Sci. J. Fac. Sci. Minufia Univ., 2009, Vol.XXIII(2).1-13

EFFECT OF THE VENOM OF THE COBRA *NAJA HAJE* ON THE HISTOLOGICAL AND ULTRASTRUCTURAL PATTERN OF SKELETAL MUSCLES.

L.I. KHAYYAT.
Department of Biology, Faculty of Applied Sciences, Umm Al-Qura University, Makkah, Kingdom of Saudi Arabia.
L.I.Khayyat@hotmail.com

ABSTRACT

Envenemotion caused by the venom of the cobra *Naja haje* has many serious problems. The present study aimed to assess the effect of sublethal dose of the *Naja haje* venom on the structure of skeletal muscles in male rabbits. Male native rabbits were intramuscullary injected with sublethal dose (0.015 μg/g body weight) of cobra venom . After three hours of injection small pieces of gastrocnemius muscle were prepared for histological and ultrastructural examination. Histological analysis revealed myofibre degeneratio, necrosis , swelling of some myofiber, edema and migration of leucocytes. Several cytological alterations such as disorganized myofibrils, abnormal outline of nuclei, peripherally located heterochromatin, disorganization and dilation of sarcoplasmic reticulum, swelling and degeneration of mitochondria and fibrosis in some peripheral areas were noticed in the muscle of treated rabbit. The results obtained show that *Naja haje* venom presents serious impacts on the histological and cytological structure of rabbits skeletal muscles.

Key-Words: *Naja haje*, gastrocnemius, cobra venom, histology, ultrastructure.

INTRODUCTION

Snake venom causes serious medical problems. It was reported by Russell (1996), that the damage effects of venom are due to the presence of at least 25 types of enzymes responsible for the catalysis of many specific biochemical reactions in the living organisms.

Among families of venomous snakes is family Elapidae which includes the cobras, mambas, kraits and coral snakes ((Harris,1991)). Cobras are the most dangerous snakes in the world (Theakston, *et al.*, 1990). The Egyptian cobra

Naja haje is widely distributed in Northern Africa and parts of the Arabian Peninsula.

Cobra venom is a mixture of many different proteins and other lethal toxins (Tu, 1977). Neurotoxins are the principal lethal components of cobra venom (Mebs, 1986 and Ueno E.*et al.*, 1996). Also cardiotoxins or cytotoxins are the most toxic components in cobra venom (Gasanov, *et al.*, 1997; and Chang, *et al.*, 1998). In addition to the above toxins, cobra venom contains many other enzymes and the most potent of them is phospholipase A2 (PLA_2) (Tsai, 1997). Cobra venom phospholipases are known by its cytotoxicity inducing around 67% of death in culture cells after 24 hours of incubation. (Martikainen, *et al.*, 1993). Moreover, was reported that cobra venom affects different body organs in the victims (Tonsing, *et al.*, 1983).

Vishwanath *et al.*(1987) reported that the mainly target of PLA_2 is cell membrane causing dysfunction through membrane phospholipids hydrolysis, for example in erythrocyte membrane (Condera, *et al.*, 1980) and damage muscle cell membrane resulting in myonecrosis (Ownby, *et al.*, 1988).

Muscle damage is one of the most common symptoms produced by venoms from different snake species. Many studies have shown that crude venoms or isolated toxins can affect in different ways the structure and functions of muscles (Preston, *et al.*, 1990; Johnson and Ownby, 1993, 1994; Samson, *et al.*, 1994; Harvey, *et al.*, 1994; Yudkowsky, *et al.*, 1994; Fatchi, *et al.*, 1994; Faiz, *et al.*, 1994, 1995).

However, few studies were done to elucidat the action of the *Naja haje* venom on skeletal muscle of mammalian animals.

The present work aimed to study the effect of *Naja haje* venom on the histological and ultrastructure pattern of the gastrocnemius muscle in rabbit.

MATERIALS AND METHODS

Experimental animals:

Male rabbits *Oryctolagus cuniculus,* weighing (1.200Kg ± 200g) were maintaind, one per cage, in a ventilated room under controlled conditions of temperature, humidity, natural dark/light cycle. The animals had free access of food and water.

***Naja haje* venom:**

The *Naja haje* crude venom was obtained from Zoology Department, Faculty of Science, Ain Shams University, Egypt.

