

Safety Assessment of *Aconitum*-Derived Bulleyaconitine A: A 91-Day Oral Toxicity Study and a Tissue Accumulation Study in Rats

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Abstract

Background: Bulleyaconitine A (BLA) is a diterpenoid alkaloid from the rhizomes of *Aconitum bulleyanum* Diels and has been clinically used for chronic pain treatment in China for many years. However, the newly reported adverse events of BLA indicated that BLA still has potential safety issues. **Materials and Methods:** To assess the safety of BLA, analgesic tests, acute toxicity studies, repeated-dose oral toxicity studies, and tissue distribution studies after single and repeated administration of BLA were carried out. **Results:** Administration of 0.14 mg/kg BLA showed potent analgesic effects in both analgesic tests. In acute toxicity study, the LD₅₀ value of BLA was calculated to be 3.4434 mg/kg. In the subchronic toxicity study, the no observed adverse effect level was 0.25 mg/kg, and the lowest observed adverse effect level was 0.5 mg/kg. The spleen, liver, and kidneys are newly identified target organs of BLA toxicity after long-term administration. Moreover, unlike a single BLA administration, repeated administration showed BLA redistribution from organs with an abundant blood supply to immune and metabolic organs. **Conclusions:** These results suggested that BLA itself would be nontoxic at a dosage of 0.25 mg/kg in rats and should be carefully used when combining BLA with medications that can cause spleen, liver, or kidney injury.

Keywords: Adverse reaction, bulleyaconitine A, repeated-dose (91-day) toxicity, tissue distribution

INTRODUCTION

Aconitum bulleyanum Diels (Ranunculaceae) is a perennial herb that is usually distributed in the mountain bush of a plateau (altitude 3.2–3.5 km) in China. As the ancient Chinese book “Compendium of *Materia Medica*” recorded, dried rhizomes of *A. bulleyanum* were a poisonous medicine that could be used to relieve pain. Bulleyaconitine A (BLA) is the extracted diterpenoid alkaloid from the rhizomes of *A. bulleyanum*.^[1] With analgesic and anti-inflammatory effects, BLA has already been used in China to treat different types of arthritis, itching and the pain of muscle strain, sprain, and other diseases.^[2-4] As the main active ingredient of *A. bulleyanum*, the underlying analgesia mechanisms of BLA have been reported to be related to blockage of neuronal voltage-dependent sodium channels,^[5,6] inhibition of presynaptic transmitter release,^[7] and direct stimulation

of dynorphin A expression in the spinal cord.^[8] Since there was no relationship with the opioid receptor and no physical dependence was observed in an experiment performed on monkeys,^[9] BLA is classified as a nonnarcotic analgesic. To date, BLA is still the sole clinically used analgesic developed in China;^[5] an application has already been filed to register BLA with the Food and Drug Administration, and BLA has obtained approval for clinical research in the United States.

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Chronic pain is a kind of pathological pain that results from disease injuries that can persist for quite a long time, even for decades after the injury or disease has been healed.^[10,11] This type of pain is usually caused by rheumatoid arthritis, strain, and cancer, which is related to peripheral or central sensitization.^[12-14] It affects not only the working ability of patients but also their emotional health, which can severely reduce the patients' quality of life. Due to the side effects of addiction, tolerance, dizziness, gastrointestinal (GI) side effects, and renal damage,^[15] the adverse reactions of analgesics such as opioids, antidepressants, and nonsteroidal anti-inflammatory agents (NSAIDs) limit their application in the clinic to treat chronic pain. Therefore, chronic pain management remains a major challenge for clinicians. In recent decades, BLA has been used to treat chronic pain in China and has shown remarkable clinical effects, especially for rheumatoid arthritis and cancer pain.^[4,16-19] On the other hand, an increasing number of adverse event (AE) reports on BLA reported to the Chinese National Center for Adverse Reaction Monitoring indicated that BLA might also have potential adverse reactions, and the safety of BLA still needs to be considered.^[20]

From January 2009 to September 2017, a total of 570 AEs of BLA were reported to the Chinese National Center for Adverse Reaction Monitoring.^[20] In addition to adverse reactions such as dizziness, nausea, rash, and palpitation related to the nervous, GI, skin, and cardiovascular systems, there are also many AEs involved in injury to other organs such as injuries to the respiratory system, administration site, and immune system.^[20] As chronic pain persists long and often presents with complex problems, BLA has long been a long-term or overdose medication for many patients or is taken in combination with other medicines such as NSAIDs, opioids, and muscle relaxants as an adjuvant medicine.^[17,20] Long-term and large-dose exposure could result in medicine accumulation in tissues, which might cause injury to vital organs. Moreover, the combination medication might aggregate the same adverse reaction to both medications. Since toxicity and tissue accumulation of BLA under long-term exposure have not been investigated, it is hard to tell if these AEs were caused by BLA itself or were the result of the combination with other drugs. Therefore, a safety evaluation of BLA after long-term exposure would be helpful to discover the potential adverse reactions caused by BLA.

This study aimed to evaluate the toxicity of BLA during a 91-day oral toxicity study. We tried to determine the no observed adverse effect level (NOAEL) and the lowest observed adverse effect level (LOAEL) of BLA under long-term exposure combined with tissue distribution using high-performance liquid chromatography with tandem mass spectrometry (HPLC-MS/MS) to determine the novel toxic effects and accumulation in target organs. First, the analgesic activity of BLA was examined by a mouse hot plate test and acetic acid writhing test to determine the effective dose. According to the effective dose, an acute toxicity study was carried out in mice to calculate the LD₅₀ value and preliminarily

confirm the toxicity of BLA. According to the LD₅₀ value, different dosages of BLA were selected to carry out the subchronic toxicity study for 13 weeks in rats. In the subchronic toxicity study, we monitored the survival states, clinical signs, and indexes of biochemical and hematological parameters while the medication was administered. At the end of the subchronic toxicity study, the tissue distribution of BLA in 25 vital organs, reproductive organs, and plasma was examined for both single-dose and repeated-dose administration of BLA for 13 weeks. These organs were also harvested to carry out gross pathological and histopathological examinations. The findings of this study provided experimental evidence that BLA is a beneficial medication and sheds light on how clinicians can help chronic pain patients avoid AE occurrences.

