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BIO-PHYSIOLOGICAL EFFECTS OF LD50 OF CRUDE VENOM OF BLACK PAKISTANI COBRA (*Naja Naja karachiensis)* IN MICE

Z. Riaz, M. Q. Zaman, [1]Z. Ullah, Habib-ur-Rehman, M. S. Yousaf, I. Rabbani, [2]M. I. Khan, M. S. Hussain, S. Tahir and [*]K. A. Majeed

Department of Physiology, [1]Department of Clinical Medicine and Surgery, University of Veterinary and Animal Sciences, Lahore, Pakistan, [2]Department of Animal Health, The University of Agriculture, Peshawar, Pakistan
[*]Corresponding Author's email: Khalid.majeed@uvas.edu.pk

ABSTRACT

The present study evaluated the acute bio-physiological effects of Lethal dose 50 (LD 50) of crude venom of Black Pakistani Cobra, *Naja naja karachiensis*, collected from Southern Punjab, Pakistan. Adult mice (n=20) were injected intramuscularly with normal saline (n/s) or LD50 of crude venom (1.2mg/kg). Blood was collected by cardiac puncture after anesthesia at intervals 0 (n/s), and 1, 1.5, 2 (LD50 venom) hours. Complete blood count, glucose, serum Alanine aminotransferase (ALT), albumin, total proteins and urea were measured to assess the physiology. One way ANOVA was used to analyze the data ($P<0.05$). A significant increase in erythrocyte count, its indices and thrombocytosis was observed during the study. Leukocyotsis was observed after a transient leucopenia at 1 hour post envenomation. No significant difference was observed for lymphocytes and monocytes. Serum concentrations of urea, total proteins, albumin and ALT were also increased significantly with all time intervals. Hypoglycemia was observed initially followed by hyperglycemia at 1.5 and 2 hours. The changes indicate that venom has severe hemotoxic potential and disrupts physiological processes by affecting vital organs like kidneys and liver in mice.

Key words: *Cobra venom, Hematology, Bio-physiological effects, Lethal dose 50, Mice.*

INTRODUCTION

Snakes are important members of class *Reptilia*. There are more than 3000 species of snakes that are divided into 402 genera and 18 families, out of which 500 species are considered venomous (Zug *et al.* 2001). Snakebites are common throughout the world and about 5.5 million people are bitten by snakes annually all over the world (Kasturiratne *et al.* 2008), out of which 40,000 bites are reported annually in Pakistan, which cause up to 20,000 fatalities (Chippaux, 1998; Junaid *et al.* 2014). In Pakistan, two species of Cobra snakes that belong to genus Naja are present, named as *Naja naja naja* (Indian cobra) and *Naja naja oxiana* (Brown cobra). In Southern Pakistan, *Naja naja karachiensis* (Black Pakistani cobra), sub-specie of *Naja naja oxiana*, is widely present. Cobra snake is highly venomous and contributes to most of snake-bite related deaths in Asia (Wuster, 1996).

Snake venom, a complex fluid, is composed of polypeptides (Hider *et al.* 1991), purines, amines, lipids, organic molecules, metal ions, carbohydrates and lipids (Mebs, 2001; Aird, 2002). It is secreted from parotid glands of snakes and causes edema, vomiting, cardiac failure, respiratory arrest, bleeding wounds, headache, altered consciousness, systemic myolysis and necrosis at the site of bite (Koh *et al.* 2006). Hematopoietic effects are also observed on administration of venom in mice (Maria *et al.* 2003). Several factors, apart from venom components, are important in determining whether the victim of snake bite will survive or not. Post-bite complications, like sepsis can aggravate the situation of the patient. In addition to this, microbial contamination from environment, health status, handling and site of snake bite are important aspects to be considered (Junaid *et al.* 2014). Snake venoms are also variable in their toxic, biochemical and pharmacological characteristics based on their geographic location (Shashidharamurthy *et al.* 2001). There is no data that describes the acute bio-physiological effects of black Pakistani Cobra present in Southern Punjab, Pakistan. Keeping in view, the demographic variability of snake venom and their ability to exert particular systemic effects, the present study was conducted to determine the acute bio-physiological effects of crude venom of Black Pakistani Cobra, collected from Mian Channu region, Southern Punjab, Pakistan. Complete hematology, blood glucose, urea, total proteins, albumin and serum Alanine aminotransferase (ALT) were performed in order to assess the effects of Cobra snake venom in mice.

