Systemic bee venom exerts anti-arthritic and anti-inflammatory properties in a rat model of arthritis

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Received October 12, 2019; Accepted April 16, 2020

DOI: 10.3892/br.2020.1327

Abstract. Bee venom (BV) is widely used as a traditional China medicine to treat various conditions, including rheumatoid arthritis (RA). The aim of the present study was to evaluate the effects of systemic BV (60 mg/kg) as an anti-arthritic natural product, compare it with Methotrexate and determine the possible underlying mechanisms of BV action using complete Freund's adjuvant-induced arthritic rats. The development of signs of RA signs (knee joint circumference and arthritis scoring index) was evaluated. Erythrocyte sedimentation rate, serum tumor necrosis factor-α (TNF-α) and serum interleukin-1β (IL-1β) levels were measured at the end of the study. Histopathological examination followed by immunostaining of NF-κB (P65) was performed on the affected knee joints. Additionally, in vitro cyclooxygenase (COX) inhibition activity, carrageenan paw edema test and acetic acid writhing tests were performed to evaluate the anti-inflammatory and analgesic effects of the assessed dose and compared with diclofenac. An acute toxicity test was performed to establish the safety of BV at high doses. The results of the present study highlighted the potential of systemic BV on preventing the development of signs of RA. BV also significantly reduced serum levels of TNF-α, IL-1β and NF-κB in the affected joints. In addition to its potent analgesic activity, BV exhibited favorable inhibitory activity of the COX pathway in both in vivo and in vitro models. Therefore, high dose administration of systemic BV displayed safe and promising anti-arthritic, anti-inflammatory and analgesic properties through regulation of different mechanisms associated with the pathogenesis of RA.

Introduction

Rheumatoid arthritis (RA) is a common form of arthritis which primarily affects multiple joints, but can also cause damage to other organs, known as extra-articular manifestations (1). The symptoms associated with such conditions include pain, swollen joints, stiffness with synovitis, and progressive cartilage and bone erosion. RA can lead to serious functional disability if it is not properly managed (2).

Although the etiology of RA remains incompletely understood, previous studies have suggested that the activation of T cells potentiates a subsequent activation of other immune cells such as B cells, fibroblasts and macrophages resulting in a complex network of continuously secreted pro-inflammatory cytokines (3-5).

The overproduction of pro-inflammatory cytokines, particularly tumor necrosis factor (TNF-α) and interleukin-1β (IL-1β) are crucially involved in the pathogenesis and progression of RA (6,7). Nuclear factor-κB (NF-κB) activation has also been shown to be associated with the pathogenesis of RA, resulting in cartilage and bone destruction (8). The NF-κB pathway was demonstrated to result in upregulation of pro-inflammatory cyclooxygenase-2 (COX-2) levels and inducible nitric oxide synthase (iNOS), leading to the subsequent production of pro-inflammatory prostaglandins (PGs) and nitric oxide, which lead to further articular damage and induced hyperalgesia (9-11).

The use of alternative natural products for treatment of RA is gaining increasing interest. Bee venom (BV), obtained from Apis mellifera, contains different peptides, including melittin, apamin, adolapin and mast cell degranulating peptide (12). BV is widely used in traditional Chinese medicine for the treatment of inflammatory diseases, such as RA and to alleviate the associated pain (13,14).

Additionally, BV has been reported to exert promising anti-inflammatory and immunomodulatory properties (15-17).

Thus, the aim of the present study was to evaluate the anti-arthritic activity of BV, and the possible underlying immunomodulatory mechanisms of systemic BV in RA using an in vivo experimental model.

Materials and methods

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Key words: bee venom, rheumatoid arthritis, Complete Freund's adjuvant, methotrexate, nuclear factor-κB, cyclooxygenase

Chemicals and solvents. Methotrexate was purchased from Mylan N.V. BV was purchased from Apis Injeel®
Scoring index of arthritic manifestations. Rats were evaluated every 6 days from day 1 for symptoms of RA and other signs of inflammation. The severity of the symptoms in each rat was evaluated by grading the four knee joints on a scale of 0-3 according to the variations in erythema, edema, presence of nodules and the involvement of other non-injected joints with a total score of 12 per rat. The scores were defined as follows: 0, erythema, no swelling with no nodules; 1, erythema, mild swelling with no nodules. 2, erythema, moderate swelling with or without nodules; and 3, erythema with severe swelling limiting the overall movement and presence of nodules or lesions, as described previously (23,24).

