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Alterations in the ultrastructure of cardiac autonomic nervous system triggered by crotoxin from rattlesnake (*Crotalus durissus cumanensis*) venom

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Abstract

This study explored the toxic effects of crotoxin isolated from *Crotalus durissus cumanensis* venom on the ultrastructure of mice cardiac autonomic nervous system. Mice were intravenously injected with saline (control group) and crotoxin diluted in saline venom (study group) at a dose of 0.107 mg/kg mouse body weight. Samples from the inter-ventricular septum were prepared for electron microscopy after 6 h (G1), 12 h (G2), 24 h (G3) and 48 h (G4). The G1 group showed some cardiomyocyte with pleomorphic mitochondria. Capillary swollen walls, nerve cholinergic endings with depleted acetylcholine vesicles in their interior and other depletions were observed. A space completely lacking in contractile elements was noticed. The G2 group demonstrated a myelinic figure, a subsarcolemic region with few myofibrils and nervous cholinergic terminal with scarce vacuoles in their interior. The G3 group demonstrated a structure with a depleted axonic terminal, mitochondrias varying in size and enhanced electron density. In addition, muscular fibers with myofibrillar structure disorganization, a depleted nervous structure surrounded by a Schwann cell along with an abundance of natriuretic peptides, were seen. An amyelinic terminal with depleted Schwann cell and with scarce vesicles was also observed. Finally, axonic lysis with autophagic vacuoles in their interior and condensed mitochondria was observed in the G4 group. This work describes the first report of ultrastructural damage caused by crotoxin on mice cardiac autonomic nervous system.
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Keywords: Autonomic nervous system; Crotalus durissus cumanensis; Crotoxin; Electron microscopy; Rattlesnakes; Ultrastructure

Introduction

Snakebites represent a serious public health problem in developing countries due to their high incidence, severity and sequel (Rengifo and Rodríguez-Acosta, 2004). In Venezuela, cases of *Crotalus durissus cumanensis* bites are high, corresponding to 20% of hospital

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cases submitted for specific treatment (Rodríguez-Acosta et al., 1995). *Crotalus* venom produces neurotoxicity, coagulation disorders, systemic myotoxicity and acute renal failure (Aguilar et al., 2001; Girón et al., 2003; Yoshida-Kanashiro et al., 2003), along with heart and liver damages (Pulido-Mendez et al., 1999; Rodriguez-Acosta et al., 1999). This venom contains toxins such as crotoxin, crotamin, gyroxin and convulxin and a number of other toxic peptides (Barraviera et al., 1995). Crotoxin is the major component of the *C.d. cumanensis* venom. In addition to being neurotoxic, crotoxin also exerts ultrastructural muscular cardiotoxic changes when inoculated into mice (Hernández et al., 2005, 2006). Equally, several studies have reported the occurrence of human lethal acute cardiac failure after snakebites from *C.d. cumanensis* (Van Aswegen et al., 1996; Tibballs, 1998; Aroch et al., 2004; Cher et al., 2005). The main objective of this work was to determine ultrastructural alterations in the cardiac autonomic nervous system produced by crotoxin from rattlesnake (*C.d. cumanensis*) venom.

Materials and methods

Venom and snakes

A pool of *C.d. cumanensis* venom was used. Snakes were collected from Caruachi, Bolívar state, Venezuela and maintained at the Pharmacy Faculty's Serpentarium of the Universidad Central de Venezuela. The venom was extracted and desiccated in a glass desiccator with calcium carbonate as the drying agent and stored at −70 °C until use.

Mice

Albino Swiss NIH strain male mice ranging between 18 and 22 g were obtained from the National Institute of Hygiene "Rafael Rangel," Caracas, Venezuela. The investigation complies with the bioethical norms taken from the guide "Principles of laboratory animal care" (Anonymous, 1985).

Determination of lethal dose 50 (LD50) of crotoxin from *C.d. cumanensis* venom

Venom lethality was determined by intravenous injections in mice at different concentrations, and the LD_{50} value calculated according to the method of Spearman–Karber (WHO, 1981).

Crotoxin purification from *C.d. cumanensis* crude venom by size exclusion chromatography

Two hundred and fifty milligrams of *C.d. cumanensis* crude venom was fractionated using Sephadex G-100 molecular exclusion chromatography in a K-26/100 (Pharmacia, Uppsala, Sweden) 95×2.5 cm column. An eluent of 0.1 M acetic acid, with an 8 mL/h flow rate at 4 °C was used. Collected fractions were immediately frozen at −70 °C and lyophilized.

After identifying the fraction with phospholipase A_2 activity by biological, enzymatic (Nakazone et al., 1984) and polyacrylamide gel electrophoresis (PAGE), the crotoxin fraction was further purified (74 mg of the fraction IV) on a Sephadex G-50 molecular exclusion column. A K-16/50 (Pharmacia, Uppsala Sweden) 45×1.5 cm column using 0.1 M acetic acid as eluent, with 8 mL/h flow rate at 4 °C was employed.

