Histopathological Changes Induced in Mice after Intramuscular and Intra Peritoneal Injections of Venom from Spine-bellied Sea Snake, *Lapemis curtus* (Shaw, 1802)

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**Abstract:** The venom of the *Lapemis curtus* was tested for its ability to induce histopathological changes in mice intraperitoneal injection of the venom (LD$_{50}$ of 0.65 mg kg$^{-1}$), by light microscopic examination of some organs (liver, kidney and spleen). *L. curtus* venom induces changes including necrosis and edematous appearance with cellular infiltration and vacuolation. The injury of kidneys includes significant changes of the glomerular apparatus. Venom treated mice liver shows congestion, micro vesicular fatty changes and infiltration of inflammatory cells around the portal vein. Where as, spleen showed hemorrhage, congested and inflammation were observed. Areas of hemorrhagic, vascular congestion and cloudy swelling in renal tubules were observed in the kidney. No myoglobinuria was noted in any group of animals. The crude venom was also administered intraperitoneally into the experimental animals and tissue samples were taken at several time intervals. The venom of the sea snake *L. curtus*, was tested for its ability to induce myonecrosis changes in albino mice. Induction of myonecrosis was demonstrated by their ability to release Creatine Kinase (CK) from damaged muscle fibers and direct histopathological examination of the injected muscles (i.m.). Crude venom exhibits intense myonecrosis characterized by the changes including, necrosis and edematous appearance with cellular infiltrate, vacuolation and degenerated muscle cells with delta lesions and heavy edema in between the cells.

**Keywords:** Sea snake, *Lapemis curtus*, histopathological changes, myonecrosis

**INTRODUCTION**

Sea snakes are the most venomous reptilian group in the world. They are encountered around coasts and reefs, in estuaries, rivers and at sea. Their venoms are more toxic than those of terrestrial snakes. However, they are rarely aggressive or menacing. Bites have become unusual with the advent of modern fishing methods (Semanayake *et al.*, 2005), but potentially serious hazard of the marine environment as their venoms contain potent neurotoxins, more lethal than venoms of many terrestrial snakes (Acott and Williamson, 1996).

The venom of the sea snake contains potent neurotoxins, which are some of the most toxic substance in the world (Tu and Tu, 1970). The venom of sea snake is more toxic than the terrestrial snakes, including rattlesnakes, copperheads and cobras (Manour *et al.*, 2005). Sea snakes produce venom from a venom gland that acts on the victim's nervous system. Its venom contains neurotoxins which act on the nerve cells of the victim. Neurotoxin paralyses the respiratory system, ultimately causing death. The phenomenon is the same as in the case of Cobra and Krait poisoning. However,
the venom of a sea snake is severer than that of a Cobra or a Krait. Bites often remain unnoticed due to the absence of or, very little pain being experienced with little swelling or inflammation and the fang marks are sometimes barely visible. There are usually four fang marks on the victim, however there may be as few as one or as many as twenty. Sometimes the fangs may remain in the wound (Senanayake et al., 2005).

Acute renal dysfunction (Soc-Soc et al., 1990; Zimmerman et al., 1992; Gopalakrishnakone et al., 1993), myonecrosis, skeletal muscle damage (Preston et al., 1990), pneumonitis and hepatitis (Alam et al., 1995) are the few common features resulting from accidental and experimental snake envenoming (Marsden and Reid, 1961; Reid, 1961). These pathological conditions have been ascribed to the effects of necrotic (Onrat et al., 1993) and cytotoxic components (Ponraj and Gopalakrishnakone, 1995; Gopalakrishnakone, 1997) in snake venom (short/long chain neurotoxins, cyto/cardiotoxins, necrotic and hemorrhagic factors/toxins and several enzymes), including important and the major class of enzyme (1995; Huang and Gopalakrishnakone, 1996).

It has been reported that venom act on hemostatic system, on thromboeytes to inhibit adhesion and aggregation (Ouyang et al., 1987; Kini and Evans, 1990), red cells to cause hemolysis (Condrea, 1979), cardiac cells to induce cardiotoxicity (Huang et al., 1993), muscle cells to cause myonecrosis (Mebs and Owenby, 1990; Gutierrez and Lomonte, 1995), nerve cells to prevent neurotransmission and cause cell damage in general (Kini and Iwanga, 1986; Harris, 1991; Tsai et al., 1995). Despite their wide variety of pathophysiological and pharmacological effects, either dependent or independent of their catalytic activity (Rosenberg, 1986; Kini and Evans, 1989), sea snake venoms show close structural similarities and constitute a large family of a homologous proteins (Armi and Ward, 1996).

