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Toxicity and toxicokinetics of *Amanita exitialis* in beagle dogs

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ABSTRACT

In this study, the toxicology of *A. exitialis*, a lethal mushroom found in China, and the toxicokinetics of peptide toxins contained in it were evaluated. Beagles were fed *A. exitialis* powder (20 or 60 mg/kg) in starch capsules, after which they were assessed for signs of toxicity, as well as biochemical and pathological changes. Ultra-performance liquid chromatography-electrospray ionization-tandem mass spectrometry was used to assay the peptide toxins. The total peptide toxins in *A. exitialis* was 3482.6 ± 124.94 mg/kg. The beagles showed signs of toxicity, such as vomiting and diarrhea, at 12–48 h following ingestion of *A. exitialis*. Furthermore, alanine transaminase and aspartate transaminase levels in plasma, as well as prothrombin time and activated partial thromboplastin time peaked at 36 h post *A. exitialis* ingestion. Furthermore, total bilirubin and alkaline phosphatase levels peaked at 48 h after *A. exitialis* ingestion. Three dogs that were administered 60 mg/kg *A. exitialis* died at 24–72 h after ingesting the capsules. Additionally, liver histopathological examinations showed hemorrhagic necrosis of hepatocytes. α-Amanitin, β-amanitin, and phallacidin were rapidly absorbed and eliminated from plasma after *A. exitialis* was ingested. A long latency period (12–24 h post *A. exitialis* ingestion) was observed in the dogs before the onset of gastrointestinal symptoms. There was acute liver damage thereafter. Gastric lavage and enhanced plasma clearance methods such as hemodialysis, hemoperfusion, or plasma exchange may be ineffective in removing amatoxins from blood at 12 h post *A. exitialis* ingestion. Enhanced excretion of amatoxins in urine could be effective within 2 days after ingestion of A. exitialis because amatoxins in 0–2 d urine accounted for more than 90% of the total urine excretion.

1. Introduction

From 2004 to 2014, the Chinese Center for Disease Control and Prevention (CDC) reported 576 incidents of mushroom poisoning, involving 3701 patients and 786 deaths (Zhou et al., 2016). *Amanita* species from the section Phalloideae are responsible for more than 70% of fatalities from mushroom poisoning among Chinese victims (Chen et al., 2014).

Amanita exitialis is a newly described lethal *Amanita* species that has been implicated in several mushroom poisoning incidents and 20 deaths (Chen et al., 2014; Yang and Li, 2001). Some mycologists have focused on detecting, quantifying, separating, and purifying the toxins in *A. exitialis*. The genomic diversity of the amatoxins in *A. exitialis* has also been studied (Hu et al., 2012; Li et al., 2013; Zhang et al., 2005). However, there have been few case reports on *A. exitialis* poisoning due to the unexpectedness of mushroom poisoning.

Toxicokinetic research has shown that amatoxins disappear rapidly from the human plasma (Jaeger et al., 1993). It is difficult to collect biological samples from victims of mushroom poisoning; therefore, there is usually not enough data to calculate the required toxicokinetic parameters in humans. In a previous study, the radioactivities of labeled amatoxins in the serum of dogs were measured after an intravenous injection of the toxins was

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administered to the dogs. Five experiments were performed, after which the toxicokinetics of the amatoxins were determined (Faulstich et al., 1985). Different patterns of exposure to these toxins might result in different toxicokinetic data being obtained. ^{14}C-methyl-γ-amanitin (^{14}C-A) and ^{3}H-O-methyl-dehydroxymethyl-α-amanitin (^{3}H-A) were used in the toxicokinetic study conducted by Faulstich et al. (1985). The data obtained for ^{14}C-A and ^{3}H-A is suggested to be different from those of γ-amanitin and α-amanitin.

The abovementioned findings from previous studies indicate that it is necessary to investigate the characteristics of *A. exitialis* poisoning with an animal model displaying closer analogies to the human situation Dogs were chosen for this study for four main reasons: (1) mice are not affected by oral administration of the mushroom (2) in contrast to humans where fatalities occur after some days, mice succumb to a lethal intraperitoneal dose of Amanita phalloides after 8–10 h (Floersheim et al., 1978) (3) mice, rats and rabbits don't have vomiting reflexes whereas vomiting is one of the most common initial symptom of amatoxins poisoning in humans (4) dogs are affected by oral ingestion of the mushrooms containing amatoxins and they have shown clinical and biochemical signs closely resembling amatoxins intoxication in man (Puschner et al., 2007).

Therefore, in our present study, the toxicology of *A. exitialis* and the toxicokinetics of peptide toxins contained in it were investigated in dogs after the animals were orally administered *A. exitialis*.