Fractions IV (Sephadex G-100) and II (Sephadex G-50) PAGE from *C.d. cumanensis* venom

Venom fractions were run on PAGE under reducing conditions. Gels were stained with Coomassie blue solution. The gel bands densitometry was carried out using a Densitometer GS-690 (Bio-Rad, USA) and the profile protein analysis and its molecular weights were determined with the Multi-Analyst version 1.1 (Bio-Rad, USA) program.

Determination of *C.d. cumanensis*, peak IV (Sephadex G-100) and peak II (Sephadex G-50) protein concentration

The protein concentration was determined by the method of Lowry et al. (1951).

Crotoxin neurotoxic activity

The crotoxin neurotoxic activity was carried out by electron microscopy techniques. Four working groups, of four mice per group, were intravenously injected with a sub-lethal dose of crotoxin (0.105 mg/kg body weight).

Routine transmission electron microscopy (TEM)

Cardiac tissues from envenomed and control mice were used for TEM studies. Sections from the interventricular cardiac septum were immediately removed from CO_2 sacrificed animals. Samples were sliced at 1-mm thickness, and prefixed at 4 °C in 2.5% glutaraldehyde in PBS for 2 h. They were washed twice in cold PBS for 10 min, and post-fixed in cold 1% osmium tetraoxide in PBS for 2 h. Specimens were then washed three times in cold distilled water, stained with 1% uranyl acetate,

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1 3 5 7 9 11 13 15 17 19 21 23 25 27 29 31 33 35 37 39 41 43 45 47 49 51 53 55

dehydrated in a series of alcohol, and embedded in epoxy resin. Ultrathin sections were cut and stained with uranyl acetate and lead citrate. Samples were observed in a Hitachi H-500 transmission electronic microscope with 100 kW voltages.

Results

LD_{50} intravenous determination of crotoxin and *C.d. cumanensis* crude venom in mice

C.d. cumanensis crude venom presented an intravenous LD_{50} of 0.144 mg/kg body weight. Whereas, the LD_{50} of crotoxin was 0.107, Crotoxin was 74.3% more toxic than crude venom.

Molecular exclusion chromatography purification of fraction with crotoxin activity

Six well defined *C.d. cumanensis* venom fractions obtained by Sephadex G-100 molecular filtration chromatography were observed (Fig. 1). Fraction IV containing phospholipase A_2 activity (detected by biological and enzymatic tests) contained the highest protein concentration of 78.15 mg (39.25% of the crude venom), followed by fractions I, II, VI, III and V.

Fraction IV was suspended in buffer of 0.1 M acetic acid, and run in a Sephadex G-50 column in which two peaks were obtained (Fig. 2). The peaks were tested on biological and enzymatic tests, focusing on phospholipase A_2 (crotoxin) activity. Peak II starting from tube number 60 had crotoxin activity, and its purity was determined by electrophoresis (Fig. 3).

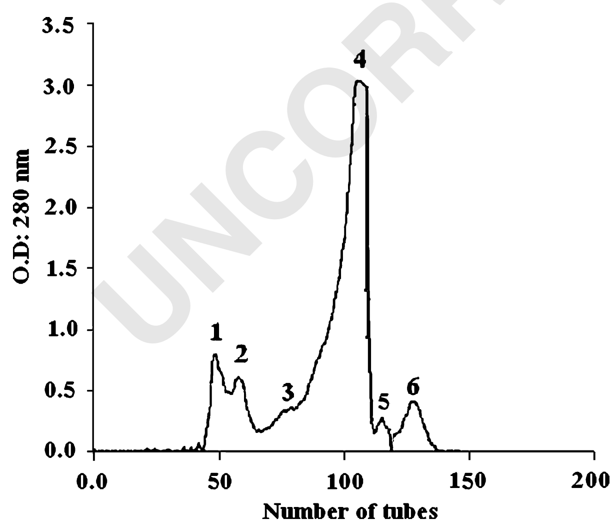

Fig. 1. Sephadex G-100: molecular exclusion chromatography purification of fraction with crotoxin activity.

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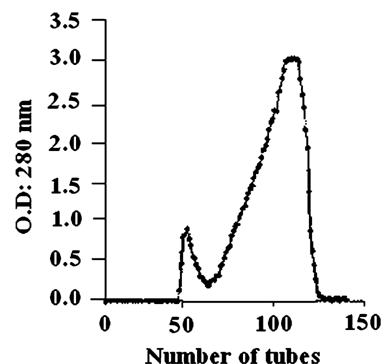

Fig. 2. Sephadex G-50: molecular exclusion chromatography purification of fraction IV (from Sephadex G-100) with crotoxin activity.

PAGE of fraction IV (Sephadex G-100) and fraction II (Sephadex G-50)

The *C.d. cumanensis* crude venom showed seven bands of molecular weights between 225 and 10 kDa (Fig. 3).