MATERIALS AND METHODS

Venom Collection and Preparation: Crude venom was obtained from the black spectacled cobra (*Naja Naja karenchiasis*) from Mian Channu region, Southern Punjab, Pakistan. Venom was obtained by massaging the gland below the eye area in dim light. Collected venom was freeze dried (lyophilized) using (Thermofreeze dryer

PL-6000), and then stored at 2°C to 7°C in a light resistant container till LD50 calculation and envenomation.

Determination of LD50 Dose: Lethal dose 50 (LD50) was calculated by method described by Reed and Muench (1938). Briefly, one mg of lyophilized venom was taken into 1.0 ml of 0.9% normal saline (n/s). Ten fold dilutions were made from this test tube in n/s, so as to make dilutions from 10^1, 10^2 to 10^{10}. 0.1ml from each dilution was injected in mice by intramuscularly (I/M) route. The number of dead mice was recorded 24 hours post-inoculation. The calculated LD50 was $10^{2.41}$, which is 0.257µg/mouse (20g) or 1.2mg/kg body weight by I/M route.

Experimental animals and design: Adult male albino mice having average weight of 20.0±3.5 g were used in this study. They were housed at animal shed of Department of Physiology, University of Veterinary and Animal Sciences, Lahore, for one week before the start of the study for acclimatization. The mice were kept in cages and given free access to regular rat chow and water. Environmental conditions included 12-h light–12-h dark cycle, with temperature of 25.0 ± 2.0 °C. The experimental protocol was approved by the University ethical committee. Mice (n=20) were randomly divided into four time intervals; 0, 1, 1.5 and 2 hours, with 5 mice per time interval. The mice were injected I/M with 1.2mg/kg body weight of n/s (0 hours) or LD50 Cobra venom (1, 1.5 and 2 hours). Blood samples were collected by cardiac puncture under inhalation anesthesia, and animals from each time interval were euthanized after collection of blood. Inhalation anesthetic, chloroform was administered with inspired air by means of a special chamber in a proportion of 1:5000, after which animal was placed in chamber for 60 to 90 seconds (Koladev, 1979).

Hematology and Biochemical Assays: Complete blood count was evaluated using Veterinary Hematology Analyzer (Diatron Abacus, Messtechnik, Ges.M.B.H Wein, Austria). Blood glucose (Cinex Diagnostics, CAT No. CD2409), urea (Kashef Diagnostics, CAT No. 3010-304), total proteins (Pioneer Diagnostics, CAT No. PD4401), albumin (Crescent Diagnostics, CAT No. CS600) and ALT (Singapore Biosciences, CAT No. GPT2195/3) were measured using commercially available enzymatic kits.

Statistical analysis: Data was analyzed for normal distribution using Kolmogorov-Smirnov test. One way analysis of variance (ANOVA) was performed for analysis of the studied parameters using SPSS software (Version 13, SPSS Inc., Chicago, IL, USA). Data was expressed as mean with standard error. Probability value at $P < 0.05$ was considered significant.

RESULTS AND DISCUSSION

The present study was conducted to determine the acute bio-physiological effects ofLD50 of crude venom of Black Pakistani Cobra, collected from Mian Channu region, Southern Punjab, Pakistan. The effects were observed at different time intervals after I/M envenomation in mice.

