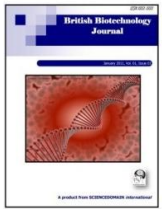

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Histopathological Alterations Induced by *Naja naja* Crude Venom on Renal, Pulmonary and Intestinal Tissues of Mice Model

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Authors' contributions

This work was carried out in collaboration between all authors. Authors MAAM, MAH and AR designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Authors MKZ and ZF managed the analyses of the study. Author KMFH managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Aim: Snake bite causes a significant number of mortality and morbidity throughout the world. So, the current study was carried out to estimate the extinct of damage caused by intraperitoneal introduction of cobra venom on kidney, lung and intestinal tissues of mice model using histological technique.
Place and Duration of Study: The entire study including the treatment along with preparing histological slide was conducted in protein science laboratory, Department of Genetic Engineering and Biotechnology, University of Rajshahi, Bangladesh between December 2013 to July 2014.
Methods: Twenty five mature female albino mice were divided mainly into two groups as control and envenomated group. Lyophilized *Naja naja* venom was dissolved in 0.9% NaCl solution and injected intraperitoneally into the mice of the envenomated group at dosages equivalent to LD_{50}

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(0.25 mg/kg). Whereas the animals from control group were not received any venomous component. Both groups of animal were sacrificed for histological study and visualized under light microscope.

Results: Injection of cobra venom induced a range of histological changes in all envenomated mice comparing with their control. Results from the histopathological examination showed mainly inflammatory cellular infiltration, vacuolation in renal tubules, shrinking of glomeruli, raising space between the walls of Bowman's capsule in renal tissue and alveolar haemorrhage, inflammatory cellular infiltration and edema in pulmonary tissue. No significant histopathological alterations in intestinal tissue were observed without infiltration and mild hemorrhage.

Conclusion: The findings from the current study revealed that, cobra venom at lethal dose causes multiple organ failure in experimental animal which could be considered among the factors that lead to death. By observing the site and the mode of action on tissue level, these findings may help to allay the severity of damage by discovering novel anti venom drug.

Keywords: Cobra; histology; intestine; kidney; lung; Mus musculus and venom.

ABBREVIATIONS

SRCC (Snake Rescue and conservation centre); NaCl (Sodium chloride); LD_{50} (Lethal dose); PLA2 (Phospholipase A2).

1. INTRODUCTION

Snake bite is a familiar term throughout the world especially in the rural areas of Bangladesh and causes a significant clinical impact on human health. Snakebite frequency has been estimated at about 500,000 and mortality between 30000–40000 annually throughout the world [1]. However, the degrees of envenomation related injury relies mainly on the nature of toxic polypeptide in the venom. Venom is a highly modified salivary secretion mainly used to initiate the breakdown of food stuff into soluble compounds for proper digestion [2]. Biochemically snake venom is a complex mixture of several protein riches substances including toxins, enzymes, growth factors, activators and inhibitors with broad spectrum biological activities, stored in venom glands at the back of the head [3-5]. The venomous components which are mainly responsible for severe clinical injury of the victims are pro-coagulant enzymes, anticoagulant enzymes, cytolytic or necrotic toxins, hemolytic and myolytic phospholipases A2, pre- and post-synaptic neurotoxins, and haemorrhagins [6]. A number of these toxic proteins singly or jointly interact with components of important physiological system of envenomated individual and producing diverse clinical effects [7].

Cobra (*Naja naja*) is the most venomous snake of Elapidae family, responsible for a significant number of deaths yearly in our country. Elapidae comprises approximately 325 species of venomous snake, including cobras, mambas, sea snakes and coral snakes widely distributed in the tropical and subtropical regions of the world, especially in southeastern Asia [8]. The symptoms of cobra envenomation mainly are local pain, severe swelling, blistering, necrosis and other non-specific effects [6]. Cobra envenoming cause's multiple-organ failure resulting death in case of severe envenomation [9]. The neurotoxin of cobra venom exerts action by blocking the transmission of nerve signals to muscles leading to the subsequent stopping of nerve transmission to the heart and lungs and death occurs rapidly due to complete failure of respiratory function. Histological, histochemical and biochemical fluctuations triggered by the venom of African black-necked spitting cobra (*Naja nigricollis)* and Egyptian cobra (*Naja haje*) have already been evaluated on rodent animal [10-12]. Whereas toxic effects of *Naja naja* on tissue level have not ever been recorded in the Bangladesh. Since *Naja naja* is the most venomous snake in this region and their venom compounds are slightly different from the other places. So, there was a paramount need to evaluate the mode of action of cobra venom on tissue level of the most important organs of human body using rodent animal as model. Hence, the current study was aimed to detect the intensity of damage resulting intraperitoneal injection of cobra venom on rodent animal.

