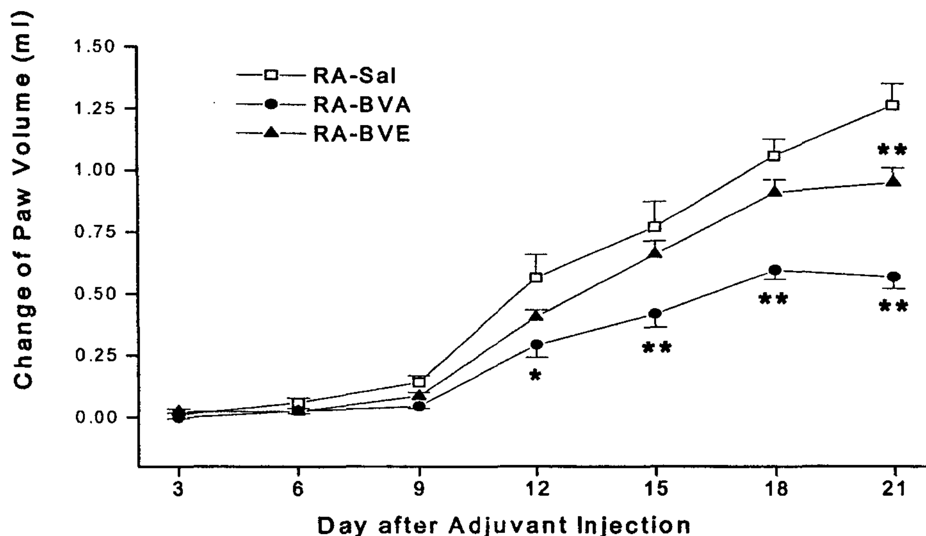


Fig. 2. The X-ray images at 3 weeks after arthritis induction in normal animals (A), saline-treated arthritic animals (B), the water soluble fraction of BV (BVA) treated arthritic animals (C) and the ethylacetate fraction of BV (BVE) treated arthritic animals (D).

