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General pharmacological profiles of bee venom and its water soluble fractions in rodent models

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Recently, the antinociceptive and anti-inflammatory efficacy of bee venom (BV, *Apis mellifera*) has been confirmed in rodent models of inflammation and arthritis. Interestingly, the antinociceptive and anti-inflammatory effect of whole BV can be reproduced by two water-soluble fractions of BV (>20 kDa:BVAF1 and <10 kDa: BVAF3). Based on these scientific findings, BV and its effective water-soluble fractions have been proposed as potential anti-inflammatory and antinociceptive pharmaceuticals. While BV's anti-inflammatory and antinociceptive properties have been well documented, there have been no careful studies of potential, side effects of BV and its fractions when administered in the therapeutic range (BV, 5 μg/kg; BVAF1, 0.2 μg/kg: BVAF3, 3 μg/kg; subcutaneous or intradermal). Such information is critical for future clinical use of BV in humans. Because of this paucity of information, the present study was designed to determine the general pharmacological/physiological effects of BV and its fractions administration on the rodent central nervous, cardiovascular, respiratory and gastrointestinal system. Subcutaneous BV and its fractions treatment did not produce any significant effects on general physiological functions at the highest dose tested (200-fold and 100-fold doses higher than that used clinically, respectively) except writhing test. These results demonstrate that doses of BV or BV subfractions in the therapeutic range or higher can be used as safe antinociceptive and anti-inflammatory agents.

Key words: bee venom, general pharmacology, antinociception, anti-inflammation

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Introduction

For several centuries, bee venom (BV) of *Apis mellifera* has been used in oriental medicine to treat a number of inflammatory diseases including tendonitis, bursitis and rheumatoid arthritis [1]. BV therapy has been considered as an alternative to more traditional acupuncture and moxibustion therapy. Recently, we have demonstrated that BV therapy also produces potent therapeutic effects on osteoarthritis [7]. Subsequently, the anti-inflammatory and antinociceptive effects of BV were further verified using several animal models with acute and chronic nociception. For example, subcutaneous treatment of BV produced a dramatic anti-inflammatory and antinociceptive effect on Freund's adjuvant-induced arthritis in rats [8]. In addition, subcutaneous BV treatment significantly suppressed the paw edema and hyperalgesia associated with carrageenan-induced acute inflammation in rats [11]. Moreover, subcutaneous BV treatment produced significant visceral antinociception in mice following abdominal acetic acid injection [6] and it suppressed pain behaviors and spinal Fos expression in rats induced by hindpaw formalin injection [5].

As a crucial step towards determining the specific antinociceptive and anti-inflammatory components of BV, whole BV constituents were fractionized according to their solubility (i.e. water-, ethylacetate-, and hexane-soluble fractions) and subsequently tested for their antinociceptive and anti-inflammatory properties. The results of this study indicated that the water-soluble fraction of BV (BVA) is responsible for producing BV's anti-inflammatory and antinociceptive effects in a rodent model of rheumatoid arthritis [9]. BVA contains high molecular weight enzymes (glycoproteins >20 kDa) including phospholipase A_2 and hyaluronidase as well as low molecular weight polypeptides

(<10 kDa) that include melittin, apamin, adolapin and mast cell degranulating (MCD) peptide [10]. There appear to be fewer constituents with molecular weights between 10 and 20 kDa in whole BV and these substances have not been well characterized. BVA has been purified in our laboratory and separated into the following three molecular weight fractions: BVAF1 (>20 kDa), BVAF2 (<20 kDa and >10 kDa), and BVAF3 (<10 kDa). Each fraction has been tested for pharmacological efficacy in previous studies in our lab. The results of this study indicate that subcutaneous injection of the BVAF1 and BVAF3 fractions produce the greatest suppressive effect on Freunds adjuvant-induced paw edema and on the mechanical/thermal hyperalgesia associated with Freund's adjuvant-induced inflammation in rats. In addition these two fractions alleviated radiological changes (i.e. bone proliferation and soft tissue swelling) in rat model with joint arthritis.

Despite the accumulating evidence showing a profound antinociceptive and anti-inflammatory effect of subcutaneous BV and BVA treatment, there have been very few studies that have examined the effect of BV or BVA therapy on a variety of physiological systems. Such information is important with respect to drug safety issues and is critical for the predicted increasing use of BV or its fractions for treating human patients. Because of the paucity of information related to these issues, the present study was designed to investigate the general pharmacological effects of BV, BVAF1 and BVAF3 on several physiological parameters of the central nervous, digestive, cardiovascular and respiratory systems in rodents.

Materials and Methods

Test reagents

Bee venom (BV) of *Apis mellifera* was purchased from Sigma (USA). The water-soluble fraction of BV was partitioned from whole BV and the water-soluble partition was subsequently fractionated by molecular weight into BVAF1 (>20 kDa) and BVAF3 (<10 kDa) using Minitan Filter plates (Millipore, USA) as previously described [12]. Each fraction was completely dried and then stored at refrigerator temperature. A single clinical dose of BV is 5 μg/kg when administered by either an intradermal or subcutaneous route in human patients in Korea. Since BV subfractions have not been administered to human patients, the theoretical dose was calculated by considering the partial ratio of the subfractions to whole BV. Based on this ratio we calculated the clinical dose of the BVAF1 and BVAF3 subfractions to be 0.2 μg/kg and 3 μg/kg, respectively. BV and the BVAF1 and BVAF3 subfractions were dissolved in saline and then administrated subcutaneously to the animals in each experimental group. In order to examine dose-response characteristics, a high dose of BV or its fractions was selected in terms of the range from 10-fold to 100-fold the effective clinical dose.