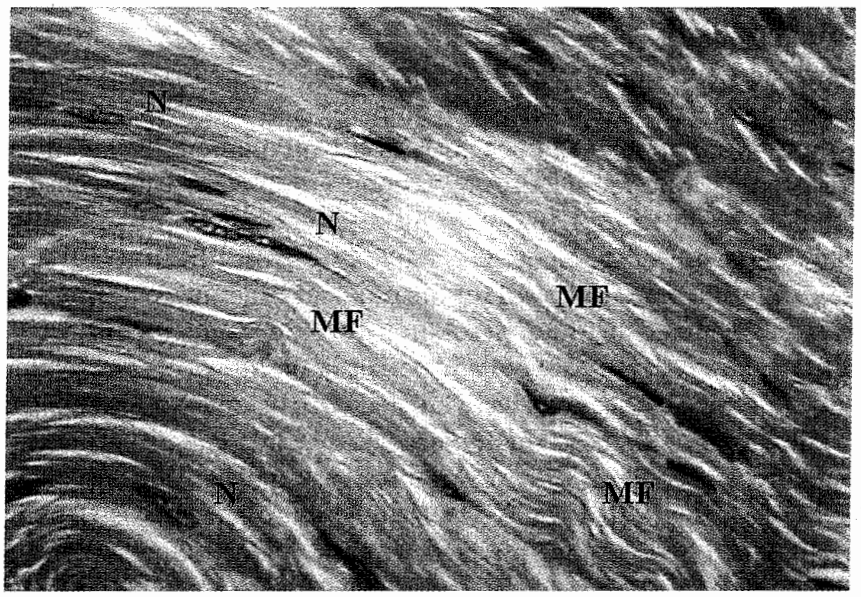

Fig. (1): Light micrograph of longitudinal section in gastrocnemius muscle from control rabbit .MF: Myscle fibers, N: Nuclei. (X600).

Fig. (2): light micrograph of longitudinal section in gastrocnemius muscle from treated rabbit 3 hrs after injection with venom showing necrotic muscular fibers , edema area (arrows) and delta lesion (*). (X400).

Experimental designe:

After one week of acclimatization in the laboratory environment, 10 rabbits were divided into two groups of 5 animals each. Animals of the first group (G1) were intramuscularly (i.m.) injected with 0.1 ml mammalian saline and used as a control group, while animals in the second group (G2) were (i.m.) injected with sublethal dose (0.015 μg/g body weight) of crude venom dissolved in 0.1 ml of saline.Three hours after the treatment, rabbits were sacrificed and fragments of gastrocnemius muscle were removed for histological examination. Small pieces of these muscles were fixed in Bouin's solution for 48h, and processed by routine histological techniques. Tissues were embedded in paraffin and 5m sections were made in rotry microtome. Sections were stained with haematoxylin-eosin.

For ultrastructural study, small pieces of the chosen muscle were fixed in 2% glutaraldehyde, buffered with 0.1M phosphate buffer (pH7.6) at 4°C. The specimens were post-fixed in 1% OsO4 (Osmium tetroxide), dehydrated in increasing ethanol concentrations , treated with propylene oxide and embedded in Epon-Araldite mixture Thin sections were cut on an LKB ultra microtome , double stained with uranyl acetate, lead citrate and investigated in a Jelo 100CX electron microscope.

RESULTS

Light microscopic examination of sections from the gastrocnemius muscle of control rabbit revealed the characteristic features of skeletal muscle fibers (Figure 1).

The gastrocnemius muscle of rabbit treated with cobra venom showed myofiber degeneration and necrosis, including the peripheral loose connective tissue that surrounds the fascicles and fibers (Figures 2, 4).

Many muscle cells were vacuolated while some myofibers exhibited swelling (Figure 3). There were some areas of edema dividing the fibers as well as delta lesions which appeared as clear triangular spaces with the base of the triangle at the plasma membrane and the head point nearer the middle of the cells (Figure 2). Considerable number of leucocytes resulting of migration to the effected area could be observed throughout the muscle fibers (Figures 3, 4).

Electron microscopic observations of longitudinal sections from gastrocnemius muscle of the control animals showed normal skeletal muscle structure with typical striated appearance (Figure 5). The A band, H band, I band, M line and Z line were clearly visible in the sections. Sarcoplasmic reticulum and mitochondria were located between the myofibrils (Figure 6). The nucleus appeared at the periphery with predominated heterochromatin and surrounded by an irregular envelope (Figure 5). Ultrastructural alterations in skeletal muscle

Fig. (3): light micrograph of longitudinal section in gastrocnemius muscle from treated rabbit 3 hrs.after injection with venom showing swolling of some myofibers , delta lesions (arrows) (X 400).

