AN ELECTRON MICROSCOPIC ANALYSIS OF CARDIAC MUSCLE MYONECROSIS INDUCED BY TWO MYOTOXIC COMPONENTS ISOLATED FROM RATTLESNAKE (*CROTALUS VIRIDIS VIRIDIS*) VENOM

By

LAVETA CAROLE BENNETT

Bachelor of Science

Central State University

Edmond, Oklahoma

1977

Submitted to the Faculty of the Graduate College
of the Oklahoma State University
in partial fulfillment of the requirements
for the Degree of
MASTER OF SCIENCE
July, 1979

AN ELECTRON MICROSCOPIC ANALYSIS OF CARDIAC MUSCLE MYONECROSIS INDUCED BY TWO MYOTOXIC COMPONENTS ISOLATED FROM RATTLESNAKE (CROTALUS VIRIDIS VIRIDIS) VENOM

Thesis Approved:

Charlotte L. Dunlap
Thesis Adviser

Eugene L. Gaines

Theodore E. Hall

Norman N. Durham
Dean of the Graduate College

ACKNOWLEDGMENTS

I wish to express my sincere appreciation to Dr. Charlotte L. Ownby, Dr. Duane L. Garner and Dr. Theodore E. Staley for their guidance and encouragement while engaged in this study. Appreciation is also extended to Lisa Seidman, Mike Woods, Vanessa Hunholz, and Denise Rhode for technical assistance.

I am extremely grateful for the cooperation from the Department of Physiological Sciences in Veterinary Medicine.

Special gratitude is expressed to my family and close friends for frequent help, understanding, enduring love, and patience.

TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER I

INTRODUCTION

In the United States Wyeth Polyvalent Antivenin (antiserum) is the common treatment for snakebite. The use of antivenin has resulted in a lower mortality rate in the United States than the rest of the world. The current number of bites by venomous snakes is about 1,500 annually; 1000 of these bites were inflicted by rattlesnakes (family Crotalidae), and 30 of these bites were fatal (Klauber, 1972). Antivenin is very useful in the preservation of people's lives but fails to prevent local tissue damage (Minton and Minton, 1969; Ownby, 1975; Ownby et al., 1976; Huang et al., 1978). Because antivenin does not prevent local tissue damage, dysfunction of the limb upon which the bite occurred, or complete amputation of the limb may result. Thus snakebite remains a serious medical concern today.

In studying the effects of snakebite poisoning considerable research has been done in describing the resulting local symptoms. The most outstanding local effects of rattlesnake (Crotalidae) poisoning are pain, swelling, hemorrhage and myonecrosis (Minton and Minton, 1969; Homma and Tu, 1971; Ownby et al., 1974; Ownby et al., 1976; Huang et al., 1978). Several studies have been conducted testing the effects of crude venom (Stringer et al., 1972; Ownby et al., 1974) and of isolated pure myotoxic components (Ownby et al., 1976; Cameron and Tu, 1977) on skeletal muscle in mice. These studies were conducted at

both light and electron microscopic levels in order that the specific cellular areas of sensitivity could be examined and the pathogenesis of necrosis understood. The sarcoplasmic reticulum in particular was found to be extremely sensitive to crude venom (Stringer et al., 1972) and myotoxin a, a pure myotoxic component of Crotalus viridis viridis venom (Ownby et al., 1976). As a result of exposure of muscle to crude venom (Crotalus viridis viridis) the sarcoplasmic reticulum was dilatated, mitochondrial matrix was condensed, and mitochondria was rounded and enlarged; myofilamentous components were also disoriented and coagulated. There was also disruption of the external lamina of the muscle fiber and sarcolemma (Stringer, et al., 1972). On the other hand, upon exposure to myotoxin a there was a disruption of the external lamina and sarcolemma. The sarcoplasmic reticulum and perinuclear space was dilatated and eventually myofibrils were disrupted. Even with complete myofibrillar breakdown severe mitochondrial damage did not occur, although slight swelling of mitochondria and area devoid of cristae were noted (Ownby, et al., 1976). At all time periods studied T-tubules were structurally intact. Ownby et al. (1976) concluded that skeletal muscle cells were specifically affected with the sarcoplasmic reticulum being the initial site of action either through a direct or indirect action of the toxin.

Cardiac muscle is a striated muscle similar to skeletal muscle. Skeletal muscle cells have many peripherally located nuclei, distinct striations and is under voluntary control, whereas cardiac muscle cells have centrally located nuclei, less distinct striations and are under involuntary control. Contraction occurs in both skeletal and cardiac muscle by a mechanism of excitation-contraction coupling of the individual

myofilaments. The sarcoplasmic reticulum in both muscle types contains an ATP-dependent pump responsible for calcium ion movement against a considerable gradient. As a result of nervous stimulation, calcium is released from the sarcoplasmic reticulum into the sarcoplasm. The increased calcium ion concentration in the sarcoplasm causes interaction between actin and myosin filaments which results in muscle contraction. In skeletal muscle an increase in calcium ions from the sarcoplasmic reticulum is the primary initiator of contraction whereas in cardiac muscle contraction results from an increased calcium ion concentration in the extracellular fluid (Endo, 1977). There exists two distinct populations of muscle cells in the ventricle of the heart. The myocardial cells of one population have transverse tubules, working fibers, and provide the contractile force of the ventricle. The other population, Purkinje fibers, do not have transverse tubules and constitute the conduction system (Sommer, et al., 1972). Somlyo (1979) showed the existence of a very close communication between sarcoplasmic reticulum and T-tubules in skeletal muscle. As in skeletal muscle the sarcoplasmic reticulum of ventricular fibers forms similar couplings with the transverse tubules but without cisternal dilations of the sarcoplasmic reticulum. Cardiac sarcoplasmic reticulum differs in structure and distribution from that in skeletal muscle in that cardiac sarcoplasmic reticulum is widely continuous from one sarcomere to the next. Close apposition between specialized regions of cardiac sarcoplasmic reticulum occurs both at the transverse tubules and the surface sarcolemma, whereas in skeletal muscle close apposition is only at the transverse tubules (Sommer et al., 1972). Transverse tubular diameters of ventricular fibers is two times larger than those found in skeletal muscle.

Although both skeletal and cardiac muscle cells contain mitochondria located beneath the sarcolemma and between myofibrils, the number present per cell is greater in cardiac muscle, and the cells are joined by intercalated discs comprised of macula adherens and fascia adherens.

The close structural and functional similarities between skeletal and cardiac muscle prompted us to determine if cardiac muscle was affected at all by myotoxin a, and if so whether the resulting damage was the same as that reported for skeletal muscle. The specific objectives of this study were to determine and compare the effects of myotoxin a and a newly isolated myotoxic component on cardiac muscle *in vivo*.

CHAPTER II

MATERIALS AND METHODS

Isolation and Homogeneity of Myotoxin

The procedure used to isolate the myotoxin a was essentially the same as that reported first by Ownby et al. (1976) and subsequently by Cameron and Tu (1978). Since our experiments involved myotoxin a and a newly isolated myotoxic component, the procedures will be described briefly. Gel-filtration on a Sephadex G-50 column (81 x 2 cm) was the initial fractionation process to which crude Prairie Rattlesnake (Crotalus viridis viridis) venom was subjected. The crude venom was purchased in lyophilized form from Miami Serpentarium Laboratories, Miami, Florida. Three to four milliliters of a buffer composed of 0.05 M 2-amino-2(hydroxymethyl)-1,3-propandiol (Tris) buffer (pH 9.0 at 21^{o}C) and 0.1 M KCl was used to dissolve a sample of crude venom (250 mg). The column was developed with this buffer at a flow rate of 30 ml/hr and 5 ml fractions were collected. Eluate absorbance at 280 nm was monitored with a Perkin-Elmer Model Coleman 101 spectrophotometer. Four fractions were obtained from this column, and after lyophilization each was tested for myotoxic activity. Only fractions 2 and 3 caused myonecrosis when injected into mice.

Fractions 2 and 3 were further fractionated by cation exchange chromatography on a CM Sephadex (C-25) column (56 x 1.5 cm), five

fractions resulted from this column, and fractions 1 and 3 caused muscle necrosis in mice upon injection. Fraction 3 corresponded to myotoxin a (Ownby et al., 1976) and was used along with fraction 1 in all these experiments. Although fraction 1 has not yet been characterized as to its specific properties, much work has been done with myotoxin a resulting in many known characteristics of this compound. Myotoxin a is a basic protein having an isoelectric pH of 9.6. The molecular weight considering amino acid components is 4,270, while the molecular weight determined by SDS-electrophoresis is 4,070 (Ownby et al., 1976).

Light Microscopy

Forty female mice were used in three separate experiments for the light microscopic study. The mice were divided into three groups in order that a comparative study of the effects of physiological saline solution (PSS), fraction 1, and fraction 3 (myotoxin a) at 24, 48 and 72 hours post-injection could be conducted.

