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Acute Toxicity Study of Alpha-Amanitin in Wistar Albino Rat

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ABSTRACT: The present study was designed to determine the acute oral toxicity of Alpha-amanitin (α-amanitin) in Wistar albino rats. Acute oral toxicity study was carried out to estimate median lethal dose (LD_{50}) of α-amanitin. LD_{50} is the dose of a chemical, calculated as per the concentration of chemicals that produce death in 50% of a population of test animals to which it is administered by any of a variety of methods. A single oral dose of α-amanitin were dissolved in phosphate buffer saline (PBS) and administered orally at a concentration of 0.05, 0.1, 0.2, 0.4 mg/kg body weight to experimental animals. LD_{50} was calculated according to the method described by Miller and Tainter (1944) and was observed as 0.2 mg/kg body weight. Single dose of α-amanitin at 0.05 mg/kg body weight did not reveal any toxic signs or behavioral alterations, hence considered as No Observed Adverse Effect level (NOAEL). Histopathology of liver tissue showed morphological abnormalities such as multiple focal necrosis, fragmentation of nuclear materials, fatty changes characterized by focal accumulation of fat droplets and distortion of hepatic cord with necrosis.

Keywords: Alpha-amanitin, Median lethal dose, No observed adverse effect level, Histopathology, Necrosis, Fragmentation.

INTRODUCTION

Mushroom poisoning is a common environmental medical emergency. Northeastern region of India has a rich macro-fungal floral due to optimal ecological conditions. Although only 50-100 of about 5000 known mushroom species are toxic, it is difficult for the untrained common people to differentiate between the toxic and non-toxic mushroom. In Assam there has been an increase in seasonal incidences of mushroom poisoning in different locations like Tinsukia, Dibrugarh, Sivsagar, Jorhat, Lakhimpur, Golaghat districts and few locations in lower Assam (Sharma *et al*., 2013). A wide clinical spectrum of symptoms can be observed, ranging from simple gastroenteritis to life-threatening hepatic and renal failure. 95 % of the mushroom poisoning is caused by *Amanita phalloides* (Deniz and Saygun 2008; Akilli *et al*., 2014; Koyuncu *et al*., 2014). *A. phalloides*, is also known as death cap (Garcia *et al*., 2015a). Diagnosis of these intoxication is challenging due to the delayed onset of symptoms.

α-amanitin plays a major role in *A. phalloides* poisoning, showing toxic effects on multiple organs particularly in liver and kidneys. The molecular mechanism responsible for Amanita species intoxication was due to inhibition of RNA polymerase II in eukaryotic cells (Karlson-Stiber & Persson 2003). In histopathology, the liver reveals hepatocellular necrosis extending from central to peripheral areas with vacuolation, renal necrosis, and hemorrhages in different organs (Cope, 2007; Puschner *et al*., 2007).

α-amanitin poisoning are fatal after one large dose (acute toxicity). The commonly used term to describe acute toxicity is $LD_{50,}$ where LD means lethal dose deadly amount and the subscript 50 means that the dose is acutely lethal to 50% of the animals to whom the test compound was administered under controlled laboratory conditions. In other words, LD_{50} is the statistically derived single dose of a substance that produces death in 50% of a population of test animals to which it is administered by any of the methods like oral, dermal, inhalation or intravenous. Determination of this test is to examine the relationship between dose and the most extreme response-death. The most potent or toxic the chemical, lower is the LD_{50} and smaller dose is needed to cause death. Normally LD_{50} is expressed in milligrams of substance per kilogram of animal body weight (mg/kg body weight). It provides information on health hazards likely to rise from short term exposure. This date serves as a basis for labelling and classification and also helpful in establishing a dosage regimen in toxicity studies.

The LD_{50} is determined by any accepted methods, e.g. (Miller and Tainter 1944; Bliss 1934; Litchfield and Wilcoxon 1919; Finney 1971; Weil 1952; Thompson 1947).