MATERIALS AND METHODS

Reagents

BLA (the structural formula and mass spectrogram are shown in Figure 1a, purity $\geq 98.3\%$) was a kind gift from Kunming Pharmaceutical Group Co., Ltd. Escitalopram oxalate was purchased from Sigma-Aldrich (E4786). Morphine was purchased from Northeast Pharmaceutical Group Co., Ltd. (Lot. No. 100509-1). Acetic acid and anhydrous methanol were purchased from Tianjin Damao Chemical Reagent Factory.

Animals

Kunming mice (KM mice) weighing 18–22 g and Sprague-Dawley (SD) rats weighing 180–220 g were obtained from the Experimental Animal Center of Shenyang Pharmaceutical University (Shenyang, China). The animals were acclimated to laboratory conditions maintained at a temperature of $25^{\circ}\text{C} \pm 1^{\circ}\text{C}$ with a relative humidity of $55\% \pm 5\%$ on a 12 h light-dark cycle. Animals were given water *ad libitum* and standardized food pellets. This study was carried out according to the Chinese animal welfare legislation for the care and use of animals and approved by Animal Ethical Committee of Shenyang Pharmaceutical University Shenyang, China (No. SYPU-IACUC-C2019-9-20-107). Every effort was made to minimize the number of animals and relieve their suffering. All animal experiments in this study complied with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments.

Solution preparation

After precise weighing, BLA was added to anhydrous ethanol. Then, the BLA was completely dissolved in anhydrous ethanol after stirring and grinding. The mixture was stirred while adding the BLA anhydrous ethanol solution to purified water for dilution. After dilution, the final concentration of ethanol in the BLA solution was 1%. Therefore, the 1% ethanol solution was used for the vehicle control group.

Hot plate test

The aim of this experiment is to examine the analgesic effect of the BLA. This experiment was performed at 10 a.m. The temperature of the hot plate apparatus (R-200, Chengdu

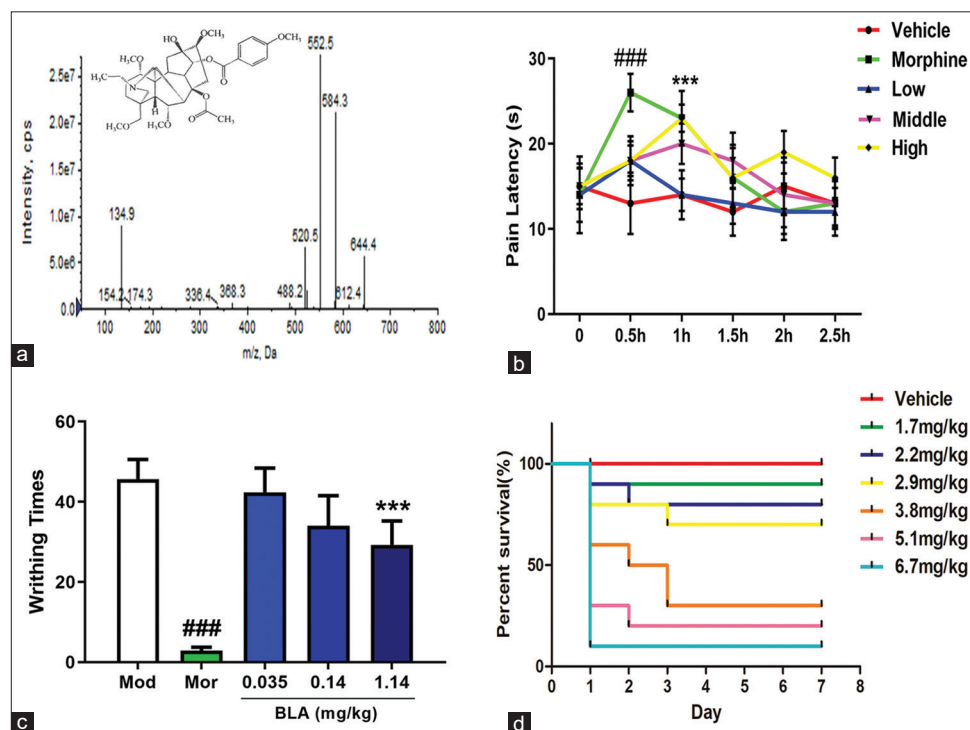


Figure 1: The analgesic effect and acute toxicity of bulleyaconitine A (a) the chemical structural formula and product ion scan of bulleyaconitine A (m/z 584.3) measured on API 4000 QTrap triple quadrupole MS equipped with an electrospray ion source operating in the positive ion mode. Bulleyaconitine A showed analgesic effect in the (b) hot plate test and (c) acetic writhing test with $***P < 0.001$; $***P < 0.001$. (d) The survival percentage of mice in acute toxicity study

Taiwang Technology Co., Ltd.) was set to 55°C. The time between putting mice on the hot plate apparatus and mice licking rear feet was recorded as the pain latency.^[21] After a preliminary screening, sixty female KM mice were selected to examine the analgesic effect of BLA. These mice were randomly divided into five groups (vehicle control; morphine 2 mg/kg; and BLA with the following concentrations 0.035 mg/kg, 0.14 mg/kg, and 1.14 mg/kg). The BLA solution was administered by oral gavage, whereas morphine was administered by intramuscular injection. The latency time of BLA or morphine was examined after administration for 0.5 h, 1.0 h, 1.5 h, 2.0 h, and 2.5 h. This experiment was repeated two times. All animals from each group were included in the analysis.

