Compositional and toxicological investigation of pooled venom from farm-raised *Naja atra*

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**Abstract**

**Background:** *Naja atra* is a venomous snake species medically relevant in China. In the current study, we evaluated the composition and toxicological profile of venom collected from farm-raised *N. atra*.

**Methods:** Venom was collected from third-generation captive bred *N. atra* on a snake farm in Hunan Province, China. The venom was analyzed using sodium dodecyl sulfate polyacrylamide gel electrophoresis and nano-liquid chromatography with electrospray ionization tandem mass spectrometry. In addition, hemolytic activity, median lethal dose, serum biochemical and histopathological parameters were accessed.

**Results:** *N. atra* venom proteome was dominated by phospholipase A₂ (46.5%) and three-finger toxins (41.4 %), and a set of common low relative abundance proteins, including cysteine-rich secretory proteins (4.7%), NGF-beta (2.4%), snake venom metalloproteinase (1.5%), glutathione peroxidase (0.6%), vespryn (0.3%), and 5'-nucleotidases (0.2%) were also found. Furthermore, the venom exhibited direct hemolytic activity, neurotoxicity, myotoxicity, and high lethal potency in mice, with a subcutaneous median lethal dose of 1.02 mg/kg. Histopathological analysis and serum biochemical tests revealed that venom caused acute hepatic, pulmonary and renal injury in mice.

**Conclusion:** This study revealed the composition and toxicity of venom collected from farm-raised *N. atra*, thereby providing a reference for the analysis of venom samples collected from captive-born venomous snakes in the future.
Background
China possesses an extremely rich and diverse snake fauna, consisting of approximately 209 known species of land and sea snakes. Of these, Chinese cobra (Naja atra), ranked as one of the top ten venomous snakes in the country, is considered one of the most medically important snake species [1]. To meet the increasing demand for snakes and their products, as well as to protect wild snake resources, farm breeding or snake farming is conducted to breed snakes of economic and medical relevance, such as N. atra. In Hunan Province, China, farm-raised venomous snakes are a stable source of venom used to produce cosmetics and snake venom hemagglutinase injections.

N. atra belongs to the Elapidae family, whose members contain venom that induces neurotoxic, hemolytic, proteolytic, hemorrhagic, and necrotizing activities in experimental models [1–3]. Patients bitten by N. atra usually develop necrosis, tremors, blurred vision, tachypnea, arrhythmia, and rarely hemorrhage. Victims may also suffer from acute respiratory, circulatory and renal failure [4]. Recently, several novel proteins with important biological activities have been characterized and isolated from this venom. For example, α-elapitoxin-Na1a, a novel short-chain postsynaptic neurotoxin, was isolated from N. atra venom and exhibits relatively strong binding activity with the skeletal muscle nicotinic acetylcholine receptor (nAChR) [5]. Moreover, atrase B, a novel metalloproteinase isolated from N. atra venom, may be a potential adjuvant therapeutic drug with application in xenotransplantation owing to the dual anti-complement and anti-coagulation activities [6].

Studies [7,8] have previously analyzed the protein composition of N. atra venom from Zhejiang and Taiwan, China, and have identified three-finger toxins (3FTx) and phospholipase A₂ (PLA₂) as the main components of this venom. Jiang et al. [9], found that the venom transcriptome of N. atra from Zhejiang mainly contains 3FTxs, exhibiting cytotoxicity and neurotoxicity (short chain α-NTX). Moreover, Li et al. [10] assessed the venom proteomic profiles from N. atra from Yiwu County, Zhejiang Province, China using four different approaches. Furthermore, previous studies have reported geographic and age-related variations in the proteome and enzymatic and toxicological activities of N. atra venom [4,8,11]. Gao et al. [11] found that venom yield of N. atra was related to the gender, age and geographic location.

Studies of other snake species have found differences between venom collected from snakes in captivity and in the wild [12]. Intraspecific variation in venom composition is an important cause of differences in venom function and the clinical pathophysiology of envenomation [13–16]. However, the current critical problem is that the knowledge of venom proteins in snakes from completely different regions is incomplete, and the clinical pathophysiology of patients envenomed by N. atra from completely different regions remain unknown. Proteomic approach has been used to characterize Naja venoms [17]. Therefore, it is necessary to study the venom proteome, toxicological activities and pathophysiology of N. atra from vital areas that include key habitats of this snake.

In the present study, we investigated the venom proteome and toxicity of farmed N. atra venom in Hunan Province, China. Our results provide important information on the composition and activities of N. atra venom from this province.