Erythrocyte sedimentation rate (ESR). ESR is a non-specific test that indirectly measures the presence of inflammation in a whole blood sample. ESR was evaluated using the Westergren method (25). Blood was drawn from each rat and was placed into disposable vacuum Westergren ESR tubes containing sodium citrate from Wei Hai Kangzhou Biotechnology Co. Ltd. The tubes were allowed to stand vertically for 1 and 2 h. The drop in erythrocyte level was measured and was considered to represent the ESR value (26).

Measurement of the serum levels of TNF-α and IL-1β using ELISA. TNF-α and IL-1β levels were assessed using ELISA. An antibody specific for TNF-α or IL-1β had been pre-coated separately onto microplates, then both the standard and the serum samples were placed into wells to form an immobilized antibody. Subsequent addition of biotin-conjugated antibody then avidin conjugated horseradish peroxidase, followed by a substrate solution was added to the wells resulting in the development of a color if the tested antibody was present in the serum. The intensity of the color was measured and was relative to the quantity of TNF-α or IL-1β bound antibody (27).

Histopathological examination of the knee joints in adjuvant-induced arthritic rats. The preserved knee joints were decalcified in EDTA for 4 weeks, then embedded in paraffin blocks. The blocks were sliced into sections 4 µm thick, and the joint sections were stained with Mayer hematoxylin solution for 8 min and eosin Y solution for 1 min, both at room temperature.

H&E stained sections were scored for changes in cell infiltration, synovitis, synovial proliferation and cartilage or bone erosion. The sections were assessed on a scale of 0-3, and classified as follows: 0, No cell infiltration, no synovitis, intact synovial lining and no damage to the cartilage or the bone; 1, small count of cell infiltration, mild synovitis, limited pannus formation and no apparent damage to the cartilage or the bone; 2, moderate density of infiltrating cells, moderate synovitis, moderate pannus formation and moderate lesions in the cartilage or the bone; and 3, large quantities of infiltrating cells, severe synovitis, severe pannus formation and extensive damage to the cartilage or the bones (28).
Determination of in-vitro COX activity. COX activity was evaluated using a COX (ovine) Inhibitor Screening assay kit that included both ovine COX-1 and human recombinant COX-2 enzymes. The assay was used to screen isozyme-specific inhibitors by direct measurement of PGF2α which is produced by the reduction of COX-derived PGH2 by SnCl2. Finally, the yield was evaluated using enzyme immunoassay for quantification, as described previously (30,31).

Carrageenan paw edema. Acute anti-inflammatory activity was evaluated using a carrageenan-induced rat paw edema test. A total of 15 male Wistar rats were divided into 3 groups. After injecting BV (60 mg/kg, i.p.), rats were treated with diclofenac sodium as a standard drug (5 mg/kg, i.p.) (32). The rats were challenged by subcutaneous injection of 0.1 ml 1% carrageenan solution into the plantar side of the right hind paw. The paw volume was measured using a micrometer caliper, before, and 1, 3 and 4 h after the injection of the carrageenan solution (33,34).

Acetic acid writhing test. To measure the analgesic activity, 20 male albino mice (weighting 20-25 g) were divided into 4 groups (n=5 per group). One group contained healthy rats. Another group served as a control that only received saline as a treatment. The other two groups were treated with wither diclofenac (5 mg/kg, i.p.) (32) or BV (60 mg/kg, i.p.), prior to administration of 0.1 ml/10 g 1% acetic acid (i.p.). The number of writhes or abdominal stretches were counted for 20 min in the mice (35,36).

Acute toxicity study in mice. Acute toxicity in mice was assessed as described previously (37). A total of 20 male albino mice were sorted into 4 groups (n=5). The mice were housed 5 days prior to the start of the study, and the animals were maintained as described above. BV was administrated as a single i.p. dose (60, 600 or 1,200 mg/kg), respectively, to 3 of the groups, and the remaining group served as the control. Following BV treatment, the mice were observed continuously every 2 h for 6 h, then daily for 3 days, for any changes in the general behavior and any signs of toxic manifestations, such as tremors, convulsions, loss of right reflex, muscle spasm, decreased motor activity, sedation, writhing, respiration and mortalities. Blood samples were collected after 3 days for further assessment of liver function using an alanine aminotransferase (ALT) test using a spectrophotometric assay with diagnostic kits purchased from Sigma-Aldrich; Merck KGaA as described previously (38). In addition, kidney function tests were guided by the determination of serum creatinine levels as described previously (39).