The Peak IV obtained by Sephadex G-100 electrophoretic run showed four bands, corresponding to 25, 21, 15 and 11 kDa (Fig. 3). Peak II of Peak IV obtained from Sephadex G-100 was run on Sephadex G-50 obtaining only one peak containing 14 and 13 kDa bands as determined by gel electrophoresis (Fig. 3).

Transmission electron microscopy

Normal controls of cardiac tissue after 48 h of intravenously saline solution injections were analyzed by TEM. The samples showed a normal axonic terminal with acetylcholine and nor-epinephrine granules. An axon with normal mitochondria and normal nervous terminals was observed (Fig. 4).

Group 1 (G1) presented several cardiomyocytes with large electron-dense and pleomorphic mitochondria, 6 h after crotoxin injection. A capillary with swollen walls was seen. Cholinergic nerve endings with scarce acetylcholine vesicles in their interior were observed along with a space completely lacking in contractile elements. Dilated cisterns of rough endoplasmic reticulum were also observed (Fig. 5).

Group 2 (G2) contained a myelinic figure and areas with muscular fiber atrophy 12 h after crotoxin injection. Vacuolization of the sarcotubular system and capillary lumen occlusion were also observed (Fig. 6). Sarcoplasmic edema and autophagic vacuole were noticed (Fig. 7). Subsarcolemmic regions with few myofibrils and cholinergic nerve endings with scarce vacuoles in their interior were observed. Different widths of endothelia were seen (Fig. 8).

Group 3 (G3) contained a structure with depleted axonic nerve ending 24 h after crotoxin injection.

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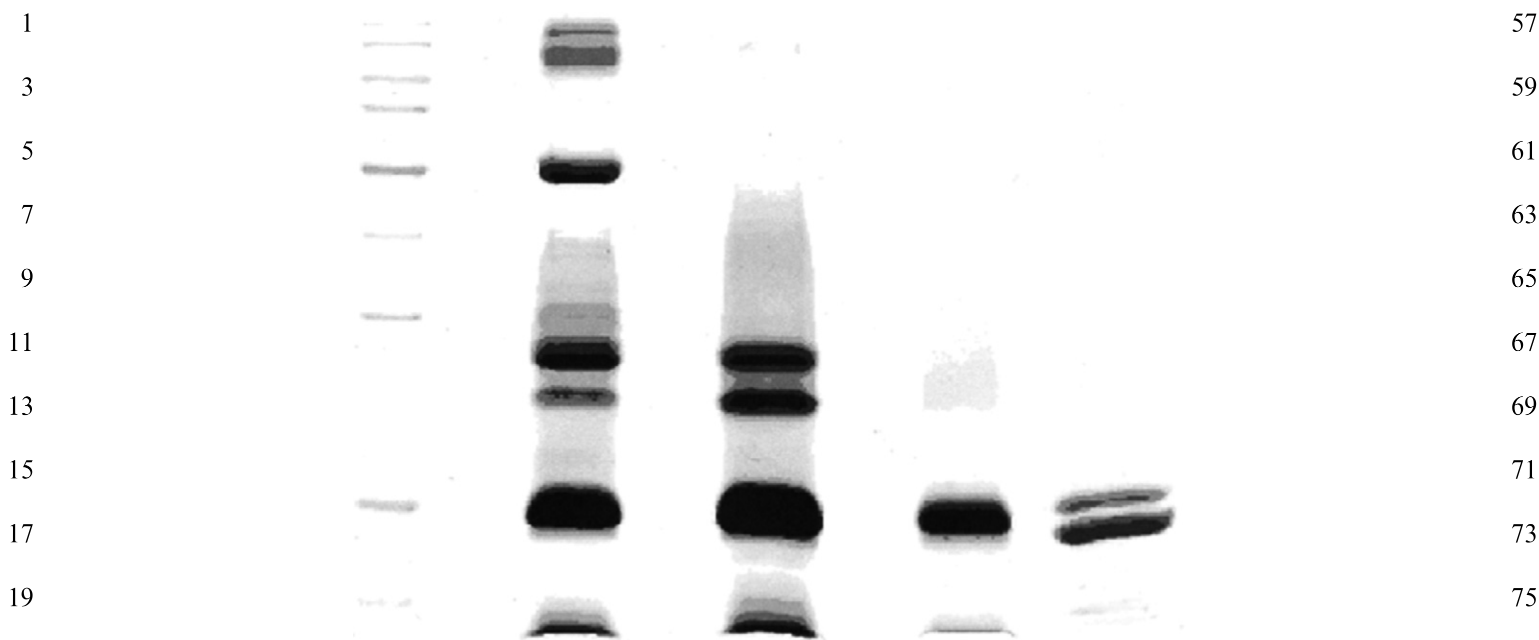

Fig. 3. SDS-PAGE of *Crotalus durissus cumanensis* crude venom (CV). Sephadex G-100 Fraction (peak IV) from CV. Sephadex G-50 Fraction: non-reduced (NR) and reduced (R) peak II. MW: molecular weight markers.