Acetic acid and atropine sulfate were purchased from Fluka (CH-9471, Buchs, Switzerland). Acetylsalicylic acid, aminopyrine, activated charcoal, chlorpromazine HCl and sodium pentobarbital were purchased from Sigma (USA). These positive drugs were administered simultaneously with vehicle or with BV or its subfractions. A standard physiological saline solution was used as the vehicle for all experiments.

Animals

These experiments were performed on male ICR mice (25-30 g), Sprague-Dawley rats (200-300 g) and New Zealand White rabbits (2-2.5 kg). All laboratory animals were obtained from the Hallym Laboratory of Animal Sciences (Korea). The protocol for animal care used in the present study were approved by the Animal Care and Use Committee at Seoul National University and its methodology conforms to the published guidelines of the USA National Institutes of Health (NIH publication No. 86-23, revised 1985). In addition, the ethical guidelines of the International Association for the Study of Pain for investigating experimental pain in conscious animals were also followed [17]. Animals were housed under the conditions of constant temperature ($23 \pm 2^{o}C$), relative humidity ($55 \pm 5\%$), and light/dark cycle (12 h/12 h: illumination at 7 : 00 AM) until the day of the experiment (a minimum 7 day acclimation period).

Effect of BV fractions on the central nervous system

General behavior in mice: Each dose of BV, BVAF1 or BVAF3 was administrated subcutaneously in separate groups of ICR mice (total n = 90). In the control group, physiological saline was injected into corresponding site. Two experimenters, blinded to the animal treatment, observed and recorded details of behavior at 5, 15, 30, 60, 120, 180 min and 24 h after BV or saline treatment using a modification of the approach described by Irwin [3]. Animals were checked daily for mortality, gross signs of toxicity and abnormal behavior for 7 days post-treatment.

Sleep-induction time and duration in mice: Vehicle, and BV, BVAF1 and BVAF3 were administered subcutaneously 30 min prior to sleep induction (total n = 90 mice; n = 10 mice/group). Sodium pentobarbital (32 mg/kg), sedative/anesthetic drug was injected intraperitoneally in each group of mice to induce sleep. One group of mice (n = 10) was intramuscularly injected with chlorpromazine HCl (1 mg/kg) as a positive control because the aliphatic phenothiazine drugs, such as chlorpromazine, are highly sedative. The effect of different doses of BV, BVAF1 and BVAF3 on sleep induction time and on sleep duration produced by sodium pentobarbital was subsequently analyzed. The loss of the

mouses righting reflex was selected as a marker of sleep-induction. The duration of sleeping time was calculated as the time from disappearance to reappearance of the righting reflex. Loss of the righting reflex was defined as an inability of a mouse to right itself 3 times within 30 sec, whereas recovery of the righting reflex was defined as the point at which the mouse could right itself during a timed 30 sec period.

Spontaneous activity in mice: The distance that a mouse traveled during a 65 min test period was measured using a spontaneous activity chamber (MED Associates, USA, Model# SG-506). The activity test was initiated just after the administration of test drugs and was stopped 65 min later. Spontaneous ambulatory activity was determined in an open field (43 × 43 cm) plexiglass box with height of 30 cm, equipped with infra-red photocells located in the walls 2 cm above a grid floor. The 16 photocells were spaced 2.5 cm apart, measured from center to center. Ambulatory activity was expressed as the distance traveled, calculated on the basis of the number of interruptions of the photobeams. Several doses of BV, BVAF1 and BVAF3 were evaluated to determine their effect on ambulatory activity (total n = 90). As a positive control, chlorpromazine HCl (5 mg/kg) was administered intramuscularly (n = 10).

Motor function in mice (rota-rod test): After drug treatment, forced motor performance was tested using a standard rota-rod apparatus (Dae-Jong Engineering & Clean Technology, Korea Model# DJ-4009). The rota-rod test is usually used to examine possible deficits in motor function including motor incoordination and ataxia in rodents [2]. Mice were placed on a rotating rod (12 cm wide; 6 cm diameter) suspended 33 cm above the bottom of the apparatus. Escape to either side was prevented by a plexiglas wall. After placing each mice on the rod, the unit was activated and set at a speed of 4 revolutions per min. Each animal was tested three times and each time trial lasted for 60 sec or until the animal fell from the platform. Animals were tested before and at 0.5, 1, 2 and 4 h after the administration of BV, BVAF1 or BVAF3. Quantification of the number of mice that fell from the rota-rod during each 60 second trial was performed rather than using the more traditional accelerating rota-rod and analyzing latency to fall. This approach was used to allow us to test the mice four times within a short time period following injection of BV, BVAF1 and BVAF3. Moreover, using 60 sec trial cutoffs does not result in muscle fatigue and therefore more accurately tests motor coordination. As a positive control, chlorpromazine HCl (5 mg/kg) was intramuscularly injected (total n = 100).