Acetic acid writhing test

The aim of this experiment is to examine the analgesic effect of the BLA. This experiment was performed at 10 a.m. In this test, sixty mice (half male and half female mice) were randomly divided into five groups (vehicle control, 2 mg/kg morphine, 0.035 mg/kg BLA, 0.14 mg/kg BLA, and 1.14 mg/kg BLA). After administration, 0.2 mL of acetic acid (v/v 0.6%, 24°C) was intraperitoneally injected into each mouse. The clinical signs of complete twisting response include the concave abdomen, stretched trunk and limbs, and raised buttocks. After injection, the writhing times were observed and recorded within 15 min.^[22] This experiment was repeated two times. All animals from each group were included in the analysis.

Acute toxicity study

The aim of this experiment is to examine the acute toxicity of BLA. This experiment was performed at 10 a.m. An acute toxicity study was performed in seventy mice (half male and half female mice) to preliminarily examine the toxicity of BLA. These mice were randomly divided into seven groups and orally administered different dosages of BLA (vehicle control and the following BLA concentrations: 1.7 mg/kg, 2.2 mg/kg, 2.9 mg/kg, 3.8 mg/kg, 5.1 mg/kg, and 6.7 mg/kg). The clinical signs, body weight, changes in fur appearance, eyes, mucosa, respiratory system, circulatory system, nervous system, physical activity, and behavior were observed and recorded. The time of occurrence, remission, and disappearance for each toxic sign were also recorded. On the 14th day, the mice were sacrificed, and vital organs such as the heart, lungs, spleen, kidneys, and liver were taken for gross pathological examination.^[23] The Bliss method was used to calculate the LD₅₀ (the unit of each dataset is group of animals). This experiment was repeated two times. All animals from each group were included in the analysis.

Subchronic toxicity study in rats

The aim of this experiment is to examine the chronic toxicity of BLA. A subchronic toxicity study was performed in eighty SD rats (randomly divided into four groups, half male and half female rats). These rats were orally administered different dosages of BLA (0.25 mg/kg, 0.5 mg/kg, and 1.0 mg/kg) or the vehicle control (1% ethanol solution) for 13 weeks (every

morning, from 8:30 a.m. to 11:00 a.m.). These dosages were two, four, and eight times the clinical equivalent dosage of BLA, respectively. The clinical signs of toxicity were observed daily, whereas body weight, food, and water consumption were recorded once every 2 days. The other evaluation indexes and the detailed method are described below. The study was performed followed the guidance of Good Laboratory Practice guidelines of People's Republic of China.

Hematological analysis

Before the experiment (−1 day), whole blood from the orbital venous plexus was collected to obtain the baseline values of hematological analysis. Then, these rats were administered different dosages of BLA (0.25 mg/kg, 0.5 mg/kg, and 1.0 mg/kg) or the vehicle control (1% ethanol solution) orally for 13 weeks (91 days, every morning, from 8:30 a.m. to 11:00 a.m.). After fasted for 24 h, we collected whole blood (1.5 mL) from the orbital venous plexus to carry out hematological analysis on the 46th day and 92nd day. The hematological parameters were examined with the hematological analyzer Mindray BC-2900 (Shenzhen, China). The parameters analyzed were white blood cell number, red blood cell number, hemoglobin concentration, hematocrit, mean cell volume, mean cell hemoglobin (MCH), mean corpuscular hemoglobin concentration, platelets counts, and clotting time.^[23,24] All animals from each group were included in the analysis.

Biochemical analysis

The serum of rats before the experiment (−1 day) was first collected to obtain the baseline values of biochemical analysis. Then, these rats were orally administered different dosages of BLA (0.25 mg/kg, 0.5 mg/kg, and 1.0 mg/kg) or the vehicle control (1% ethanol solution) for 13 weeks (every morning, from 8:30 a.m. to 11:00 a.m.). After fasted for 24 h, we collected the serum of rats (0.15 mL) to carry out biochemical analysis on the 46th day and 92nd day. The serum obtained was further analyzed using a DIRUI automatic biochemical analyzer CS-1200 (Changchun, China). The parameters assessed included alanine transferase (ALT), aspartate transferase (AST), blood urea nitrogen (BUN), and creatinine.^[24] All animals from each group were included in the analysis.

Gross pathological and histopathological analyses

After administration for 13 weeks, the rats were sacrificed by cervical dislocation on the 92nd day (at 9:00 a.m.). Then, 25 vital organs (tongue, submandibular lymph, esophagus, stomach, jejunum, colon, liver, pancreas, spleen, intestinal lymph, heart, aorta, lung, trachea, kidney, bladder, thyroid, thymus, adrenal gland, brain, bone marrow, bone, optic nerve, muscle, and fat) and reproductive organs (uterus, ovary, breast, prostate, testis, and epididymis) were removed for gross pathological examination and histopathological examination. The morphology, size, and color of these organs were observed for gross pathological examination. Then, the organs were fixed in 10% buffered formalin before being subjected to processing procedures using a TKY-TSF

automatic tissue processor (Taikang Medical Equipment Co. Ltd., Hubei, China). The tissues were embedded into paraffin wax using a TKY-BMD paraffin embedding station (Taikang Medical Equipment Co., Ltd., Hubei, China). A Leica RM2245 semiautomatic rotary microtome (Leica, Germany) was used to section the tissues into 5 μm-thick sections. These sections were mounted onto histological slides and dried overnight. Tissues were manually stained with hematoxylin and eosin. Histological analyses were performed under × 200 magnification using a Nikon DS-Ri2 light microscope (Nikon, Japan).^[25]