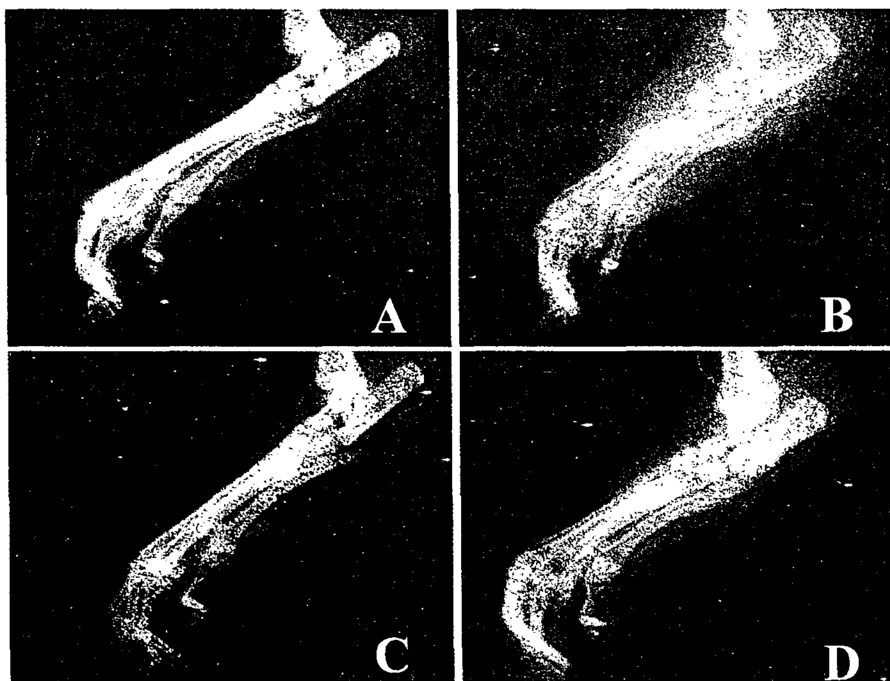
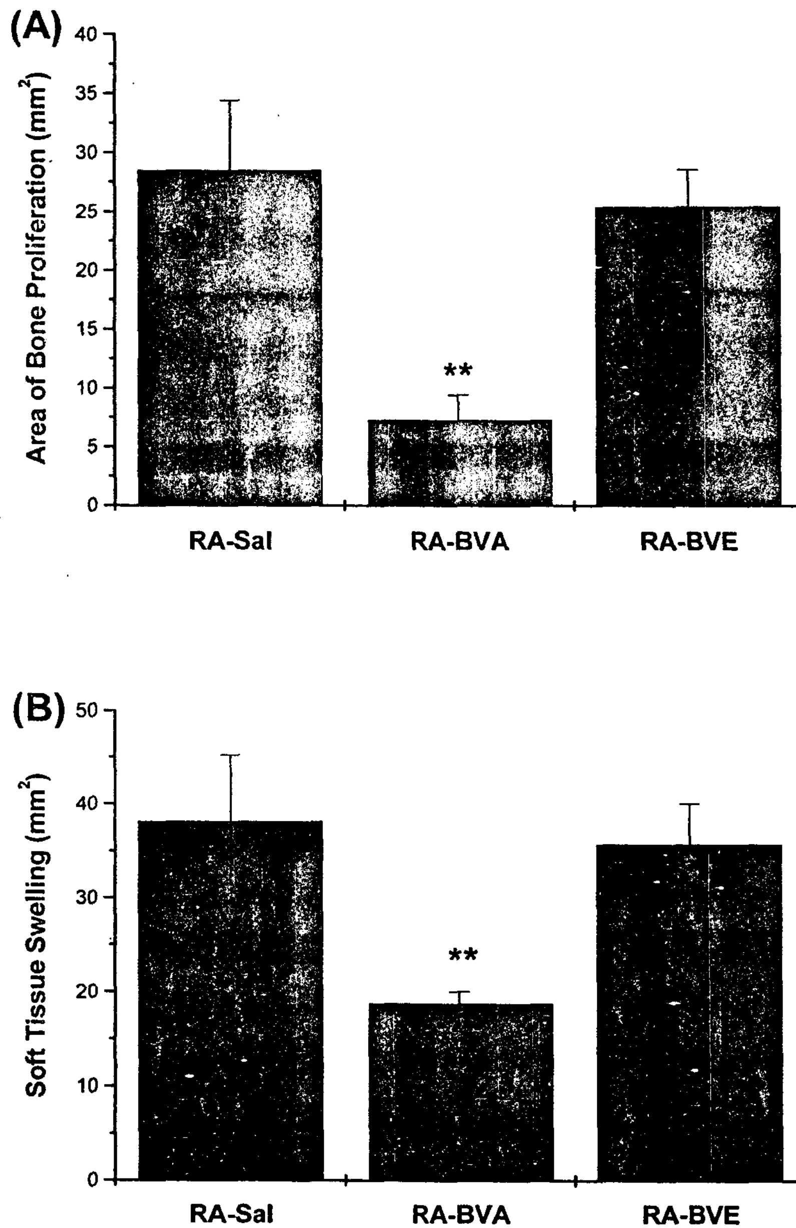


Fig. 3. The image analysis values at 3 weeks after arthritis (RA) induction in saline-treated arthritic animals (RA-Sal, n=5), the aqua fraction of BV treated arthritic animals (RA-BVA, n=5) and the ethylacetate fraction of BV treated arthritic animals (RA-BVE, n=5). A: depicts the change of new bone proliferation B: depicts the change of soft tissue swelling. Each data was evaluated at the level of tibio-tarsal joint in the contralateral left hind limb. $**p<0.01$: significantly different from RA-Sal group.



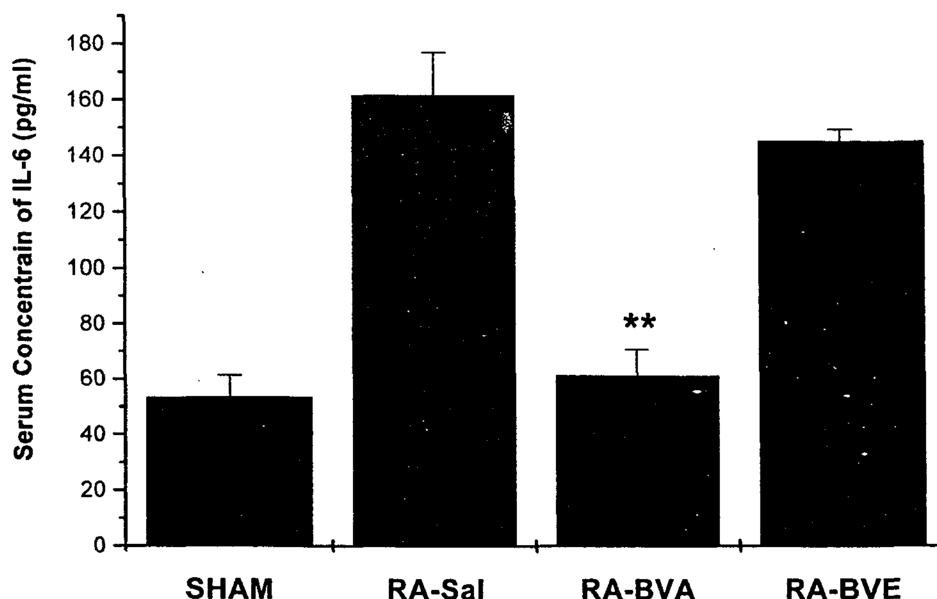
3.3. Evaluation of serum interleukin-6 level