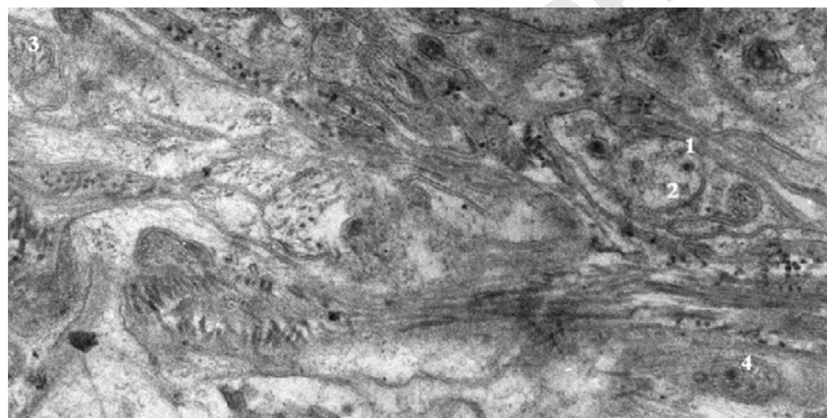

Fig. 4. Forty-eight hours after saline solution injection (normal control) shows a normal axonic nerve ending with acetylcholine (1) and nor-epinephrine (2) granules. An axon with normal mitochondria (3) and normal nerve ending (4) magnification × 24,000.

Pleomorphic mitochondria varying in different sizes with enhanced electron density were noticed (Fig. 9). Abundant natriuretic peptides were detected along with a depleted axonic nerve ending (Fig. 10). An amyelinic nerve ending with a depleted Schwann cell or with scarce vesicles, as well as a degenerated axonic nerve ending was observed (Fig. 11). Furthermore, an amyelinic nerve ending with no membrane depleted vesicles containing severe edema was also observed. The disappearance of the sarcomeric structure around the nerve ending was apparent. Pleomorphic mitochondria with different electron density and loss of cristae and intense edema were also noticed (Fig. 12).

Group 4 (G4) contained an axonic ending surrounded by a Schwann cell 48 h after crotoxin injection. Axonic lysis with autophagic vacuoles in their interior, condensed mitochondria, a large vesicle in the axon and an autophagic vacuole were seen. Rough endoplasmic reticulum was dilated and smooth endoplasmic reticulum was vesiculated (Fig. 13).

Discussion

The majority of snake venoms exert their activities on almost all tissues or cells and their pharmacological actions are determined by a number of biologically active fractions (Sanchez et al., 1992). Cardiotoxicity is an observed problem in a large number of snakebites (Cupo et al., 1990) and the phospholipase A_2 (PLA_2)

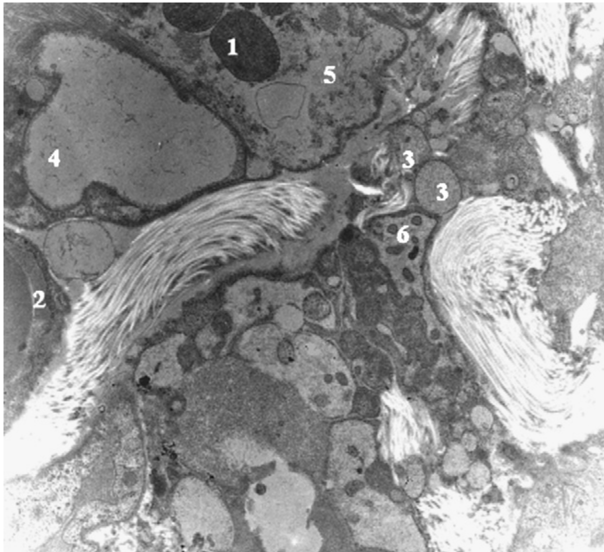

Fig. 5. Six hours after crotoxin injection (G1) shows several cardiomyocytes with large electron-dense and pleomorphic mitochondria (1); a capillary showing swollen walls (2); cholinergic nerve ending with scarce acetylcholine vesicles in their interior, and other cholinergic nerves endings totally depleted (3); big vacuolar structure (4) and disappearance of myofibrils (5). Dilated cisterns of rough endoplasmic reticulum (6) magnification × 20,000.