Tissue distribution/accumulation analysis

The tissue distribution/accumulation of BLA in rats was researched at a dose of 1 mg/kg. After a single or repeated dose of BLA, the organs were harvested for sample preparation (At 9:00 a.m.). Then, LC-MS/MS analysis was carried out to examine the concentration of BLA in tissues. In brief, after homogenization, 50 μl of tissue homogenate supernatant was placed in a 1.5 mL Eppendorf (EP) tube. Then, 10 μL internal standard working solution (escitalopram oxalate) and 110 μL methanol were added. The mixture was vortexed for 1 min and then centrifuged (14,000 rpm) for 5 min (4°C). Then, 100 μL of supernatant was aspirated and added to a 2 mL EP tube. The supernatant was air dried at 40°C; then, 100 μL of the initial mobile phase was added to reconstitute the residue, which was vortexed for 2 min and centrifuged at 12000 rpm for 10 min. Then, we took a 10 μL sample solution for HPLC-MS/MS analysis. HPLC-MS/MS analysis was performed using a Venusil ASB C18 column (2.0 × 50 mm I.D., 3 μm). We used different proportions of the methanol-water mobile phase for gradient elution. The flow rate was set to 0.3 mL/min, and the column temperature was 30°C. The mass spectrometry analysis was performed in positive ion multiple reaction monitoring mode with precursor ion to product ion transitions from *m/z* 644.4-*m/z* 584.3 (BLA) and *m/z* 325.1-*m/z* 262.2 (escitalopram). All animals from each group were included in the analysis.

Statistical analysis

The results are expressed as the means ± SDs. We used one-way ANOVA followed by Dunnett's *t*-test for statistical analysis (SPSS 19.0 software, SPSS, USA). Probability values of *P* < 0.05 were considered statistically significant. The unit of each dataset is group of animals.

RESULTS

Bulleyaconitine A showed an analgesic effect in classic pain animal models

The hot plate test was first carried out to examine the analgesic effect of BLA. As Figure 1b shows, compared with the vehicle control, BLA (0.035 mg/kg, 0.14 mg/kg, and 1.14 mg/kg) dose dependently and time dependently prolonged the latency time of mice. After administration for 1 h at dosages of 0.14 mg/kg and 1.14 mg/kg, BLA significantly increased the pain threshold of mice. In the acetic acid writhing test, compared with the

vehicle control, BLA dose dependently decreased the writhing times of mice. As Figure 1c shows, a 1.14 mg/kg dose of BLA significantly increased the pain threshold of mice. These results not only confirmed the analgesic effect of BLA but also indicated the effective dosage of BLA in classic pain animal models.

Acute toxicity study of bulleyaconitine A

According to the effective dose, we selected a series of BLA dosages (1.7 mg/kg, 2.2 mg/kg, 2.9 mg/kg, 3.8 mg/kg, 5.1 mg/kg, and 6.7 mg/kg) to preliminarily examine the toxicity of BLA in acute toxicity studies. Each BLA treatment group showed mortality, and the mouse deaths generally occurred during the first 7 days, with the survival curve shown in Figure 1d. The mortality of each group was recorded, and the Bliss method was used to obtain the regression equation. With the regression equation $Y = 2.5304 + 4.5990X$; ($r = 0.9657$), the LD_{50} value of BLA was calculated as 3.4434 mg/kg. The highest dose of animals without death was 1.1103 mg/kg, and the lowest dose causing all animals to die was 8.7675 mg/kg. Clinical signs such as convulsion, vomiting, reflex reduction, and piloerection were observed in the BLA-treated mice (2.9 mg/kg, 3.8 mg/kg, 5.1 mg/kg, and 6.7 mg/kg). The adverse reactions disappeared within 24 h. There was no significant difference between the body weights of each treatment group. These results preliminarily confirmed the toxicity of BLA.

Subchronic toxicity study of bulleyaconitine A

Bulleyaconitine A could affect the clinical signs in rats

During the 13 weeks of subchronic toxicity, neither the vehicle control group nor the 0.25 mg/kg BLA group showed a remarkable change in clinical signs. The fur appearance, physical state, locomotor activity, breathing, gland secretion, urination, and defecation of these two groups were normal. The rats from the 0.5 mg/kg group showed body curl up, a lack of energy, and a decrease in locomotor activity. Moreover, the rats from the 1.0 mg/kg group showed messy fur, weakness, gait instability, and salivation. Several rats in this group even had hematuria. Food and water consumption in the rats in the 0.25 mg/kg BLA and 0.5 mg/kg BLA treatment groups were not different from food and water consumption in the rats in the vehicle control group on the 13th week [Figure 2a and b]. However, 1.0 mg/kg BLA significantly reduced the food and water consumption of rats [Figure 2d and e]. Although the body weight of rats from each group was increased during the 13 weeks [Figure 2c], the weight gain of rats from the 1.0 mg/kg group was lower than that of rats from the other BLA groups during the 13th week [Figure 2f]. However, the difference was slight and was not significant. Therefore, these results suggested that orally administered 0.25 mg/kg BLA for 13 weeks was relatively safe for the rats, whereas 0.5 mg/kg BLA and 1.0 mg/kg BLA might have a toxic effect on the rats.

Bulleyaconitine A could affect the liver function of rats

In a biochemical analysis, the concentrations of ALT and AST and the albumin/globulin ratio in serum are considered to be important indexes that can reflect liver function in the

clinic.^[26,27] On the 46th and 92nd days of the study, the results of the 0.25 mg/kg BLA group showed that the AST/ALT and albumin/globulin ratios of rats were still within the normal range. For the 0.5 mg/kg BLA and 1.0 mg/kg BLA groups, the ALT and AST levels were significantly increased on the 46th and 92nd days and the albumin/globulin ratio was significantly decreased [Figure 3a-c]. The histopathological results also confirmed the toxic effect of 0.5 mg/kg and 1.0 mg/kg BLA on the liver [Figure 3d]. The pathological sections of the vehicle group and 0.25 mg/kg BLA group under a microscope showed clear structures of the hepatocyte cord and hepatic sinusoid. Observation of the 0.5 mg/kg group showed hepatocyte atrophy, hepatic sinus swelling, and partial hepatocyte dissolution. For the 1.0 mg/kg group, the pathological changes in rat hepatocytes included swelling, ballooning, degeneration, and hepatic sinus congestion. The organ index of the liver was also significantly increased in these two groups (3.1 and 3.2 vs. 2.8, respectively, $P < 0.001$). These results suggested that orally administered 0.25 mg/kg BLA for 13 weeks was relatively safe for the rats, while administration of 0.5 mg/kg and 1.0 mg/kg BLA were toxic to liver function.