All the mice were injected with 4 μg/g of the appropriate myotoxic fraction or PSS, using a volume of 0.2 ml for a 20 gram mouse. The mice were injected intrathoracically (IT) via the right side of the ribcage. The first time this was done without any anesthesia, but this was found to be too traumatic for the mice. Subsequent IT injections were made after the mice had been slightly anesthetized with ether. The mice were killed at varying times after injection by cervical dislocation after which the whole heart was removed, washed in PSS, and cut longitudinally before being placed in 10% phosphate buffered formalin for 24 hours. After formalin fixation these samples were washed in tap

water, dehydrated in a series of graded ethanols, cleared in chloroform and embedded in paraplast. Cross sections (6 μm) of the muscle were obtained with an AO Spencer "820" microtome and stained with hemotoxylin and eosin for light microscopic examination.

Electron Microscopy

Fifty female mice were used in three separate experiments for the electron microscopic study. The mice were divided into three groups in order to study the effects of PSS, fraction 1, and fraction 3 (myotoxin a) at 24, 48, and 72 hours post-injection. The mice were injected in the same manner as for the light microscopic study. Upon cervical dislocation the thoracic cavity was opened and a few milliliters of 2% glutaraldehyde in 0.27 M cacodylate buffer were used to wash the heart before its removal. After the whole heart was removed from the cavity it was cut longitudinally and pieces of tissue were taken from the ventricular wall and placed in 2% glutaraldehyde in 0.27 M cacodylate buffer, pH 7.4 for 2 hours at $4^{o}C$. After primary fixation the tissue was washed for 12 hours in a buffered wash of sucrose and cacodylate buffer before secondary fixation in 1% osmium tetroxide for 1 hour. Dehydration in a series of graded ethanols was followed by propylene oxide as a transition solvent before embeddment in Epon 812.

Thick sections (1 μm) were taken on an LKB ultratome I, stained with Mallory's Azure II methylene blue (Richardson et al., 1960), and observed with a Zeiss photomicroscope. Thin sections (600-800 nm) were taken also on an LKB ultratome I using glass knives, placed on 300 mesh uncoated copper grids, stained with aqueous uranyl acetate and lead citrate (Venable and Coggeshall, 1965) and observed with a Philips EM 200 electron microscope.

CHAPTER III

RESULTS

Light Microscopy

Preliminary light microscopic examination of mouse hearts was done to determine the location and extent of damaged areas within the myocardium. Muscle from control mice (PSS-injected) was normal in histology at all time periods studied. Hemotoxylin and eosin-stained paraffin sections showed the normal cross-sectional appearance of cardiac muscle: cell limits were intact, cytoplasm was typical, and the centrally located nuclei were normal in appearance (Figure 1A). Plastic (1 μm) sections showed cardiac muscle of normal morphology: distinct and intact cell limits, typical nuclei containing prominent nucleoli, indistinct striations, intercalated discs and mitochondria (Figure 1B). The cross-sectional appearance is shown in Figure 1C and is normal in all aspects.

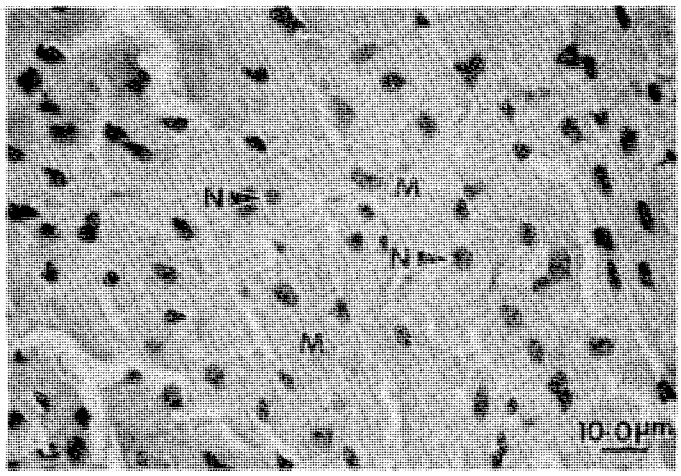
In hearts from experimental animals lesions were found to be focal in occurrence in every region examined of the ventricular wall of the heart at each time period examined. Myotoxin a induced a series of degenerative events in cardiac muscle cells beginning with slight vacuolation and progressing to cell swelling and lysis. Numerous vacuoles were observed in affected cells at 24 hours in sections stained with hemotoxylin and eosin (Figure 2A) and also in swollen cells

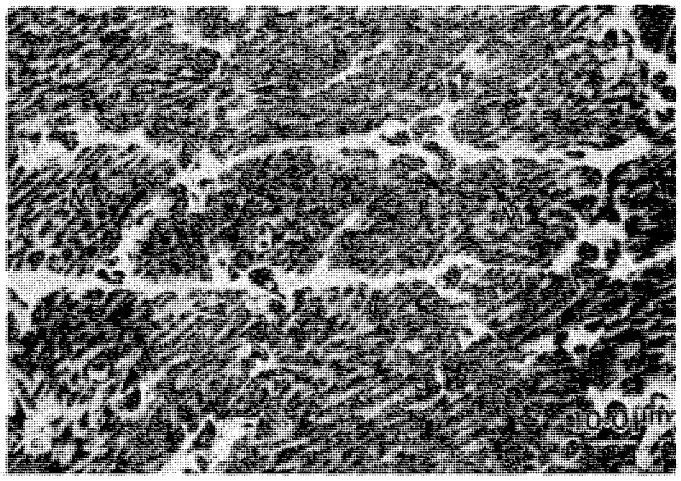
Figure 1. Light Micrographs of Cardiac Muscle from Control Mice Taken at Various Times After Injection of Physiologic Saline Solution (PSS)

A. 24 hours post-injection, H&E-stained paraffin section. Normal cross-sectional appearance of muscle. M, muscle cells; note the centrally located nuclei, N.

B. 48 hours post-injection, plastic (1 μm) section. Normal longitudinal to oblique sectional appearance of muscle. M, muscle cells; note the striations, arrowheads.

C. 72 hours post-injection, plastic (1 μm) section. Normal cross-sectional appearance of muscle is noted. M, muscle cells.


N
M
N
M
10·0μm


10·0μm

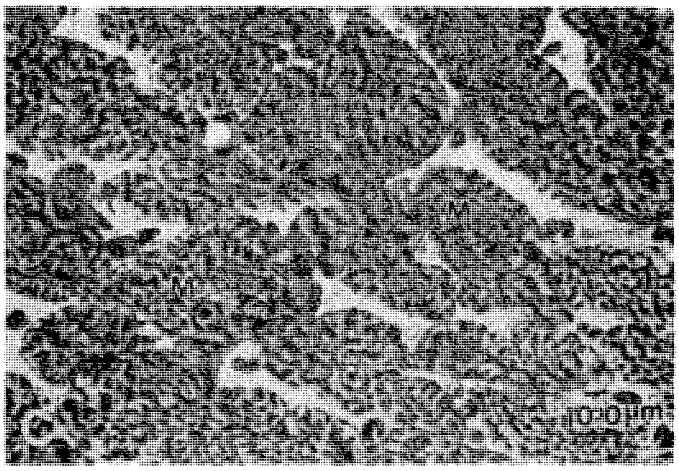
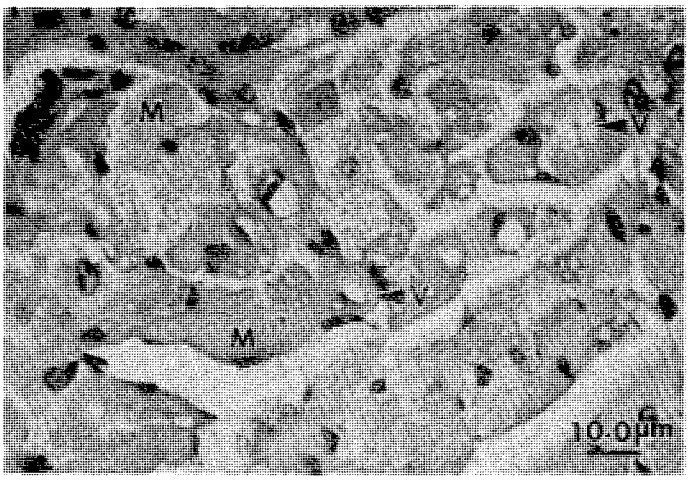

M
N
10·0μm

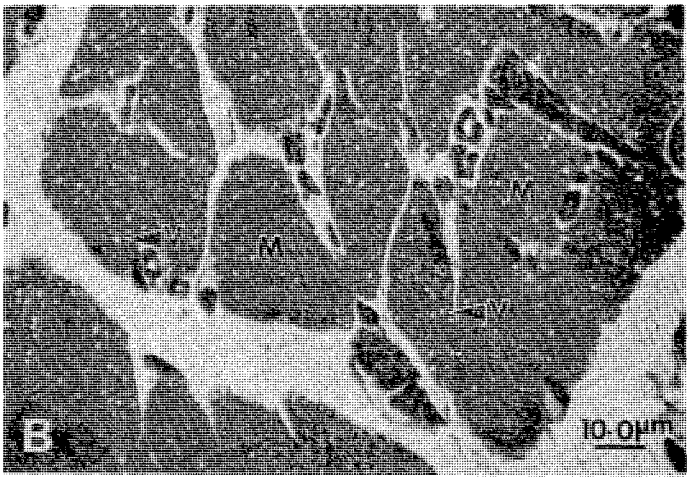
Figure 2. Light Micrographs of Cardiac Muscle from Experimental Mice Taken at Various Times After Injection of Myotoxin a

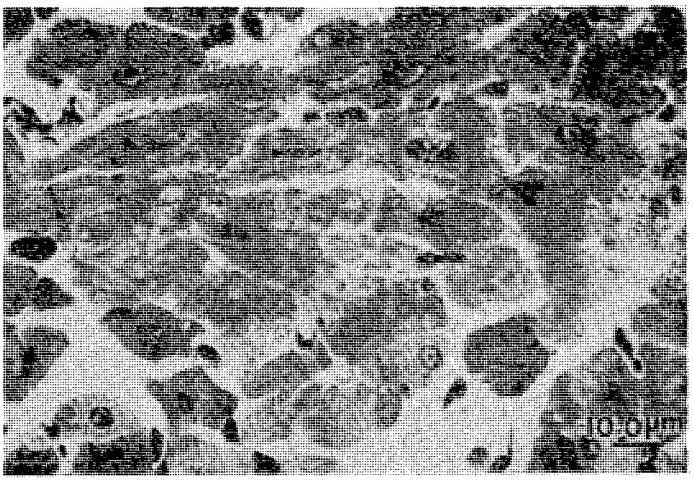
A. 24 hours post-injection, H&E-stained paraffin section. Note the swollen muscle cells, M, and numerous vacuoles, V.