The main toxin of these mushroom includes amatoxins (α and β-amanitin) and phallotoxins (phalloidin) with the heat-stable bicyclic octapeptide alpha-amanitin (α-amanitin) as the primary toxin (Ward *et al*., 2013; Mas, 2005). In previous study, they found out that the i.v. median lethal dose (LD_{50}) of α-amanitin in dogs is 0.1 mg/kg body weight. Based on the oral dosing study in dogs, the oral LD_{50} for methyl-γ-amanitin was estimated to be 0.5 mg/kg body weight. Guinea pigs and rabbits are considered to be equally sensitive to amanitin as dogs with LD_{50}'s of α-amanitin of 0.1 and 0.2 mg/kg body weight respectively. The estimated oral LD_{50} of α-amanitin in human is 0.1 mg/kg body weight. Considering the average concentration of amanitins per mushroom. One *A. phalloides* has the potential to kill an adult, while in dog it may require two mushrooms to cause death (Puschner and Wegenast 2018). Based on the oral dosing study in human, our study was designed to estimate in vivo LD_{50} in Wistar albino rats and to investigate the extend of liver affections by histopathology.

MATERIAL AND METHODS

Test chemicals: α-amanitin (*Amanita phalloides* ≥ 85 % HPLC) was purchased from Sigma-Aldrich chemicals Pvt. Ltd., Bengaluru.

Animals and experimental design: The study was performed in accordance with the guidelines for the use and care of laboratory animals approved by Institutional Animal Ethical Committee (Approval No. 770/GO/Re/S/03/CPCSEA/FVSc/AAU/IAEC 19-20/780). A total of 20 numbers Wistar albino rats weighing 150-200 g are taken for estimation of median lethal dose (LD_{50}). All the rats were maintained under standard laboratory conditions.

Dose preparation and administration: Rats were fasted for 12 hours prior to dosing. α-amanitin was administered once orally by 22-gauze oral feeding needle to the rats. The volume of the dose depends on the size of the animals. In rodents, it should not exceed 1ml/100 g body weight (Ghosh, 1984; Turner, 1965). In this study, the dose was mixed in 2 ml of PBS.

Estimation of the dose range and percentage of mortalities: Initially, dosing at 0.05 mg/kg body weight to two rats did not produce any toxicological signs and symptoms. Keeping the above criteria of dosing and facts, four different doses were given orally to four groups of rats (n=5) with increase in two times of each dose for determination of LD_{50} of α-amanitin starting from 0% to 100% mortality (Randhawa, 2009) [Table 1]. The animals were observed for 2 hr and then at 4 hr, 6 hr and 24 hr for toxic signs and symptoms. After 24 hr, the numbers of deceased rats in each group were counted and the percentage of mortality was calculated using the graphical method (Miller and Tainter 1944).

Table 1: Total dose given orally to rats.

Groups	No. of Rats (n)	Alpha-amanitin (mg/kg)	Total dose (mg/kg)
I	5	0.5 × 0.1mg/kg	0.05
II	5	1.0 × 0.1 mg/kg	0.1
III	5	2.0 × 0.1mg/kg	0.2
IV	5	4.0 × 0.1mg/kg	0.4

Histopathological study: For histopathology, representative tissue samples from liver will be collected in 10% formalin. Formalin fixed tissues will be processed for histopathological studies as per the standard procedure (Culling 1974). LD_{50} of Alpha-amanitin was calculated by linear regression analysis by extrapolation of probit units.

RESULTS

Signs recorded during experiment: Initially, α-amanitin did not produce any significant effect on rats at 0.05 mg/kg body weight. However, when the doses of 0.1, 0.2 and 0.4 mg/kg body weight were administered, signs of toxicity like laboured breathing, gasping, and death were observed in some rats. The percentage of animals that died at each dose was then transformed to probit [Table 1] using Finney's method [Table 2]. The percentage dead for 0 and 100 were corrected before the determination of probits.