Bulleyaconitine A could affect the kidney function of rats

In a biochemical analysis, the concentrations of creatinine, BUN, and alkaline phosphatase (ALP) in serum are considered to be important indexes that can reflect kidney function in the clinic.^[28] In our study, the three parameters of both the vehicle control and 0.25 mg/kg BLA groups on the 46th and 92nd days were within the normal range. In addition, 0.5 mg/kg and 1.0 mg/kg BLA significantly increased the creatinine and BUN levels in the rats. Moreover, the ALP levels of these two groups were significantly decreased after BLA treatment [Figure 4a-c]. The results of histopathological examination also confirmed the toxic effect of BLA on the kidney. In the 0.25 mg/kg BLA group, the histopathological sections showed mild renal interstitial dilation. Microscopy analysis showed that the 0.5 and 1.0 mg/kg BLA treatment groups showed different degrees of damage. The glomeruli of rats showed atrophy, congestion, and interstitial dilation after BLA treatment [Figure 4e]. In addition, pink staining and unstructured protein-like secretion could be observed in the renal tubules from these two groups [Figure 4f]. The gross pathological changes in kidneys showed decreased kidney size, hard texture and dark red color for the 0.5 and 1.0 mg/kg groups [Figure 4d]. Although the organ indices of the kidneys of these two groups dose dependently decreased, the decrease was slight and without significance. These results suggested that orally administered 0.25 mg/kg BLA for 13 weeks was relatively safe for the rats, whereas 0.5 mg/kg and 1.0 mg/kg BLA were toxic to kidney function.

The toxic effect of bulleyaconitine A on other organs

In addition to the liver and kidney, the spleen was also a targeted organ of BLA toxicity. Although 0.25 mg/kg BLA did not cause a change in the histopathological section of the spleen, hemosiderin pigments could be observed after

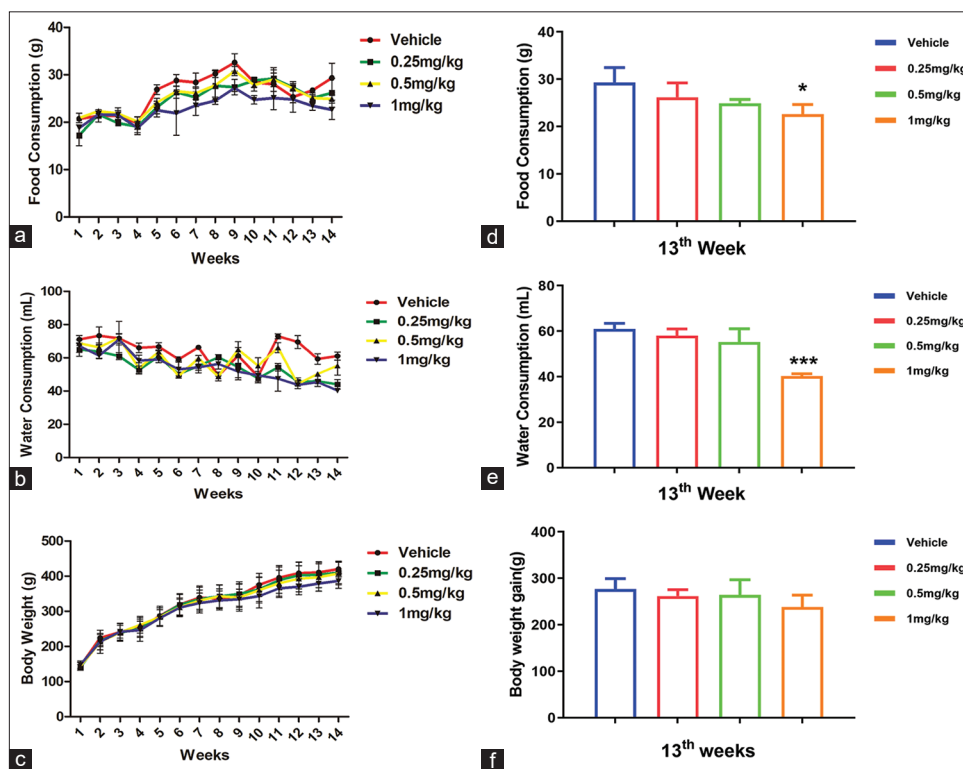


Figure 2: Bulleyaconitine A could affect the clinical signs in rats of chronic toxicity study the food consumption (a), water consumption (b), and body weight (c) of rats were affected by bulleyaconitine A in chronic toxicity study. Both food (d) and water (e) consumption of Bulleyaconitine A 1.0 mg/kg treated group in the 13th week were significantly lower than that of the vehicle control with * $P < 0.05$ and *** $P < 0.001$, respectively. (f) The body weight gains of rats in 13th week were also dose dependently decreased by Bulleyaconitine A administration with $P > 0.05$