After 2 hours post-envenomation, all the erythrocytic indices were found to be higher compared to time intervals 0, 1 and 1.5. A significant increase in erythrocyte count and hemoglobin (Hb) values was observed. Packed Cell Volume (PCV), Mean Corpuscular Volume (MCV), Mean Corpuscular Hemoglobin (MCH) and Mean Corpuscular Hemoglobin Concentration (MCHC) were also significantly increased after I/M envenomation. The results are indicated in Table 1. Khalil *et al.* (1958) observed a decrease and then an increase in erythrocytes with *Naja haje* venom. We hypothesize that a decrease in erythrocyte count could be due to stress of envenomation, which was not observed in our study. Moreover, the increase in erythrocytes could be due to impairment in kidney and/or liver function, which cause a decrease in plasma volume thus increasing the PCV (Schafer, 2004). An increase in Hb concentration corresponded to the erythrocyte count in the present study. The increase in erythrocytic indices, like MCV along with erythrocyte count and Hb content, indicates that erythrocytes were trying to carry maximum amount of Hb (Al-Sadoon and Fahim, 2012a), as an integral part of homeostatic mechanisms to cope with effects of envenomation. Hemoglobinemia, observed is probably due to hemolytic nature of the cobra venom (Soto *et al.* 1988), which may be causing intravascular hemolysis and releasing Hb. The increase in erythrocytic indices, MCH and MCHC indicated that the physiological mechanisms for maintaining Hb weight and concentration could be compromised.

In our study, number of leukocytes was decreased at 1 hour post-envenomation and increased afterwards. At 2 hours, leukocytes count was found to be significantly increased compared to 0 hour. The results of leukocytes and platelets counts in envenomated mice are presented in the table 2. Leukopenia observed initially was probably as an effect of peripheral destruction of cells in reticuloendothelial system or liver impairment as both of the effects were observed in our study. The increase in leukocytes count afterwards is consistent with other studies (Amin *et al.* 2008; Al-Sadoon *et al.* 2012b) and this leukocytosis could be due to inflammation and toxicity in the kidneys as a result of venom components (Al-Sadoon *et al.* 2012a). No change in the number of lymphocytes and granulocytes was observed during the study. Platelets count was increased significantly during the time intervals and was highest at 2 hours post-envenomation. Platelets play an important role in

coagulation, and thrombocytosis may depict that clotting process was initiated in response to intravascular hemorrhages and bleeding.

Table 3 shows the results of biochemical profile in envenomated mice. In the present study, blood glucose concentration decreased at 1 hour and increased afterwards with highest level observed at 2 hours post-envenomation. Most of the literature supports this finding that different types of snake venoms are responsible for hyperglycemia in different species (Ezzat and El-aal, 1989; Hyslop and Marsh, 1991). However, hypoglycemia observed acutely in this study, could be due to stimulatory effects of phospholipase A, a component of *Naja naja* venom, on facilitated transport of glucose, indicating that the venom exhibits kinetics resembling insulin (Blecher, 1969). An increase in glucose concentrations afterwards is probably because of impaired metabolism in liver and muscle tissue (Marsh *et al.* 1997). A neurotoxic fraction of Egyptian cobra, *Naja haje* has been shown to modulate insulin and glucose turnover and cause sustained hyperglycemia (El-Fiky, 1999). Apart from this, release of tissue catecholamines and altered neurotransmission of adrenergic mechanisms may be responsible for increased glucose levels.

A linear increase in serum urea concentration was noticed with significant difference observed at 1.5 and 2 hours post-envenomation. The findings were supported by Ibrahim and Jammaz (2001). Histopathological lesions, like acute tubular necrosis, acute cortical necrosis and glomerular lesions, have been observed with snake bites (Pal *et al.* 2010). Some snake venoms have also been shown to enhance Na^+/K^+-ATPase expression and activity in this early phase of renal damage (Linardi *et al.* 2011). In the present study, the rapid increase in urea concentrations post-envenomation strongly suggests that kidneys are instantly affected by venom components.

The serum total proteins concentration was not affected at 1 and 1.5 hours time intervals but increased at 2 hours post-envenomation. Similar findings were observed in rabbits by viper snakes venom (Hyslop and Marsh, 1991). However, another study reported that intra-peritoneal injection in rabbits decreased serum total proteins (Ibrahim and Jammaz, 2001), which may be due to dose and time dependant reactions inside the body. Albumin, an important part of total serum proteins, was not affected at 1 hour but increased slightly at 1.5 and 2 hours post-envenomation. The increase in total serum proteins and albumin could also be due to disturbances in metabolism, which is evident with liver and renal damage. If further time intervals could be investigated, a decrease in total serum proteins and albumin might have been observed.

Table 1. Effects of LD50 dose of Black Pakistani Cobra venom on Erythrogram in envenomated mice.

