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Acute toxicity of bee venom from *Apis mellifera* L. in mice: A histopathological study

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Although the toxic effects of bee venom (BV) in humans are known, there are only a few systematic studies are available in literature. Here, we have evaluated the potential toxicity of BV from *Apis mellifera* L. (Hymenoptera: Apidae) by studying acute toxicity in mice receiving subcutaneous injections of high (120 mg/kg), medium (60 mg/kg) and low (8 mg/kg) doses of BV. General toxicity symptoms and histopathological changes were recorded for 14 days. The results indicated that high-dose BV could be fatal. The mental state, appetite, and respiration of mice in the medium- and high-dose groups changed in varying degrees; higher doses led to more prominent symptoms (LD_{50}, 119.7 mg/kg). BV in medium- and high-dose groups significantly inhibited weight gain in mice within 2 days, which did not significantly differ between the dose groups and control at the later stage. The hemolysis rate was significantly higher in the low-dose, while in the medium- and high-dose groups, the coagulation time significantly prolonged in male mice. Histopathological changes showed that medium- and high-dose groups exhibited toxicity to tested organs. Our study showed that BV exhibited strong toxicity at higher concentration and is relatively safe when the BV concentration is lower than 8 mg/kg.

Keywords: Acute toxicity, Coagulation, Hemolysis

Bee venom (BV) is a light yellow, aromatic and transparent liquid secreted by the venom glands of worker and queen bees. Humans use it as an anti-inflammatory drug to relieve pain and treat chronic inflammatory diseases, such as rheumatoid arthritis and multiple sclerosis[1-3]. In addition, BV is a complex mixture of substances that enables bees to resist other invaders[4,5]. The components are polypeptides, enzymes, amines, lipids, and amino acids[6-8], some of which have been found to have antibacterial, anti-inflammatory, analgesic, anticancer, and antiviral properties[9-13]. However, BV also causes pain, allergy, and neurotoxic reactions in the body[14,15].

BV will cause skin irritation and allergic reaction after bee stings. There are diverse clinical manifestations of BV allergic reactions; mild ones comprise local skin reactions, such as redness, swelling, heat, and pain, and severe ones can cause anaphylactic shock and may be life-threatening[16-19].

Evaluating the safety of traditional Chinese medicine injection has always been a concern. Studying drug toxicity is an important aspect in drug research and is a key hindrance in the clinical application of drugs[20]. Timely research on the potential toxicity of drugs will reduce the cost of drug development.

Although the main toxic effects of BV in the human system have been discussed in many clinical case reports and certain autopsy studies[21,22], to the best of our knowledge, there have been only a few systematic studies on BV toxicity[23]. To avoid adverse reactions of BV during clinical application, and elucidate the safety of BV injection, thorough insight on BV toxicity is necessary. In this study, we simulated bee stinging by inducing systemic BV poisoning in mice by subcutaneous injection and analysed its acute toxicity from multiple angles through behavioural observation, median lethal dose (LD_{50}) determination, hemolysis and coagulation tests. We did histopathological observations as well to for possible evidences.

Materials and Methods

Experimental animals

SPF Kunming healthy mice, half male and half female (18-22 g body wt.), were purchased from SBF Biotechnology Co., Ltd. (Beijing, China; production license no.: SCXK 2019-0010). All mice were fed according to the international rules of animal

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experimentation and internationally recognized ethical principles of laboratory animal use and nursing. The feeding temperature ranged 20-25°C, and the relative humidity between 50 and 70%. The mice received natural light, special feed, and unlimited drinking water. Mice were fed adaptively for 1 week before the acute toxicity test. Animal experiments were approved by the Ethics Committee of the Shanxi Agricultural University (Taigu, China).

Drugs and reagents

Dried BV powder (85% protein) obtained from *Apis mellifera* L. by an electro-shock method was purchased from Anhui Jinfeng BV Biotechnology Development Co., Ltd (Anhui, China). Anticoagulant EDTA.2K was purchased from Solarbio (Beijing, China). BV was dissolved in 0.9% physiological saline for further use.

Calculation of LD_{50}

Fifty mice were fed adaptively for 1 week, and then randomly divided into five groups (10 mice per group with equal number of males and females). Each group received injections of different doses of BV solution (75, 101.34, 136.93, 185.02 and 250 mg/kg) in a volume of 0.2 mL. The dose groups showed an equal ratio series and equal ratio coefficient of r = 0.75. Deaths in each group were recorded after administration, and the LD_{50} value was calculated using the modified Karber method[24].