Statistical analysis of the data. Results are expressed as the mean ± the standard error of the mean. Analysis was performed using SPSS version 15.0 (SPSS, Inc.). One-way ANOVA followed by a Bonferroni post hoc test was used to compare the differences between the groups. P<0.05 was considered to indicate a statistically significant difference.

Analysis of immunostaining was analysed using ImageJ version 1.45f 112_1.8.0 (National Institutes of Health). The percentage of stained area was compared between groups using a one-way ANOVA followed by a Bonferroni post hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

Knee joint circumference. Immunization of the rats with CFA resulted in the induction of prominent arthritis, as shown by the significant increase in knee joint swelling and an increase in the knee joint circumferences compared with the healthy controls (P<0.05). Individual treatments with methotrexate and BV significantly reduced the knee joint swelling circumferences compared with the control arthritic group (Fig. 1A; both P<0.05).

Arthritic index. Rats injected with CFA showed a significant increase in the arthritis index compared with the healthy rats.
Rats treated with methotrexate or BV showed a significant reduction in the arthritis index at the end of the study (day 21) compared with the arthritic control group (Fig. 1B; both P<0.05).

**ESR.** The arthritic group showed a significant increase in ESR values compared with the normal control (P<0.05). Methotrexate and BV significantly reduced the increase in ESR levels (Fig. 2A and B; both P<0.05).

**Serum TNF-α and IL-1β levels.** The arthritic group showed a noticeable increase (~3-fold) in both the levels of TNF-α and IL-1β, and this increase was consistent throughout the entire duration of the study compared with the normal control group. Treatment with methotrexate significantly reduced TNF-α and IL-1β levels compared with the arthritic control group. Similarly, treatment with BV resulted in a reduction in serum TNF-α and IL-1β levels compared with the methotrexate group, reaching the levels of the normal control rats Fig. 2C and D.
Table I. Assessment of in vitro COX inhibition IC\textsubscript{50} values of bee venom.

| Treatment       | COX-1 IC\textsubscript{50}, µM | COX-2 IC\textsubscript{50}, µM | COX-2 selectivity index |
|-----------------|--------------------------------|-------------------------------|-------------------------|
| Celecoxib       | 15.1                           | 0.049                         | 308                     |
| Indomethacin    | 0.041                          | 0.51                          | 0.08                    |
| Diclofenac sodium | 3.8                            | 0.84                          | 4.5                     |
| Bee venom       | 9.41                           | 0.15                          | 63                      |

Histopathological examination. Histopathological examination of sections of the knee joint showed apparent infiltration of mononuclear inflammatory cells, and cell debris with a high incidence of cartilage destruction and pannus development in the arthritic group. Treatment with methotrexate or BV significantly reduced the histopathological score (P<0.01), and the sections showed notably reduced inflammatory cell infiltration and pannus formation in the synovium and surrounding tissues, and the sections maintained most of the normal joint and tissue construction and integrity when compared with the arthritic control group (Fig. 3).

Immunostaining for NF-κB (p65) expression in arthritic rats. Healthy normal rats showed nearly no staining for NF-κB p65, whereas the arthritic control knee joint sections exhibited the highest intensity of immunostaining and was significantly greater compared with the normal, methotrexate and BV treated groups (P<0.01). Methotrexate treatment resulted in moderate levels of staining, and treatment with BV resulted in a mild density of NF-κB immunostaining in the affected knee joints. The percentage of the area stained was scored to validate the difference in the immunostaining intensity (Fig. 4). A greater intensity of the color represented an increase in NF-κB(p65) expression.

In vitro COX inhibition, IC\textsubscript{50} and COX-2 selectivity index. BV inhibited COX-2 at relatively lower IC\textsubscript{50} doses compared with indomethacin and diclofenac sodium. The COX-2 selectivity index of BV was higher compared with both indomethacin and diclofenac sodium (Table I).

In vivo anti-inflammatory and analgesic activity of BV. BV exhibited systemic anti-inflammatory activity that was shown by the 40.74% reduction in hind paw carrageenan-induced edema compared with diclofenac sodium (37.03%) at 4 h. BV also demonstrated notable analgesic activity as shown by the comparatively lower number of abdominal writhes when compared with the control or diclofenac sodium groups (Table II).

Acute toxicity study. Administration of BV in the albino mice did not exhibit any toxic effects up to and including a dose of 1,200 mg/kg. The mice did not demonstrate any signs of toxicity and there were no mortalities.