Body temperature in mice: All animals were first fasted for 24 h prior to the measurement of body temperature. This was done because the digested contents within the large intestine could interfere with the determination of rectal temperature which would increase the variance of the temperature readings. Body temperature was determined using a thermistor thermometer (Cole-Parmer, USA, Model# 8402-00) to measure rectal temperature. Mice were gently restrained and then a lubricated thermistor probe was inserted 3 cm into the rectum for 20 sec to stabilize rectal temperature. As a positive control, aminopyrine (50 mg/kg) was intramuscularly injected. Body temperature was measured before and at 0.5, 1, 2, 3, 5, and 7 h after the administration of BV, BVAF1 and BVAF3 (total n = 100).

PTZ-induced convulsions in mice: Pentetrazole (pentyleneteterazole, PTZ) has been commonly used to induce convulsions in rodents [14]. All animals used for this phase of the study were fasted for 24 h before PTZ administration to minimize variability. PTZ (85 mg/kg) was subcutaneously administered into the back 30 min after vehicle, BV, BVAF1 or BVAF3 treatment (n = 90). The number of convulsions that occurred during an one-hour period following PTZ administration was counted. As a positive control, pentobarbital sodium (5 mg/kg) was injected intramuscularly (total n = 10) 30 min prior to PTZ injection.

Analgesic activity in mice (writhing test): A group of mice were placed in a temperature-regulated Plexiglas observation chamber (60 cm height; 40 cm diameter) and acclimated for 30 min before the test. Acetic acid (0.9%, 200 μl/10 g B.W.) was then injected intraperitoneally and the number of abdominal constrictions (writhing reflex) was counted. In order to obtain an unimpeded view of the abdomen, a mirror was attached underneath the transparent glass floor of the chamber and set to an angle of 45°. Acetic acid solution was injected 30 min post-injection of vehicle, BV, BVAF1 or BVAF3 (total n = 90). For the next 30 min the number of abdominal constrictions was counted. Abdominal constrictions were characterized by strong contractions of the abdominal musculature accompanied by dorsiflexion of the back and extension of the hindlimbs. As a positive control, calcium acetylsalicylic acid (100 mg/kg) was administered intramuscularly 30 min prior to acetic acid injection (n = 10).

Effect of BV fractions on the digestive system

GI propulsion of charcoal in mice: A modification of the method of Takemori *et al*. was utilized for this test [13]. Before the test, all experimental mice were fasted for 24 h. Following the fasting period active charcoal (5%, 200 μl, in 0.5% CMC suspension) was administered orally using a gastric probe, 30 min after the injection of vehicle, BV, BVAF1 or BVAF3 (total n = 81). Four hours after the charcoal injection, animals were euthanized by cervical

dislocation. Intestinal motility was determined by measuring the distance that the charcoal traveled from the pylorus. The distance was expressed as a percentage of the distance from the pylorus to the rectum. As a positive control, atropine sulfate (5 mg/kg) was given 30 min prior to charcoal administration (n = 9).

Secretion of gastric juice: To minimize contamination of the gastric juice, rats were fasted for 48 h prior to the test (total n = 72). Rats were initially anesthetized with 3% isoflurane (Baxter, USA) in 70% O_2/30% N_2O and then maintained on 1.5% isoflurane during the surgical procedure to ligate the gastric pylorus. After the surgery, vehicle and BV fractions were subcutaneously injected and 5 h post-injection, the rats were euthanized and their gastric juice was collected. The gastric content was centrifuged and the resulting supernatant was used for analysis. Samples grossly contaminated with blood or bile juice were discarded. Potentiometric measurements were performed at 25°C with a pH meter (Istek, Korea, Model# 720P). For conductometric titrations, 2 ml of gastric juice were pipetted into a suitable titration vessel and diluted to 1000 ml with distilled water. A standardized titration reagent, 0.1 M NaOH, was slowly added to the diluted gastric juice and the amount added was used to calculate the total acidity of the gastric juice [15].

Effects on the cardiovascular and respiratory system

Blood pressure and heart rate in awake rats: Animals were acclimated for 1 h in a test room prior to starting the test (total n = 27). The tail of each rat was pre-heated using a heating chamber for 10 min and the rats were subsequently fitted with a tail cuff pulse sensor (Narco Bio-systems, USA) and the systolic blood pressure was then measured. This experiment was repeated 3 times and the mean value for each animal was recorded. Blood pressure and heart rates were recorded at 0, 0.5, 1 and 2 h after treatment with vehicle, BV, BVAF1 or BVAF3.

Respiratory rates in anesthetized rabbits: Male New Zealand White rabbits were anesthetized by intraperitoneal administration of pentobarbital sodium (50 mg/kg) and fitted with a respiration belt (Narco Bio-systems, USA). Respiratory rates were analyzed at 0, 5, 15, 30 and 60 min after each drug treatment (total n = 27).

Statistical analysis: Data are presented as the mean ± the standard error of the mean (SEM). Statistical analyses were performed using a paired *t*-test for most assays. Test values for body temperature, spontaneous activity and blood pressure were statistically analyzed using a two-way repeated measure analysis of variance (ANOVA). A *P* value of < 0.05 was considered to be significant.