A basal systemic IL-6 concentrations (53.57.9 pg/ml) was observed in normal (Sham) animal (Fig 4). Although the systemic IL-6 concentration (161.415.3 pg/ml) in RA-Sal was significantly increased at 3 weeks after RA induction, BVA treatment (61.19.1 pg/ml) inhibited the increase of IL-6 concentration up to Sham level (Fig 4). However, BVE treatment was not different from that of RA-Sal group (Fig 4).

3.4 Thermal hyperalgesia test

The paw withdrawal latency (PWL) of animals in the Sham group did not change significantly throughout the 21 days of the experiment. In contrast both the ipsilateral and contralateral PWL of animals in the RA-Sal group decreased significantly and remained lower throughout the experiment. There was a significant decrease in the PWL of the

Fig. 4. The change of serum concentration of interleukin-6 (IL-6) in normal animals (SHAM, n=5), saline-treated arthritic animals (RA-Sal, n=5), the aqua fraction of BV treated arthritic animals (RA-BVA, n=5) and the ethylacetate fraction of BV treated arthritic animals (RA-BVE, n=5). Serums were obtained at 3 week after arthritis induction. **p<0.01: significantly different from RA-Sal group.



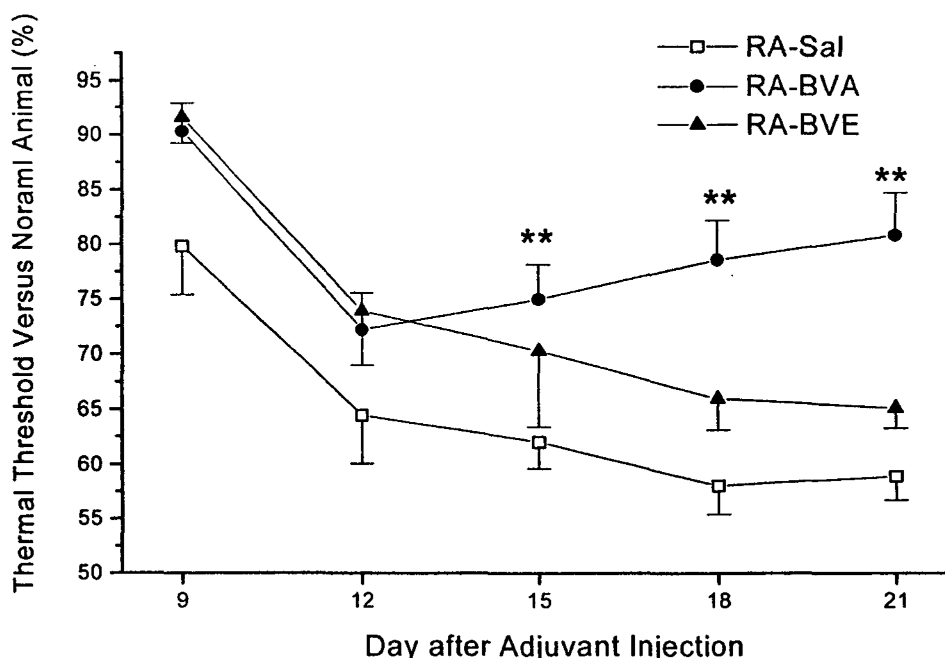
contralateral hind paw of all the arthritis-induced groups compared to the Sham group, beginning 9 days after adjuvant injection (Fig. 5a).

However, the PWL of the RA-BVA group showed a significantly higher thermal threshold from 15 days post RA induction as compared with those of the RA-Sal and RA-BVE groups ($p<0.01$, Fig. 5a).