Liver function was assessed by performing an alanine aminotransferase (ALT) test. ALT levels in the normal control group were 21.8±0.8 U/l; in the mice treated with 60 mg/kg BV, 27±0.89 U/l; mice treated with 600 mg/kg BV, 36.6±0.5 U/l; and in the mice treated with 1,200 mg/kg, 71.6±1.96 U/l.

Kidney function was preliminarily assessed based on the concentration of serum creatinine. In the normal control group, serum creatinine levels were 0.27±0.01 mg/dl; in the mice treated with 60 mg/kg BV, 0.31±0.008 mg/dl; mice treated with 600 mg/kg BV, 0.39±0.007 mg/dl; and in the mice treated with 1,200 mg/kg BV 0.47±0.009 mg/dl.

Discussion

RA is one of the most common autoimmune diseases. The overall age-standardized prevalence and incidence rates have been increasing globally (40) to almost affect ~1% of the worldwide population (2). The pathophysiology of RA involves the contribution of several complex and connected inflammatory pathways (4).

BV has been widely used in traditional Chinese medicine to alleviate pain and inflammation during chronic inflammatory conditions such as RA (13,41).

Several studies have assessed the effects of administration of different doses of BV via different routes of administration to determine a suitable dosing regimen for management of arthritis. For example, BV treatment was administered subcutaneously at zusani acupoint, and it exhibited potent anti-arthritic activity, although it was not compared with methotrexate (42). In addition, another study demonstrated that concurrent treatment of BV at zusanli acupoint with methotrexate in adjuvant-induced arthritis was more effective than methotrexate alone (29). Additionally, BV administered i.p. 20 µl/100 g/day in collagen induced arthritis (14), and up to 20 mg/kg intraperitoneal for adjuvant-induced arthritis (43) both showed good anti-arthritic properties, although these were not compared with a standard treatment.

The primary purpose of the present study was to evaluate the activity of i.p. BV, and the results of BV treatment showed it may exhibit potential for treatment of RA.

In the present study, induction of arthritis was performed using unilateral intra-articular injection of CFA. Previous studies reported the competence of CFA to induce systemic arthritis in rats (22,44,45). CFA-induced arthritis models in rats were found to result in histological and immunological manifestations of RA. This provided a practical model for investigating systemic BV as a potential anti-arthritic agent (46).

Injection of CFA in the knee joint was shown to produce significant edema in the affected joint and a corresponding increase in the arthritis scoring index within 2 days when compared with the healthy control. Histopathological examination demonstrated the presence of a high density of cell infiltration, alterations in joint integrity and overexpression of NF-κB in the affected joint. Additionally, the arthritic group also showed a significant increase in ESR values, serum TNFα and IL-1β levels. All these changes showed successful induction of arthritis by CFA.

BV was administered by i.p. injection of 60 mg/kg. The dose was selected according to preliminary testing of a range of doses. BV 60 mg/kg was selected as it showed the most prominent anti-arthritic activity based on biochemical and histopathological examination (data not shown). Systemic injection of BV...
was previously reported to exhibit potent anti-arthritic activity at different doses (14,43,47). Previous studies proposed the use of BV at different doses which varied between 1-50 mg. Doses ≤20 mg/kg, i.p, have been evaluated for their anti-arthritic potential (13,43), and another study used 50 mg whole BV in dogs to determine the effect of BV on plasma cortisol levels (48).

The results of the present study showed that individual treatment of BV (60 mg/kg) and methotrexate (a widely-used standard treatment for RA) exhibited potent anti-arthritic activity, as demonstrated through a significant reduction in knee joint swelling circumferences, and a corresponding low arthritic index score. These findings agree with previous studies examining the use of methotrexate and BV (21,43).

Systemic BV was found to significantly reduce ESR values compared with the arthritic group to a similar degree as the methotrexate group. Methotrexate was previously shown to be an effective agent in reducing ESR associated with RA (21,43). This highlights the potential of BV to reduce RA-associated inflammation.

BV significantly reduced TNF-α and IL-1β serum levels compared with the arthritic and methotrexate groups. This may explain the effectiveness of BV in management and alleviation of RA, as both TNF-α and IL-1β are key pro-inflammatory cytokines in the pathogenesis of RA (3).