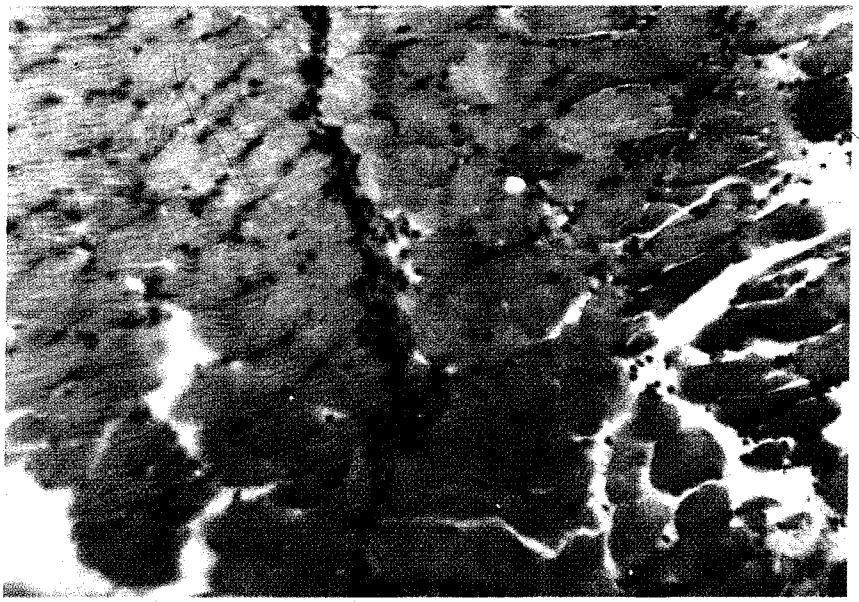

Fig. (4): light micrograph of longitudinal section in gastrocnemius muscle from treated rabbit 3 hrs. after injection with venom showing infiltration of muscle fibers with leucocytes. (X 400).

tissues were observed in animals injected with *Naja haje* crude venom. Some of the myofibrils were broken, disorganized and demonstrated lack of typical striated appearance. Also numerous large vacuoles in many muscle cells could be easily identified (Figure 7). The cytoplasm between myofibrils was expanded so that adjacent myofibrils were no longer in contact (Figure 7). The changes included also dilatation and disorganization of the sarcoplasmic reticulum as well as swelling and degeneration of mitochondria (Figure 8).

The nuclei appeared with abnormal outline marginated nucleoli and peripherally located heterochromatin (Figure 7). Fibrosis could be clearly observed in some peripheral areas (Figure 9).

DISCUSSION

In the present study histological and ultrastructural alterations were observed in the gastrocnemius muscle of rabbits injected with crude venom of *Naja haje* after three hours of infection.

The histological alterations included myofiber degeneration and necrosis, areas of edema, delta lesions and migration of leucocytes.

Extensive necrosis in skeletal muscles was also recorded in patients envenoming by the sea snake *Enhydrina Schistosa* (Sitprija, *et al.* 1971), and by *Crotalus durissus terrifcus* venom (Sangiorgio, *et al.* 2008).

A study carried out by Hood and Johnson (1974) demonstrated systemic myonecrotic action of snake venom, which attack several skeletal muscles of an Australian patient. Also, Azevedo-Maroques, *et al.* (1985) demonstrated that the venom of Brazilian snakes of the genus crotalus can induce systemic myonecrosis. Edema and myonecrosis were also noticed in the limb of dogs bitten by crotalus snake (Azevedo-Marques, *et al.*, 1985). Muscular alterations were reported by Salvini *et al.* (2001) after intramuscular administration of crotoxin to mice, with the presence of hypercontractivity of muscular fibers and edema three hours after such administration.

Moreover, neutrophilic inflammation observed myofiebrillar edema, hemorrhage and myonecrosis was noticed in gastrocnemius muscle of rats after envenomation by *Bothrops neuwiedi* snake. (Dourado, 2008) .

Destruction of muscle fibers and cellular inflammatory infiltration in between the damaged fibers were also recorded in mice treated with lithium carbonate (Essawy, *et al.*, 2004).