B. 24 hours post-injection, plastic (1 μm) section. Note the numerous vacuoles, V, present in the swollen muscle cells, M.

C. 72 hours post-injection, plastic (1 μm) section. Note the loss of striated appearance in damaged muscle cells, arrows.


M
V
M
10.0μm


M
M
10.0μm


10.0μm

at 24 (Figure 2B) and 48 hours in sections stained with Methylene Azure II. Extensive vacuolation was evident at 72 Hours and some cells appeared to be undergoing lysis as indicated by the loss of striated appearance; entire cells were swollen and contraction bands were present in some cells (Figure 2C). Slight hemorrhage was present at all time periods examined.

Fraction 1 also induced a series of degenerative events in cardiac muscle cells. Swelling of the entire cell and complete lysis of cardiac muscle cells were observed at 24 and 48 hours. At 24 hours vacuoles were seen in the cardiac muscle cells (Figure 3A). Striations were less distinct at 48 hours and vacuoles were present in swollen cells (Figure 3B). Although vacuolation was observed at all time periods in plastic (1 μm) sections, vacuolation was not observed in the paraffin sections at any time period studied. Vacuolation produced by Fraction 1 was more pronounced at 72 hours in the swollen cardiac muscle cells (Figure 3C).

Electron Microscopy

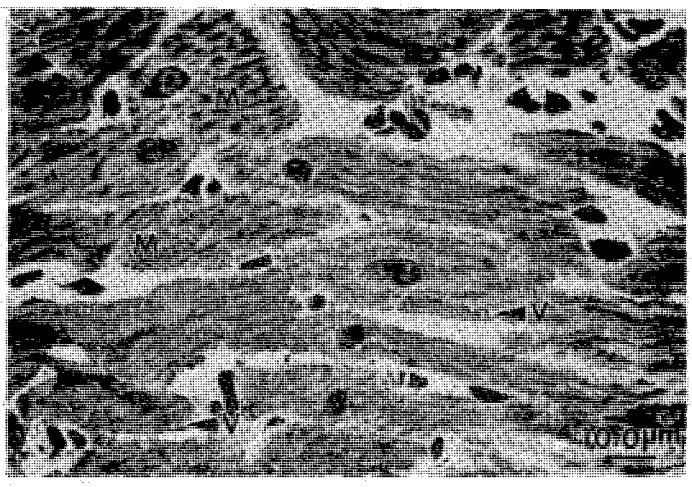
Electron microscopic examination of myocardium showed that muscle from control mice (PSS-injected) was normal in morphology for the fixation procedure used except for a slight swelling of the sarcotubular system which was observed at all time periods studied (Figure 4A). Mitochondria were also normal in appearance, as were the myofilaments comprising the sarcomeres, the length of which was as expected for contracted muscle; intercalated discs were also normal and intact (Figure 4B).

Figure 3. Light Micrographs of Cardiac Muscle from Experimental Mice Taken at Various Times After Injection of Fraction 1

A. 24 hours post-injection plastic (1 μm) section. Note the slightly swollen muscle cells, M, and presence of vacuoles, V.

B. 48 hours post-injection, plastic (1 μm) section. Note the evidence of vacuolation, V, in swollen muscle cells, M.

C. 72 hours post-injection, plastic (1 μm) section. Note vacuoles, V, in muscle cells are larger and more distinct than earlier time periods studied; some cells appeared as an amorphous mass, AM.


M
M
V
V
1.0.0µm


M
M
M
V
V
B
1.0.0µm

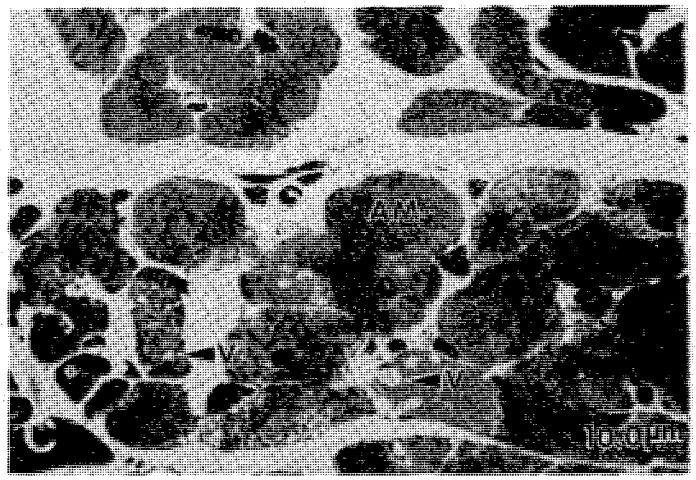
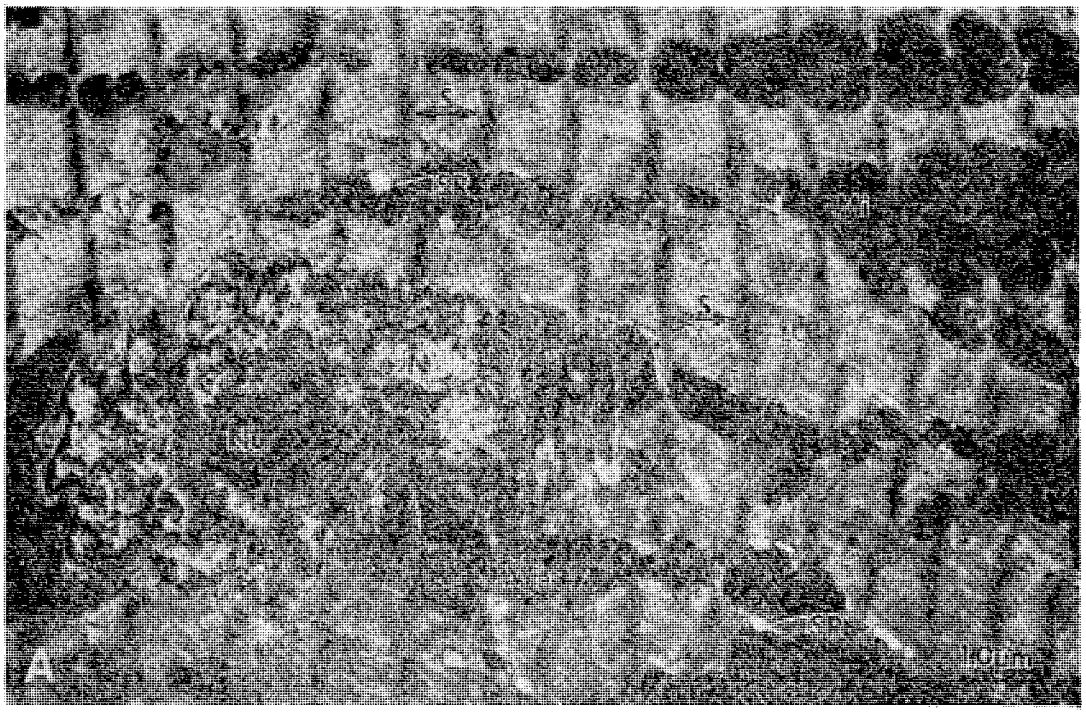
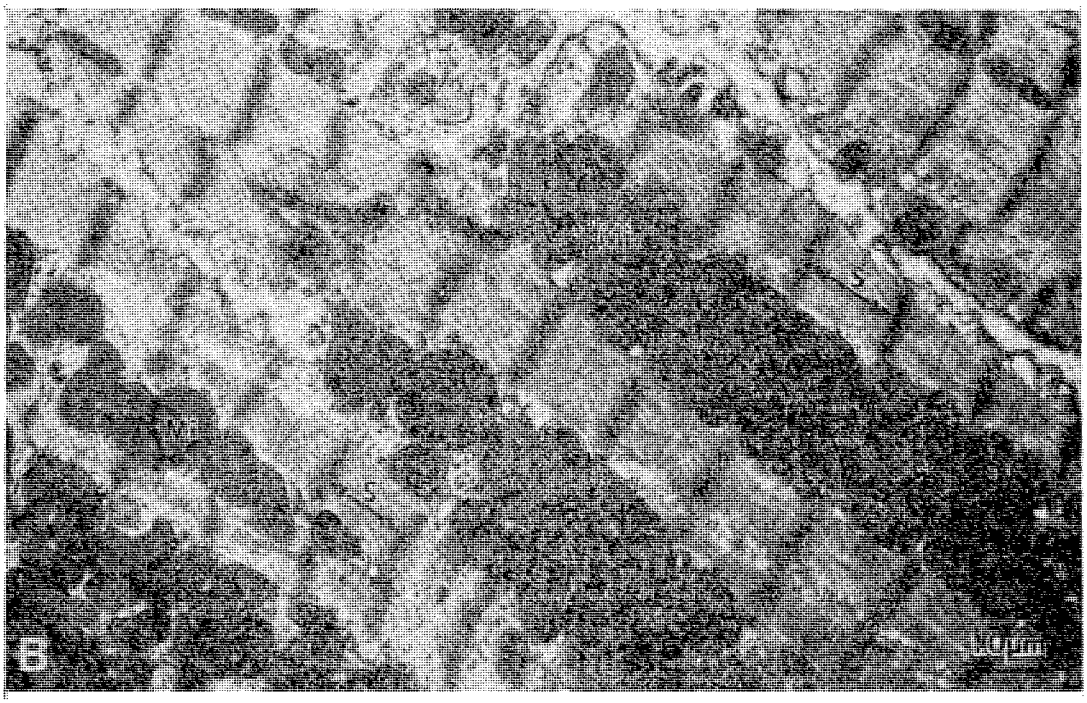