Table 1. Effect of bee venom (BV), BVAF1 and BVAF3 on pentobarbital sodium induced sleep-induction time and sleep duration in mice

Treatment	Dose (mg/kg)	N	Sleep induction time (min)	Sleep duration (min)
Vehicle	-	10	4.3 ± 0.4	40.2 ± 6.3
BV	0.005	10	5.4 ± 0.7	28.5 ± 3.5
	1	10	6.2 ± 1.9	28.5 ± 3.8
BVAF1	0.0002	10	5.0 ± 0.4	36.6 ± 5.5
	0.002	10	5.8 ± 0.4	39.5 ± 7.1
	0.02	10	3.0 ± 0.6	34.4 ± 5.0
BVAF3	0.003	10	3.9 ± 0.3	44.7 ± 3.9
	0.03	10	4.3 ± 0.4	39.0 ± 4.6
	0.3	10	4.1 ± 0.2	44.5 ± 6.0
Chlorpromazine HCl	1	10	4.0 ± 0.2	65.6 ± 5.7*

Each value represents the mean ± SEM. N = number of animals.
Statistical significance of difference from the vehicle group (*$p < 0.05$).

Results

Effect on the central nervous system

General behavior in mice: Animals that received subcutaneous injections of BV or BV fractions (BVAF1 and BVAF3) showed normal behavior during the 7-day examined post-injection. There was no evidence of abnormal behavior nor any signs of toxicity observed during this 7-day period.

Sleep-induction time and sleep duration in mice: The mean time to induction of sleep in the vehicle group was 4.3 ± 0.4 min post-injection of sodium pentobarbital (32 mg/kg). The mean sleep duration time was 40.2 ± 6.3 min in the vehicle-injected group (Table 1). BV and BVA fractions (BVAF1 and BVAF3) administered subcutaneously at various doses did not alter the sleep-induction time or sleep duration time as compared to that of animals subcutaneously injected with vehicle. In contrast, the injection of chlorpromazine HCl (1 mg/kg) significantly increased the time of sleep duration (65.6 ± 5.7, $p < 0.05$).

Spontaneous activity in mice: During the initial period following placement in the activity box, animals of all groups showed increased ambulation that was related to the exploratory phase of being placed in the novel environment of the activity chamber (Table 2). Spontaneous ambulatory activity (indicated by the distance traveled in Table 2) gradually decreased over the 65 min recording period as the animals became acclimated to the new environment. The distance traveled by the animals in the BVAF1 group (0.2

Table 2. Effect of bee venom (BV), BVAF1 and BVAF3 on spontaneous ambulatory activity

Treatment	Dose (mg/kg)	N	Distance traveled (cm)						
			0-5	5-15	15-25	25-35	35-45	45-55	55-65
Vehicle	-	10	524.5±66.2	598.7±55.9	311.5±64.3	317.1±74.9	226.6±47.7	194.2±60.4	188.2±48.4
BV	0.005	10	553.0±80.0	644.3±53.7	597.8±66.3	642.8±24.0	426.1±59.9	257.3±84.5	156.0±27.5
	1	10	343.7±96.6	236.1±94.7	184.8±71.4	191.3±52.8	249.1±79.1	126.7±71.8	135.2±66.1
BVAF1	0.0002	10	639.4±53.3	773.6±105.2	538.4±83.9	402.2±55.3	423.8±40.9*	298.8±37.1	277.1±44.7*
	0.002	10	605.9±41.1	646.1±95.3	422.0±103.7	229.8±84.9	352.8±119.9	283.4±109.7	158.9±79.9
	0.02	10	632.5±86.2	656.4±100.0	406.5±72.3	368.5±85.4	353.6±109.5	219.7±98.0	166.6±80.2
BVAF3	0.003	10	714.9±53.4	700.2±97.1	529.4±90.6	438.8±128.0	326.3±112.3	346.5±131.8	236.1±76.1
	0.03	10	569.5±45.0	702.7±78.6	546.9±72.8	434.7±80.8	364.4±89.7	237.2±102.4	239.7±75.3
	0.3	10	555.1±49.4	490.3±76.0	281.3±57.2	153.7±70.1	198.6±54.9	234.7±96.5	100.8±59.1
Chlorpromazine HCl	5	10	105.3±52.3^{+}	33.7±19.2^{+}	11.8±5.5^{+}	30.4±16.5**	12.4±9.6^{+}	21.2±15.5**	14.2±7.0*

Each value represents the mean ± SEM. N = number of animals.
Statistically significant differences compared to the vehicle group (*p<0.05, **p<0.01 and $^{+}$p<0.001).