2. Materials and methods

2.1. Animals

Approval to conduct the study was obtained from the National Institute of Occupational Health and Poison Control review board for animal experiments. Twelve male beagle dogs (age, 8–10 months; weight, 10 ± 1 kg) were obtained from Beijing Institute of Xieerxin Biology Resource (Beijing, China) for the study. The dogs were acclimatized for a week before they were used in the experiments. After determining the baseline values of biochemical indicators, the dogs were fasted for 24 h but received water ad libitum. All the animals were orally administered a lyophilized powder of *A. exitialis* fungus in starch capsules (20 mg/kg, n = 6; 60 mg/kg, n = 6). Immediately after the dogs were dosed, they were placed in their cages and food and water were provided ad libitum. All procedures were performed in accordance with the ethical standards of the National Institutes of Health Guide for Animal Welfare, as approved by the Institutional Animal Care and Use Committee (EAWE-2017-005).

2.2. Chemicals

Lyophilized *A. exitialis* (100 g) was given by Professor Chen (College of Life Sciences, Hunan Normal University, Changsha, China). Peptide toxins (α-amanitin, β-amanitin, γ-amanitin, and phallacidin) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ammonium acetate, acetonitrile, and water, all of high-performance liquid chromatography grade, were also obtained from Sigma-Aldrich. Normal saline (0.9% NaCl), formalin, ethyl alcohol, and hematoxylin and eosin (H&E) were purchased from Sangon Biotech (Shanghai, China).

2.3. Peptide toxins in A. exitialis

The lyophilized mushroom was ground to fine powder and 0.2 g of the powder was taken from each point as shown in Fig. 1. Ultra-performance liquid chromatography-electrospray ionization-tandem mass spectrometry (UPLC-ESI-MS/MS) was then used to detect the peptide toxins in the powder.

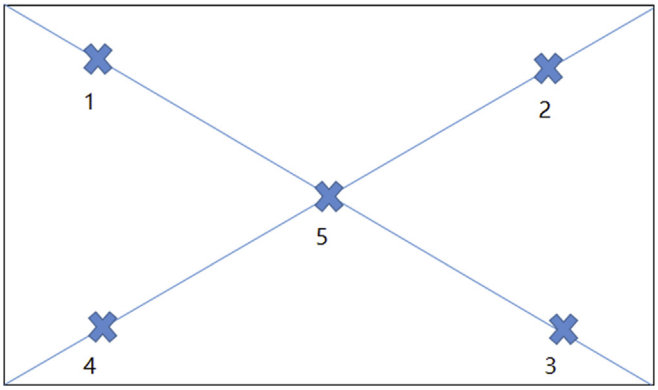

Fig. 1. Distribution of sampling points.

2.4. Toxicology study

2.4.1. Signs of toxicity

At 0, 6, 12, 24, 36, 48, 60, 72, 96, 120, 144, and 168 h after dose administration, the dogs were observed for clinical signs such as appetite, vomiting, diarrhea, weakness, hematemesis, and hematochezia. All observations were recorded.

2.4.2. Biochemical analysis of blood

Blood samples (2 mL) were collected at 0, 6, 12, 24, 36, 48, 72, 96, and 168 h from the cephalic vein after dose administration into tubes containing sodium citrate. Prothrombin time (PT) and activated partial thromboplastin time (APTT) were then determined using Coatron M2 (TECO Medical Instruments, Neufahrn i.NB, Germany) and the appropriate kits according to the manufacturer's instructions. Blood samples (2 mL) were collected at 0, 6, 12, 24, 36, 48, 72, 96, 168, 336, and 504 h from the cephalic vein. The samples were stored in tubes without anticoagulants and immediately centrifuged at 11,200 ×g for 15 min to obtain plasma. The plasma samples were stored at −80 °C until analysis for the levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), total bilirubin (TBIL), Direct Bilirubin (DBIL), blood urea nitrogen (BUN), and creatinine (CRE) using the corresponding kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

2.4.3. Histopathological examination

The livers were surgically removed after the dogs were euthanized. Portions of the liver were washed with ice-cold normal saline, cut into slices (approximately 2-mm thick), and fixed immediately in 10% neutral-buffered formalin for 48 h. The tissues were stored in 70% ethyl alcohol until they were processed. The specimens were then infiltrated with and embedded in paraffin, sectioned at 4 μm, and stained with H&E for histological examination.

2.5. Analysis of peptide toxins by UPLC-ESI-MS/MS

Blood samples (2 mL) were collected at 0, 0.25, 0.5, 1, 1.5, 2, 3, 4, 5, 6, 8, 10, 12, 14, 24, 36, and 48 h from the cephalic vein, stored in tubes without anticoagulants, and immediately centrifuged at 11,200 ×g for 10 min to obtain plasma. 0–1 d, 1–2 d, 2–3 d, 3–4 d and 4–5 d urine samples were collected, stored in urine container, and urine volume were recorded. The plasma and urine samples obtained were stored at 2–8 °C until analysis for peptide toxins.