Methods
Snakes
The snakes from which venom samples were collected from third-generation Naja atra captive bred on the Hunan Yongzhou Yishe Science and Technology Industrial, Lingling District, Yongzhou City, Hunan Province, China. The snakes were all hatched at the same time, and the feeding environment and other aspects in the later feeding process were the same for all specimens. The average weight of the snakes was 1.5 kg, with an approximate age of one year. A total of 20 snakes (10 females and 10 males) were maintained at constant temperature and humidity conditions (25 ºC, 60%) and fed with thawed rodents.

Snake venom
Snakes were provided with water for a week before venom collection; during this period, food was not provided to the snakes. Venom extraction was performed during July-August in a glass beaker covered with a thick plastic sheet and immersed in an ice bath. July-August was the peak period for venom collection in the snake farm, and the interval between each collection was 25 days. The freshly collected venom samples was pooled, freeze-dried in a vacuum dryer and stored at -20 ºC.

Mammals and reagents
Male and female Kunming mice (20 ± 0.7 g; licensed: SCXK [Xiang] 2016-0002) and rabbits (male; approximate weight: 2.0 kg) were purchased from Hunan SJA Laboratory Animal, Hunan, China. Kits (batch number: 2200424) for analysis of serum creatinine (SCR), urea nitrogen (UN), aspartate aminotransferase (AST), alanine aminotransferase (ALT), and creatine kinase (CK) levels were purchased from Jiangsu Sinnowa Medical Science & Technology, Jiangsu, China. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels were purchased from Sigma-Aldrich (St. Louis, MO, USA), and protein endonuclease trypsin (sequencing grade) was purchased from Promega (Madison, WI, USA). Other chemicals for proteolysis were purchased from Sigma-Aldrich.

SDS-PAGE
SDS-PAGE was done as described by Laemmli [18]. The Spectra Multicolor Broad Range Protein Ladder (11–245 kDa) (Beijing Solarbio Science &Technology, Beijing, China) was used for calibration. Crude venom (20 µg) was loaded onto a 15% gel,
**Identification of N. atra venom proteins**

**Sample preparation**
Venom was dissolved in ultrapure water and the concentration was adjusted to 1 mg/mL. Subsequently, the sample was centrifuged (3,000 × g; 10 min, 4 °C), and the supernatant was used for further analysis. Venom samples were subjected to treatments as per protocols described by Shevchenko et al. [19]. The sample solution was first denatured in 8 M urea, the disulfide linkages were reduced with 10 mM dithiothreitol, and all cysteine residues were alkylated using 55 mM iodoacetamide in the dark at 37 °C for 45 min. The sample was then subjected to cleaning in a C18-based spin column (Thermo Fisher, USA) and was digested using sequencing-grade modified trypsin (Promega, USA) with a digestion buffer (ammonium bicarbonate 100 mM, pH 8.5). The peptides obtained after digestion were dried in a Speed Vac dryer (Thermo Fisher, USA). The dried sample was then resuspended in 2% acetonitrile, 97.5% water, and 0.5% formic acid and analyzed using the nano-LC-ESI-MS/MS system.

**Nano-liquid chromatography-electrospray ionization-mass spectrometry (nano-LC-ESI-MS/MS) analysis**
Nano-LC-ESI-MS/MS analysis of the digested protein samples was performed using a high-pressure liquid chromatography (HPLC) system (Agilent, USA) with a reverse phase C18 column (inner diameter: 75 μm; length: 8 cm; particle size: 3 μM; and pore size: 300 Å). The injection time was 20 min. HPLC solvent A comprised 97.5% water, 2% acetonitrile, and 0.5% formic acid, and solvent B contained 9.5% water, 90% acetonitrile, and 0.5% formic acid. The time required for the gradient to run from 2% solvent B to 90% solvent B was 60 min, in addition to 20 min for sample loading and 20 min for column washing. The column flow rate was approximately 800 nL/min after the completion of splitting, and the typical injection volume was 3 μL.

The HPLC system was coupled to an LTQ linear ion trap mass spectrometer (Thermo, USA) such that the sample eluted from the HPLC column was directly subjected to ionization by ESI before sample entry into the mass spectrometer. The ionization voltage was optimized before the conduction of every step and maintained within a range of 1.2–1.8 kV. The capillary temperature was set at 110 °C. The mass spectrometer was used in a data-dependent mode to acquire MS/MS data via a low-energy collision-induced dissociation (CID) process. The default collision energy was 33% and the default charge state was 3. One full scan using a microscan with a mass range of 550–1800 Da was acquired; subsequently, one MS/MS scan of the most intense ion was acquired, which included a full mass range and three microscans. The dynamic exclusion feature was set as follows: repeat count of 1 within 0.3 min and exclusion duration of 0.4 min. The exclusion width was 4 Da.