Observation of general toxic symptoms

Forty mice were randomly divided into four groups with 10 replications for each group (half male and half female). High (120 mg/kg, BV-H), medium (60 mg/kg, BV-M), and low (8 mg/kg, BV-L) doses of BV and physiological saline (CK) were subcutaneously injected (volume: 0.2 mL) into the mice. General toxic effects on diet, water drinking, respiration, mental state, and behavior were monitored for 14 days after treatment. The mortality and time of death were recorded daily. The body weight was measured every 2 days.

Organ index and histopathological analysis

The mice from the abovementioned test were fasted at night for 12 h and then euthanized using carbon dioxide. Various organs, including the heart, liver, spleen, lung, and kidney, were weighed, and the organ index was calculated. Organ index (%) = organ mass / body mass × 100. After weighing, each organ was fixed in a 4% paraformaldehyde solution and then embedded in paraffin and sectioned continuously (5 μm thick). After dehydration and dewaxing, Hematoxylin and eosin (HE) staining was applied and samples were observed using an optical microscope to determine the general structure and degenerative changes.

Hemolysis *in vitro*

First, 100 μL of anticoagulant EDTA.2K was placed in a 2 mL EP tube. Approximately 1 mL of blood was collected from the retro-orbital plexus of six male and six female mice and then added to the tube, and the fibrin was removed. Subsequently, the blood was centrifuged at 1,000 r/min for 3 min. Then, 1 mL of saline was added to the solution, which was mixed and centrifuged at 1,000 r/min for 3 min, following which the supernatant was discarded. The procedure was repeated three to four times until the supernatant was pellucid. The supernatant was discarded and saline was added to prepare 2% red blood cell suspension.

Three different concentrations of BV solution (1, 7.5 and 15 mg/mL) were prepared. First, 0.5 mL of the BV solution was mixed with 0.5 mL of erythrocyte suspension, shaken gently, and then incubated at 37°C. Hemolysis was observed during incubation for 15, 30, 45 min, 1, 2 and 3 h. The mixtures were centrifuged at 10,000 r/min for 3 min after 3 h of incubation, and 200 μL supernatant solution was added to a 96-well plate. The absorbance value (A) was detected at 545 nm. Distilled water and saline with erythrocyte suspension were used as positive control and negative control, respectively. Each sample was subjected to three technical repetitions.

The hemolysis rate was calculated as follows:

$$\text{hemolysis rate (\%)} = \frac{(A_{sample} - A_{negative})}{(A_{positive} - A_{negative})} \times 100$$

A hemolysis rate exceeding 5% was considered to indicate hemolysis.

Coagulation test

The animal grouping method was that the same as mentioned earlier. The capillary glass tube was inserted into the retro-orbital plexus to collect blood after subcutaneous injection of BV solution for 30 min. The duration of the blood flow into the tube was determined. The capillary glass tube was broken off at 5 mm every 30 s and then gradually retracted to determine whether there were clotting strands. The duration from the opening to the appearance of hemagglutination filament was regarded as the coagulation time.

Table 1 — Death and general toxic symptoms of mice

Bee venom conc. (mg·kg^{-1})	Deaths/Total Male	Deaths/Total Female	Symptom performance
8	0/5	0/5	Food intake, activity, and mental performance were normal.
60	0/5	0/5	Mice exhibited reduced activity, curling up, squinting, flocking, and sleepiness; all mice exhibited tail shaking (swing 3–5 times each time, swing again at an interval of more than 10 s), intermittent tremor. These symptoms were alleviated the following day, and all the symptoms disappeared on the third day.
120	3/5	2/5	With respect to abdominal breathing, when inhaling, the abdomen clearly collapsed, and the respiratory rate slowed. Poor mental state, curled up, and sleepy. Tail shaking phenomenon with high amplitude and frequency was observed (swing 6–7 times each time and swing again at an interval of 3–5 s). Intermittent convulsions, difficulty in crawling, and slow response were observed.

Statistical analysis

The data were analysed using SPSS 26.0 software. The normality and homoscedasticity of data were verified using Shapiro–Wilk and homogeneity of variance tests. Body weight data were analyzed using the paired-samples t test. Other data, including the organ index, hemolysis rate, and coagulation time, were analyzed by one-way analysis of variance (ANOVA), and Duncan's test was used for multiple group comparisons. All data are presented as the mean ± standard error ($\bar{x} \pm$ SE). Figures were created using GraphPad Prism 8.0.