We have noted intense myonecrosis induced by a single dose of a myotoxic venom from the sea snake L. macrops (Fig. 1). Associated acute nephritis, hepatitis and mild pneumonitis were also apparent in all test groups of animals. The present paper describes the histopathological changes induced by venom in different organs, pathogenesis and possible mechanisms of such myonecrotic, nephrotic and hepatic syndromes have been discussed.

Fig. 1: Shows live spine-bellied sea snake L. macrops

308
Venom Collection

Sea snake L. curtus was collected from Kalpakam to Cuddalore waters of Coramandal coast (India) during 2005. The identification of live snake was carried out in the zoological survey of India. Venom from live snake was squeezed out manually, lyophilized immediately and stored at -4°C until further use (Ali et al., 1999).

Animals

Adult male Swiss albino mice (22±2 g) were obtained from the Central Animal House, Rajah Muthiah Medical College, Annamalai University. The animals were housed five per cage in a room with 12:12 h light-dark cycle at a room temperature (28°C) and allowed to consume commercial standard pellet diet and water ad libitum.

Lethality and Toxicity Assay

The toxicity of the crude Venom (CV) was quantitatively assayed by intraperitoneal injection (i.p.) in albino mice (n = 8–10 per dose) using 0.1 to 1.0 mg of crude venom in 0.9% NaCl (w/v). LD₅₀ was determined at five dose levels and observed during a 24 h period following i.p. injection.

Histopathological Assay

The mice divided into five groups (I to V). Group I (10 mice) given physiological saline (i.p.) serve as control. Group II and III (10 mice each) were injected (i.p.) with 1.5 mg of crude venom in 100 mL saline and sacrificed after 24 and 48 h, respectively. Group IV and V (10 mice each) received 3.0 mg of crude venom in 100 mL saline (i.p.) and were sacrificed at 24 and 48 h, respectively.

The animals were autopsied and all internal organs were macroscopically examined and fixed in 7% formalin. Liver, kidneys and spleen were embedded in paraffin and sliced. The slides for light microscopy were stained with hematoxylin and eosin (H & E). All preparations were made and analyzed as described earlier (Alam and Qasim, 1997; Alam et al., 1995). Kidney sections from rat and mice were also stained with Masson's trichrome stain to monitor myoglobin casts (Ponraj and Gopalakrishnan, 1995). Blood was also collected from group I through V and the Creatine Kinase (CK) level was determined to examine tissue destruction.

Determination of the Ability of Crude Venom to Induce Myonecrosis and Myoglobinuria

Enzyme Assay

The liberation of creatine kinase from damaged muscle cells was followed by using the enzymatic UV method (Deutsche Gesellschaft für Klinische Chemie) as described in the assay manual (Boehringer-Mannheim, Germany) to measure enzyme activity in plasma. Male Swiss Wistar mice (approximately 30 g) were injected in the right flugh (gastrocnemius muscle) with 1.5 mg of CV in 25 mL 0.9% sodium chloride (w/v). The CV injected mice were sacrificed at various time intervals at t = 30 min, 1, 6, 12, 18, 24, 48, 72 h and 6 days (groups A to I, respectively (n = 4) and blood was collected from the abdominal cava and the plasma separated by centrifugation at 15,000 rpm for 3 min at room temperature. Control animals (n = 6) received 0.9% sodium chloride only (Mancuso et al., 1995; Ponraj and Gopalakrishnan, 1995).

Assay for Myonecrosis

For the myonecrosis, induced by CV, a small portion of the central region of gastrocnemius muscle was excised from all groups (groups A to I and control) and post-fixed overnight in 10% formalin, dehydrated through a series of ethanol treatments and embedded in wax. Sections of 5 mm thickness were cut (Shandon AS 325, USA), stained with H & E and examined under the light
microscope (Nikon, Japan). Areas exhibiting pathology were photographed with a Nicon digital camera. Kidneys were also removed from all groups, processed as described above and stained with Masson's trichrome stain. Whereas, lungs, hearts, livers and spleens were removed, processed similarly and stained with H & E.