Table 3. Effect of bee venom (BV), BVAF1 and BVAF3 on rota-rod performance in mice

Treatment	Dose (mg/kg)	N	Number of mice that fell				
			Before	0.5 h	1 h	2 h	4 h
Vehicle	-	10	0.3 ± 0.2	0.3 ± 0.2	0.1 ± 0.1	0.4 ± 0.3	0.4 ± 0.2
BV	0.005	10	0.3 ± 0.2	0	0.2 ± 0.2	0.5 ± 0.2	0.5 ± 0.5
	1	10	0	0.7 ± 0.5	0.7 ± 0.3	0.2 ± 0.2	0.3 ± 0.2
BVAF1	0.0002	10	0.3 ± 0.2	0	0	0.2 ± 0.2	0
	0.002	10	0.1 ± 0.1	0	0.2 ± 0.1	0.1 ± 0.1	0
	0.02	10	0.1 ± 0.1	0	0	0	0
BVAF3	0.003	10	0.1 ± 0.1	0	0	0	0.4 ± 0.2
	0.03	10	0	0.2 ± 0.1	0	0	0.1 ± 0.1
	0.3	10	0.2 ± 0.1	0	0.1 ± 0.1	0.1 ± 0.1	0.3 ± 0.2
Chlorpromazine HCl	5	10	0.2 ± 0.2	7.3 ± 0.7^{+}	7.3 ± 1.3^{+}	6.3 ± 0.8^{+}	3.5 ± 0.9**

Each value represents the mean ± SEM. N = number of animals.
Statistical significance of difference from the vehicle group (**p<0.01 and ^{+}p<0.001).

μg/kg) was found to be temporarily increased at the 35-45 and 55-65 min post-injection intervals when compared to the vehicle-injected mice at each time point using a *t*-test ($p < 0.05$). However, when these data were analyzed using a two-way repeated measure ANOVA, this dose of BVAF1 did not significantly affect the spontaneous activity in this experiment. There were no significant changes in spontaneous activity in the BV or BVAF3 groups. On the other hand, in the positive control group, chlorpromazine HCl (5 mg/kg) significantly suppressed spontaneous locomotor activity as predicted based on its known sedative effect.

Motor function in mice (rota-rod test): In comparison with the vehicle group, BV and BVA fractions (BVAF1 and BVAF3) did not produce any significant changes in motor function at any doses tested in the present study (Table 3). However, chlorpromazine HCl (5 mg/kg), used as a positive control drug, significantly decreased motor performance on the rota-rod ($p < 0.01$ and $p < 0.001$).

Body temperature in mice: Animals in the vehicle group showed small but non-significant changes in body temperature over the 7 h time-course of this experiment (Table 4). Similarly the mice that received BV or one of the BVA fractions (BVAF1 or BVAF3), showed no significant alterations in body temperature over the 7 h test period. Conversely, the positive control drug, aminopyrine (50 mg/kg), significantly decreased body temperature at nearly all the observation time period, except for the final, time-points tested ($p < 0.05$ and $p < 0.001$).

Table 4. Effect of bee venom (BV), BVAF1 and BVAF3 on body temperature for 7 h

Dose (mg/kg)		Vehicle	aminopyrine	BV		BVAF1			BVAF3		
		-	50	0.005	1	0.0002	0.002	0.02	0.003	0.03	0.3
N		10	10	10	10	10	10	10	10	10	10
Body temperature (°C)	Before	37.1±0.2	36.9±0.3	38.6±0.2	37.9±0.2	36.8±0.2	37.1±0.2	37.3±0.4	36.8±0.3	36.9±0.2	36.7±0.3
	0.5 h	37.0±0.2	35.2±0.2^{+}	38.2±0.1	37.3±0.1	36.8±0.2	37.3±0.2	37.1±0.4	36.3±0.2	36.8±0.3	37.2±0.2
	1 h	37.5±0.1	35.8±0.3^{+}	38.1±0.1	37.6±0.1	37.0±0.2	37.4±0.2	37.3±0.3	37.0±0.2	37.5±0.2	37.7±0.1
	2 h	36.8±0.2	36.4±0.2	37.3±0.2	37.2±0.1	36.3±0.2	37.1±0.2	36.5±0.4	35.7±0.3	37.0±0.1	37.1±0.1
	3 h	37.1±0.2	36.4±0.3*	36.9±0.2	36.9±0.2	36.6±0.2	37.1±0.2	36.6±0.4	36.6±0.2	37.9±0.4	37.5±0.2
	5 h	37.0±0.1	36.4±0.2*	37.1±0.2	37.1±0.2	36.4±0.2	36.7±0.1	36.5±0.4	36.5±0.1	36.8±0.1	37.2±0.1
	7h	36.7±0.3	36.6±0.3	37.5±0.2	37.4±0.2	36.1±0.2	36.3±0.1	36.0±0.4	35.9±0.2	36.9±0.2	36.9±0.1

Each value represents the mean ± SEM. N = number of animals.
Statistically significant from the vehicle group (*p<0.05 and ^{+}p<0.001).

Table 5. Effect of bee venom (BV), BVAF1 and BVAF3 on pentyltetrazole-induced convulsions in mice

Treatment	Dose (mg/kg)	N	No. of convulsions
Vehicle	-	10	1.3 ± 0.4
BV	0.005	10	1.0 ± 0.1
	1	10	1.3 ± 0.4
BVAF1	0.0002	10	1.3 ± 0.4
	0.002	10	1.0 ± 0.3
	0.02	10	1.1 ± 0.3
BVAF3	0.003	10	1.4 ± 0.5
	0.03	10	1.3 ± 0.4
	0.3	10	1.3 ± 0.3
Pentobarbital sodium	5	10	0.3 ± 0.2*

Each value represents the mean ± SEM. N = number of animals.
Statistical significance of difference from the vehicle group (*p<0.05).