**Database search and validation**
Mass spectrometric data were used to search against the UniProt protein database with ProtTech’s ProtQuest software suite (Norristown, PA, USA). The parameters for data acquisition and processing were as follows: Data acquisition parameters: instrument, LTQ; software, Xcalibur 2.0; and centroid mode minimum MS signal for precursor ion: 5 × 10^4 counts. Data processing parameters: software, ProtQuest 2.0; signal-to-noise-ratio, ≥ 5; De-isotoped, yes; filtered, yes; missed cleavages, ≤ 5; modifications, Cys +57; and database, Serpentex protein database from UniProt (accession date: November 23, 2018). Validation parameters: for proteins identified based on 1 peptide (score ≥ 15); for proteins identified with more than 1 peptide (score ≥ 13).

**Protein quantitation**
Protein abundance was calculated using the label-free protein quantitation method described by Griffin et al. [20]. The scoring function was based on the MS abundance recorded in both MS and MS/MS data sets, spectral count (SC; number of MS/MS spectra per peptide), non-duplicate peptide number (PN), and fragment ion (MS/MS) intensities. The normalized spectral index (SI_n) was calculated for venom solution using the following equation:

\[
SI_n = \frac{\sum_{i=1}^{n} \left( \sum_{j=1}^{sc} \frac{i_j}{s_i} \right)}{\sum_{j=1}^{n} SI_j} / L
\]

where \(pn\) represents the PN, \(sc\) represents the SC, \(i\) represents the fragment ion intensity of peptide \(k\), \(j\) represents the \(j\)th spectral count of \(sc\) total spectral counts for peptide \(k\), \(n\) represents the total number of proteins identified, and \(L\) represents the number of amino acids in a protein.

**Hemolytic activity**
The *in vitro* hemolytic activity of the venom was determined as described by Accary et al. [21], with slight modifications. Blood was collected from healthy rabbits into tubes without anticoagulant, and the erythrocytes were separated by centrifugation (2,000 × g, 5 min). Before analysis, the erythrocytes were washed three times with normal saline and then incubated with increasing normal saline. The erythrocytes were prepared as described above and aliquots were incubated with increasing concentrations (10–100 μg/mL) of venom for 60 min, after which the tubes were
centrifuged and the absorbance of the supernatant read at 530 nm. The extent of hemolysis was expressed as a percentage of the absorbance obtained by incubating erythrocytes with distilled water (positive control, 100%). The negative control consisted of erythrocytes incubated with 0.9% saline alone.

**Venom lethality**

Mice were injected subcutaneously with venom at doses of 0.5, 0.7, 1.1, 1.7 and 2.5 mg/kg (10 mice/dose) in the dorsal region of the neck in a fixed volume of 100 uL of saline solution. The general condition of the mice, including the manifestations of envenomation and number of deaths, was monitored over the next 24h, during which the animals had free access to food and water. All mice were necropsied to assess the internal venom-induced pathological alterations. The median lethal dose (LD$_{50}$) was calculated using probit analysis [22].

**Histopathological analysis**

Kunming mice were subcutaneously injected with an LD$_{50}$ of venom. Randomly selected mice were killed by cervical dislocation at 6, 12, and 24h (n = 3), and their liver, lungs, kidneys, and heart were dissected immediately and fixed using a fixative solution (Wuhan Servicebio Science &Technology, Beijing, China). Tissue sections 5 μm thick [Indicate thickness] were stained with hematoxylin and eosin (H&E) and examined under a microscope.

**Serum biochemical tests**

Kunming mice were injected s.c. with one LD$_{50}$ of venom whereas control mice received the same volume of saline. When the venom-injected mice showed signs of envenomation (~1 h after injection), blood samples were collected from the orbital plexus into tubes without anticoagulant and the mice then killed by cervical dislocation. The blood samples were centrifuged (2,800 x g, 4 °C) and the resulting serum was used for the quantification of ALT, AST, UN, SCr and CK using commercial assay kits.

**Statistical analysis**

Experimental data are presented as mean ± SEM. Statistical analyses were performed using the GraphPad Prism 5 software (La Jolla, CA, USA). Two-tailed Student’s t-test and analysis of variance were used to analyze different groups, with $P < 0.05$ indicating significance.