RESULTS

Lethality

Crude venom was found to be mice (LD₃₅ i.p. 0.65 mg kg⁻¹).

Pathological Changes

In general the histopathological changes observed in liver, kidney and spleen of crude venom (cv) treated mice (LD₃₅) are compared with the normal mice. Liver of venom treated mice shows congestion, micro vesicular fatty changes and infiltration of inflammatory cells around the portal vein. Where as, spleen showed hemorrhage, congested and inflammation were observed. Areas of hemorrhage, vascular congestion and cloudy swelling in renal tubules were observed in the kidney.

After i.p. injection, CV was highly toxic to animals causing reduced mobility within a few hours. Generalized paralysis appeared after 4 to 5 h. Most of the animals did not survive after 30 h in all test groups, except in groups III, where 4 out of 10 mice survived up to 30 h. Macroscopic examination of autopsied organs in mice showed congested livers, spleen and swollen kidneys. Changes in the wet weight of affected kidneys were also noticed which was slightly higher as compared to the control group. In addition, kidneys were tender on touching. Likewise, slightly swollen and discolored liver was noted, especially from those animals surviving up to 30 h. No visible change was observed in spleens. Notably, spleens showed no pathological changes in any group. Intramuscular injection in mice also did not induce any notable change in all organs except the area of muscle injected. Morphologically, mild changes were only seen in kidneys of groups sacrificed after 48 h.

Light microscopic examination of kidney sections, from all test groups showed generalized tubular degeneration (Fig. 2B and C). However, tubular degeneration was more prominent in groups surviving up to 30 h, specially exhibiting a profound degenerated epithelial lining of tubules. Complete disquamation of epithelial lining was also noted with sporadic focal tubular necrosis (Fig. 2B and C). Marked nephro toxicity in the present study was possibly due to the fact that the kidneys are the main filtration organs of the blood and thus the main source of elimination of toxemia which is experimentally induced by CV administration. Kidney sections, stained with Masson's trichrome stain, do not reveal the presence of any myoglobin casts, but do exhibit similar pathological findings in both animal groups, i.e., mice and rats.

Microscopic examination of liver and spleen showed considerable changes in animals surviving up to 30 h. Marked fatty changes in liver were noted in all subjects (Fig. 3B and C). Hepatic injury is also characterized by degenerated hepatocytes and identified by swollen edematous hepatocytes with clumped cytoplasm and large clear spaces (Fig. 3C). The spleen showing poorly differentiated cell clumps around the capsule (Fig. 4B and 3C).

However, analysis of plasma for creatine kinase liberation by lysed tissues and cells revealed a several-fold increase in its level, both, at 24 and 48 h.

Myonecrosis

The maximum peak of CK liberation from damaged muscle, induced by CV in mice by intramuscular injection (i.m.) in groups A to I, was obtained at different time intervals. CV induced gradual increase in CK liberation within 24 h. Three peaks of high CK liberation were detected, the first peak at t = 12 h, the second at t = 18 h and the third at t = 24 h. However, after 48 h, the CK level gradually normalized (Fig. 5).
Fig. 2. (A) Light microscopy of kidney from control rats receiving 100 mL of normal saline (p.) The section shows normal kidney tubules with intact epithelial linings (arrow head), magnification 20x H. & E stain. (B) Photomicrograph from test group V, receiving 5.0 mg of CV (p.) The section shows focal tubular necrosis (arrow head), complete disorganization of epithelial lining (arrows) and epithelial degeneration of tubules (double arrow heads), magnification 20x H. & E stain. (C) Photomicrograph from test group VI, receiving 5.0 mg of PLA2-HI (p.) and showing marked epithelial degeneration of tubules (arrow heads), magnification 20x H. & E stain.
Fig 3 (A) Light micrograph of liver from control group I receiving 100 mL saline (p.) The section shows normal hepatocytes (arrowheads) and uniform parenchyma, magnification 20x H & E stain. (B) Photomicrograph from test group I receiving 6.0 mg of purified PLA2-H1 and sacrificed after 24 h. The section shows generalized fatty infiltration in parenchyma (arrowheads) and squashed hepatocytes (arrow), magnification 20x H & E stain. (C) Photomicrograph from test group V, receiving 6.0 mg of purified PLA2-H1 and sacrificed after 48 h. The section shows fatty vacuolation of parenchyma (arrowheads) in a generalized pattern, magnification 20x H & E stain.
Fig 4 (A) Light micrograph of lung from control group receiving 100 mL saline (p.) Section shows normal bronchus (arrowhead) and intact alveoli (arrow) (B) Photomicrograph of test group V receiving 60 mg PLA2-H1 (p.) and sacrificed after 48 h. Section shows dilated bronchus (arrowhead) and marked infiltration of inflammatory cells within alveoli (arrows) magnification 20x H & E stain
FIG. 5: Myotoxic activity of crude venom in mice after intramuscular (i.m.) administration followed in terms of plasma creatine kinase liberation.