Table 6. Effect of bee venom (BV), BVAF1 and BVAF3 on acetic acid-induced writhing reflex in mice

Treatment	Dose (mg/kg)	N	No. of writhes
Vehicle	-	10	13.3 ± 1.1
BV	0.005	10	8.8 ± 1.2*
	1	10	1.4 ± 0.9^{+}
BVAF1	0.0002	10	13.1 ± 1.4
	0.002	10	11.6 ± 1.5
	0.02	10	12.2 ± 2.6
BVAF3	0.003	10	11.1 ± 2.2
	0.03	10	10.6 ± 2.0
	0.3	10	7.7 ± 1.4**
Calcium acetylsalicylic acid	100	10	7.4 ± 1.7*

Each value represents the mean ± SEM. N = number of animals.
Statistical significance of difference from the vehicle group (*p<0.05, **p<0.01 and ^{+}p<0.001).

Drug-induced convulsion time in mice: The number of convulsions evoked by pentyltetrazole over the 1 h test period was 1.3 ± 0.4 in vehicle group (Table 5). There were no significant differences in the number of convulsions induced by pentyltetrazole among the vehicle group and the BV, BVAF1 or BVAF3 injected groups. However, sodium pentobaribital treatment at a dose of 5 mg/kg significantly decreased the number of convulsions (0.3 ± 0.2, $p < 0.05$).

BV-induced analgesic activity in mice (writhing assay): The mean number of abdominal stretches in animals that received a subcutaneous injection of vehicle 30 min prior to an intraperitoneal injection of 0.9% acetic acid was 13.3 ± 1.1 (Table 6). In the whole BV treatment group, the lowest dose of BV (0.005 mg/kg) tested significantly suppressed the abdominal stretch reflex (8.8 ± 1.2, $p < 0.05$). The highest dose of BV (1 mg/kg) tested produced a much more decrease in the number of abdominal stretches elicited by intraperitoneal acetic acid injection (1.4 ± 0.9, $p < 0.001$). Between the two BVA fraction groups (BVAF1 and BVAF3), only the highest dose of BVAF3 (0.3 mg/kg) tested significantly reduced the number of abdominal stretches (7.7 ± 1.4, $p < 0.01$). In the positive control group, calcium acetylsalicylic acid (100 mg/kg) also significantly suppressed the number of abdominal stretches (7.4 ± 1.7, $p < 0.05$).

Effect of BV and its fractions on the digestive system

Charcoal propulsion in mice: The peristaltic distance traveled by the activated charcoal during the 4 h test period was 93.7 ± 1.2% of whole gastrointestinal length in the vehicle treatment group (Table 7). In comparison with the vehicle group, BV and BVA fractions (BVAF1 and BVAF3) did not produce any significant changes in the gastrointestinal

Table 7. Effect of bee venom (BV), BVAF1 and BVAF3 on gastrointestinal motility in mice

Treatment	Dose (mg/kg)	N	% Peristaltic distance[1)]
Vehicle	-	9	93.7 ± 1.2
BV	0.005	9	91.2 ± 2.1
	1	9	92.3 ± 1.9
BVAF1	0.0002	9	88.9 ± 4.8
	0.002	9	95.7 ± 1.1
	0.02	9	93.3 ± 1.3
BVAF3	0.003	9	92.5 ± 1.4
	0.03	9	91.6 ± 5.7
	0.3	9	91.7 ± 1.1
Atropine sulfate	5	9	71.4 ± 5.5**

Each value represents the mean ± SEM. N = number of animals.
[1)]% Peristaltic distance = (peristaltic distance of charcoal from the stomach/total gut length.)×100
Statistical significance of difference from the vehicle group (**p<0.01).

transit distance at any doses tested in the present study. However, the positive control drug, atropine sulfate (5 mg/kg), significantly suppressed gastrointestinal motility (gastrointestinal transit distance = 71.4 ± 5.5%, $p < 0.01$).

Secretion of gastric juice: In the vehicle control group, pH, gastric volume and total acidity was 2.1 ± 0.3, 2.5 ± 0.3 ml and 100.0 ± 8.6 mEq/L HCl, respectively, at 5 h post-treatment (Table 8). As compared to vehicle-injected animals, the values obtained for pH, gastric volume and total acidity were not significantly different in the animals treated with BV or BVA fractions (BVAF1 and BVAF3) at any doses tested.

Effect of BV and BV fractions on the cardiovascular and respiratory system

Blood pressure and heart rate in awake rats: Treatment with BV or BVA fractions (BVAF1 and BVAF3) did not

Table 8. Effect of bee venom (BV), BVAF1 and BVAF3 on gastric secretion in rats.