Results

LD_{50} estimations and the general symptoms

Five BV doses were subjected to the LD_{50} test, which was calculated to be 119.7 mg/kg with 95% confidence intervals of 109.2-131.1 mg/kg. Based on the LD_{50} value, high, medium, and low BV doses in this study were selected.

The death rates and general toxic symptoms of mice in each dose group during the 14 days of observation are presented in Table 1. In the BV-H group, five mice died within 12-48 h after injection (three males and two females). No mice died in the other groups. In addition, the mice showed certain toxic symptoms with respect to their mental state, respiration, behavior, and appetite in different BV groups. Generally, toxic symptoms increased as the dose increased. The toxic symptoms in males were stronger than those in females. In addition, these symptoms were most severe at 1 day after injection and then alleviated at the following day before gradually disappearing.

Change in body weight

The body weight of all mice (control mice and treated mice observed on day 2 and 14) is presented in Table 2. During the 14 days of monitoring, the weight gain of females was slower than that of males (Fig. 1). On the 2nd day after administration, the BV-H groups exhibited significant inhibition of body weight for both males and females (P_{male} <0.01, P_{female} <0.05). In addition, there was no significant difference in body weight change in the BV-M and BV-L group between both males and females (P = 0.085, P = 0.693; Table 1). Four days after injection, the weight of the BV-H and the BV-M group gradually recovered (Fig. 1). At the end of the observation period, there was no significant difference in weight between the BV groups and control group (Table 2).

Table 2 — Changes in mouse weight pre-and post bee venom injection

Bee venom conc. (mg·kg^{-1})	Weight gain 2 days/g Male	Weight gain 2 days/g Female	Weight gain 14 days/g Male	Weight gain 14 days/g Female
0	3.26±0.35	0.86±0.35	10.62±0.66	6.51±0.40
8	3.48±0.38	1.43±0.32	12.81±1.46	6.71±0.28
60	1.11±0.49	0.21±0.49	8.63±1.47	6.72±0.96
120	-2.11±0.03**	-2.43±0.47*	9.33±0.13	6.08±1.02

[Paired-samples t test was used to analyze the data between groups of test and control males or females at 2 and 14 days]

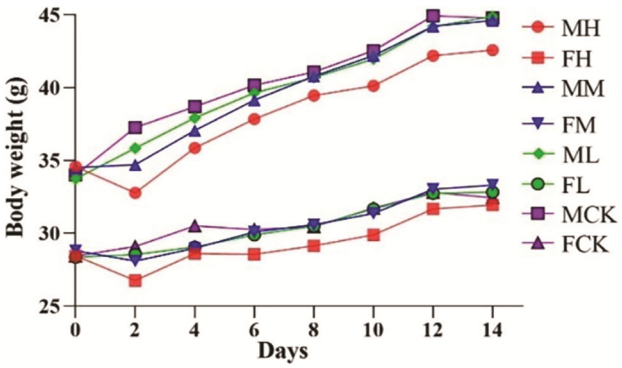

Fig. 1 — Change trend of mouse weight during the observation period of 14 days. [MH & FH, MM & FM high and medium doses of bee venom injected into male and female mice, respectively; FL, low doses of bee venom in female mice; MCK & FCK, normal saline injected into male and female mice, respectively]

Effect on organ index

After injecting different doses of BV for 14 days, the mice were sacrificed and dissected. The five organs (heart, liver, spleen, lung and kidney) were

observed and weighed, and the organ index was calculated (Table 3). Compared with the control group, the organ indexes did not change significantly in all BV groups for both males and females (P >0.05). However, the value of indexes from the liver, spleen, and kidney in the BV-H group were higher than those in the CK group. In addition, pulmonary hemorrhage was evident in the BV-H group. The kidneys of the BV-H and BV-L groups were darker than those of the CK group.

Histopathological analysis

The organs of mice in the control group were normal, but they changed to varying degrees in the BV groups (Fig. 2). In the control group, the myocardial striations were clear. No obvious lesions were observed in the BV-M and BV-L groups. In the BV-H group, broken myocardial fibers, nucleolysis, interstitial widening, a small amount of hyperemia, and inflammatory cell infiltration were observed.