The muscle tissue from the control group was normal, whereas the tissue from test groups D to F (12, 18 and 24 h, respectively) exhibited intense myonecrosis throughout the muscle sections. The changes include necrosis and edematous appearance with cellular infiltrate, vacuolation degenerated in the muscle cells with delta lesions and heavy edema in between the cells. None of the tissue of the animals in groups A to C (30 min, 1 and 6 h, respectively) showed prominent myonecrosis. Similarly, there are no significant myonecrotic areas observed in groups G to I (48, 72 h, 6 days, respectively). There were no pathological changes observed in the lungs, spleen and heart in any of the groups examined. However, the kidney sections of the groups sacrificed at 48 and 72 h (groups G and H, respectively) showed mild tubular degeneration. Furthermore, fatty infiltration in liver was observed, but only in animals of the group sacrificed after 24 h, probably due to the lipolytic action of CV. No myoglobinuria was noted in any of the groups of animals during the whole period.

DISCUSSION

We have examined the generalized histopathological and myonecrotic activities of a crude venom from the sea snake *Lopenis curtis* venom in mice. The lethal dose was determined to be 0.65 mg kg⁻¹ in mice which makes a potent lethal toxin. Previous report has been reported that the LD₅₀ value can show the same results in the sea snakes venom (Mebs and Ownby, 1990).

The results of our studies indicate that CV causes pathological changes to kidneys, livers and spleen after i.p. injection, whereas additionally myonecrosis was induced with mild nephritis and hepatitis after i.m. injection. Interestingly, no myonecrosis was noted in groups injected intraperitoneally with CV. Likewise, no myoglobinuria was detected in any of the animal groups. Masson's trichrome staining of kidneys also did not reveal any myoglobin cast in kidney sections examined. However, induced nephrotoxicity was possibly due to the fact that the kidneys are the main filtration organ of blood and thus the main source of elimination of toxemia. During the elimination of CV by kidney compartments (viz., tubules and glomerulus) which depend on blood supply, CV caused tubular and interstitial disorders through its cytotoxic potencies.

We have noticed marked tubular degeneration and necrosis in our study, as described in the preceding section, having a generalized feature and possibly caused by a generalized origin. However, various types of nephritis were characterized by several basic tissue reactions, of which hypercellularity or inflammatory disease are more common (Cotran et al., 1994). The inflammatory disease is characterized by cellular proliferation of mesangial and endothelial cells and leukocytic
infiltration. We have observed mild hypercellularity of lymphocytic origin in all kidney sections, both, at 24 and 48 h intervals in groups receiving i.p. treatment. These inflammatory cells release specific mediators (viz histamine, 5-HT, oxygen metabolites and nitric oxide) upon activation, which cause vasoconstriction, endothelial damage and cytotoxicity. Other mechanisms may also be responsible in contributing towards nephrotoxic injury, e.g., injury of epithelial cells, renal ablation, glomerulopathy or interstitial inflammation. Of these four, epithelial cell injury is often the result from antibody-antigen reactions (Emancipator, 1992), toxins (Ashkenazi, 1993) or and cytokines (Border and Noble, 1993). Previously, necrotic debris and degenerated tubular epithelium was also noted in kidneys of mice injected with myotoxic PLA2 (Porraj and Gopalakrishnakone, 1995). Crude snake venoms also induce tubular degeneration as reported for rabbits by the injection of Vipera russelli’s venom (Soe-Soe et al., 1990). Acute renal tubular degeneration and proliferative glomerulonephritis was also noticed in mice by the venom of the sea snake Apisaurus laevis (Zimmerman et al., 1992). Terao et al. (1994) also reported morphological changes in kidney tubules induced by an algal toxin, cylindrospermopsin, from Umezakia natans, including single cell necrosis in distal and proximal urinary tubules. Furthermore, earlier studies by Marsden and Reid (1961), Reid (1961) on sea snake envenomation victims revealed renal damage, especially tubular necrosis, edematous interstitial necrosis and, both, diffuse and focal cellular infiltration. Regarding the basis of hepatic injury as seen in our study, several reactions have been documented preceding the hepatic injury regardless of the nature of the causative agents (Cotran et al., 1994). These reactions are necrosis, degeneration, inflammation, regeneration and fibrosis. In necrosis, this is mediated by toxic agents, isolated hepatocytes round up to form shrunken, pyknotic and intensely eosinolytic councilman bodies. We have noted sporadic hydropic degeneration, where hepatocytes osmotically swell and rupture.