**Results**

**Characterization of *N. atra* venom proteome**

*Figure 1A* illustrates the protein components in venom based on 15% SDS-PAGE under reducing conditions. An intense low-molecular-weight protein band at < 15 kDa, as well as multiple protein bands with molecular weights ranging from 40 kDa to > 100 kDa, could be observed. To identify the major protein components in *N. atra* venom samples, we conducted a comprehensive proteomic analysis using nano-LC-ESI-MS/MS. A total of 47 different proteins were identified in the venom, belonging to 21 protein families (*Table 1*); the main toxin protein families were PLA$_2$ (45.6%), 3FTx (41.4%), CRISP (4.7%), NGF-beta family (2.4%), and SVMP (1.5%) (*Figure 1B*). Additional file 1 shows more detailed data on such proteins.

**Lethality and hemolytic activity of *N. atra* venom**

*Table 2* and *Figure 2* shows the data on lethality and hemolytic activity of *N. atra* venom, respectively. The LD$_{50}$ for Kunming mice injected subcutaneously with venom was 1.02 mg/kg, the 95% confidence interval was 0.82-1.22 mg/kg, and the clinical symptoms were muscle weakness and respiratory paralysis. As shown in *Figure 2*, a venom concentration of 100 μg/mL caused 32% hemolysis in rabbit erythrocytes.

**Histological analysis of tissue damage**

Tissue sections of vital organs were stained with hematoxylin-eosin for histological analysis of venom-induced tissue damage (*Figure 3*). Hepatic tissues in the control groups exhibited normal cellular structures with distinct hepatic cells, hepatic portal canals, and bile ducts. However, in the experimental groups, inflammatory infiltrate (at 6h), cell border obscurity, and hepatocyte necrosis (at 12h and 24h) were clearly distinguishable. The renal tissues in the control group showed no histological abnormalities, whereas those in the experimental groups showed histological alterations such as the formation of glomerular and renal cysts (at 6h), proteinaceous material deposition in renal tubules (at 12h), epithelial cells of renal tubule edema, and cytoplasmic vacuolation (at 24h). The pulmonary tissues of control mice showed the presence of homogeneous air sacs and alveolar septa, as observed in normal lungs. However, histological alterations were noted in the lung tissues of experimental mice, including hyperplasia of peripheral lymphoid tissues and perivascular edema (at 6h), alveolar fusion, thickening and rupture of alveolar walls, and pulmonary interstitial edema (at 12h and 24h). Heart tissues of envenomed mice showed muscle fiber degeneration (see Additional file 2).

**Serum biochemical parameters**

Significant increases were noted in the serum biochemical parameters of venom-injected mice compared to saline-treated (control) mice. The parameters indicative of hepatic function are shown in *Figure 4A–4B*: AST and ALT activities were markedly higher in the experimental group than those observed in the control group. The levels of serum UN and SCr, which are important indices of renal function, have been presented in *Figure 4C–4D*. CK activity, a biomarker for systemic myotoxicity, was significantly higher in envenomed mice than in the control group (*Figure 4E*). These parameters were markedly higher in the experimental group than those observed in the control group.
Table 1. Summary of venom proteins of *N. atra* assigned by protein families.