2. MATERIALS AND METHODS

2.1 Experimental Animals

A total of twenty five female albino mice (26-30 gm) were collected from the Department of

Pharmacy of Jahangirnagar University, Dhaka, Bangladesh. The mice were kept in metal cages (5 per cage) and allowed to feed ordinary poultry food and tap water and kept at the animal house of Biochemistry Department, Faculty of Science, Rajshahi University. All mice were handled in accordance with the typical guide for the care and use of laboratory animals (under the license of Institutional animal, medical ethics, bio-safety and bio-security committee (IAMEBBC) for experimentations on animal, human, microbes and living natural sources . No.33/320/IAMEBBC/IBSC).

2.2 Preparation of *N. naja* Venom

Cobra venom was collected from the Snake Rescue and conservation centre (SRCC) at Darusha, Rajshahi. In protein science lab, collected venom was milked, lyophilized and stored in a refrigerator at 4°C. Prior to use the venom was dissolved in 0.9% NaCl solution. LD_{50} dose of crude venom was determined by a technique previously described by Meier et al. [13]. The LD_{50} of cobra venom from our current study was calculated was 0.25 mg/kg of mice body weight.

2.3 Experimental Design

The animals were randomly distributed into two groups including control and envenomated group.

2.3.1 Control group

A total of five mice were injected intraperitoneally with only 200 µL physiological saline (0.9% NaCl) solution and serve as control.

2.3.2 Envenomated group

Twenty mice of the envenomated group were intraperitoneally injected with crude cobra venom as a single dose equivalent to LD_{50} of *Naja naja* venom (0.25 mg / Kg).

2.4 Histopathological Examination

Histopathological analysis of three organ from both groups of animal were conducted based on the technique previously described by Carleton et al. [14]. The animals were anesthetized using chloroform and sacrificed by cervical dislocation. Kidney, lung and intestine were collected and washed with normal saline, then fixed with Bouin's fluid (fixative) for -16-18 hour and subsequent washing under running tap water for one hour until complete removal of all traces of chemical. Followed by washing, dehydration of the tissues was conducted by immersing the tissue in a series of gradually increasing concentrations of alcohol (50%, 70%, 80%, 95% and absolute alcohol) and embedded into paraffin wax for making blocks. The block was to be trimmed by removing of wax from the surface of block to expose the tissue. Sectioning of the tissue was performed by using a microtome (The microtome machine was sold from Tokyo, Japan by the trade name of SHIBUYA produced by optical. Co LTD). The microtome was pre-set to cut the tissue as thicknesses around 6 µm. Blocks Small ribbons of tissue sections were placed on microscopic slide with the help of warm distil water containing few drops of Mayer's albumen and deparaffinized with xylene solution. Haematoxylene and alcoholic eosin solution was used to stain the tissue for preparing permanent slide. Histopathological changes were observed under 20 x and 40 x magnification of a light microscope (The microscope was purchased from Italy by the trade name optika) and snaps were taken.

3. RESULTS AND DISCUSSION

3.1 Histopathological Changes in Kidney

Renal tissue of control mice (Fig. 1A) showed normal organization of the renal corpuscles with intact Bowman's capsules each enclosing by a tuft of glomerular capillaries. The typical renal tubule consists of intact proximal and distal convoluted tubules, loop of Henle and collecting tubules. Injection of cobra venom induced a range of histopathological alterations in renal tissues after 6 hours of the envenomation. Histological investigation of renal tissue showed that *N. naja* venom caused an inflammatory cellular infiltration, vacuolation in renal tubules, shrinking of glomeruli, degeneration of the distal and proximal convoluted tubules and raising space between the walls of Bowman's capsule (Fig. 1B). Since the toxic substances are circulated throughout the body with blood flow and whole blood samples of higher organism are filtered in the kidney. So kidney injury is among the common and most serious symptoms of cobra envenoming. The result of the present observations (Fig. 1B) were in agreement with findings reported by Amany et al. [15] which indicated that inflammatory cellular infiltration, vacuolation in the tubule and shrinkage of glomeruli in most cases in renal structure of envenoming mice injected with 1/2 LD_{50} *N. haje* venom.