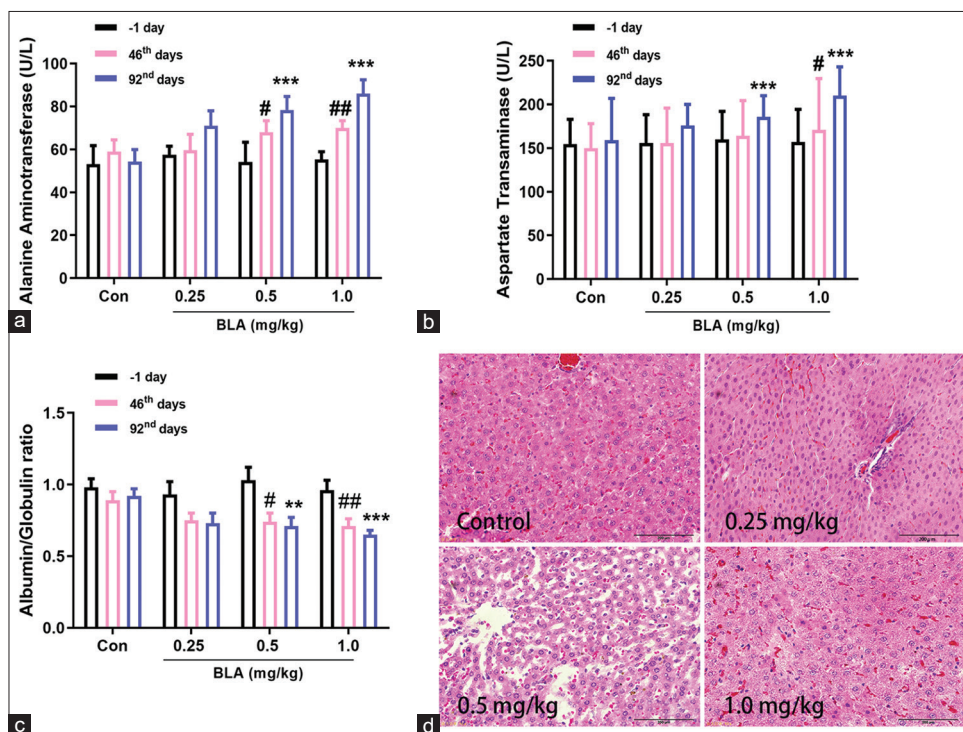


Figure 3: Bulleyaconitine A could affect the liver function of rats in chronic toxicity study both 0.5 mg/kg and 1.0 mg/kg Bulleyaconitine A could significantly increase the alanine transferase (a) and aspartate transferase (b) levels and decrease the A/G ratio (c) of rats with # $P < 0.05$; ## $P < 0.01$; ** $P < 0.01$ and *** $P < 0.001$. (d) These two dosages of Bulleyaconitine A could also cause pathological changes of liver

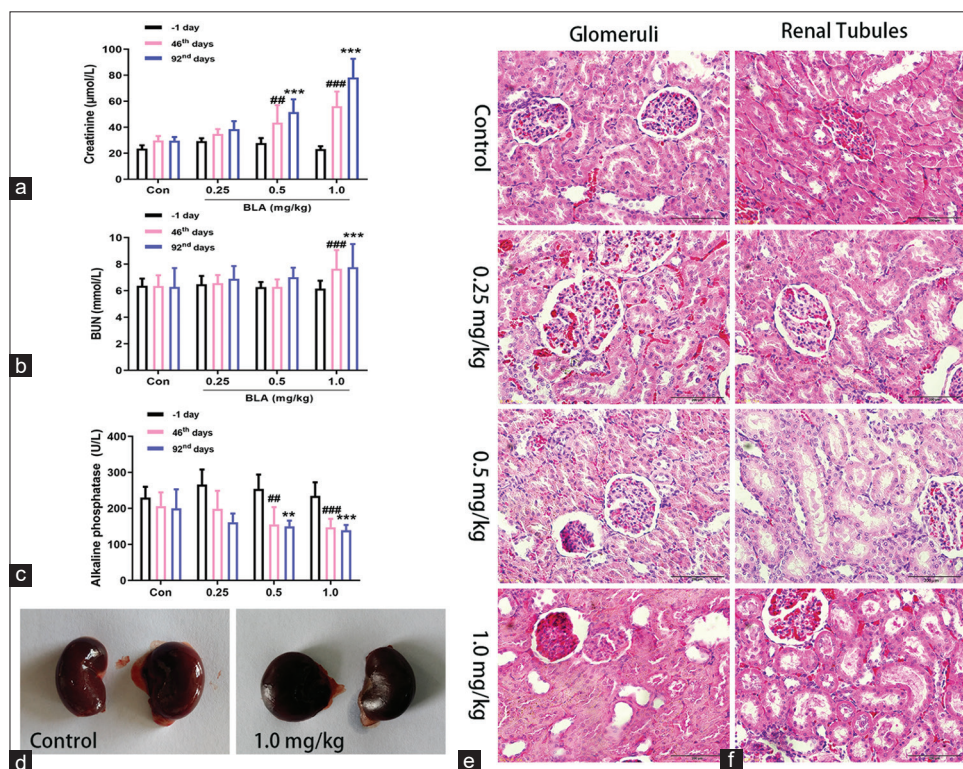


Figure 4: Bulleyaconitine A could affect the kidney function of rats in chronic toxicity study Both creatinine (a) and blood urea nitrogen (b) were significantly increased after administered with Bulleyaconitine A 0.5 mg/kg and 1.0 mg/kg for 13 weeks with $^{##}P < 0.01$; $^{###}P < 0.001$ and $^{***}P < 0.001$. Bulleyaconitine A 1.0 mg/kg could also decrease the alkaline phosphatase (c) level of rats with $^{##}P < 0.01$; $^{###}P < 0.001$; $^{**}P < 0.01$ and $^{***}P < 0.001$. Bulleyaconitine A 0.5 mg/kg and 1.0 mg/kg could cause gross pathological changes of kidney (d) and histopathological changes of glomeruli (e) and renal tubules (f)