| Family               | Protein name                          | Protein mass (kDa) | Source organism | Database accession | Length | No. of peptide | No. of non-duplicate peptide | Probability Score | Relative abundance (%) |
|----------------------|---------------------------------------|--------------------|-----------------|--------------------|--------|----------------|-----------------------------|-------------------|------------------------|
| PLA$_2$              | Acidic phospholipase A$_2$ natratoxin | 14.0               | *Naja atra*     | A4FS04             | 119    | 188           | 10                          | 99.0%             | 2745                   | 46.5                  |
| 3FTx                 | Cytotoxin KJC3                        | 7.2                | *Naja sputatrix*| P60311             | 60     | 91            | 8                           | 99.0%             | 1010                   | 21.0                  |
| 3FTx                 | Neurotoxin homolog NL1                | 10.5               | *Naja atra*     | Q9DEQ3             | 86     | 14            | 4                           | 99.0%             | 227                    | 1.3                   |
| 3FTx                 | Long neurotoxin 3                     | 8.4                | *Naja naja*     | P25671             | 71     | 8             | 3                           | 99.0%             | 108                    | 2.1                   |
| 3FTx                 | Neurotoxin 3                          | 7.4                | *Naja sputatrix*| Q9PSN6             | 62     | 29            | 4                           | 99.0%             | 319                    | 6.3                   |
| 3FTx                 | Cobrotoxin-b                          | 7.4                | *Naja kaouthia* | P59275             | 61     | 14            | 4                           | 99.0%             | 203                    | 0.5                   |
| 3FTx                 | Cytotoxin-like basic protein          | 7.5                | *Naja atra*     | P62377             | 62     | 7             | 3                           | 99.0%             | 93                     | 0.6                   |
| 3FTx                 | Muscarinic toxin-like protein 2       | 7.7                | *Naja kaouthia* | P82463             | 65     | 14            | 7                           | 99.0%             | 210                    | 2.2                   |
| 3FTx                 | Weak toxin S4C11                      | 8.0                | *Naja melanoleuca* | P01400             | 65     | 6             | 2                           | 96.9%             | 66                     | 0.2                   |
| 3FTx                 | Weak toxin CM-9a                      | 8.0                | *Naja kaouthia* | P25679             | 64     | 22            | 1                           | 84.5%             | 277                    | 5.3                   |
| 3FTx                 | Weak neurotoxin 6                     | 10.4               | *Naja sputatrix*| O42256             | 86     | 46            | 4                           | 99.0%             | 534                    | 1.1                   |
| 3FTx                 | Muscarinic toxin-like protein 1       | 7.8                | *Naja kaouthia* | P82462             | 65     | 9             | 4                           | 99.0%             | 109                    | 0.7                   |
| CRISP                | Cysteine-rich venom protein kaouthin-2| 27.1               | *Naja kaouthia* | P84808             | 238    | 40            | 10                          | 99.0%             | 587                    | 0.7                   |
| CRISP                | Cysteine-rich venom protein kaouthin-1| 27.8               | *Naja kaouthia* | P84805             | 239    | 35            | 10                          | 99.0%             | 501                    | 2.2                   |
| CRISP                | Cysteine-rich venom protein (Fragment)| 39.1               | *Naja naja*     | P86543             | 33     | 22            | 3                           | 99.0%             | 289                    | 1.4                   |
| CRISP                | Cysteine-rich venom protein TR1 (Fragment)| 27.5             | *Trimorphodon biscutatus* | Q2XXP4 | 236 | 2 | 1 | 85.9% | 24 | <0.1 |
| CRISP                | SCP domain-containing protein         | 15.8               | *Micrurus corallinus* | A0A2D4G403 | 138 | 10 | 2 | 99.0% | 191 | 0.3 |
| NGF-beta             | Venom nerve growth factor              | 13.4               | *Naja atra*     | P61898             | 116    | 34            | 10                          | 99.0%             | 490                    | 2.4                   |
| SVMP                 | Zinc metalloproteinase-disintegrin-like atragin | 71.4               | *Naja atra*     | D3TTC2             | 613    | 63            | 20                          | 99.0%             | 980                    | 0.6                   |
| SVMP                 | Zinc metalloproteinase-disintegrin-like kaouthiagin-like | 68.2               | *Naja atra*     | D3TTC1             | 593    | 44            | 17                          | 99.0%             | 629                    | 0.6                   |
| SVMP                 | Zinc metalloproteinase-disintegrin-like atrase-A | 70.4               | *Naja atra*     | D5LMJ3             | 607    | 41            | 15                          | 99.0%             | 526                    | 0.2                   |
| SVMP                 | Zinc metalloproteinase-disintegrin-like mikarin (Fragments) | 19.5               | *Micrurus ikaheca* | P0DJH3 | 169 | 1 | 1 | 90.5% | 15 | <0.1 |
| SVMP                 | SVMP-Den-9                            | 70.5               | *Denisonia devisi* | R4FIC4 | 613 | 7 | 1 | 83.9% | 93 | <0.1 |
| SVMP                 | Metalloproteinase 12 (Fragment)       | 73.9               | *Ahaetulla prasina* | A0A346CM42 | 614 | 2 | 2 | 98.5% | 28 | <0.1 |
| UP                   | Uncharacterized protein (Fragment)     | 33.5               | *Micrurus corallinus* | A0A2D4GFB1 | 291 | 9 | 1 | 81.8% | 95 | 1.0 |