The results of the present study agree with a previous study which showed that subcutaneous BV treatment reduced serum TNF-α and IL-1β levels in a dose-dependent manner, although they did not compare the effects of BV with a standard treatment (43). BV at a dose of 60 mg/kg reduced serum TNF-α levels to levels similar level as that observed in the healthy group. Previous studies suggested that methotrexate reduced NF-κB expression with limited effect on TNF-α and IL-1β levels (49-51). BV and methotrexate treatment reduced NF-κB levels to a similar degree, which was upregulated in the joints of the arthritic rats. This result was consistent with a study by Darwish et al (29), where methotrexate and BV were administered at the zusanli point (an acupuncture point located below the knee). These findings confirmed the effect of systemic

![Figure 4. Effect of bee venom and methotrexate on NF-κB (p65) expression in knee joints of adjuvant-induced arthritic rats. Representative images of NF-κB immunostaining in knee sections of (A) normal control group; (B) arthritic control group, (C) methotrexate treated group and (D) bee venom treated group. (E) Percentage of area showing NF-κB (p65) expression. **P<0.01 vs. healthy group; ##P<0.01 vs. arthritic control group. NF-κB, nuclear factor-κB.](image-url)
BV on the inhibition of a key inflammatory pathway which contributes to the aggravation of RA manifestations.

The anti-inflammatory activity of BV was evaluated using an *in vitro* assay of COX inhibition and confirmed in the *in vivo* model, carrageenan-induced paw edema. Both results suggested that BV is a more potent anti-inflammatory agent for use of inhibition of COX-2 than the previously well-established anti-inflammatory standards (diclofenac sodium and indomethacin) (52,53), and supports the hypothesis that BV is pivotal in suppressing the pro-inflammatory COX-2 levels. COX-2 inhibition was found to result in subsequent inhibition of pro-inflammatory PGs, particularly PGE2, which are partly involved in the progression of the inflammatory cascade. Previous studies showed *in vitro* inhibition of COX-2 activity using BV and its sub-fractions (54,55).

COX-2 selectivity index of BV was higher compared with diclofenac sodium and indomethacin, which may also explain the increased anti-inflammatory properties, whilst exhibiting acceptable relative COX-1/COX-2 inhibition (56,57).

The results of the acetic acid writhing test demonstrated that systemic BV had potent analgesic activity, which was evident by the lower number of abdominal writhes compared with diclofenac sodium. These findings agree with a previous study that suggested that a high dose of BV treatment by acupuncture produced potent anti-nociceptive effects, regardless of the site of BV injection in an abdominal stretch assay (42).

BV as an anti-nociceptive agent may explain the collective properties of BV on the inhibition of TNF-α levels, which results in desensitizing nociceptive primary afferents (58,59). In addition, BV-mediated inhibition of NF-κB, results in subsequent inhibition of pro-inflammatory COX-2 and iNOS expression, and thus inhibition of PGE2 and NO as demonstrated previously *in vitro* (55). BV inhibited the COX-2 signaling pathway in both the *in vitro* assay and in the carrageenan induced paw edema, and NF-κB and COX-2 pathways have been studied previously for their role in the induction of pain and hyperalgesia (60).

Regarding the safety of BV, doses of 60-1,200 mg/kg, i.p. showed no apparent toxicological manifestations or mortalities. Previous studies also showed that there were no adverse toxic or fatal outcomes detected using a single 1,500 mg/kg dermal dose of BV (61,62). However, a dose of 1,200 mg/kg (20-fold higher than the selected dose) may exert certain pathological effects on the liver and kidneys as shown by the liver and kidney function tests. Previous studies proposed similar concerns, particularly regarding the kidneys (29,63).

The findings of the present study highlight the potential anti-arthritis, anti-inflammatory and anti-nociceptive mechanisms of action of BV (60 mg/kg/day, i.p.) for treatment of RA, BV exerted its effects through inhibition of basic inflammatory axes, including the combined reduction of serum TNF-α, IL-1β and NF-κB expression levels, and inhibition of the COX-2 signaling pathway, all of which are considered cornerstones in the pathophysiology of RA.

**Acknowledgements**

Not applicable.

**Funding**

No funding was received.

**Availability of data and materials**

The datasets used and/or analysed during the present study are available from the corresponding author on reasonable request.

**Authors’ contributions**

All authors contributed to the study conception and design. DMET wrote the original draft of the manuscript and performed the experiments. MMAA reviewed the manuscript. MWH performed the data analysis. AIG supervised the study. All authors read and approved the final manuscript.

**Ethics approval and consent to participate**

Ethical approval for all the procedures was granted by the Ethics Committee of the Faculty of Pharmacy, Damanhour University, (Damanhour, Egypt) (approval no. 717PO5).

**Patient consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests and all authors confirm its accuracy.

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