Treatment	Dose (mg/kg)	N	pH	Gastric vol. (ml)	Total acidity (mEq/L HCl)
Vehicle	-	8	2.1 ± 0.3	2.5 ± 0.3	100.0 ± 8.6
BV	0.005	8	2.2 ± 0.3	3.0 ± 0.3	93.0 ± 6.8
	1	8	2.5 ± 0.3	2.7 ± 0.2	94.0 ± 6.8
BVAF1	0.0002	8	2.8 ± 0.4	2.6 ± 0.5	68.8 ± 5.8
	0.002	8	3.0 ± 0.6	2.0 ± 0.4	79.2 ± 12.0
	0.02	8	2.2 ± 0.2	1.8 ± 0.2	74.4 ± 6.5
BVAF3	0.003	8	2.2 ± 0.2	2.4 ± 0.6	87.9 ± 9.6
	0.03	8	2.4 ± 0.5	3.9 ± 0.9	100.6 ± 12.4
	0.3	8	1.5 ± 0.1	3.0 ± 0.8	108.3 ± 10.9

Each value represents the mean ± SEM. N = number of animals.

Table 9. Effect of bee venom (BV), BVAF1 and BVAF3 on systolic blood pressure in rats.

Treatment	Dose (mg/kg)	N	Mean arterial blood pressure (mmHg)			
			Before	30 min	1 h	2 hrs
Vehicle	-	3	97.0 ± 5.5	95.7 ± 4.4	102.3 ± 2.7	103.0 ± 6.4
BV	0.005	3	91.6 ± 6.8	104.9 ± 1.5	109.0 ± 4.4	104.8 ± 6.1
	1	3	91.4 ± 10.5	102.1 ± 0.5	104.8 ± 12.8	99.3 ± 16.2
BVAF1	0.0002	3	94.0 ± 2.5	102.0 ± 9.5	93.7 ± 8.2	93.0 ± 9.0
	0.002	3	101.3 ± 8.8	107.7 ± 2.6	96.7 ± 3.2	95.3 ± 4.9
	0.02	3	93.0 ± 12.1	98.3 ± 8.6	92.0 ± 5.0	92.3 ± 7.8
BVAF3	0.003	3	107.0 ± 5.7	102.0 ± 11.5	105.3 ± 2.8	98.3 ± 3.3
	0.03	3	94.3 ± 8.3	108.7 ± 1.7	106.3 ± 2.2	99.7 ± 7.7
	0.3	3	96.3 ± 13.2	97.7 ± 8.1	95.7 ± 6.5	111.3 ± 10.0

Each value represents the mean ± SEM. N = number of animals.

Table 10. Effect of bee venom (BV), BVAF1 and BVAF3 on heart rates in rats

Treatment	Dose (mg/kg)	N	Heart rates (beats/min)			
			Before	30 min	1 h	2 h
Vehicle	-	3	412.7 ± 8.4	383.7 ± 11.7	418.7 ± 8.7	412.0 ± 8.1
BV	0.005	3	346.7 ± 4.8	365.0 ± 9.5	374.0 ± 16.6	385.7 ± 16.7
	1	3	346.7 ± 9.2	360.7 ± 13.8	370.3 ± 9.8	360.7 ± 3.9
BVAF1	0.0002	3	372.0 ± 15.3	409.0 ± 11.9	411.3 ± 4.8	417.7 ± 10.5
	0.002	3	371.0 ± 17.5	361.7 ± 15.4	355.0 ± 15.1	376.7 ± 18.2
	0.02	3	362.7 ± 13.1	383.3 ± 8.8	393.7 ± 7.4	398.0 ± 10.7
BVAF3	0.003	3	351.3 ± 6.4	393.0 ± 16.3	400.3 ± 10.4	415.3 ± 4.7
	0.03	3	367.3 ± 18.1	360.7 ± 16.7	358.3 ± 10.5	387.3 ± 10.5
	0.3	3	361.0 ± 11.7	381.7 ± 16.8	386.3 ± 6.4	373.7 ± 14.7

Each value represents the mean ± SEM. N = number of animals.

Table 11. Effect of bee venom (BV), BVAF1 and BVAF3 on respiratory rates in rabbits

Treatment	Dose (mg/kg)	N	Respiratory rates (times/min)				
			Before	5 min	15 min	30 min	60 min
Vehicle	-	3	58.0 ± 8.7	58.0 ± 8.7	50.0 ± 4.4	55.3 ± 5.2	62.0 ± 8.5
BV	0.005	3	51.3 ± 2.4	46.3 ± 11.1	48.0 ± 2.6	43.7 ± 8.4	50.7 ± 4.3
	1	3	50.3 ± 2.3	54.7 ± 1.5	59.3 ± 2.6	56.3 ± 7.2	51.0 ± 2.1
BVAF1	0.0002	3	52.0 ± 1.0	48.0 ± 3.0	50.7 ± 4.9	51.0 ± 5.2	61.7 ± 4.4
	0.002	3	45.0 ± 1.7	41.0 ± 2.6	45.7 ± 5.6	51.0 ± 7.5	45.0 ± 3.0
	0.02	3	63.0 ± 4.6	54.0 ± 6.9	57.0 ± 3.5	60.3 ± 0.3	66.0 ± 4.6
BVAF3	0.003	3	49.0 ± 2.0	45.0 ± 0	48.0 ± 3.0	47.0 ± 2.6	59.3 ± 6.4
	0.03	3	47.0 ± 3.6	46.0 ± 6.1	47.7 ± 6.3	54.0 ± 6.9	50.0 ± 4.4
	0.3	3	47.0 ± 5.0	43.0 ± 1.0	53.0 ± 6.1	57.0 ± 6.2	51.0 ± 3.5

Each value represents the mean ± SEM. N = number of animals.

alter the systolic arterial blood pressure or the heart rate as compared to the rats treated with vehicle during the 2 h test period (Tables 9 and 10).