M
V
V
10.0µm

Figure 4. Electron Micrographs of Cardiac Muscle from Control Mice Taken at Various Times After Injection of Physiologic Saline Solution (PSS)

A. 72 hours post-injection. Note normal appearance of nucleus, N, containing nucleolus, Nu. Sarcomeres, S, comprised of myofilaments are of normal length for contracted muscle. Mitochondria, Mi, are normal in appearance. Slight swelling of the sarcotubular system, SR, was observed at all time periods studied.

B. 24 hours post-injection. Mitochondria, Mi, and sarcomeres, S, appear normal. Intercalated disc, in upper left hand corner of micrograph, is also normal and intact.


A
10μm


B
10μm

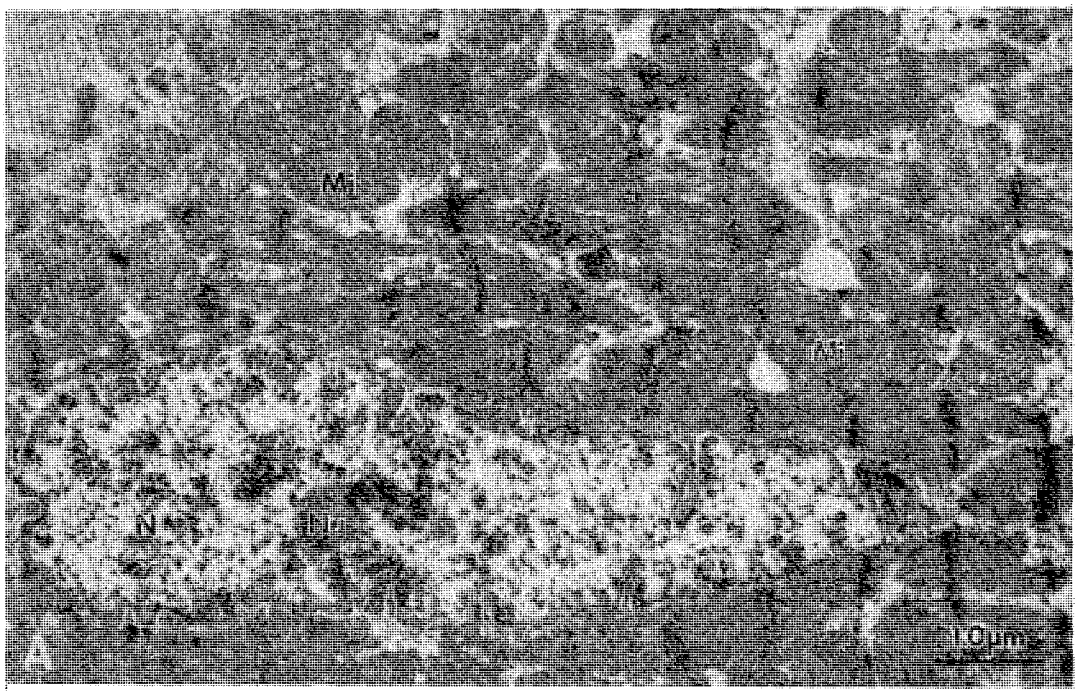
Myotoxin a induced a series of degenerative events within cardiac muscle cells beginning with slight vacuolation and swelling of the entire cell followed by myofibril and mitochondrial rupture. At 24 hours the nucleus no longer had the typical pattern of condensed chromatin but was slightly swollen; swollen mitochondria were also apparent (Figure 5A). Swollen cells containing mitochondria with ruptured cristae and disrupted myofibrils (Figure 5B) were also observed at 24 hours. At 48 hours dilatated sarcoplasmic reticulum and mitochondria approximately twice normal size were observed (Figure 6A and 6B). Membrane whorls were also evident at this time (Figure 6B). At 72 hours increased dilatation of sarcoplasmic reticulum was observed. Increased separation of myofibrils caused by swelling of the entire cell as indicated by a decrease in electron density of the sarcoplasm also occurred at 72 hours. Nucleoplasm was washed-out indicating more diffuse heterochromatin (Figure 7A); there was some apparent lipid accumulation (Figure 7A and 7B). Mitochondria containing cristae arranged in a "fingerprint" pattern were also observed (Figure 7A and 7B). Dilatation and separation of intercalated discs were also seen at this time (Figure 7B). The desmosomes of the discs were typical in appearance and intact, but dilatation occurred between the desmosomes and involved the apposed membranes of adjacent cardiac muscle cells.

Fraction 1 also induced a series of degenerative events in cardiac muscle. At 24 hours swelling of the cell and dilatation of the sarcoplasmic reticulum were observed, along with hemorrhage (Figure 8A). Sarcolemmal rupture was also observed at this time (Figure 8B). Internal changes of the cell at this time period were not prominent. At 48 hours there was increased dilatation of the sarcoplasmic reticulum and

Figure 5. Electron Micrographs of Cardiac Muscle from Experimental Mice Taken 24 Hours After Injection of Myotoxin a

A. Nucleoplasm looks "washed-out" due to swelling of nucleus, N; nucleolus, Nu, and swollen mitochondria, Mi.

B. Swelling of entire cell is indicated by loss in electron density of the sarcoplasm, SP; mitochondria, Mi, are swollen; cristae are ruptured; myofibrils, MF, are disrupted.