| Family         | Protein name                                      | Protein mass (kDa) | Source organism         | Database accession | Length | No. of peptide | No. of non-duplicate peptide | Probability | Score | Relative abundance (%) |
|----------------|--------------------------------------------------|--------------------|-------------------------|--------------------|--------|----------------|-----------------------------|-------------|-------|------------------------|
| UP            | Uncharacterized protein (Fragment)               | 14.8               | Micrurus surinamensis   | A0A2D4Q7C6         | 131    | 4              | 3                           | 99.0%       | 59    | 0.1                    |
| UP            | Uncharacterized protein (Fragment)               | 18.8               | Ophiophagus hannah      | V8N885             | 157    | 3              | 3                           | 99.0%       | 31    | 0.1                    |
| UP            | Uncharacterized protein                          | 40.5               | Micrurus corallinus     | A0A2D4F1T3         | 349    | 1              | 1                           | 91.1%       | 15    | <0.1                   |
| GLU           | Glutathione peroxidase (Fragment)                | 29.8               | Ophiophagus hannah      | V8P395             | 264    | 20             | 8                           | 99.0%       | 277   | 0.6                    |
| peptidase S1  | Plasminogen activator                            | 61.6               | Crotalus adamanteus     | A0A0F7Z9B1         | 526    | 11             | 6                           | 99.0%       | 148   | 0.1                    |
| peptidase S1  | Snake venom serine protease NaSP                 | 31.9               | Naja atra               | A8QL53             | 282    | 4              | 3                           | 99.0%       | 58    | 0.1                    |
| peptidase S1  | Tissue-type plasminogen activator (Fragment)     | 32.5               | Ophiophagus hannah      | V8NYC7             | 283    | 3              | 2                           | 99.0%       | 45    | 0.1                    |
| OV            | Ohanin                                            | 23.7               | Phalotris mertensi      | A0A182C6D0         | 213    | 7              | 4                           | 99.0%       | 89    | 0.2                    |
| OV            | Ohanin                                            | 24.6               | Micrurus surinamensis   | A0A2D4NZ30         | 220    | 4              | 2                           | 95.4%       | 40    | 0.1                    |
| PDE           | Snake venom phosphodiesterase                    | 96.4               | Naja atra               | A0A2D0TCE04        | 830    | 50             | 29                          | 99.0%       | 730   | 0.2                    |
| S'-NU         | Ecto-5'-nucleotidase                             | 63.7               | Micrurus tener          | A0A194AS98         | 574    | 32             | 16                          | 99.0%       | 466   | 0.2                    |
| FMO           | Amine oxidase (Fragment)                         | 58.3               | Naja atra               | A0A2R4N4Q6         | 507    | 25             | 19                          | 99.0%       | 352   | 0.1                    |
| ENDOD1        | Endonuclease domain-containing 1 protein         | 19.8               | Ophiophagus hannah      | V8NYC7             | 160    | 8              | 6                           | 99.0%       | 114   | 0.2                    |
| MCM           | DNA helicase                                      | 97.9               | Micrurus spixii         | A0A2D4MKN7         | 863    | 4              | 1                           | 79.3%       | 40    | 0.1                    |
| PI3/P4-kinase | Phosphatidylinositol-4,5-bisphosphate 3-kinase   | 27.3               | Ophiophagus hannah      | V8NEE1             | 235    | 22             | 1                           | 83.8%       | 230   | <0.1                   |
| S-100         | Protein S100                                     | 10.3               | Boiga irregularis       | A0A0B8RWRC4        | 90     | 1              | 1                           | 89.8%       | 15    | <0.1                   |
| S-100         | Protein S100                                     | 10.2               | Micrurus fulvius        | U3FBA4             | 90     | 1              | 1                           | 85.8%       | 13    | <0.1                   |
| Type-B        | Carboxylesterase/lipase                          | 69.0               | Ahaetulla prasina       | A0A346CLZ4         | 605    | 4              | 4                           | 99.0%       | 53    | <0.1                   |
| TMEM131       | Transmembrane protein 131                        | 182.2              | Crotalus adamanteus     | A0A0F7Z222         | 1592   | 2              | 2                           | 95.3%       | 20    | <0.1                   |
| PLBL          | Phospholipase-B 81                               | 64.4               | Drysdalia coronoides    | FBJ2D3             | 553    | 2              | 2                           | 99.0%       | 40    | <0.1                   |
| Cystatin      | Cystatin                                         | 16.1               | Naja kaouthia           | E3P6P4             | 141    | 2              | 1                           | 92.7%       | 32    | <0.1                   |
| CSA           | Cobra serum albumin                              | 71.7               | Naja naja               | Q91134             | 614    | 1              | 1                           | 90.6%       | 17    | <0.1                   |
Figure 1. SDS-PAGE and proteomes of *N. atra* venom. (A) Protein separation of *N. atra* venom (20 μg) on 15% SDS-PAGE under reducing conditions. (B) Composition of *N. atra* venom according to protein families, expressed as percentages of the total protein content. *N. atra*: *Naja atra*; PLA₂: phospholipase A₂; 3FTx: three-finger toxin; CRISP: cysteine-rich secretory protein; NGF-beta: venom nerve growth factor; SVMP: snake venom metalloproteinase; PDE: phosphodiesterase; 5'NUC: 5'-nucleotidases; GLU: glutathione peroxidase; UP: uncharacterized protein; SNTX: short-chain α-neurotoxin; LNTX: long-chain α-neurotoxin; WNTX: weak neurotoxin.