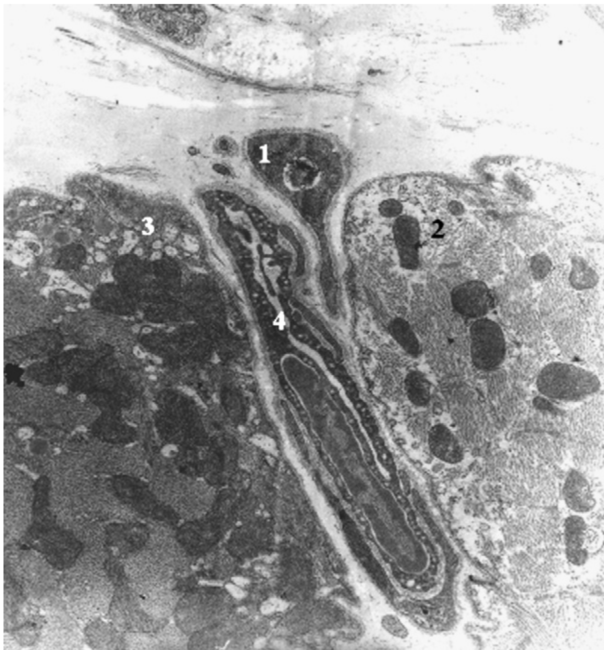

Fig. 6. Twelve hours after crotoxin injection (G2) shows myelinic figure (1) and areas with intense muscular fiber necrosis (2). Vacuolization of sarcotubular system (3) and capillary light occlusion (4) magnification × 20,000.

enzymes have been responsible for such action (Siqueira et al., 1990). PLA_2 have been described in several animals, but only a few, which includes snakes and bees,

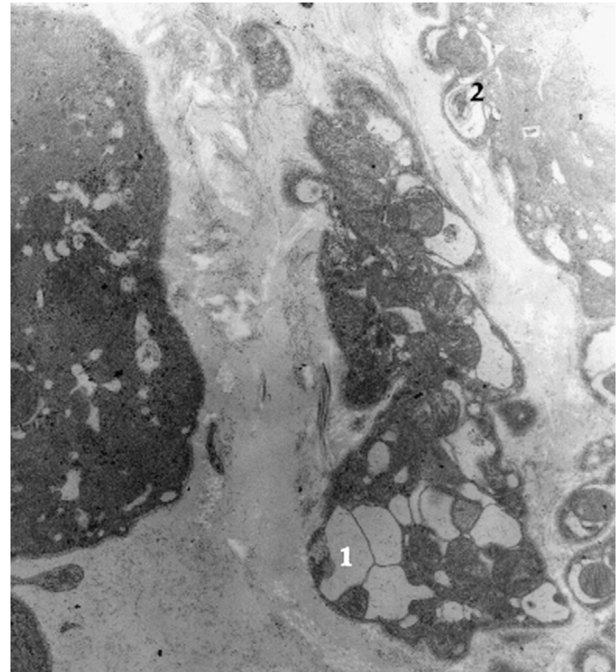

Fig. 7. Twelve hours after crotoxin injection (G2) shows sarcoplasmic edema (1) and autophagic vacuole (2) magnification × 22,000.

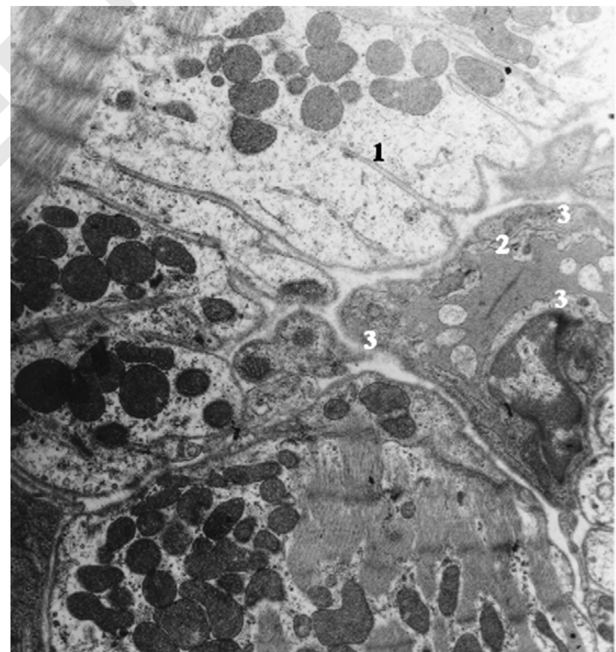

Fig. 8. Twelve hours after crotoxin injection (G2) shows subsarcolemmic region with few myofibrils (1) and cholinergic nerve ending with scarce vacuoles (2). Different widths of endothelia (3) magnification × 22,000.

use it as a toxin. Crotoxin, which is a PLA_2, is acid and heat resistant and is a remarkable venom toxin because it can sustain many different natural environments and