Mi
Mi
N
1.0μm

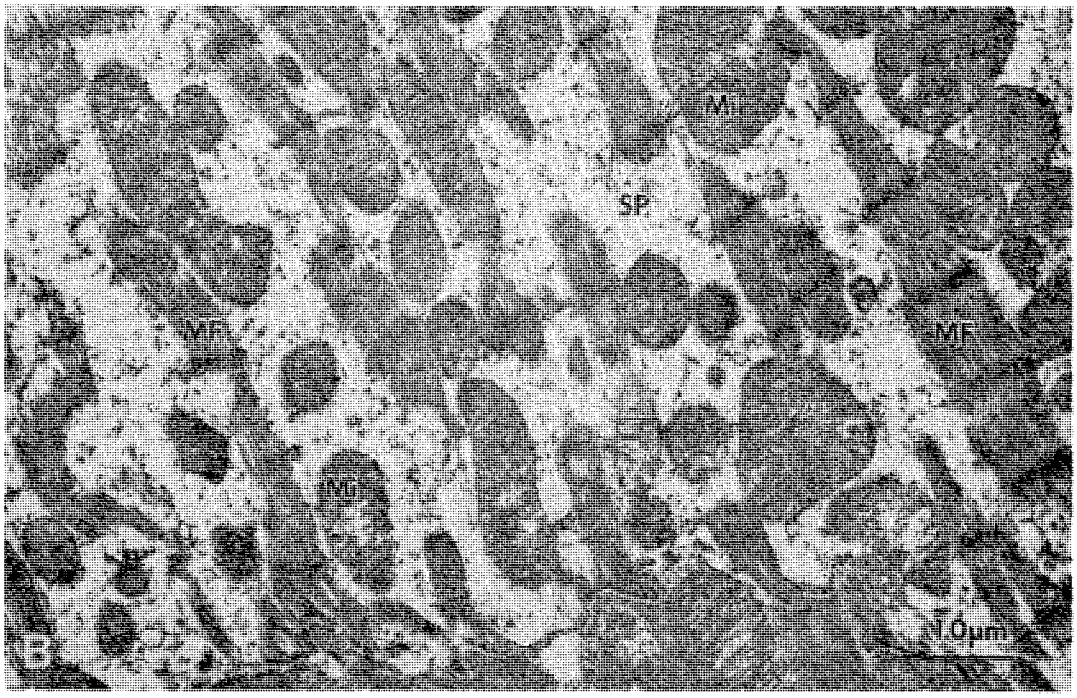
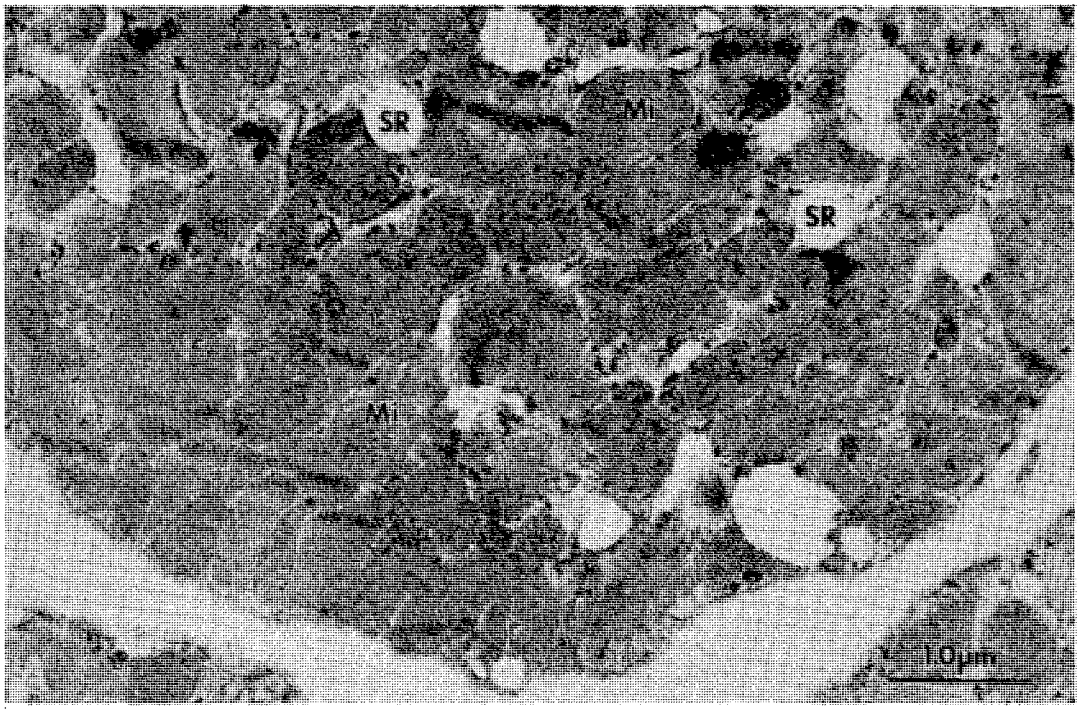

Mi
SP
MF
MF
Mi
1.0μm

Figure 6. Electron Micrographs of Cardiac Muscle from Experimental Mice Taken 48 Hours After Injection of Myotoxin a

A. Note dilatated sarcoplasmic reticulum, SR, and very large mitochondria, Mi, containing ruptured cristae.

B. Swollen areas of sarcoplasm containing swollen sarcoplasmic reticulum, SR, membrane whorls, W, and disrupted myofibrils, MF.


SR
M
SR
M
1.0μm

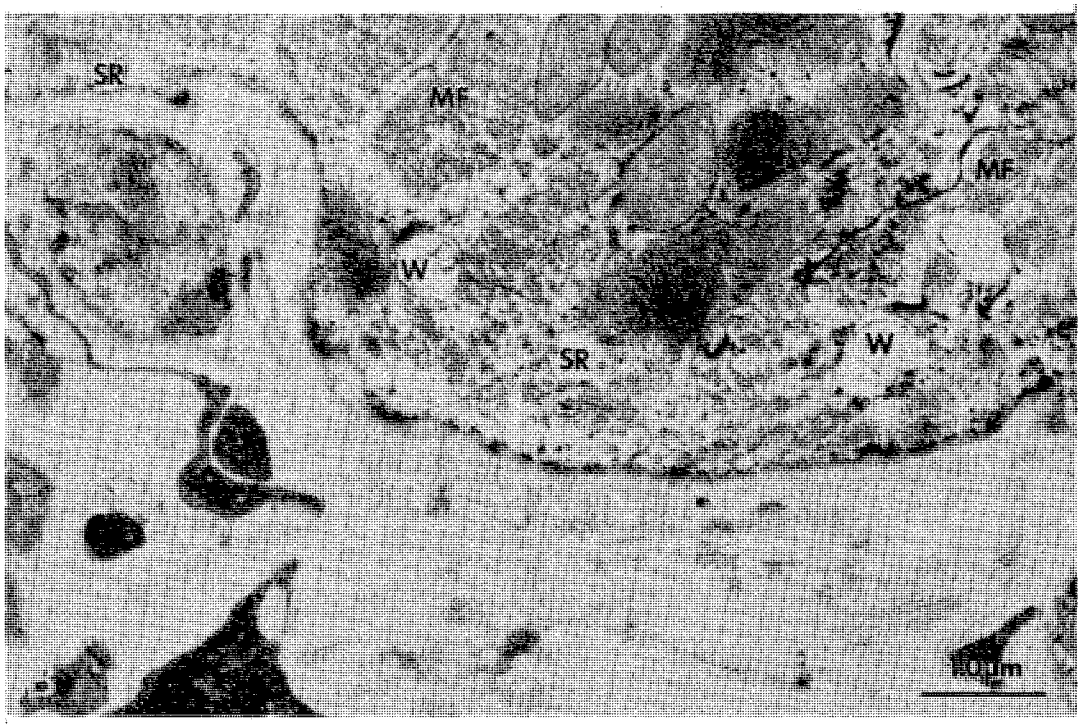
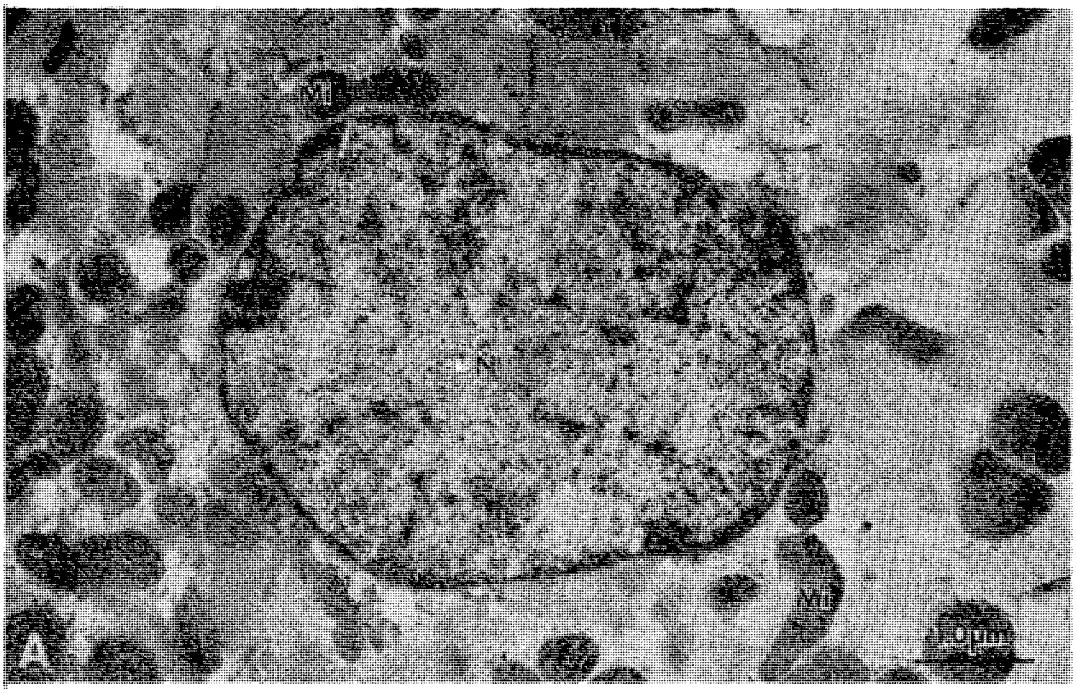

SR
MF
MF
W
SR
W
1.0μm

Figure 7. Electron Micrographs of Cardiac Muscle from Experimental Mice Taken 72 Hours After Injection of Myotoxin a

A. Nucleus, N, is slightly enlarged. Note mitochondria, Mi, containing cristae in "fingerprint" pattern.

B. Dilatation, D, of intercalated disc occurs between desmosomes, DE, which are intact; mitochondria, Mi, containing cristae arranged in "fingerprint" pattern and some apparent lipid accumulation, L, are also present.