In the control group, the outline of hepatocytes was clear, the intercellular matrix was filled, the arrangement was dense, and the nucleus was complete. There was slight vacuolation in the nuclei of BV medium- and low-dose groups. In the high-dose group, hepatocytes were enlarged, hepatic sinuses were disorderly, the boundary between cells was not clear, and granular degeneration and nucleolysis were observed.

In the control group, the red and white pulp boundaries of the spleen, trabeculae, and germinal center were evident. There were no abnormal changes in spleen parenchyma and no clear histopathological

Table 3 — Changes in mouse organ index pre-and-post bee venom injection

Bee venom conc.	Heart		Liver		Spleen		Lung		Kidney	
(mg/kg)	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
0	0.65±0.05	0.62±0.02	5.90±0.24	5.53±0.05	0.35±0.05	0.41±0.05	0.68±0.04	0.75±0.05	1.76±0.05	1.35±0.02
8	0.69±0.09	0.71±0.07	5.99±0.20	5.83±0.17	0.38±0.03	0.44±0.03	0.63±0.03	0.79±0.06	1.72±0.06	1.44±0.06
60	0.71±0.02	0.66±0.05	6.43±0.29	6.30±0.23	0.55±0.03	0.51±0.02	0.71±0.05	0.77±0.05	1.82±0.08	1.48±0.08
120	0.60±0.01	0.66±0.04	6.49±0.10	6.36±0.46	0.57±0.01	0.57±0.06	0.79±0.11	0.71±0.05	2.10±0.02	1.54±0.07

[One-way ANOVA was used to analyze the data in groups of males or females, and Duncan's was used for multiple range test]

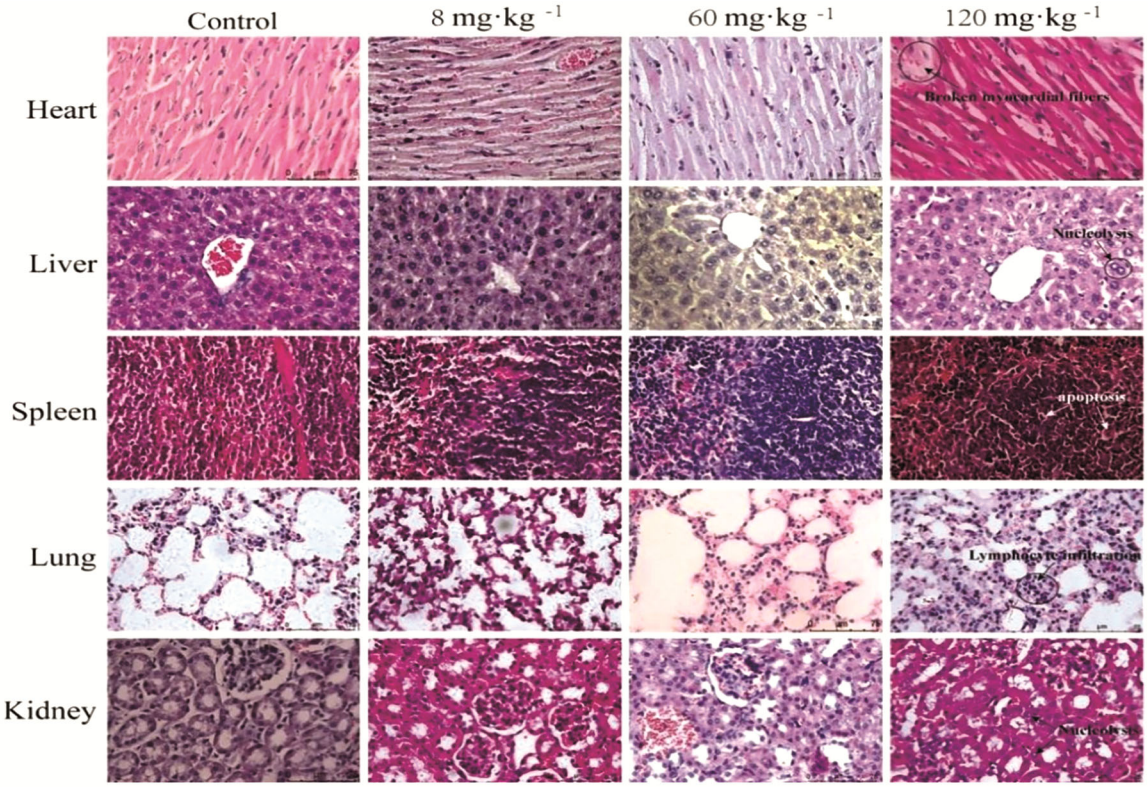

Fig. 2 — Histopathological results of means low doses of bee venom (ML) injected into selected organs in male mice. [Five different organs viz. heart, liver, spleen, lung and kidney were arrayed in different rows. Different columns indicate different groups, including control group and treatment groups. HE 400X]

damage in the BV-L group. In the BV-M and BV-H groups, the boundary between the red and white pulp was blurred and apoptotic nucleosomes, disintegration of a large number of lymphocytes, and necrosis were observed.