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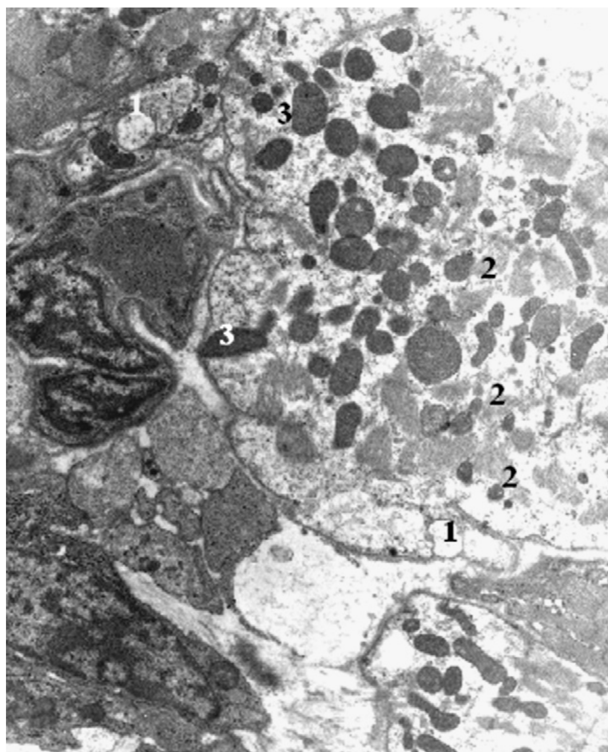

Fig. 9. Twenty-four hours after crotoxin injection (G3) shows a structure with a depleted axonic nerve ending (1); necrotic muscular fibers with membrane lost (2) and pleomorphic mitochondria varying in size with enhanced electron-density (3) magnification × 20,000

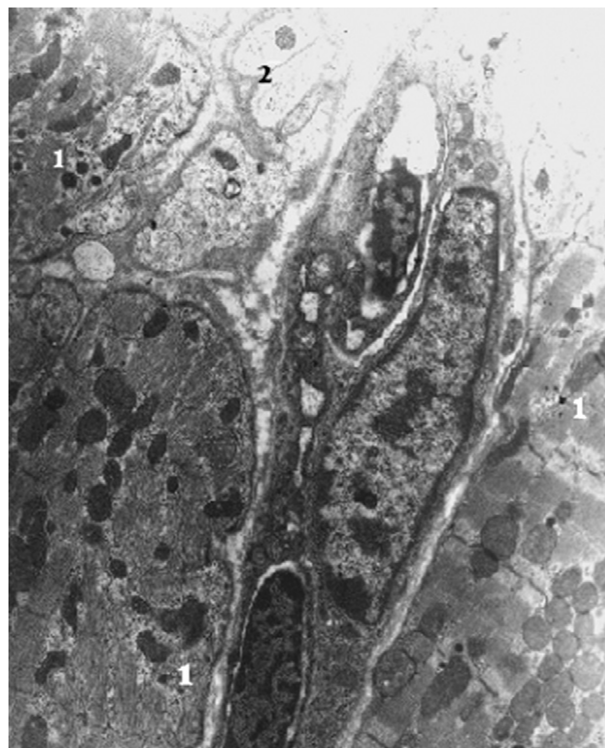

Fig. 10. Twenty-four hours after crotoxin injection (G3) shows abundant natriuretic peptides (1) and depleted axonic nerve ending (2) magnification × 20,000.

maintain their activity (Yates and Rosenberg, 1991). It seems that the metabolite fatty acids from PLA_2 activity interfere with cellular respiration (Valente et al., 1998). The PLA_2 found in snake venom are analogous to the nontoxic mammalian pancreatic PLA_2; in effect, if the amino acid Phe in bovine pancreatic PLA_2 is changed to Tyr, the nontoxic enzyme becomes neurotoxic (Tzeng et al., 1995). Yates and Rosenberg (1991) proposed that the difference in activity between PLA_2 comes from the modification to a hydrophobic area in the protein, which is essential for the neurological activity. Crotoxin, given all it can do, may be the most remarkable constituent of the *Crotalus* venom.

Crotoxin works on both presynaptic and postsynaptic neuromuscular membranes to inhibit signal transmission in an unknown way. Montecucco and Rossetto (2000) proposed that PLA_2 enters the lumen of synaptic vesicles following endocytosis and hydrolizes phospholipids of the inner leaflet of the membrane. Phospholipase A_2 hydrolyzes the sn-2 ester bond of 1,2-diacyl-3-sn-phosphoglycerides producing fatty acids and lysophospholipids (Kini, 1997). The transmembrane pH gradient compels the translocation of fatty acids to the cytosolic monolayer, leaving lysophospholipids on the lumenal layer. Such vesicles are extremely fusogenic and