Electron micrographs of the current work revealed marked alterations in the gastrocnemius muscle of rabbits administered with *Naja haje* venom. These alterations included disorganized myofibrils, abnormal outline of the nuclei,

Fig. (5): Electron micrograph of normal gastrocnemius muscle from control rabbit showing sarcomeres . A: A band, H: H band , I: I band, M: M line, Z: Z line, N: nucleus (X 7500).

Fig (6): Electron micrograph of normal gastrocnemius muscle from control rabbit. M: mitochondria, G: glycogen particles, SR: sarcoplasmic reticulum (X 7500).

peripherally located heterochromatin, disorganization and dilatation of sarcoplasmic reticulum and swelling and degeneration of mitochondria.

Disorganized myofibrils and washed striation of muscle fibers in skeletal muscle may lead to loss of myofibrils as reported by Ghadially (1978).

The changes of the shape of the myonuclei observed in the present study may be an indication of alteration in the nucleoplasmic exchange and decrease in the metabolic activity which is associated with an increased complexity of nuclear form (Abdel-meguid, *et al*, 2002).

Moreover, the observed marked margination of the heterochromatin probably produced largely by accumulation of chromatin granules at the nuclear envelope as the nucleus shrinks (Latta, *et al*, 1965).

In the present work the mitochondria of envenomous animals infected, appeared shrunk or swollen. Senger (1986) stated that the mitochondrial changes may represent a very clear demarcation of future pathological development. Novikoff and Holtzmann (1976) claimed that mitochondria morphological changes may be releated to mitochondrial function. They suggested that the conformational changes reflect structural rearrangement of membrane inner proteins that are directly related to ATP formation.

In the present work, the observed pathological changes in the envenomed rabbits by Naja *haje* could be attributed to the acidic phospholipase ($A-PLA_2$) present in this venom (Mebs& Samejima, 1986).

It is well established that many snake venoms PLA_2s are destructive to skeletal muscles (Mebs& Samejima, 1986; Mebs & Ownby, 1990). In their study on the effect of $A-PLA_2$ isolated from the venom of the King Cobra, Hung and P.Gopalakrishnakone (1996) stated that , injection of mice with $A-PLA_2$ (8mg/Kg) caused several pathological changes that included dissolution of actin and myosin filaments , dilatation and disorganization of sarcoplasmic reticulum and degeneration of mitochondria. They added that, some of the myofibrils were severity disorganized, present lack of typical striated appearance, sarcomeres disrupted and most of the mitochondria containd vesicles or were destroyed.

From the results obtained in this work, it could be concluded that *Naja haje* venom induces serious detrimental impacts on the histological and cytological structures of skeletal muscles of mammalian animals.

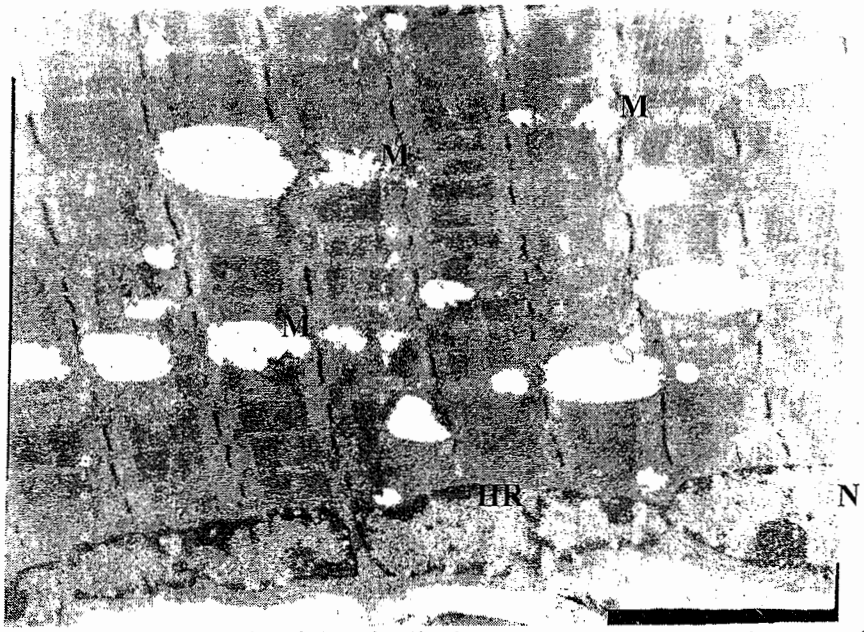

Fig. (7): Electron micrograph of longitudinal section in gastrocnemius muscle from treated rabbit at 3 hrs. of injection with venom showing disorganized myofibrils. N: Nucleus, HR: Heterochromatin, M: Swollen Mitochondria (X 5000).