Table 2. The median lethal dose (LD₅₀) of *N. atra* venom.

| Venom dose (mg/kg, s.c.) | Dose logarithm | Number of mice | Number of dead mice | Mortality (%) |
|--------------------------|----------------|----------------|---------------------|---------------|
| Control                  | –              | 10             | 0                   | 0             |
| 0.5                      | –0.3010        | 10             | 0                   | 0             |
| 0.7                      | –0.1549        | 10             | 2                   | 20            |
| 1.1                      | 0.0414         | 10             | 6                   | 60            |
| 1.7                      | 0.2304         | 10             | 9                   | 90            |
| 2.5                      | 0.3979         | 10             | 10                  | 100           |

Figure 2. Direct hemolysis caused by *N. atra* venom in rabbit erythrocytes. N: negative control (saline); P: positive control (distilled water). Data are presented as mean ± SEM (n = 6 each).
Figure 3. Histological analysis of hepatic, renal and pulmonary tissues of mice 6h, 12h and 24h after the injection of one LD$_{50}$ (1.02 mg/kg, s.c.) of *N. atra* venom. The black arrows indicate the lesion area (scale bar: 50 µm).

Figure 4. Increases in the serum (A) ALT, (B) AST, (C) UN, (D) SCr and (E) CK levels of mice 1h after the injection of one LD$_{50}$ (1.02 mg/kg, s.c.) of *N. atra* venom. The columns represent the mean +/- SEM (n = 6 each). **p < 0.01 and ***p < 0.001.
Discussion

With growing demand for snake venom products, the economic and medicinal value of snake venom is becoming more and more prominent. However, the hidden danger is that intraspecific variation in venom composition may affect the clinical pathophysiology of envenomation, venom toxicity, and the function of venom products [23]. Hence, understanding the compositional and toxicological properties of snake venom from completely different regions is crucial. Importantly, venom used in this study was collected from the same group of snakes living in the same conditions, which is vital to guarantee a quality sample for this research [24].

In this study, SDS-PAGE of *N. atra* venom samples showed the presence of prominent protein bands, similar to those previously reported [4,7,8,10] and also similar to those documented for other elapids, including *Naja* and *Bungarus caeruleus* [25]. The venom proteomic results obtained here based on direct tryptic digestion supported this hypothesis. Similar to the proteomic profile of *N. atra* venom from other regions, such as Taiwan and Zhejiang province, China, venom proteins consisted mainly of 3FTx and PLA₂ [2,8,26,27]. However, the relative abundances of these two families of toxins were not significantly different from each other, in contrast to previous studies [2,8,26]. Geographic variation in *N. atra* venom proteomic profiles has been observed, with 3FTx showing a surprising geographical variation [8]. However, these studies have focused mainly on factors that influence venom variation, an aspect that we did not address in our study. Future studies should examine the factors that influence intraspecific variation in venom composition and the mechanisms involved.

Consistent with previous work, the main component of 3FTx in *N. atra* venom is cytotoxin, known as cardiotoxin, followed by short chain α-neurotoxin, weak neurotoxin and long chain neurotoxin [2,8,28]. Cytotoxins are the principal toxic components that cause hemolytic damage, membrane disruption, and circulatory failure [29]. Cardiotoxin isolated from *N. atra* venom induces direct hemolysis in washed erythrocytes from several animals [30]. Although we were unable to identify a single specific component of venom for inducing hemolysis, we did find that *N. atra* venom induce direct hemolysis in rabbit erythrocytes in the present study, unlike other cobra venoms [31].

Furthermore, the short-chain α-neurotoxin (type I α-neurotoxin) toxin of 3FTx in this venom binds to the muscle nAChR and inhibits the binding of acetylcholine to its receptor, thereby impairing neuromuscular transmission [32]. Orphan group II toxins showed weak α-neurotoxin activity. Long-chain α-neurotoxin (type II α-neurotoxin) of 3FTx is a peripheral nAChR antagonist that causes paralysis, respiratory failure, and death [33,34]. Overall, based on venom proteomics, *N. atra* venom would exhibit strong neurotoxicity in experimental animals [2]. In agreement with this, the lethality assay showed that the s.c. injection of *N. atra* venom caused muscle weakness and respiratory paralysis. However, for humans, neurotoxic symptoms do not seem to be a clinically vital consequence of *N. atra* envenomation [3]. The reason for the discrepancy between neurotoxic effects of *N. atra* envenomation in mice and humans remain unclear. Moreover, the LD₅₀ of venom in the present study was 1.02 mg/kg, which was higher than that of venom samples collected from Zhejiang (0.68 mg/kg, s.c.) and Taiwan (0.29 mg/kg, s.c.) [2,8]. Although the origin of the venom is different, the lethal dose of venom in this study was higher than that of other regions, suggesting that it might be less lethal.