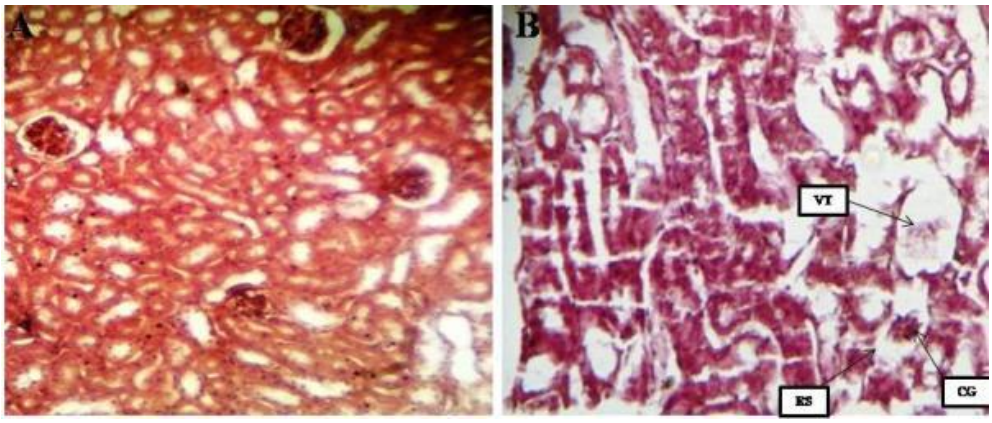

Fig. 1. Histological photograph of kidney tissue albino mice. (A) Control kidney showing compact glomerular and tubular structure. (B) Histological section from envenomated renal tissue showed vacuolation in the tubule (VT), shrinking glomeruli (CG) and raising space between the walls of Bowman's capsule (RS) .Sections were stained with hematoxylin and eosin (400X H and E)

According to Rahmy et al. [16] and Gunatilake et al. [17] a sub-lethal dose of the cobra venom was found to encourage injurious effects on the histological and histochemical patterns of the renal tissue of rabbit including complete necrosis in the glomeruli and proximal and distal convoluted tubular cell. Alterations in the glomerular architecture might be due to structural and molecular alterations in the glomerular basement membranes [18]. Renal tissue of envenomated animals showed tubular lesions with subsequent accumulation of inflammatory cells in the tubular tissues probably due to recovery of the injury by cobra itself. Cytoplasmic vacuolation appeared in renal tubules is a clinical sign of irregular lipid inclusions and fat metabolism happening under pathological cases [19]. Severe cellular swelling of the tubular epithelial lining cells resulting inflammation might be due to the action of venom phospholipase leading to cellular damage followed by scattering of the cellular contents in the tubular lumina [20]. The venom phospholipase (PLA_2) is probably the key factor responsible for tissue injury by disturbing cell membrane permeability through disorganizing of lipid bilayer on the plasma membrane resulting pore formation with subsequent influx of Na^+ and water [21]. Interaction between plasma membrane and phospholipase encourages the reduction of Na^+/K^+ ATPase activities with subsequent changes in the ionic gradients and followed by disordering the membrane lipid bilayer ultimately leading to cell death of envenomated person [22]. It is predicted that all the noticed renal injury could be the reason of injecting higher *Naja naja* venom in the envenomed mice as formerly stated by Ismail et al. [23]. Renal injuries might be due to either direct or indirect effect of the venom composition [24]. The direct effects were mainly attributed to the exerting the venomous components which have an acute effects on the function as well as organization of the renal tissue, whereas the indirect action might be due to the deadly effect brought about by reactive metabolites or mediators produced in the kidney during envenoming [25].

3.2 Histopathological Changes in Lung Tissue

Pulmonary tissues of control mice (Fig. 2A) showed the normal and compact organization of bronchii, bronchioles and terminal bronchioles followed by specialized sac-like structures called alveoli consisting surface epithelium, blood vessels and supporting tissue surrounded by a double layer membrane structure called plura. Crude cobra venom persuaded some severe changes in their histological structure by showing significant inflammatory cellular infiltration and edema (Fig. 2B). The organism of envenomated group also showed alveolar haemorrhage and mionecrosis after 6 hours of envenomation with LD_{50} dose of cobra venom.

The essentiality to maintain the integrity of lung is inevitable for any higher animal due to its gaseous exchange activity and cleaning of blood cells with oxygen.

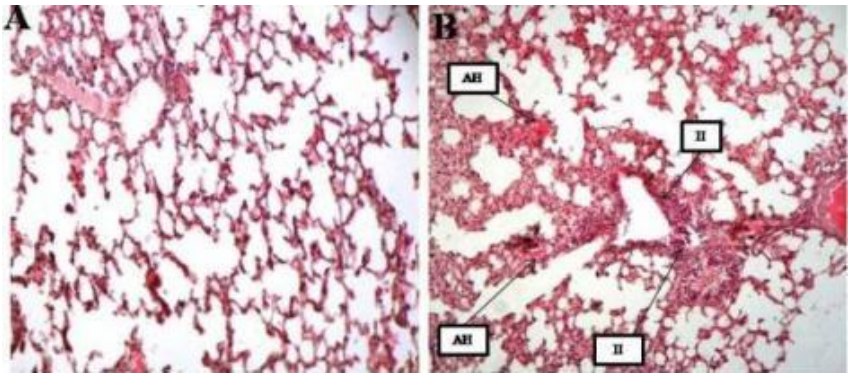

Fig. 2. Photomicrograph of histological section of lung tissue of albino mice. (A) The image from control pulmonary tissue showing compact configuration with intact alveoli and associated vessels and capillaries. (B) Lung tissue of envenomated group indicated extensive tissue damages and showing inflammatory cellular infiltration (II) and alveolar haemorrhage (AH). Sections were stained with hematoxylin and eosin (400 X H and E)