A
1.0μm

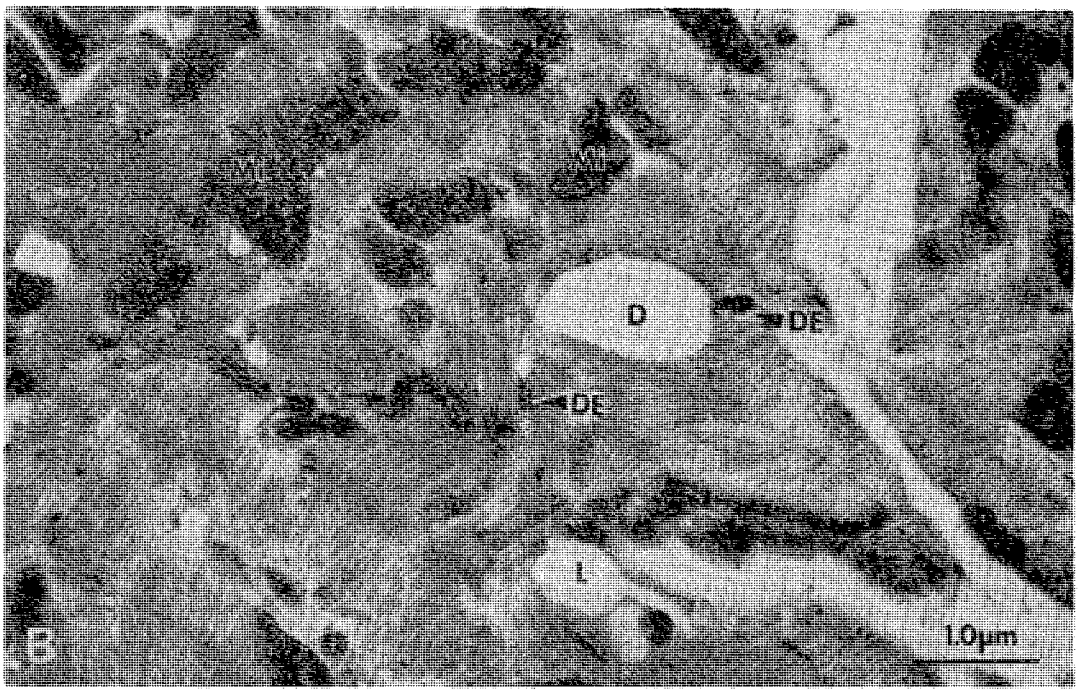
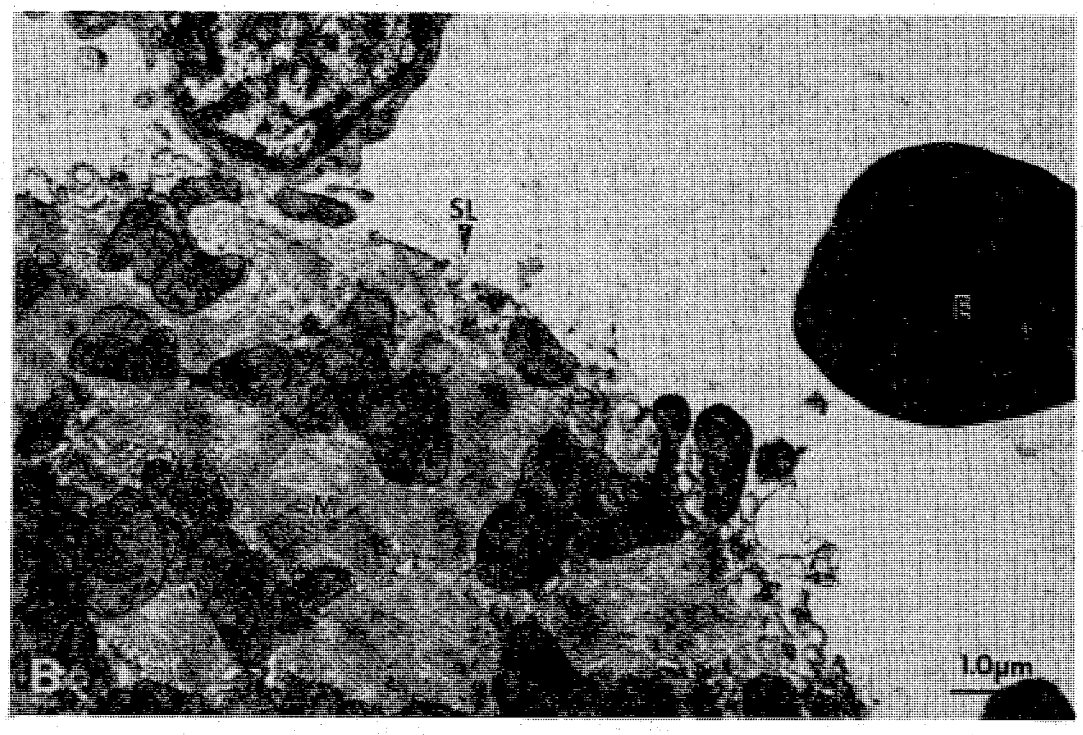

D
DE
DE
L
B
1.0μm

Figure 8. Electron Micrographs of Cardiac Muscle from Experimental Mice Taken 24 Hours After Injection of Fraction 1

A. Note swelling of the cell, M, and dilatations of the sarcoplasmic reticulum, SR. Hemorrhage is evident by presence of erythrocyte, E, and hemolyzed erythrocyte, HE, in the endomysium.

B. M, muscle cell; note sarcolemmal rupture, SL. Hemorrhage indicated by presence of erythrocyte, E, in the endomysium.


E
SR
HB
SR
1.0μm

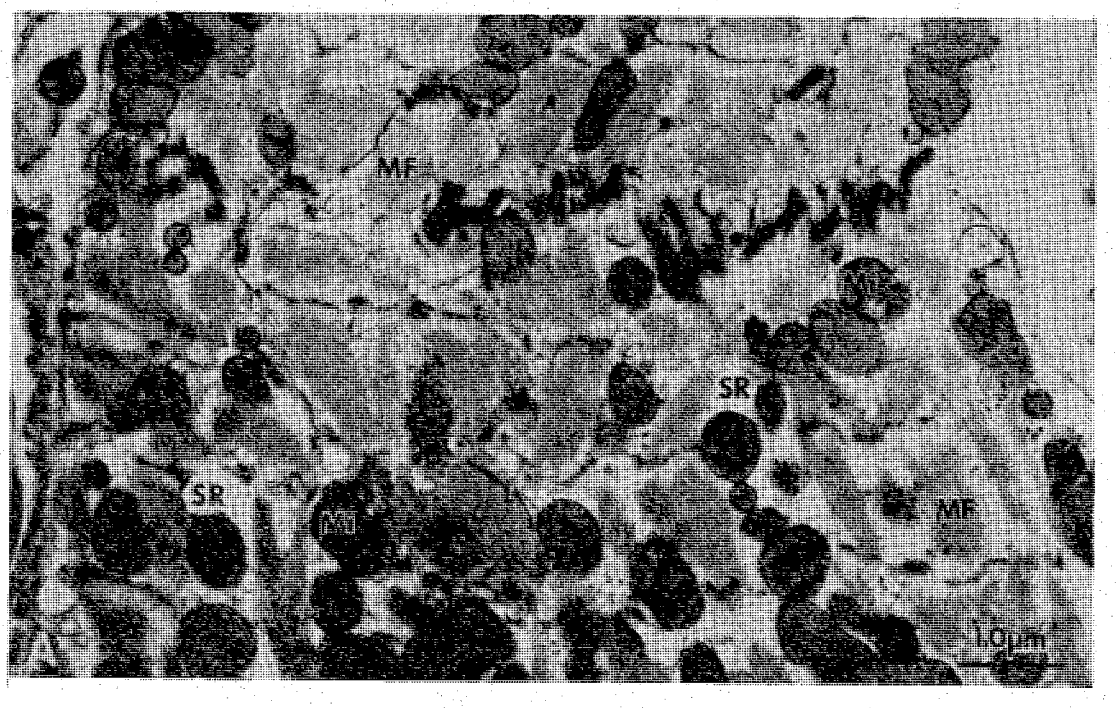

SL
E
M
1.0μm

myofibril degradation (Figure 9A). Swollen mitochondria and margination of nuclear chromatin coupled with a slight dilatation of the outer membrane of the nuclear envelope (Figure 9B) were also observed at this time. Changes occurring at 72 hours included swelling of the entire cell and myofilament degradation (Figure 10A); contraction bands were also present as were mitochondria approximately twice normal size (Figure 10A and 10B). Dilatation and separation of intercalated discs, and membrane whorls were also observed (Figure 10B). The desmosomes were typical in appearance and intact with the dilatation occurring between the desmosomes and involving the apposed membranes of the two cardiac muscle cells involved in the intercalated discs.

Figure 9. Electron Micrographs of Cardiac Muscle from Experimental Mice Taken 48 Hours After Injection of Fraction 1

A. Note dilatation of sarcoplasmic reticulum, SR, and myofibril breakdown, MF. Intercalated discs, across top of micrograph and mitochondria, Mi, are also present.

B. Note margination of nuclear chromatin, NC, and slight dilatation of the nuclear envelope, NE; sarcoplasmic reticulum, SR, is dilatated, and mitochondria, Mi, are slightly swollen.