3.5. Mechanical hyperalgesia test

There was a significant decrease in the contralateral hind paw mechanical pain threshold of animals in the RA-Sal group as compared to those in the Sham group (Fig. 6b). The pain threshold of the RA-BVE group was not statistically different from that of the RA-Sal group (Fig. 6b). In contrast, the pain threshold was significantly increased in the RA-BVA group as compared to the

Fig. 5. The changes of paw withdrawal latency (PWL) produced by noxious heat stimuli in saline-treated arthritic animals (RA-Sal, n=10), the aqua fraction of BV treated arthritic animals (RA-BVA, n=10) and the ethylacetate fraction of BV treated arthritic animals (RA-BVE, n=10). Graph depicts the percent inhibition compared to the Sham PWL in the contralateral left hind paw. **$p<0.01$: significantly different from RA-Sal group.



RA-Sal group ($p<0.01$, Fig. 6b).

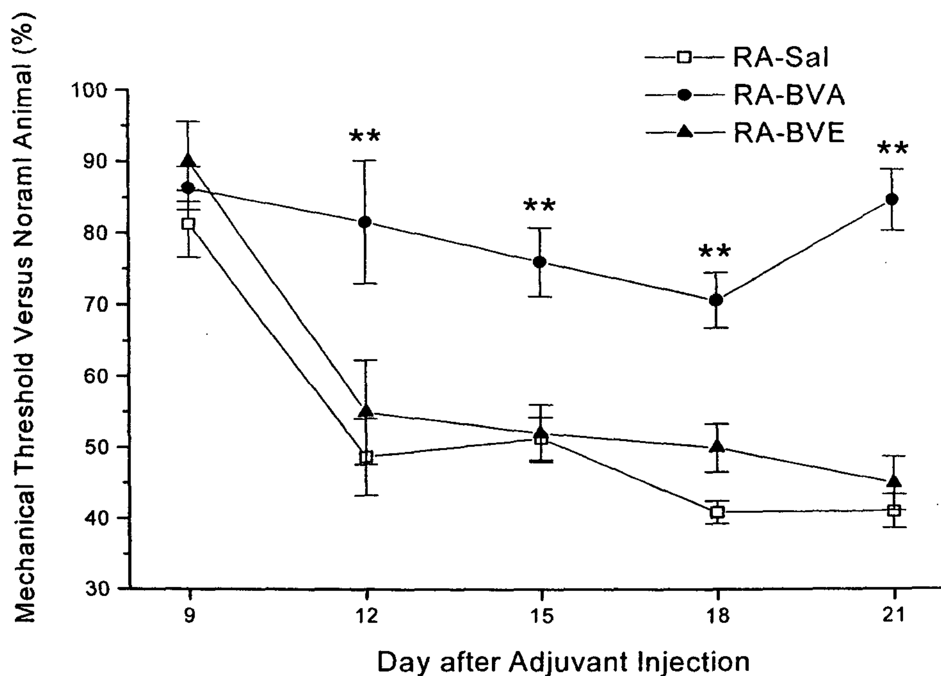
3.6. Fos immunohistochemistry

A significantly higher number of FLI neurons were observed in contralateral (left) sides of the L3-L5 lumbar spinal cord segments in rats from the RA-Sal group at 21 days after unilateral Freunds adjuvant injection as compared with Sham group (Fig 7).

The number of FLI neurons in the RA-BVE group was not significantly different from that of animals in the RA-Sal group (Fig 7).

However, the number of FLI neurons was significantly reduced in both SDH and NP region of spinal cord in the both the RA-BVA as compared with those in the RA-Sal group ($p<0.01$, Fig. 7).

Fig. 6. The changes of mechanical threshold (Randall-Selitto test) in saline-treated arthritic animals (RA-Sal, n=10), the aqua fraction of BV treated arthritic animals (RA-BVA, n=10) and the ethylacetate fraction of BV treated arthritic animals (RA-BVE, n=10). Graph depicts the percent inhibition compared to the mechanical of threshold normal animal value in the contralateral left hind paw. **$p<0.01$: significantly different from RA-Sal group.