For 0% Dead = $100 \times (0.25/n)$

For 100% Dead = $100 \times (n - 0.25/n)$, where n = 5 rats

Table 2: Graphical Method for Calculation of Ld_{50}.

Groups	Dose (mg/kg)	Log dose	Dead	% Corrected	Probits
I	0.05	-1.30103	0	5	3.36
II	0.1	-1	1	20	4.16
III	0.2	-0.69897	2	40	4.75
IV	0.4	-0.39794	4	80	5.84

Table 3: Transformation of Percentage Mortalities to Probit.

%	0	1	2	3	4	5	6	7	8	9
0	-	2.67	2.95	3.12	3.25	3.36	3.45	3.52	3.59	3.66
10	3.72	3.77	3.82	3.87	3.92	3.96	4.01	4.05	4.08	4.12
20	4.16	4.19	4.23	4.26	4.29	4.33	4.36	4.39	4.42	4.45
30	4.48	4.50	4.53	4.56	4.59	4.61	4.64	4.67	4.69	4.72
40	4.75	4.77	4.80	4.82	4.85	4.87	4.90	4.92	4.95	4.97
50	5.00	5.03	5.05	5.08	5.10	5.13	5.15	5.18	5.20	5.23
60	5.25	5.28	5.31	5.33	5.36	5.39	5.41	5.44	5.47	5.50
70	5.52	5.55	5.58	5.61	5.64	5.67	5.71	5.74	5.77	5.81
80	5.84	5.88	5.92	5.95	5.99	6.04	6.08	6.13	6.18	6.23
90	6.28	6.34	6.41	6.48	6.55	6.64	6.75	6.88	7.05	7.33

The probit values thus obtained were plotted against log-dose and then the dose corresponding to probit 5 (50%) [Table 1].

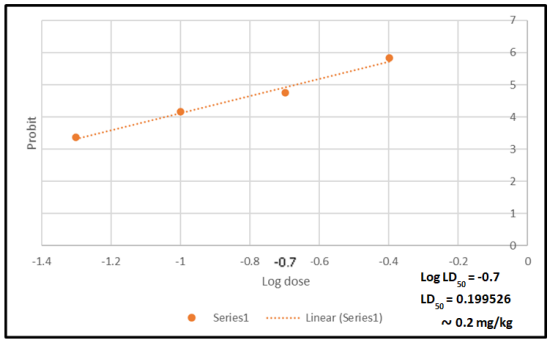

Fig. 1. Plot of log-doses versus probits.

In the present study, α-amanitin Log LD_{50} is -0.7 and LD_{50} is approximately 0.2 mg/kg [Fig. 1]. The probits of 84 and 16 from Table 3 are 5.99 and 4.01 (approximately 6 and 4) respectively. The Log-LD values for the probits 6 and 4 are obtained from the line on the graph in Fig. 1, which in the present case are –0.4 and –1 and their antilog are 0.398104 (approx. 0.4) and 0.1 respectively. Therefore, LD_{50} of α-Amanitin when given orally was observed as 0.2 ± 0.09, with 95% confidence interval (CI).

HISTOPATHOLOGY

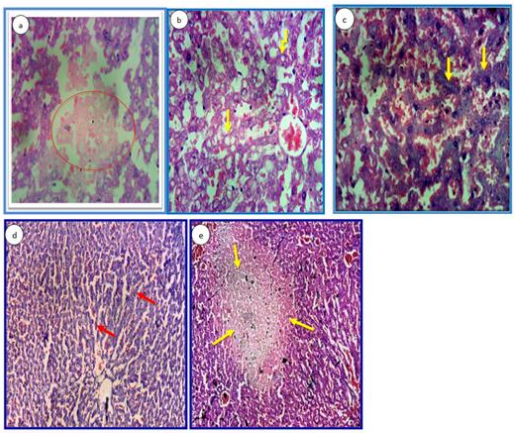

Fig. 2. (a) The hepatic parenchyma showed multiple focal areas of necrosis. (b) Fatty changes characterized focal accumulation of fat droplets. (c) Moderate hemorrhages. (d) Distortion of the hepatic cord. (e) Necrosis in hepatic parenchyma.