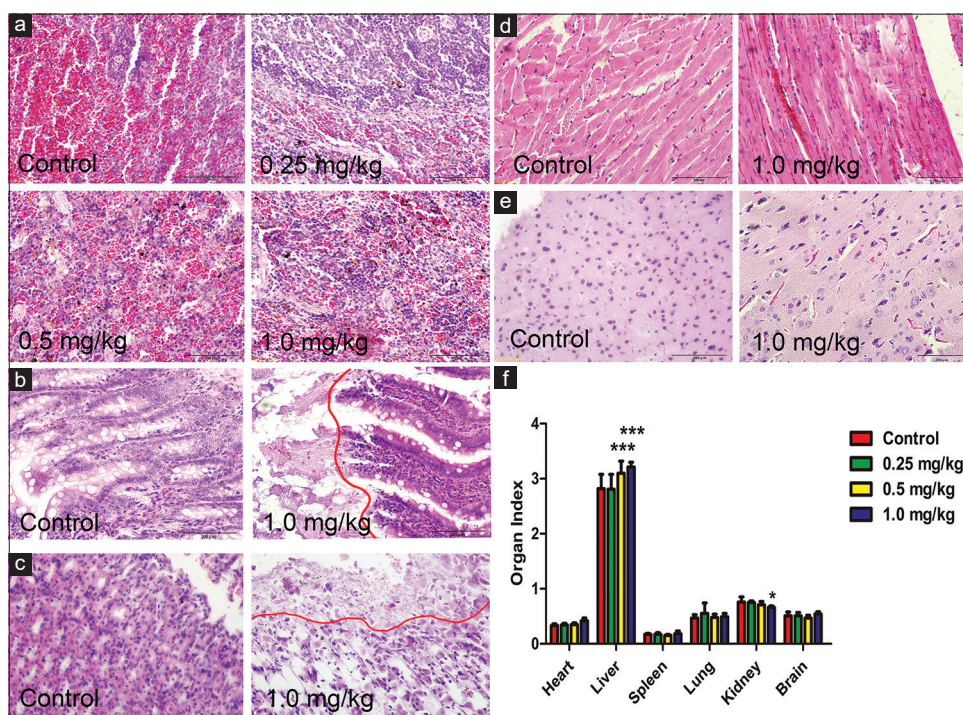


Figure 5: The toxic effect of Bulleyaconitine A on other organs spleen (a), gastric mucosa (b), jejunum (c), heart (d), and brain (e) showed histopathological changes after Bulleyaconitine A 0.5 mg/kg and 1.0 mg/kg administration in the chronic toxicity study. (f) The organ indexes of kidney and liver were significantly affected by Bulleyaconitine A with $^{*}P < 0.05$ and $^{***}P < 0.001$

long-term exposure to both 0.5 and 1.0 mg/kg BLA [Figure 5a]. Moreover, the results of our study also found GI tract, heart, and brain injuries after BLA (0.5 and 1.0 mg/kg) treatment, and these effects were consistent with the adverse reactions listed in the package insert. The gastric mucosa showed injury and necrosis, and for the jejunum [Figure 5b], the top of the villi showed necrosis and broke off [Figure 5c]. The fibril of cardiac muscle broke and unstructured substances stained pink could be observed in the sections of the heart [Figure 5d]. For the brain, nuclear pyknosis and phagocytosis could be observed in the histological sections [Figure 5e]. The results of organ indexes confirmed the toxicity of BLA; 0.5 and 1.0 mg/kg BLA treatment dose dependently affected the organ indexes of the liver, kidney, heart, spleen, and brain [Figure 5f]. However, the organ index of the lung was not affected by BLA treatment. The histological sections of other vital organs and reproductive organs did not show significant changes after BLA treatment, nor did the gross pathological results. Except for the indexes related to liver and kidney, other indexes of biochemical analysis were not affected after BLA administration.

Bulleyaconitine A is mainly distributed in the gastrointestinal tract and in organs with an abundant blood supply after a single administration

The tissue distribution of BLA in rats was researched after a single administration of 1 mg/kg BLA for 30 min. Twenty-five vital organs, all reproductive organs, and plasma samples were collected from the animals, and HPLC-MS/MS was carried out to examine the concentration of BLA in these tissues. There was a wide distribution of BLA in rat tissue, as shown in Figure 6. The sequence of the BLA concentration in tissue after a single administration was as follows: jejunum>esophagus>stomach>trachea>liver>thyroid>tongue > adrenal gland > spleen >thymus > kidney > colon > lung > submandibular lymph > heart > intestinal lymph >pancreas > fat > bone > bladder > aorta > muscle > plasma > bone marrow > optic nerve > brain. As our results showed, BLA was mainly distributed in the digestive organs such as the jejunum and esophagus. In addition, BLA was distributed

in organs with an abundant blood supply such as the trachea, liver, and thyroid. BLA could also be detected in the optic nerve and brain, which means it can cross the blood–brain barrier. For the reproductive organs, BLA could cross the testicular barrier and was mainly distributed in the testes of male rats. For female rats, BLA was mainly distributed in the breasts.

Bulleyaconitine A mainly accumulated in the gastrointestinal tract, immune system, and major metabolic organs after repeated administration

The tissue accumulation of BLA in rats was researched after repeated administration of three dosages of BLA for 13 weeks. The sequence of BLA accumulation concentrations in these organs was as follows:stomach > jejunum > spleen > kidney > intestinal lymph > liver > lung > esophagus > adrenal gland > colon > pancreas > thyroid > fat > submandibular lymph >bone marrow > thymus > muscle > trachea > heart >tongue > bone > bladder > aorta > optic nerve > brain > plasma. After long-term exposure, BLA mainly accumulated in the digestive system organs such as the stomach and jejunum, which were similar to the results after a single administration. Then, BLA redistributed and accumulated in the spleen, kidney, and liver, which is quite different from the results of a single administration. We could also detect BLA in the central nervous system. Moreover, for the reproductive system, the prostate was the target organ where BLA accumulated in male rats, which is different from that of a single administration. Therefore, these results suggest that BLA is first distributed in digestive organs and organs with an abundant blood supply. Then, as the administration frequency increased, BLA gradually accumulated in the spleen, kidney, intestinal lymph, and liver.