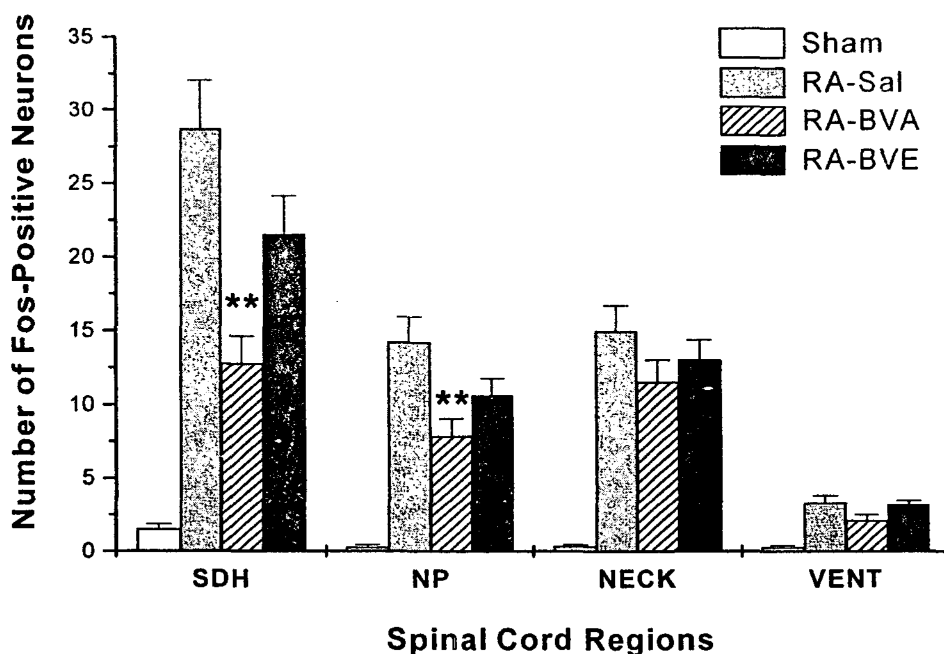
4. Discussion

4.1. Anti-inflammatoy effect of water soluble fraction of bee venom (BVA)

Unilateral Freunds adjuvant injection into the right paw was found to evoke arthritis induced paw edema initially from 12 day after adjuvant injection in contralateral hind limb as previously reported (Eiseman et al., 1982; Philippe et al., 1997).

Our recent study, we demonstrated that whole BV injection into Zusanli acupoint had an anti-inflammatory effect on RA by measuring the change of paw volume (Kwon et al., in press). In present study, we observed that only BVA among BV fraction injection into Zusanli acupoint significantly inhibited arthritis induced paw edema from 12 day

Fig. 7. The number of Fos positive neurons in the contralateral (left) lumbar spinal cord in normal animals (Sham, n=5), saline-treated arthritic animals (RA-Sal, n=5), the aqua fraction of BV treated arthritic animals (RA-BVA, n=5) and the ethylacetate fraction of BV treated arthritic animals (RA-BVE, n=5) at 3 weeks after arthritis induction. The number of Fos expression was analyzed in each of four regions (SDH: lamina I-II, NP: lamina III-IV, NECK: lamina V-VI and VENT: lamina VII-IX).



post RA induction. Accompany with paw edema, we further evaluated that the anti-inflammatory effect of BV extracts was observed by radiological change.

A number of change in bone and periarticular soft tissue occurred during course of adjuvant induced RA (Esser et al., 1995). Using image analysis of radiographs, we had measured for new bone proliferation and soft tissue swelling in the tibio-tarsal joint. It has been previously demonstrated that these measurements were correlated with the results of conventional radiological and histological evalutiaon (Esser et al., 1995). In addition, this method has a more sensitive and quantitative data analysis for radiological image as compared with conventional observation. This method clearly demonstrated that BVA injection was inhibited radiological change (i.e. new bone proliferation and soft tissue swelling) by systemic RA induction. Finally, we measured the serum concentration of IL-6 at 3 weeks after RA induction for anti-inflammatory effect of BVA. Proinflammatory cytokines including IL-6 have been implicated in the chondral degenerative process which causing by decrease of cartilage anabolism matrix in experimental arthritis (Breennan, 1994). It has been reported that systemic concentration of IL-6 in adjuvant injected rats increased by 6 h after injection, peak 12 h, returned to control concentration by 6 days (Philippe et al., 1997). According to systemic RA induction, IL-6 l

4.2. Possible mechanism of anti-inflammatoy effect of water soluble fraction of bee venom (BVA)

In general, water-soluble extract contains peptide, protein and sugar or -OH attached small molecule. Therefore, it is assumed that BVA contained several peptide (i.e. melittin, MCD peptide and adolapin) and enzymes (i.e. phospholipase A2) and some small molecule in whole BV. Melittin, a major component of whole BV and BVA, provokes local pain and edema in animal (Hartman et al., 1991) and induces pain and axon reflex in human subject (Koyama et al., 2000).