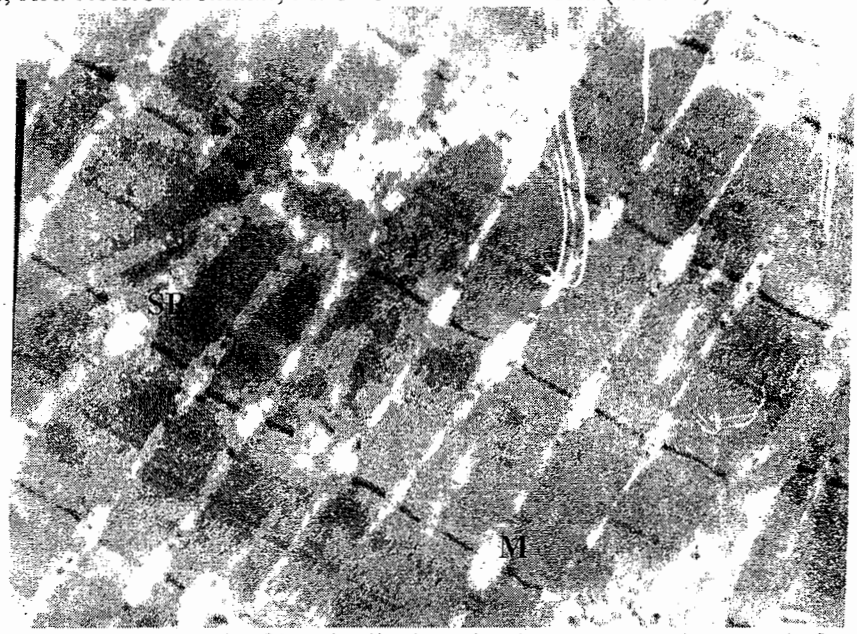

Fig.(8): Electron micrograph of longitudinal section in gastrocnemius muscle from treated rabbit at 3 hrs. of injection with venom note the breakge of myofibrils , SR: Disorganized Sarcoplasmic reticulum, M: Degenerated Mitochondria (X 10000).

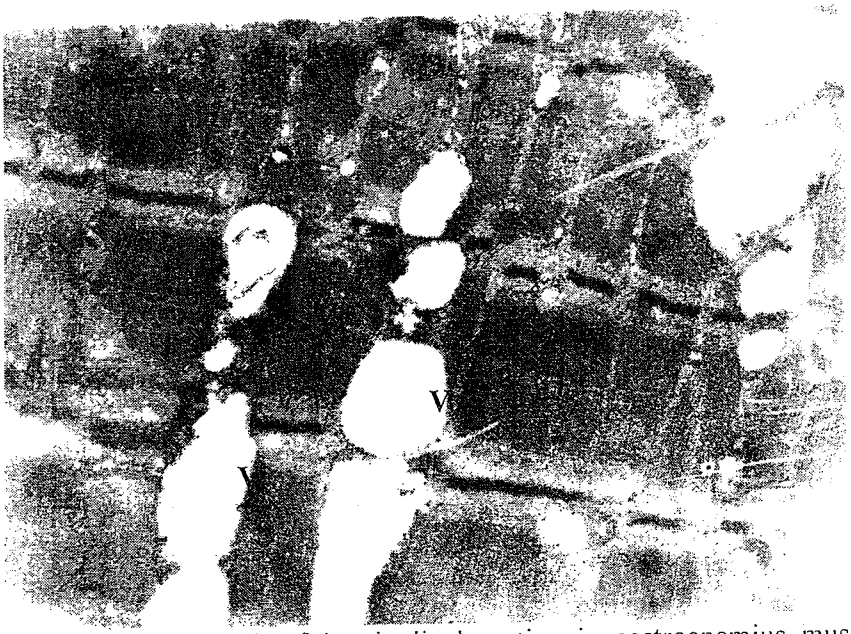

Fig. (9): Electron micrograph of longitudinal section in gastrocnemius muscle from treated rabbit at 3 hrs. of injection with venom V: Large Vacuoles (X 13000).

Fig. (10): Electron micrograph of longitudinal section in gastrocnemius muscle from treated rabbit at 3 hrs. of injection with venom fibrosis in the peripheral area (arrows), N: nucleus (X 15000).