Bulleyaconitine A did not affect the hematological indexes of rats

Hematological analysis in animal models could reflect the toxicity of drugs in the human body. The toxicity of drugs could not only decrease the full blood count of hemoglobin, erythrocytes, hematocrit, and platelets but also influence the differential count such as the MCH and percentage of neutrophils, lymphocytes, monocytes, and eosinophils.^[24,25] Both the hematological analysis results of the 46th and 92nd days showed an increase in white blood cell counts for the 0.5 mg/kg and 1.0 mg/kg BLA treatment groups. However, the changes were still within the normal range and thus considered not toxicity related. Other hematological parameters were still within the normal range after BLA treatment. The clotting time was also not influenced by BLA treatment.

DISCUSSION

Although BLA has been clinically used as an analgesic to treat chronic pain for many years, an increasing number of BLA-related AEs have been reported to the Chinese National Center for Adverse Reaction Monitoring, indicating that safety assessments about the long-term exposure and tissue distribution of BLA should be conducted to test the safety of BLA.

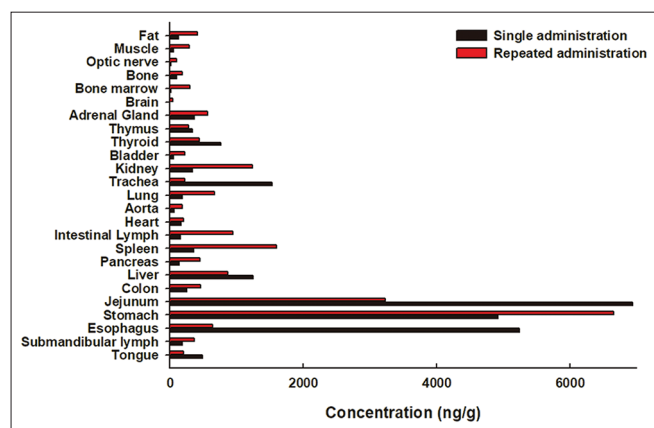


Figure 6: The tissue distribution of Bulleyaconitine A after single administration and repeated administration

In our study, we first confirmed the analgesic effect of BLA by the hot plate test and the acetic acid writhing test. BLA significantly prolonged the pain latency of mice during the hot plate test and reduced the writhing times of mice during the acetic acid writhing test. According to the effective dose of BLA in these two tests, we set seven dosages in the acute toxicity study, and the LD₅₀ value of BLA was shown to be 3.4434 mg/kg. Clinical signs of BLA toxicity in acute toxicity studies included convulsion, vomiting, reflex reduction, and piloerection, which preliminarily indicated that BLA exerted toxicity. Then, we further carried out a subchronic toxicity study of BLA after long-term exposure for 13 weeks, with dosages of 0.25, 0.5, and 1.0 mg/kg, which were two, four, and eight times the clinical equivalent dosage of BLA, respectively. As our results showed, 0.25 mg/kg BLA is relatively safe for rats after long-term exposure among the three dosages. The clinical signs, food and water intake, and body weight were not affected after 0.25 mg/kg BLA treatment for 13 weeks. The results of the biochemical and hematological analyses were still within the normal range, and the gross pathological and histological results did not show a difference between the vehicle control group and the 0.25 mg/kg BLA treatment group. Therefore, 0.25 mg/kg was considered to be the NOAEL of BLA.

After treatment with 0.5 and 1.0 mg/kg BLA for 13 weeks, the rats showed pathological clinical signs. Food and water intake were also significantly reduced during long-term administration, which caused body weight gain to decrease in a dose-dependent manner. To comprehensively analyze the results, in addition to injuries, we observed in the GI tract, heart, and brain, which are organs that were already listed to be adversely affected by BLA, the liver, kidney, and spleen were novel observable target organs of BLA toxicity in this study. Not only the biochemical indexes for liver and kidney functions but also the histopathological results confirm the hepatotoxicity and renal toxicity of 0.5 and 1.0 mg/kg BLA. The hemosiderin pigments in histopathological spleen sections also indicated direct injuries of BLA toxicity or it might be secondary to the heart failure caused by BLA. Therefore, the liver, kidney, and spleen are novel observable target organs of BLA toxicity. According to these results, 0.5 mg/kg BLA was considered to be the LOAEL.

For tissue distribution analysis, regardless of single or repeated administration, BLA primarily distributed to the GI duct. For other organs, BLA would first distribute into organs with an abundant blood supply such as the trachea, liver, and thyroid and then redistribute and accumulate in immune and metabolic organs after repeated administration of BLA for 13 weeks; the distribution sequence was spleen > kidney > liver. Since the spleen, liver, and kidney are also target organs of BLA toxicity after long-term exposure, a large amount of BLA accumulation might be responsible for injuries to these vital organs.

However, although concentrations of BLA were relatively low in the brain and heart, myocardial fibers broken, and neuronal

injuries could also be observed after BLA treatment. This might be due to the high sensitivity of cells from these two organs to BLA. Moreover, although the accumulation of BLA was relatively high in the lung, no abnormality was observed in the lung histology results. This might be due to the insensitivity of lung cells to BLA.

CONCLUSIONS

Under long-term exposure to BLA, the 0.25 mg/kg dosage did not show an adverse effect, and 0.25 mg/kg was considered to be the NOAEL while 0.5 mg/kg was considered to be the LOAEL. The spleen, liver, and kidney were found to be novel target organs of BLA toxicity under long-term exposure. Moreover, large amounts of BLA were found to accumulate in the GI tract, spleen, liver, and kidney, which might be responsible for the toxicity of BLA. These results suggest that BLA itself does not raise safety concerns at intake levels (0.25 mg/kg) no more than twice the clinical equivalent dosage. It is recommended to carefully use BLA when combining BLA with other medications that can cause spleen, liver, and kidney injuries.

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Conflicts of interest

There are no conflicts of interest.

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