Parameters	0 hour	1 hour	1.5 hour	2 hour
Erythrocytes count (x 10^{12}/l)	8.14±0.43^{c}	9.36±0.11^{b}	10.02±0.11^{b}	11.16±0.37^{a}
Hb (g/dl)	11.20±0.43^{c}	11.82±0.11^{bc}	12.60±0.28^{a}	13.88±0.41^{a}
PCV (%)	38.38±1.84^{c}	44.12±0.42^{b}	46.78±0.69^{b}	53.84±1.16^{a}
MCV (fl)	44.40±0.40^{c}	46.20±0.20^{b}	46.93±0.05^{b}	49.80±0.58^{a}
MCH (pg)	11.74±0.22^{c}	12.40±0.05^{b}	12.76±0.09^{b}	13.42±0.13^{a}
MCHC (g/dl)	25.40±0.10^{d}	26.34±0.14^{c}	26.78±0.08^{b}	27.64±0.22^{a}

LD50: lethal dose 50, Hb: hemoglobin, PCV: packed cell volume, MCV: mean corpuscular volume, MCH: mean corpuscular hemoglobin, MCHC: mean corpuscular hemoglobin concentration, l: liter. Data is presented as mean±SE. Values within a row denoted by different superscripts indicate significant difference.

Table 2. Effects of LD50 dose of Black Pakistani Cobra venom on Leukogram and platelets in envenomated mice.

Parameters	0 hour	1 hour	1.5 hour	2 hour
Leukocytes (×10^9/l)	3.69±0.48^{b}	1.48±0.24^{c}	2.43±0.68^{bc}	9.48±1.00^{a}
Monocytes (%)	19.48±6.91^{a}	17.22±0.72^{a}	16.64±1.65^{a}	19.22±7.14^{a}
Lymphocytes (%)	43.18±5.19^{a}	29.86±7.13^{a}	28.96±6.94^{a}	45.82±11.23^{a}
Granulocytes (%)	43.56±11.17^{a}	52.92±7.41^{a}	61.46±9.27^{a}	34.96±11.94^{a}
Platelets (×10^9/l)	271.16±69.10^{c}	530.20±65.82^{b}	576.36±87.15^{b}	677.60±64.27^{a}

LD50: lethal dose 50, l: liter. Data is presented as mean±SE. Values within a row denoted by different superscripts indicate significant difference.

The ALT concentration was also increased in a linear pattern in the present study. The increase correlated with several studies previously reported (Al-Sadoon *et al.* 2012b; Silva *et al.* 2011). Regardless of the differences in routes, dose and time post-envenomation, all the studies showed increased ALT, indicating that liver is the

primary target of venom components. ALT is the most commonly used clinical biomarker used in evaluating hepatocellular injury. An increase in the ALT in present study affirms that hepatic damage takes place with most of snake venoms.

In conclusion, the changes indicate that hematology, some of the kidney and liver biomarkers are severely affected by envenomation of Pakistani cobra (*Naja naja karachiensis*) in mice. Innate immune cells are not affected acutely. The venom has severe hemotoxic potential and disrupts physiological processes by affecting vital organs like kidneys and liver.

Table 3. Effects of LD50 dose of Black Pakistani Cobra venom on biochemical parameters in envenomated mice.

Parameters	0 hour	1 hour	1.5 hour	2 hour
Glucose (mg/dl)	110.00±2.74^{c}	66.40±10.71^{d}	140.89±7.08^{b}	196.60±8.70^{a}
Urea (mg/dl)	30.40±1.17^{c}	41.00±1.61^{bc}	50.33±1.77^{b}	72.80±6.91^{a}
Total proteins (g/dl)	3.26±0.13^{b}	4.02±0.11^{b}	4.56±0.07^{b}	6.48±0.87^{a}
Albumin (g/dl)	1.78±0.13^{b}	2.18±0.15^{b}	2.68±0.08^{a}	2.86±0.20^{a}
ALT (U/l)	52.60±4.43^{c}	61.55±3.32^{bc}	73.05±5.10^{b}	95.80±7.08^{a}

LD50: lethal dose 50, ALT: Serum Alanine aminotransferase. Data is presented as mean±SE. Values within a row denoted by different superscripts indicate significant difference.

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