REFERENCES

Abdel Meguid N., Essawy A. and Abdel Reheam Y. (2002) Degeneration of external flexor digitorum muscle following acrylamide administration. Folia Anatomica, 30: 41-47.

Azevedo-Marques M. M., Cupo P., Coimbra T. M., Hering S. E., Rossi M.A. and Laure C.J. (1985) Myonecrosis myoglobinuri and acute renal failure induced by South American rattlesnake (*Crotalus durissus terrificus*) envenomation in Brazil. Toxicon, 23, 4, 613- 636.

Chang, L.S., Lo, Y. and Lin, S.R. (1998). Structural factors affect the interaction of anticardiotoxin antibodies and cobra venom cardiotoxins. Biochem. Biol. Internat., 44(3), 529-534.

Condrea, E., Yang, C. C. and Rosenberg, P. (1980). Comparison of a relatively toxic phospholipase A2 from *Naja nigricollis* snake venom with that of a relatively non-toxic phospholipase A2 from *Hemachatus hemachatus* venom-I. Enzymatic activity on free and membrane bound substrates. Biochem. Pharmac. 29: 1555-1563.

Dourado D. M., Matias R., Almeida M.F., De Paula K. R., Vieira R. P., Oliveria L. V. F. and Carvalho P.T.C. (2008) The effects of low-level laser on muscle caused by *Bothrops neuwiedi* venom. J. Venom. Anim. Toxins incl. Dis vol.14 no.3 Botucatu.

Essawy A., Abdel –Maguid N., Ali A. and Sedky A. (2004) Lithium-induced changes in muscles and neuromuscular junctions. INT. J. BIOTECH., 1(4): 655-666.

Faiz M. A., Harris J. B. and Mante D. (1994) Biochemical analysis of degeneration in rat soleus muscle induced by venom from three subspecies of *Daboia russeli*. Comp . Biochem. Physiol. 107B, 231-240.

Faiz M. A., Harris J. B., Maltin C. A. and Mante D. (1995)Comparison of structural protein and proteolytic enzyme levels in degenerating and regenerating rat muscle induced by *Notachis scutatus scutatus* venom.Comp. Biochem. Physiol. 110B, 241-253.

Fatehi M., rowan E. G., Harvey A. L. and Harris J. B.(1994) The effect of Five phospholipases A2 from the venom of king brown snake *Pseudechis australis*, on nerve and muscle. Toxicon 32, 1559-1572.

Gasanov, S.E.,Alsarraj, M.A., Gasanov, N.E. and Rael, E.D. (1997). Cobra venom cytotoxin free of phospholipase-A, and its effect on model membranes and T-leukemia-cell. J. Memb. Biol., 155 (2), 133-142.

Ghadially F.H. (1978) Ultrastructural pathology of the cell. A text book and atlas of physiological and pathological alterations in cell fine structure. Butterworths, London and Boston.

Harris, J.B. (1991). Phospholipases in snake venoms and their effects on nerve and muscle. In: Snake venoms, pp. 91-129 (A.L. Harvey editor) Pergamon Press, New York.

Harvey A. L., Barfaraz A., Thomson E., Faiz A., Preston S. and Harris J. B. (1994) Screening of snake venoms for neurotoxicity and myotoxic effects using simple in *vitro* preparation rom rodents and chicks.Toxicon. 32, 257-265.

Hood V. L. and Johnson J. R. (1974) A cute renal failure with myoglobinuria following tiger snake bite. Aust. N.Z.J. Med. 4, 415.

Johnson E. K. and Ownby C. I. (1993) Isolation of a myotoxin from the venom of *Agkistrodom contortrix laticinctus* (broad-banded copperhead) and pathogenesis of myonecrosis induced by it in mice. Toxicon 31, 243- 245.

Latta H.L., Osvaldo J.D., Jackson and M. Cook (1965): changes in renal cortical tubules during autolysis. Electron microscopic observations. Lab. Inves., 14: 535-557.

Martikainen, P., Nyman, K. and Nevalainen, T.J. (1993). Toxic effects of human pancreatic and snake and bee venom phospholipases A2 in MCF-7 cells in culture. Toxicon, 31(7), 835-843.

Mebs D. and Ownby C.L. (1990) Myotoxic components of snake venoms :Their biochemical and biological activities. Pharmac Ther. 48, 223-236.