In the control group, the structure of the glomerulus and renal tubules at all levels was intact, the cells were closely arranged, and the nephron structure was clear. In the BV group, the renal tubules were swollen, the glomeruli were shrunk, the cells were disorderly, the capillaries of renal interstitium were congested, and a small number of inflammatory cells had infiltrated. The higher the BV dose, the more evident the lesion.

Hemolysis rate analysis *in vitro*

After incubation for 45 min, the solutions of the BV groups and distilled water groups with red blood cell suspension exhibited no blood cell aggregation, but there were differences in colour among these groups. By contrast, blood cell aggregation was observed in the saline group, indicating that hemolysis occurred in the BV and positive control groups. The red colour in the BV-L group was deeper than that in the BV-M and BV-H groups, which was closer to that of the positive control group, indicating that hemolysis in the BV-L group was strong. When the incubation time was prolonged, the solution color of the BV groups gradually deepened, indicating that a longer interaction time between BV and red blood cells led to more severe hemolysis.

The mixed solutions of the BV groups and the control groups were incubated for 3 h. Then, the absorbance value of each group was measured using an enzyme labeling instrument at 545 nm, and the hemolysis rate of each group was calculated. The results indicated that the different dose groups of BV had a strong hemolysis effect, with a hemolysis rate >5% (P_{male} <0.001, P_{female} <0.001), and the hemolysis rate of the BV-L group significantly exceeded that of the BV-M and BV-H groups (P_{male} <0.001, P_{female} <0.001; Table 4). The hemolysis rate measured using the enzyme labeling instrument was similar to that observed by the naked eye.

Coagulation analysis *in vitro*

As shown in Table 5, for male mice, there was no significant difference in coagulation time between the BV-L and control groups, but the duration was significantly longer in the BV-M (P = 0.015) and BV-H (P = 0.04) groups than in the control group. For female mice, there was no significant difference between each BV group and the control group, but the coagulation time of the BV-H group tended to be long. Overall, BV was observed to delay coagulation.

Table 4 — Hemolysis rate *in vitro*

Bee venom conc. (mg/kg)	male	female
8	80.0±2.6[a]	70.9±4.1[a]
60	45.6±1.7[b]	45.6±2.1[b]
120	44.4±1.1[b]	45.1±1.2[b]
8	80.0±2.6[a]	70.9±4.1[a]

[One-way ANOVA was used to analyze the data in groups of males or females, and Duncan's test was used for multiple comparisons. Same letters in the table indicate no significant differences between groups (P >0.05) and different letters indicate significant differences between groups (P <0.05)]

Table 5 — Determination of coagulation time *in vitro* (in seconds)

Bee venom conc. (mg/kg)	male	female
0	42.0±12.0[b]	66.0±14.7
8	84.0±22.04[ab]	84.0±6.0
60	96.0±7.35[a]	102.0±12.0
120	108.0±20.35[a]	108.0±18.0

[One-way ANOVA was used to analyze the data in groups of males or females, and Duncan's test was used for multiple comparisons. Same letters in the table indicate no significant differences between groups (P >0.05) and different letters indicate significant differences between groups (P <0.05)]

Discussion

Bee venom, a natural biological toxin, has important application value for used as one of the drugs for treating some diseases[25-27]. In recent years, BV has been used to treat diabetes[28], neuropathic pain[29], skin aging[30], wound healing[31], which has shown great potential in various aspects[32]. While the safety of BV is also an important issue. Some studies reported that BV could cause various toxic reactions, with severe cases leading to organ failure and even death[17]. In addition, there may also be symptoms such as immune system reactions, local reactions, allergies, etc.[33]. This study provides an evaluation of BV from the aspects of general toxicity characteristics, organ index, histopathology, hemolysis, and coagulation, in order to determining the safe dosage of BV.