DISCUSSION

α-amanitin plays a major role in *A. phalloides* poisoning, a common environmental medical emergency. Ingestion of just one mushroom can lead to toxicity, resulting in severe morbidity or mortality in a healthy individual. While in dog, it may require two mushrooms to cause death (Puschner *et al*., 2007). α-amanitin are extremely toxic, having great heat stability and this property combined with their solubility in water make them exceptionally toxic as they are not destroyed by cooking or drying (Wieland and Faulstich 1978). A fatal case was reported after consuming *A. phalloides* frozen during 7-8 months, thus demonstrating that these compounds also resist to thawing processes (Himmelmann *et al*., 2001). Additionally, amatoxins decompose very slowly when stored in open, aqueous solutions or following prolonged exposure to sun or neon light (Barceloux, 2008).

Amanitins are extremely toxic. Therefore, the present study was conducted to find out LD_{50} of α-amanitin. Initially at 0.05 mg/kg body weight α-amanitin did not produce any significant effect on rats and considered to be No Observed Adverse Effect level (NOAEL). However, when the doses of 0.1, 0.2 and 0.4 mg/kg body weight were administered, signs of toxicity like laboured breathing, gasping, and death were observed in some rats. The patterns of toxicity signs in liver like morphological abnormalities such as multiple focal necrosis, fragmentation of nuclear materials, fatty changes characterized by focal accumulation of fat droplets and distortion of hepatic cord with necrosis, strongly suggestive of acute toxicity caused by α-amanitin (Ergin *et al*., 2015; Puschner *et al*., 2007).

The mean of two doses i.e. the lowest dose that killed one animal and the highest dose that do not kill any animal determines LD_{50} of a particular substance. The graphical representation of probit versus log dose showed a typical straight line, which was in agreement with the principle of probit analysis (Fig. 1). According to Miller and Tainter probit analysis method, at 24 hr the acute oral LD_{50} was calculated as 0.2 mg/kg body weight (with 95% confidence interval) in the present study, which is 2-folds higher in terms of LD_{50} than the oral LD_{50} values of α-amanitin in human and 2-folds lesser in terms of LD_{50} than the i.v. LD_{50} values of α-amanitin in dog and guinea pig (Puschner and Wegenast 2007). Actual decrease in LD_{50} or increase in mortality are used to assess the scale of the increase in intoxication following exposure to α-amanitin.

Histopathology revealed morphological abnormalities such as multiple focal necrosis, fragmentation of nuclear materials, fatty changes characterized by focal accumulation of fat droplets and distortion of hepatic cord with necrosis in liver tissue of α-amanitin intoxication (Fig. 2). These pattern of changes in liver due to α-amanitin intoxication is in agreement with the findings by Wu *et al*. (2013); Fineschi *et al*. (1996); Puschner *et al*. (2007).

CONCLUSIONS

The strong point of our study was the intoxication model (Wistar albino rat), in which there was no death after oral dosing of α-amanitin at 0.05 mg/kg body weight. We obtained the results from all animals, so there was no data loss. The oral LD_{50} of α-amanitin is calculated as 0.2 mg/kg body weight in Wistar albino rat. The pathology results of our study showed the presence of hepatic damage in *in vivo* rat intoxication model.

Pharmacokinetics and pharmacodynamics of ingested α-amanitin orally may differ from that of intraperitoneal α-amanitin injected. An animal model may not represent the same oral median lethal dose (LD_{50}) with that of human beings.

FUTURE SCOPE

We believed that our findings provide a basis for research on the pathophysiology of hepatic damage caused by α-amanitin intoxication. This data can also be useful in the study of therapeutic potential screening for A. phalloides poisoning.

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Conflict of Interest. None.

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