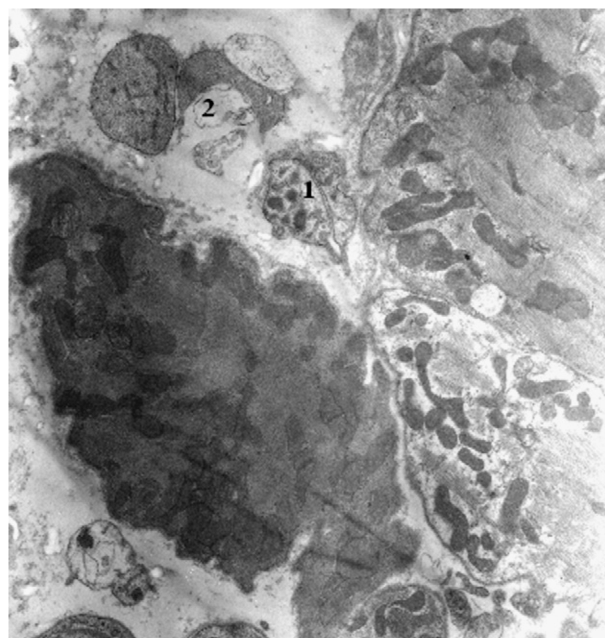

Fig. 11. Twenty-four hours after crotoxin injection (G3) amyelinic nerve ending terminal with depleted Schwann cell or with scarce vesicles (1), as well as degenerated axonic nerve ending (2) were observed. magnification × 22,000.

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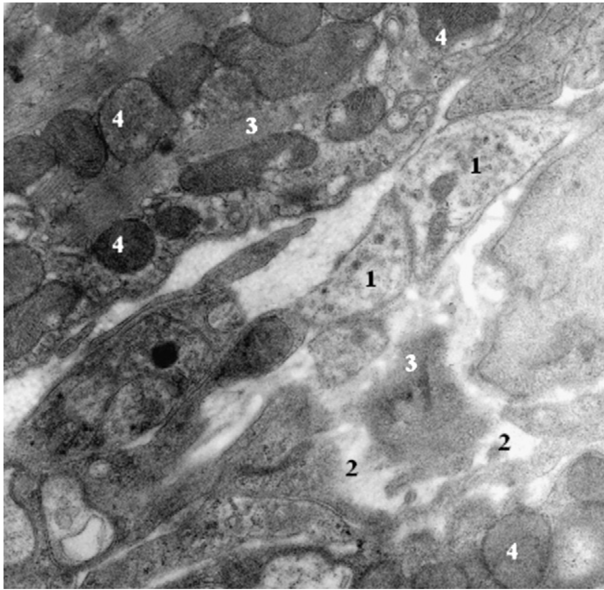

Fig. 12. Twenty-four hours after crotoxin injection (G3) shows a amyelinic nerve ending with membrane lost and depleted vesicles (1) with intense edema (2). Disappearance of the sarcomeric structure (3) around the nerve ending. Pleomorphic mitochondria with different electron-density and lost of cristae and intense edema (4). magnification × 22,000.

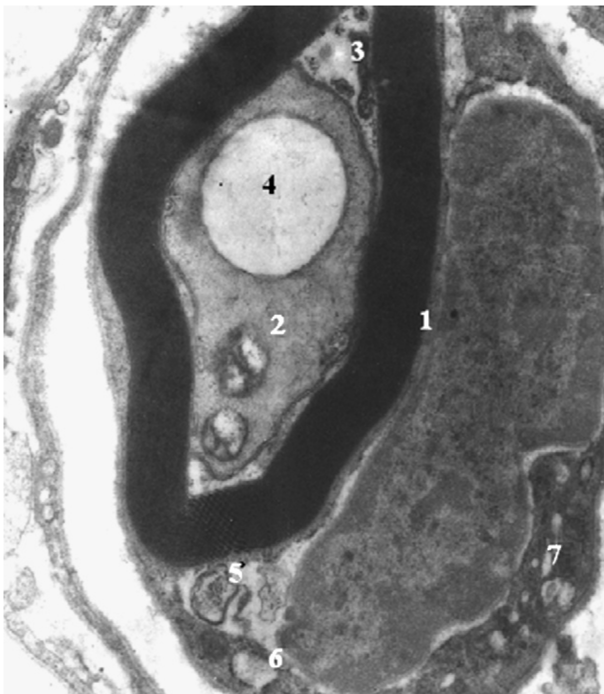

Fig. 13. Forty-eight hours after crotoxin injection (G4) shows an axonic nerve ending (1), surrounding by a Schwann cell. Axonic lysis with autophagic vacuoles (2) in their interior and condensed mitochondria (3); big vesicle in the axon (4) and autophagic vacuole (5). Rough endoplasmic reticulum (6) was dilated and smooth endoplasmic reticulum (7) was vesiculated. magnification × 24,000.

discharged neurotransmitters lead to vesicle fusion with the presynaptic membrane.

In the present work the LD_{50} of crotoxin in mice was 0.107 mg/kg body weight by intravenous injection. The crotoxin acidic sub-unit directs the basic sub-unit to receptors on the presynaptic membrane at the neuromuscular junction. The receptor where the neurological activity of crotoxin exerts its effects has not been specified and not comprehensively studied. The basic sub-unit without the acidic sub-unit binds nonspecifically to every part of the membrane (Hendon and Fraenkel-Conrat, 1971). Once at this receptor, the basic unit detaches from the acidic one and inserts itself into the cell membrane (Yates and Rosenberg, 1991).