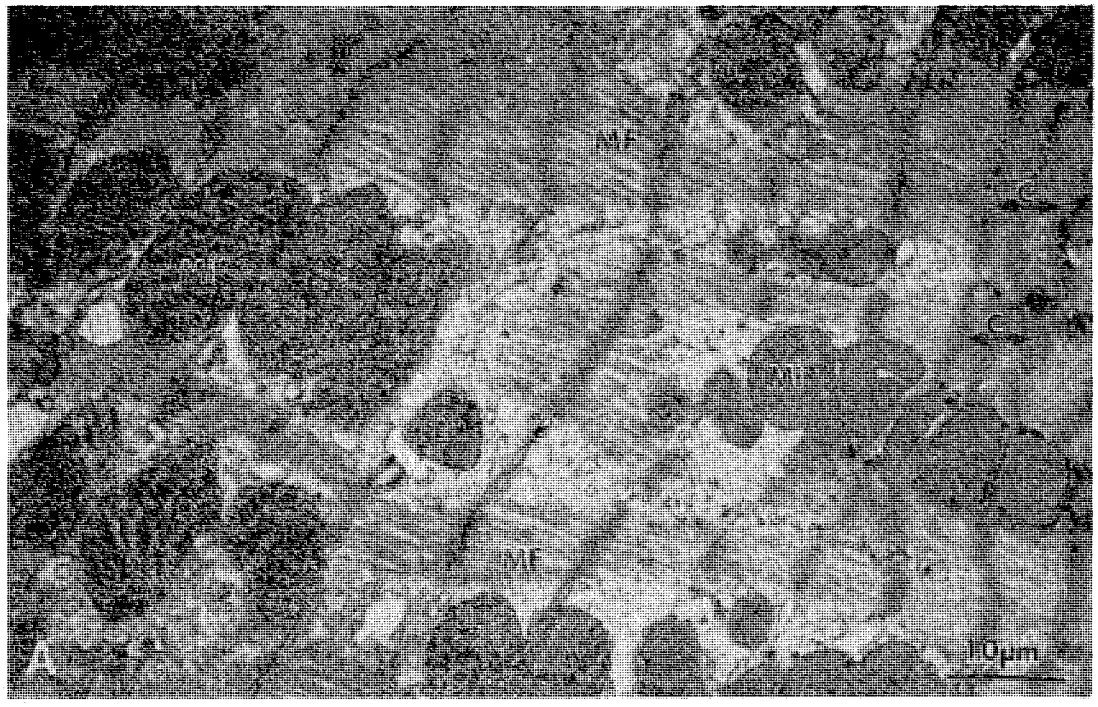

MF
SR
SR
MF
1.0μm

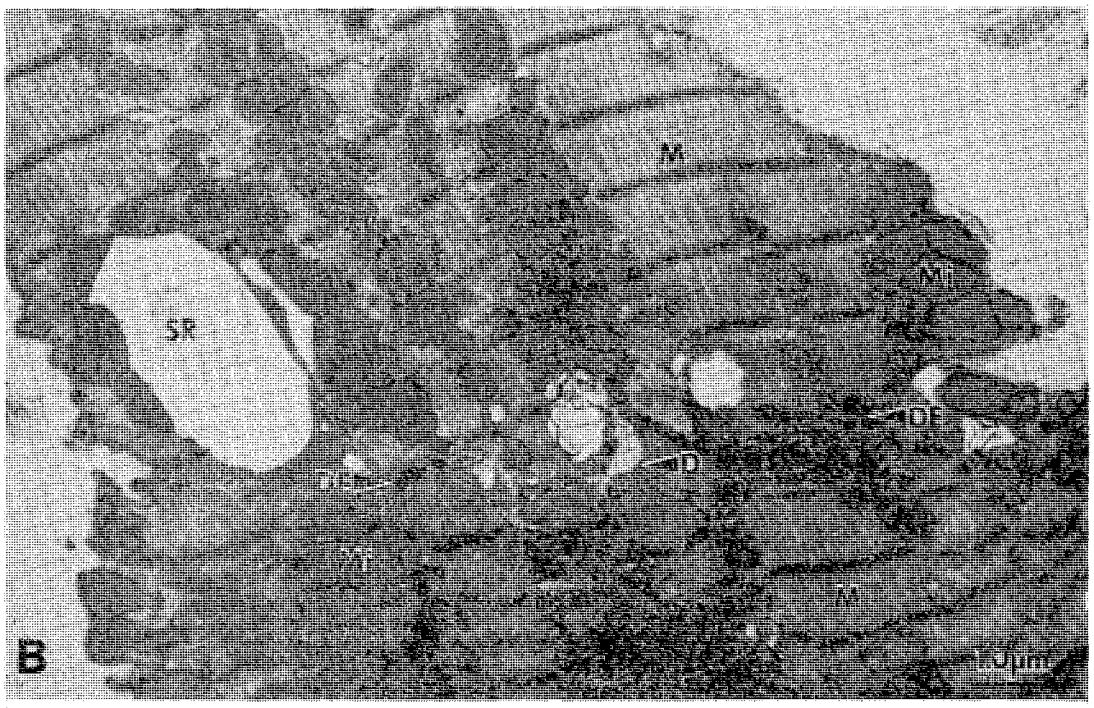

NE
NC
SR
SR
1.0μm

Figure 10. Electron Micrographs of Cardiac Muscle from Experimental Mice Taken 72 Hours After Injection of Fraction 1

A. Very large swollen mitochondria, Mi, and contraction bands, C, are present as is myofibril breakdown, MF.

B. Note area of junction between two cardiac muscle cells, M; dilatation, D, of intercalated disc between desmosomes, DE; mitochondria, Mi, are enlarged; and sarcoplasmic reticulum, SR, is dilatated.


MF
MF
A
10μm


M
SR
B
10μm

CHAPTER IV

DISCUSSION

The pathological changes induced in cardiac muscle by two myotoxic components isolated from rattlesnake (*Crotalus viridis viridis*) venom were studied with both light and electron microscopy. Lesions produced by both myotoxic components were found to be focal in occurrence in every region examined of the ventricular wall of the heart at each time period examined. The two myotoxic components induced a series of degenerative events in cardiac muscle cells beginning with diltation of sarcoplasmic reticulum followed by swelling of the entire cell, mitochondrial rupture, and eventually myofibril degradation. Dilatation and separation of intercalated discs also occurred. The effects produced by myotoxin *a* and Fraction 1 in cardiac muscle cells were similar but occurred at different rates possibly due to a difference in toxicity of the two myotoxic components.

The effects produced by myotoxin *a* and Fraction 1 in cardiac muscle cells were similar but not identical to those reported for myotoxin *a* in skeletal muscle (Ownby *et al.*, 1976). The pathological reactions which we observed with both myotoxic components in cardiac muscle occurred at later time periods than reported for myotoxin *a* in skeletal muscle (Ownby *et al.*, 1976). Differences in the response to injury by cardiac muscle versus skeletal muscle have been reported in at least one

other instance. According to Ketelsen et al. (1973) pathological changes occurring in experimental steroid myopathy appeared at a later time period in cardiac muscle than in skeletal muscle. This difference in reaction time could be due to the morphological and physiological differences of the two muscle types. In addition to the time differences, swelling of the entire cardiac muscle cells was induced by both myotoxic components. However, swelling of the entire cell was not observed in skeletal muscle (Ownby et al., 1976).

Several studies have been conducted testing the effects of cardiotoxins isolated from the venoms of snakes, scorpions and jellyfish. Cardiotoxin from Formosan Cobra (Naja naja atra) venom produced blockage of axon conduction (Chang et al., 1972) and induced muscle contracture by release of membrane calcium (Lin Shiau et al., 1976). Thailand Cobra (Naja naja siamensis) venom caused irreversible depolarization of cultured embryonic heart cells (Arms and McPheeters, 1975). Cardiotoxin isolated from Mojave rattlesnake (Crotalus scutulatus scutulatus) venom (molecular weight is 12,000 and isoelectric point is 4.7) showed effects on the cardiovascular system indicating bradycardia and resulting in cardiovascular collapse and respiratory failure (Bieber et al., 1975). Scorpion (Tityus serrulatus) venom also showed various effects on the cardiovascular system including bradycardia, tachycardia, atrioventricular (A-V) block, hypertension and hypotension (Freri-Maia et al., 1974). Jellyfish (Cyanes capillata) venom produced arrhythmias, loss of contractility and contracture in isolated rat heart cells (Walker, 1977; Walker et al., 1977). Most of the studies to date on cardiotoxins have been either biochemical, pharmacological, physiological or toxicological. However, there has been one electron microscopic

study reporting the effects of a small basic protein toxin isolated from Eastern diamondback rattlesnake (Crotalus adamanteus) venom (Abel et al., 1973). The molecular weight of this basic protein was first determined by gel filtration to be 3,256, but upon further filtration and sedimentation analyses the molecular weight was found to be 10,900. The discrepancy has not been resolved (Bonilla and Fiero, 1971; Bonilla et al., 1972). Nevertheless, the toxin induced myocardial ischemia resulting in Z band disruption and increased lipid droplet formation (Abel et al., 1973); pathological reactions not observed with our two myotoxic components. Alterations caused by this toxin in calcium availability to cell membranes, particularly of the muscle were reported by Bonilla et al. (1972).