Mebs D. and Samejima Y. (1986) Isolation and characterization of myotoxic phospholipase A2 from crotalid venom. Toxicon 24, 161-168.

Mebs, D. (1986). Myotoxic activity of phospholipase A2 isolated from cobra venom. Neutralization by polyvalent anti-venom. Toxicon, 24, 1001-1008.

Min-zhou H. and Gopalakrishnakone P. (1996) Pathological changes induced by an acidic phospholipase A2 from *Ophiophagus Hannah* venom on heart and skeletal muscle of mice after systemic injection. Toxicon, 34, 2, 201- 211.

Novikoff A.B. and Holtzmann E. (1976) Cells and organelles. Holt, Rinehart and Winstor New York, 2nd ed.

Ownby C. L. and Colberg T.R. (1988) Classification of myonecrosis induced by snake venoms: venom from the prairie rattlesnake (*Crotalus riridis riridis*). Western diamondback rattlesnake (*Crotalus atrox*) and the Indian cobra (*Naja naja naja*) . Toxicon 26,459-474.

Preston S.A., Davis C. E., and Harris J. B. (1990) The assessment of muscle fiber loss after the injection of the venom of *Notechis scutatus scutatus* (Australian tiger snake). Toxicon 28, 201-214.

Russell, F.E. (1996). Toxic effects of animal toxins. In Curtis D.Klassen editor, Casartt and Doullis Toxicology: The Basic Science of Poison, 5th edt. Ch. 26, pp 801-3-839, Mc Graw-Hill, Health Profssion Division, New York.

Salvini T. F., Amaral A. C., Miyabara E. H., Turri J. A., Danella P.M. and Selistre-De-Araujo H.S. (2001) Systemic skeletal muscle necrosis induced by crotoxin. Toxicon, 39, 1141.

Samon Z. J., Chen P., Gopatakrishn P. and Gwee M. C. E. (1994) Pharmacological effects and pathological changes induced by the venom of *Pseudechis australis* in isolated skeletal muscles preparation. Toxicon 32, 303-315.

Sangiorgio F., Sakate M., Nogueira R. M. B. and Tostes R. A. (2008) Histopathological evaluation in experimental envenomation of dogs with *Crotalus durissus terrificus* venom. J. Venom. Anim. Toxins incl. Trop. Dis Vol.14 no. 1 Botucatu.

Senger H. (1987)Response of fed and starved roach, Rutilus rutilus to sublethal copper contamination. J. Fish. Biol., 30: 423-437.

Sitprija, V., Sribhibhadh, R. and Benyajati C. (1971) Haemodialysis in poisoning by sea-snake venom. Br. Med. J. 3,218.

Theakston, R.D.G., Phillips, R.E., Warrell, D. A., Galagedera, Y., Abeysekera, D. T., Dissanayaka, P., De-Silva, A. and Aloysius, D. J. (1990). Envenomation by the common Krait (*Bungarus caeruleus*) and Srilankan cobra (*Naja naja haja*): Efficiency and complication of therapy with Kaffkine antivenom. Trans. Royal Soc. Trop. Med. Hyg., 84, 301-308.

Tonsing L., Potgieter D.J.J., Louw A.I. and Visser L. (1983) The binding of snake venom cardiotoxins to heart cell membranes. Biochem. Biophys. Acta. 732, 283- 288.

Tsai, I.H. (1997). Phospholipase A(2) of snake venoms. J.Toxivol. – Toxin Rev., 16(3), 79-113.

Tu, A.T. (1977). Chemistry and Molecular Biology. John Wiley and Sons, NewYork, London, Sydney and Toronto.

Ueno, E., Rosenberg, P., Mereish, K.A. and Wannemacher, R. (1996). Mechanism of action of beta-bungarotoxin, a presynaptically acting phospholipase A2 neurotoxin. Proceeding of the 5th Pan American system on Animal, Plant and Microbial toxins., 34: (11-12), 1219-1227.

Vishwanath, B. S., Kini, R. M. and Gowda, T. V.(1987).Characterization of three edema- inducing phosphplipase A2 enzymes from habu (*Trimeresurus favoviridis*) venom and their interaction with the alkaloid aristolochic acid. Toxicon, 25: 501-515.

Yudkowsky M.L., Beech J. and Fletcher J.F. (1994) Myotoxin reduces the threshold for calcium-induced calcium release in skeletal muscle. Toxicon. 32,273-278.