Individuals death occurred by cobra envenomation were mainly claimed due to the neurotoxic action of both presynaptic and postsynaptic neurotoxins followed by peripheral respiratory paralysis [26]. PLA_2 induces mionecrosis with subsequent infiltration of inflammatory cell to the affected tissue. The accumulation of polymorphonuclear leukocytes (mainly neutrophils) followed by macrophages indicated the establishment of neurotoxic effect of phospholipage [27]. In the current study, the rapid accumulation of polymorphonuclear cells suggesting severe attract of PLA_2 on the pulmonary tissue. Neutrophils have a significant contribution in the mediation of micro vascular damages leading to pulmonary tissue damage [28]. Leukocyte plays a key role in producing a range of inflammatory mediators contributing an important role in venom related respiratory failure. Inflammation may also be caused by reactive oxygen atom present in the venom and is also responsible for tissue damage (mionecrosis) occurring at the subsequent phases of inflammatory process. The lung integrity is maintained by the conservation of clear airspace and the regulation of fluid movement between the vascular, interstitial, and airway compartments. Edematous swelling of lung tissue with subsequent accumulation of pulmonary infiltrate blocks the alveolar airspaces disrupting gas exchange and lung mechanics, leading eventually to respiratory failure.

3.3 Histopathological Alteration in Intestinal Tissue

It was observed that the intestinal tissue from control mice was normally compact and regular structure of finger-like extensions villus, the mucosa and submucosa (Fig. 3A). But the tissue of envenomated grouped showed minor alteration in the structure (Fig. 4B). Histological evaluation of intestinal tissue demonstrated that *N. naja* envenomation caused no significant histopathological changes without cellular infiltration and mild hemorrhage (Fig. 3B). The degree of action of cobra venom on intestinal tissue was not sufficient to cause severe clinical sign compared with previously mentioned organs. Toxicological evaluation of *Naja naja* venom on intestinal tissue until precisely documented anywhere, though the toxin from other venomous animal like as scorpion have detrimental effect on intestinal tissues which showed slight bleeding (low dose), severe bleeding (high dose), mucosal necrosis and swelling and inflammation [29]. However, the current study demonstrated that mice were envenomated with cobra venom at LD_{50} producing a range of histopathological alterations such as inflammatory cellular infiltration in intestine probably due to recovery of the toxic effect by tissue itself and mild hemorrhage probably due to circulation of the venom components with blood flow in mucosa and sub mucosa (Fig. 3B).

The clinical injury, their sites and mode of action on tissue level along with the alteration in biochemical patterns must be taken in consideration in curing the victims bitten with *Naja naja* by producing the sustainable antivenom drugs for neutralizing the toxin reaction produced by venomous snake like as cobra.

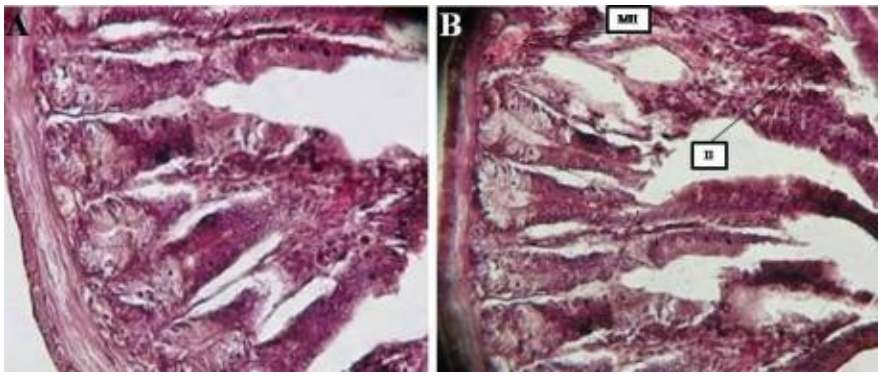

Fig. 3. Photomicrograph of histological section of intestinal tissue of albino mice (A) Intestinal tissue of control mice showed regular and compact configuration with villus, mucosa and sub mucosa. (B) Whereas, envenomated intestinal tissue appeared with inflammatory cellular infiltration (II) and mild hemorrhage (MH). Sections were stained with hematoxylin and eosin (400X H and E)

4. CONCLUSION

It can be concluded that the exposure of *Naja naja* venom encourages serious histopathological alterations on renal and pulmonary tissue and moderately on intestinal tissue of envenomed mice. Therefore, further studies need to be carried out for the isolation and purification of cobra venom and applying this valuable natural raw material in the discovery of anti-venom related drug along with other pharmaceutical valuable product.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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