PLA₂ is the most abundant component of venom in this study, indicating that it may be an important factor in the pathogenesis of *N. atra* envenomation. PLA₂ exert multiple toxic effects on the prey or victim, such as membrane damage, myotoxicity, neurotoxicity, and edema [35,36]. Moreover, PLA₂ has also been implicated in multiple pathologies, including hepatotoxicity and nephrotoxicity [37]. Additionally, PLA₂ from *Naja kaouthia* venom has been reported to promote CTX-induced cytotoxicity in cell models, leading to cell vacuolation and rupture [27], which indicates that cobra PLA₂ play a role in the pathogenesis of necrosis. *N. atra* venom triggered considerable cell necrosis in the hepatic, renal and pulmonary tissues of mice in this study, which was similar to the findings reported for venom collected from other cobra species [38,39]. Importantly, the significant changes in serum biochemical parameters observed here indicated that *N. atra* venom caused damage to the liver, kidney, and lung of the mice.

Myonecrosis is a common phenomenon in animals injected with snake venom [40]. As shown in the present study, a significant increase in serum levels of CK also indicated that skeletal and cardiac muscle necrosis occurred in the mice injected with *N. atra* venom, and similar activity was reported in other studies conducted on different types of snake venom [41–43]. Several previous studies have reported the occurrence of muscle fiber degeneration after the injection of cardiotoxin from *N. atra* [44–46], which is similar to the pathological analysis results of heart tissue obtained in the present study. The study of *Naja* venom is important due to its great potential for medical application as an anticancer among others [47].

Conclusion

In conclusion, the present study investigated the components of farm-raised *N. atra* venom and assessed its toxicity *in vivo* and *in vitro*. This work extends our knowledge regarding venom of farm-raised *N. atra*, indicating that PLA₂ and 3FTx are major protein families in venom that exhibit direct hemolytic activity, neurotoxicity, myotoxicity, and induce pathological changes in tissues. These findings provide information on the venom of captive *N. atra* from Hunan province, China, that should be useful in future comparisons with venom from wild snakes from the same geographic region.

Abbreviations

3FTx: three-finger toxin; 5’NUC: 5’-nucleotidases; ALT: alanine aminotransferase; AST: aspartate aminotransferase; CID:
collision induced dissociation; CK: creatine kinase; CRISP: cysteine-rich secretory protein; CSA: cobra serum albumin; CVF: cobra venom factor; ddH₂O: double distilled H₂O; DTT: dithiothreitol; HPLC, high-pressure liquid chromatography; ENDO1: endonuclease domain-containing 1 protein; ESI: electrospray ionization; FMO: flavin monooxygenase; GLu: glutathione peroxidase; H&E: hematoxylin and eosin; KUN: Kunitz-type serine protease inhibitors; LAO: L-amino acid oxidase; LD₅₀: median lethal dose; LNTX: long-chain α-neurotoxin; N. atra: Naja atra; Nano-LC-ESI-MS/MS: nano liquid chromatography with electrospray ionization tandem mass spectrometry; OV: ohanin/vespryn; PDE: phosphodiesterase; PL-A: phospholipase A₁; PLBL: phospholipase B-like; SCR: serum creatine; SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SNTX: short-chain α-neurotoxin; SVM: snake venom metalloproteinase; UN: urea nitrogen; UP: uncharacterized protein; WNTX: weak neurotoxin.

Availability of data and materials
All data generated or analyzed during this study are included in this article. The mass spectrometry proteomics data were deposited in the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the iProX partner repository [48] with the dataset identifier PXD027140.

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Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
GX, JQL and ZLS conceived and designed the experiments. GX, JQL and ZLS performed the experiments and analyzed the data. GX wrote the paper, which was edited by JQL, ZLS and YY. All authors read and approved the final manuscript.

Ethics approval
The animal study protocol of the present work was approved by the Ethics Committee of Hunan Agricultural University (process n. 43321503).

Consent for publication
Not applicable.

Supplementary material
The following online material is available for this article:

Additional file 1. Identification results of nano-LC-ESI-MS/MS protein spectrum of N. atra venom.

Additional file 2. Histogramical analysis of heart tissue from mice 24h after the injection of one LD50 (1.02 mg/kg, s.c.) of N. atra venom. The black arrows indicate the lesion area (scale bar: 50 μm).

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