In the present studies spleen sections showed infiltration of inflammatory cells and dilatation of bronchus (Fig. 4B). However, no conclusive mechanism was reported yet for pneumonitis occurring due to sea snake envenomation, even though edema and inflammatory changes in spleen was noted in the early 60 s by Marsden and Reid (1961). Therefore, pneumonitis observed in the present studies was not due to the direct action of the venom or its components, but probably due to factors related to renal dysfunction which induces bronchopneumonia and acute and chronic inflammatory response to broncospasm.

The myotoxicity induced by CV in mice was examined by two methods, i.e. determination of creatine kinase, released from damaged muscle fiber and the direct histological examination of the injected muscle (Mancuso et al., 1995). CV induced the highest CK release by i.m. injection and less by i.p. injection (Fig. 5). These results suggest a correlation between the levels of CK and the intensity of myonecrosis. Maximum CK liberation peaks were observed at t = 12 h, 18 h and 24 h. Marked release of CK was also noted at t = 6 h and 48 h. However, the CK level is 10 times lower than observed in former treatments. Previously, CK liberation, as induced by venoms from the snakes of the Genus Micruurus, was determined at t = 3 h and t = 6 h (Barros et al., 1994). In the present study it was found that the maximum peak of CK liberation did not occur always at such a short span of time as observed with the venoms from the snakes of the Genus Micruurus. It is indicated that the occurrence of the CK liberation peaks most likely correspond to the direct myotoxic effects of crude venom in the body (Barros et al., 1994).

The type of myonecrosis that we have observed, closely relates to those described earlier by several myotoxic fractions (Fohlman and Eaker, 1977; Porraj and Gopalakrishnakone, 1995; Huang and Gopalakrishnakone, 1996; Ownby et al., 1997) and also myotoxins devoid of detectable phospholipase activity (Gutiérrez et al., 1989, 1992; Lomonte and Gutiérrez, 1989; Mancuso et al., 1995). However, CV is a relatively slow acting myotoxin than those reported earlier, indicating variations in the myotoxic potencies of different phospholipases. Previously, Huang and Gopalakrishnakone (1996) reported on differences in the potencies of myotoxins, although having
homologous structures. For example, the PLA2(s) from elapid venom produces myonecrotic changes in muscles, but the minimum dose to induce morphological changes may vary. Similarly, *Trimeresurus flavoviridis* and *Bothrops asper* myotoxins are about 5-10 times more active than those from *Agkistrodon* venoms (Fohlman and Eaker, 1977). The absence of any myotoxic activity of crude venom in spleens might be caused by the non-susceptibility of the muscle and tissue towards the enzyme. This is well supported from another venom from the sea snake *Enhydrina schistosa* which is a highly active myotoxin, but shows no effect on cardiac and intrafusal muscles (Brooks et al., 1987; Huang and Gopalakrishnakone, 1996). In contrast, venom fraction from *Pseudechis colletti* produces pathological changes in myocardium and skeletal muscle (Weinstein et al., 1992). Thus, from these investigations the conclusions must be drawn that different crude venom cause specific actions on different muscles and tissues.

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