Crotoxin's effects have been experimentally described. The enzymatic action alone of crotoxin on membrane phospholipids can change membrane permeability. Crotoxin alters the morphology of the nerve cells as well; there is a diminution of synaptic vesicles at the neuromuscular junction, "U" shaped indentations in the axolemma and degeneration of small axons. However, removal of the crotoxin allows for a quick recovery of the nerve cells (Yates and Rosenberg, 1991).

The actions of crotoxin on the cardiac autonomic nervous system described in this study are characteristically associated with high levels of transmitter discharge and the enhanced turnover of vesicle membrane. The data in this work suggest that the depletion of synaptic vesicles may be the result of a combination of enhanced transmitter release and impaired retrieval and recycling of emptied vesicles. Cholinergic nerve endings with scarce vacuoles and myelinic figures in their interior, and nerve fibers with profound damage as well as depleted nerve structures, surrounded by a Schwann cells, or amyelinic ending with depleted Schwann cells, or with scarce vesicles maybe caused by the presynaptic effects of crotoxin or the release of mediators such as biological amines such as histamine, serotonin or prostaglandins that may take part in the pathogenesis of edema. The discharge of these compounds is associated with an increase in capillary permeability. The abnormal capillary, including the nonexistence of walls in some places, and the increase in the endoplasmic reticulum with the increment of the fenestrae, could all be the results of toxins such as crotoxin, that produce the rapid rupture of the plasmatic membrane followed by detriment of the permeability regulation for ions and macromolecules (Ownby et al., 1997). The observed muscular necrosis may have developed through an indirect mechanism, under the action of hypoxia or more likely through the ischemia that causes damage to the capillary walls (Gutiérrez et al., 1995); and thus, the ischemia must also affect the nervous system.

There are a number of investigations of the functional effects of ischemia and hypoxia on conduction tissues

(Coffman et al., 1960; Senges et al., 1981; Kohlhardt and Haap, 1980). Jennings et al. (1965) demonstrated that, when subjected to a rigorous degree of oxygen deprivation, all cell types in the specialized AV conduction tissues develop fine structural alterations, typical of ischemically injured ventricular myocytes.

Hylop and De Nucci (1993) established that the release of histamine is related to an increase in phospholipase concentrations. On the other hand, crotoxin postsynaptic effects have been discovered as well: the crotoxin binds to the acetylcholine receptor in the postsynaptic membrane and attaches it in a desensitized state (Bon et al., 1979). It has long been recognized that presynaptically active neurotoxic phospholipases A_2, *in vitro* cause an early augmentation of transmitter release before the failure of transmission (Harris, 1991; Hawgood and Bon, 1991). This is usually thought be a sign of a response to the hydrolytic action of the phospholipase, but the mechanism of action of the toxin at the molecular level, as alleged above, is not known. However, steady information that the density of synaptic vesicles in nerve endings is reduced when exposed to the presynaptically active PLA_2 (Cull-Candy et al., 1976; Strong et al., 1977), and sporadic reports of nerve ending injuries (Abe et al., 1976; Harris et al., 1980; Gopalakrishnakone and Hawgood, 1984) including the findings in our study, reinforce that nerve ending lesions may be the principal reason for the extended paralysis produced by crotoxin.

The occurrence of many natriuretic peptides has been accounted for in snake venom (Higuchi et al., 1999; Schweitz et al., 1992), including the present work. Natriuretic toxins have been shown to have potent systemic effects, such as profound hypotension (Fry, 2005). A gene has been recognized that codes for 7 bradykinin-potentiating peptides and also a C type natriuretic peptide in the venom of Viperidaes (Murayama et al., 1997) and the diuretic and natriuretic effects promoted by the peptide called DNP, isolated from Elapidaes, have been recently described (Ha et al., 2005).

The mitochondria damage (mainly condensed mitochondria) observed in this work classically occurs under conditions where respiration and/or oxidative phosphorylation are inhibited. The condensed conformation cannot actually be preserved (an alteration that also possibly correlates with the loss of the mitochondrial membrane potential) if significant injury produced by crotoxin to the inner membrane takes place (Trump and Berezesky, 1992).

It is concluded that crotoxin from *C.d. cumanensis* venom had a strong cardiotoxic action on cardiac autonomic nervous system. The ultrastructural changes were time dependent and it is suggested that the ultrastructural cardiac modifications due to crotoxin might also be the consequence of a direct action of crotoxin or by means of the release of biological mediators by several tissues. It is reported in this study that the toxin produces the reduction of synaptic vesicles and the degeneration of nerve endings. As a consequence of these findings it is believed that the most important pathological neurotoxic processes observed in envenomed experimental animals is a result of crotoxin. This study is the first report describing ultrastructural damage of the cardiac autonomic nervous system as a result of crotoxin when *Crotalus* envenomation occurs.

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Acknowledgments

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