The vasodilatory effect by the way of axon reflexes even into the deep tissues may exert a favorable influence upon chronic inflammation increasing the tissue metabolism and eliminating

endogenous and exogenous irritant or toxic substance. It has been reported that counter-irritants such as capsaicin produces antinociceptive phenomenon that may associated with vasodilation by axon reflex (Morris et al., 1995).

Furthermore, melittin binds to secretory phospholipase A2 (PLA2) and inhibits its enzymatic activity. Because PLA2 is a major inflammatory enzyme for release of arachidonic acid whose activity is enhanced in RA, it is possible that the formation of melittin-PLA2 complex by BV injection is able to suppress some of the symptoms associated with the development of arthritis.

Therefore, it is possible that the axon reflex by the peripheral stimulation of melittin and scavenger of PLA2 by melittin play an important role in the anti-inflammatory and antinociceptive effect of BVA. However, it is unclear whether melittin itself represented anti-inflammatory effect of BVA in present study.

Therefore, further fraction study about BVA was required. In addition, it is valuable to test whether melittin has anti-inflammatory effect on RA in further study.

4.3. Anti-nociceptive effect of BVA and its possible mechanism

In this study, arthritic pain induced central sensitization (thermal and mechanical hyperalgesia) was significantly reduced by long term BVA treatment.

The inhibition of nociceptive input by arthritic pain into spinal cord was further measured by the spinal Fos expression. It was previously reported that arthritis induced nociception significantly increased the number of Fos expression in the lumbar spinal cord three weeks after adjuvant injection (Abbadie and Besson, 1992; Kwon et al., in press). This increased number of spinal cord Fos expression is remarkably reduced by treatment with aspirin or whole BV (Abbadie and Besson, 1994; Kwon et al., in press). In the present study, we observed that BVA treatment dramatically suppressed the increase in the number of spinal cord Fos positive neurons induced by arthritic pain. Therefore, we concluded that BVA had antinociceptive effect on RA.

The curative properties of BV have a very long history. In old Egypt many diseases were treated with ointment made from bees. However, the very expansion and scientific research of the

BV therapy of rheumatic diseases started only in the twenties of this century (Eiseman et al., 1982; Hadjipetrou -Kourounakis and Yiangou, 1984).

Traditionally, BV was administered with live bees by stimulating them to sting in the affected area or acupuncture points. Depending on the nature of the disease, it can be used in a cream, ointment or injection form. In this study, we administrated injectable form of BV extract into the acupoint Zusanli. Clinically, acupoint stimulation is used for reducing pain with several modified methods such as mechanical (i.e. needle acupuncture, acupressure), electrical (i.e. electro acupuncture) or thermal stimuli (moxibustion). We are not fully explained why acupoint stimulation produced analgesia. One of the most popular theories is the "gate control theory". According to this theory, the perception of pain is controlled by a part of the nervous system that regulates the impulse that will later be interpreted as pain. Whole BV injection produced tonic pain response (Lariviere, et al., 1996; Chen, 1999) and a brief vocalization at injection time. BVE that did not produce any vocalization at injection time whereas BVA induced a brief vocalization.

Therefore, it is possible that BVA induced stimulation was reduced the nociceptive input according to gate control theory. Presently, other chemical stimulants such as capsaicin and mustard oil are not tested whether acupoint injection produces an antinociceptive effect on RA. However, it is plausible that chemical induced acupoint stimulation by BVA is also valuable methods for inducing the optimal analgesic effect of acupuncture. Therefore, we assumed that BV or its extract potently activated acupoint and subsequently produced antinociceptive effect.

In summary the present study demonstrated that the therapeutical effect of BV therapy was only reproduced by water-soluble fraction of BV (BVA). BVA injection into Zusanli acupoint significantly reduced RA induced inflammatory symptoms such as paw edema, radiological change and the elevation of serum IL-6. In addition, BVA administration into the Zusanli acupoint produced a significant anti -nociceptive effect on arthritis induced inflammatory pain symptoms including thermal and mechanical hyperalgesia.

Further supporting data for an anti-nociceptive effect of BV was obtained using Fos immunohistochemistry which demonstrated that arthritis induced Fos expression in the lumbar spinal cord was significantly decreased in response to BVA administration. Based on the present results, we suggested that BVA may be a effect fraction among whole BV for long-term treatment of RA-induced pain and inflammation.

Acknowledgments

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