Respiratory rate in anesthetized rabbits: The mean respiratory rate of the rabbits used in this experiment was 58.0 ± 8.7 min^{-1} before vehicle injection. Values obtained after vehicle injection were: 58.0 ± 8.7 min^{-1} at 5 min, 50.0 ± 4.4 min^{-1} at 15 min, 55.3 ± 5.2 min^{-1} at 30 min and 62.0 ± 8.5 min^{-1} at 60 min post-treatment (Table 11). The mean respiratory rate values obtained in animals following subcutaneous injection of BV or BVA fractions (BVAF1 and BVAF3) did not differ significantly from vehicle-injected controls.

Discussion

This preclinical study was designed to evaluate the potential effects of BV and BVA fractions on a number of physiological parameters in animals prior to more widespread therapeutic use in human patients. In South Korea, BV therapy has been traditionally used in oriental medical clinics to treat a number of inflammatory diseases in human patients, such as osteoarthritis [7]. As a result, the selection of clinical dose (5 μg/kg) and administration route (subcutaneous) were determined based on that recommended for clinical use in human patients. The doses of the two BV subfractions (BVAF1: 0.2 μg/kg; and BVAF3: 3 μg/kg) used in the present study were determined based on the partial ratio of each fraction to whole BV. The possible physiological effects induced by each BV subfraction was tested up to a dose that was 100-fold higher than the estimated therapeutic clinical dose of BVAF1 and BVAF3.

We have demonstrated that treatment with whole BV (at a dose that is 200 times greater than the recommended clinical dose) or with BV subfractions (BVAF1 and BVAF3, that are 100 times greater than the estimated clinical dose) did not produce any significant effect on the central nervous system [i.e. (1) general behavior, (2) sleep-induction time and duration, (3) spontaneous activity, (4) motor function, (5)

body temperature, or (6) drug-induced convulsions], aside from the anticipated antinociceptive effects on acetic acid-induced abdominal stretches. Although BVAF1 (0.2 μg/kg) appeared to temporarily increase spontaneous ambulatory activity in the activity chamber, this increase was not statistically different when analyzed by a two-way repeated measure ANOVA. In the sleep-induction time and duration assay, BV and its subfractions did not produce any significant effects when compared to that of the vehicle group suggesting that BV, BVAF1 and BVAF3 do not produce sedation. This is important since several analgesic drugs, such as codeine, which is the most widely used naturally occurring narcotic drug, have serious side effects that include sedation [4]. BV, therefore, produces a potent antinociception without the side affects associated with many of the narcotic drugs.

BV and its subfractions did not produce any alterations in normal motor functions as judged by both the activity box and rota rod tests. In addition, in the present study BV (0.005 mg/kg and 1 mg/kg) was shown to act as a potent antinociceptive agent. In this regard, BV significantly suppressed abdominal pain behavior characterized by abdominal stretches, which is consistent with previous work from our laboratories [6]. The present results strongly suggest that BV treatment produces a significant antinociceptive effect and does not affect motor activity. Thus it is likely that BV treatment is affecting the sensory (nociceptive) component of the abdominal stretch reflex rather than the motor portion of the reflex. Among the BV subfractions, only the treatment with BVAF3 at the highest dose tested (0.3 mg/kg) significantly suppressed abdominal pain behavior as compared to the vehicle-treated group. Because the BVAF1 subfraction failed to produce a significant antinociceptive effect at doses up to 0.02 mg/kg, it is supposed that the major constituents of whole BV that produce an analgesic effect are contained within BVAF3 subfraction. Further study remains to test this supposition to determine if BVAF3 is also able to mimic BV's antinociceptive and anti-inflammatory effects in other models with acute and persistent pain.

With respect to the gastrointestinal system, BV, BVAF1 and BVAF3 did not affect gastrointestinal motility as determined by the charcoal propulsion test nor did they affect gastric secretory functions (pH, volume of gastric juice and total acidity). In this regard, it is interesting that morphine, which is one of the most potent antinociceptive drugs used in human medicine, produces severe constipation as a major adverse side-effect [16]. The results of the present study show that neither BV nor its subfractions altered intestinal peristaltic function or gastric function and thus BV and its BVAF3 subfraction have potent antinociceptive effects without adverse effects in intestines. Additionally, BV and its BVA subfractions did not alter blood pressure and heart rate in rats nor respiratory rates in rabbits.

In summary, this study examined the general pharmacological effect of BV and BVA fractions (BVAF1 and BVAF3) on various physiological parameters associated with the central nervous, cardiovascular, respiratory, gastrointestinal systems. BV, BVAF1 and BVAF3 did not produce any significant physiological changes in these systems. Examination of BV and its BVA subfractions in a visceral nociceptive test (writhing test), indicated that BVAF3 reproduced the antinociceptive effect of BV, which suggests that BVAF3 contains the major constituents of BV that are responsible for pain relief. From this point of view, we hope that the results of the present study demonstrate the safety and effectiveness of BV therapy and provide therapeutic guidelines for use of the BVAF3 subfraction.

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