In the present study, three groups with different BV doses were established to elucidate the acute toxicity of BV in mice. After 14 days of observation, the mice in the BV-L group did not die and there was no significant behavioral change. Most mice in the BV-M and BV-H groups showed behavioral reactions, such as mental depression, body curling, muscle tremor, and flocking, and the BV-H group reached the half lethal dose. The behavioral symptoms may be

due to the cytotoxicity of BV, which can cause apoptosis and necrosis[34]. Most mice showed severe toxic symptoms 2 days after receiving injections of medium and high doses of BV, including changes in body weight, but the symptoms could be alleviated later. Similar phenomena were observed in the study of *Aralia elata*[35]. The weight loss may be due to the reduction of food intake and drinking water because appetite and feed consumption play an important role in weight regulation[36].

In the present study, no significant changes were observed in the weight of each organ in the BV groups compared with that in the control group, but there was evident congestion in the lung. Despite the significance of organ weight changes and sensitive parameters indicating harmful effects of chemicals, organ weight data must be interpreted along with gross pathology, clinical pathology, and histo-pathological results[37]. Microscopic examination of tissue sections showed that the low dose of BV had little effect on the organs of mice, which was similar to the research results of Prado *et al*.[38] and the subcutaneous injection of BV(20.8 μg/g) had no obvious changes on the heart, liver, spleen, and lung of mice, but slightly affects the kidney. The medium and high doses of BV significantly damaged the liver, spleen, lung, and kidney of mice, including the vacuolar degeneration of hepatocytes and cellulosic exudates in the central vein. The boundary between the red and white pulp of spleen tissue was blurred, and a large number of lymphocytes disintegrated and necrotized. Alveolar congestion, widening of alveolar septum, exfoliation and necrosis of renal tubular epithelial cells, and renal tubular degeneration were also observed. The damage to myocardial tissue was relatively small. The above pathological changes show that higher doses of BV injection have severe toxic effects on mouse organs and tissues. At the same time, there will also be severe inflammatory reactions, including edema, lipid peroxidation, production of nitric oxide, and systemic release of cytokines[39]. The dose should be strictly controlled in clinical use, and the liver and kidney function and physical state of patients should be comprehensively evaluated before medication[40].

LD_{50} refers to the drug dose that can cause the death of half of the tested animals, which is a significant indicator of the toxicity of a drug. The LD_{50} of BV and its 95% confidence limits is 119.7 (109.2-131.1) mg/kg, which is equivalent to 12 bees stinging simultaneously (calculated based on the release of 0.3 mg per bee). According to the Blach well method, the initial dose of human clinical tolerance test was 0.2 mg/kg. The toxic BV dose is significantly higher than the therapeutic dose. Therefore, BV is safe within the clinical dose range but induces significant physical differences among individuals because it contains allergens. In clinical practice, allergic reaction to BV should be considered. In addition, the drug should be administered in small doses and increased gradually.

The hemolysis reaction mechanism is complex. Melittin in BV can cause biofilm structure disorder, lyse red blood cells and degrade membrane lipid, resulting in hemolysis[41-43]. As a specific component of bee venom, PLA2 accounts for about 10-12%. It can assist melittin in breaking down red blood cells, leading to hemolysis. Therefore, PLA2 is also known as an indirect hemolytic toxin[44,45]. Unexpectedly, among the three BV dose groups selected in the hemolysis test, the hemolysis rate was low in the BV-H group but was high in the BV-L group. This result was inconsistent with the hemolysis research results of BV peptide reported by Shi *et al*.[42], indicating that the hemolysis rate of BV peptide is dose-dependent. The hemolysis results *in vitro* will be affected by many factors, such as reaction time, color, and absorbance of the test substance itself. Further studies should examine the possible distinction between the hemolysis of BV and a single component, which may be attributed to the influence of other external factors.

Conclusion

Bee venom (BV) has acute toxicity and hemolytic characteristics. Though, the anti-inflammatory, antivirus, and antitumor effects of BV are well known, for clinical applications, special attention should be paid to the dosage, rhythm, and allergic reaction. The above results showed that BV at high-dose (≥120 mg/kg) causes death, tissue lesion, and prolonged coagulation time in mice. In low-dose (≤8 mg/kg) it led to a higher hemolytic rate. However, it had no obvious effect on the organs heart, liver, lungs, spleen and kidney. Our findings provide an experimental basis for the safety evaluation and clinical application of BV. In addition, BV exhibits great potential for treating certain difficult and miscellaneous diseases in clinical practice.

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Conflict of Interest

Authors declare no competing interests.

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