The lesions produced by myotoxin a and Fraction 1 in cardiac muscle were similar to the lesions observed in several clinical or experimental cardiac muscle conditions. For example, contraction bands were observed in experimental myocardial infarction (Fishbein et al., 1978). Stemmer et al. (1973, 1975) reported swelling of the entire cell, mitochondrial distortion, disruption and dissolution of cristae in induced normothermic anoxia for 60 minutes, with longer periods of normothermic anoxic arrest resulting in disruption and dissolution of myofibrils. Dilatation of sarcoplasmic reticulum was also observed in several cardiac diseases including degenerative cardiac hypertrophy, aortic and mitral valvular disease, and two congestive cardiomyopathies-alcoholic cardiopathy and cobalt-beer cardiopathy (Maron et al., 1975; Maron and Ferrans, 1978). Mitochondrial changes similar to those induced by myotoxin a and Fraction 1 were also reported for experimental and clinical cardiac muscle conditions. The destruction of mitochondria

which occurred in experimentally induced topical hypothermia without coronary perfusion for 2 hours (Stemmer et al., 1973; Stemmer et al., 1975) was very similar to what we observed with both myotoxic components. Swelling of mitochondria and loss of cristae occurred in alcoholic cardiopathy and change in mitochondria size occurred in cobalt-beer cardiopathy (Maron and Ferrans, 1978). Maron and Ferrans (1978) also reported swelling and loss of cristae in aortic and mitral valvular disease very similar to that observed by us with both myotoxic components. The membrane whorls observed in cardiac muscle with both myotoxic components were also observed in congenital heart disease by Maron and Ferrans (1978). Congenital heart disease, alcoholic cardiopathy and cobalt-beer cardiopathy (Maron and Ferrans, 1978) all produced myofibrillar breakdown similar to that which we observed in cardiac muscle cells produced by myotoxin a and Fraction 1. Maron et al. (1975; 1978) reported dilatation and separation of intercalated discs in degenerative cardiac hypertropy very similar to what we observed with both myotoxic components in cardiac muscle cells.

On the other hand, some of the effects produced by myotoxin a and Fraction 1 in cardiac muscle cells were different than those reported for some cardiac muscle diseases. Increased Z band material formation (degenerative cardiac hypertrophy and congenital heart disease), dissolution of Z band material (aortic and mitral valvular disease), discontinuity of sarcomeres (aortic and mitral valvular disease), and loss of contractile elements (degenerative cardiac hypertrophy) were not induced by either myotoxin. Hemorrhage was produced by both myotoxic components in cardiac muscle but was not reported for any of these cardiac muscle diseases. Fraction 1 produced sarcolemmal rupture in

cardiac muscle that was not observed in reported cardiac muscle diseases (Maron et al., 1975; Maron and Ferrans, 1978).

Increased amounts of sarcoplasmic reticulum and very small mitochondria were characteristic of congenital heart disease (Maron and Ferrans, 1978) whereas, both myotoxic components resulted in very large swollen mitochondria. The swollen mitochondria produced by myotoxin a in cardiac muscle containing cristae arranged in a "fingerprint" pattern was not observed in these cardiac muscle diseases. It has been reported (Palmer et al., 1977) that subsarcolemmal and interfibrillar mitochondria have different biochemical properties, however we observed both types of mitochondria in myotoxin a-injected cardiac muscle to contain cristae arranged in a "fingerprint" pattern. Myotoxin a may act directly on the biochemically different mitochondria upon entrance into the cell. A dramatic increase in lipid droplet, lysosome and lipofusion granule number and the presence of intramitochondrial concentric lamellae were not observed with either myotoxic component, although the damages were reported as characteristic of alcoholic cardiopathy (Maron and Ferrans, 1978). Reported characteristics of cobalt-beer cardiopathy (Maron and Ferrans, 1978) were an increase in cytoplasmic glycogen and intramitochondrial localization of glycogen neither of which was observed with either myotoxic component.

The swelling of sarcoplasmic reticulum, mitochondria, nuclei and entire cardiac muscle cells induced by the myotoxic components is a common response of mammalian cells to injury (Ginn et al., 1968). These changes could result from a loss in cell volume control due to damage to the plasma membrane. For example, if the myotoxin inhibited the Na-K ATPase as does ouabain this would lead to a loss in cell

volume control and to the ultrastructural changes similar to those reported here. However, Knight (1978) demonstrated that myotoxin a has a different physiologic effect on mouse skeletal muscle than does ouabain, although the morphologic changes were similar.

Since the primary effect of the myotoxins seems to be on the sarcoplasmic reticulum and subsequently on the mitochondria and myofilaments, the ultrastructural changes observed could be due to changes in Ca^{2+} levels in the sarcoplasm. Duncan (1978) has recently proposed that muscle damage in various muscle diseases as well as in experimentally-induced muscle lesions is due to very high levels of calcium in the sarcoplasm. The intracellular Ca^{2+} concentration may be raised in various ways. The divalent cation ionophore A23187 apparently causes release of Ca^{2+} from the sarcoplasmic reticulum directly (Statham et al., 1976; Publicover et al., 1978), whereas caffeine causes a Ca^{2+}-induced release of Ca^{2+} from the sarcoplasmic reticulum indirectly (Duncan and Smith, 1978). The end result in either case is an increase in the concentration of calcium in the sarcoplasm. This can lead to degradation of myofilaments by activation of Ca^{2+}-activated proteases which breakdown myofilaments; by causing the release of hydrolases from the lysosomal system which would degrade myofilaments; or by activation of enzymes that cause disassembly of the polymers which compose the myofilaments (Duncan and Smith, 1978).

Therefore, the myotoxins could cause, by one of several mechanisms, an increase in Ca^{2+} in the sarcoplasm which would lead to myofilament degradation. Additional studies are necessary to determine the exact initial site of action of the myotoxins.

Observations at the light and electron microscopic levels has revealed that myotoxin a and Fraction 1 induced a series of degenerative events within cardiac muscle beginning with slight vaculoation and swelling of the entire cell followed by myofibril and mitochondrial rupture. The lesions produced by both myotoxins in cardiac muscle cells were similar but not identical to those reported for myotoxin a in skeletal muscle (Ownby et al., 1976). Myotoxin a and Fraction 1 produced lesions in cardiac muscle that were similar to those observed in several clinical or experimental cardiac muscle conditions.

REFERENCES

Abel, J. H., Jr., Nelson, A. W. and Bonilla, C. A.: Crotalus adamanteus basic protein toxin: electron microscopic evaluation of myocardial damage. Toxicon 11: 59-63, 1973.

Arms, K. and McPheeters, D.: Sensitivity of cultured embryonic heart cells to cardiotoxin obtained from Naja naja siamensis venom. Toxicon 13: 333-338, 1975.

Bieber, A. L., Tu, T. and Tu, A. T.: Studies of an acidic cardiotoxin isolated from the venom of Mojave rattlesnake (Crotalus scutulatus). Biochmica et Biophysica Acta 400: 178-188, 1975.

Bonilla, C. A. and Fiero, M. K.: Comparative biochemistry and pharmacology of salivary galnds secretions. II Chromatographic separation of the basic proteins from some North Ameriacan rattlesnake venoms. Journal of Chromatography 56: 253-263, 1971.

Bonilla, C. A., Fiero, M. K. and Frank, L. P.: Isolation of a basic protein neurotoxin from Crotalus adamanteus venom. IN: Toxins of Animal and Plant Origin Volume 1. deVries, A. and Kochva, E., New York, 1972, 343-359.

Cameron, D. and Tu, A. T.: Characterization of myotoxin a from the venom of prairie rattlesnake (Crotalus viridis viridis). Biochemistry 16: 2546-2553, 1977.

Chang, C. C., Chuang, S. T., Lee, C. V. and Wei, J. W.: Role of cardiotoxin and phopholipase A in the blockade of nerve conduction and depolarization of skeletal muscle induced by cobra venom. British Journal of Pharmacology 44: 752-764, 1972.

Duncan, C. J.: Role of intracellular calcium in promoting muscle damage: a strategy for controlling the dystrophic condition. Separatum Experientia 34: 1531-1535, 1978.

Duncan, C. J. and Smith, J. L.: The action of caffeine in promoting ultrastructural damage in frog skeletal muscle fibres. Evidence for the involvement of the calcium-induced release of calcium from the sarcoplasmic reticulum. Archives of Pharmacology 305: 159-166, 1978.

Endo, M.: Calcium release from the sarcoplasmic reticulum. Physiological Reviews 57(1): 71-108, 1977.

Fishbein, M. C., Maclean, D. and Maroko, P. R.: Experimental myocardial infraction in the rat. American Journal of Pathology 90: 57-70, 1978.

Freire-Maia, L., Pinto, G. I. and Franco, I.: Mechanism of the cardiovascular effects produced by pruified scorpion toxin in the rat. Journal of Pharmacology and Experimental Therapeutics 188(1): 207-213, 1974.

Ginn, F. L., Shelburne, J. D. and Trump, B. J.: Disorders of cell regulation. I. Effects of inhibition of plasma membrane adenosine triphosphatase with ouabain. American Journal of Pathology 53: 1041-1071, 1968.

Homma, M. and Tu, A. T.: Morphology of local tissue damage in experimental snake envenomation. British Journal of Experimental Pathology 52: 538-542, 1971.

Huang, T. T., Blackwell, S. J. and Lewis, S. R.: Hand deformities in patients with snakebite. Plastic and Reconstructive Surgery 2(1): 32-36, 1978.

Ketelsen, U. P., Freund, M. E. and Struck, E.: Pathomorphological changes in steroid myopathy. Ultrastructural changes within the plasmalemma of skeletal and cardiac muscle cells as compared to the intracellular reaction. Beitrage zur Pathologie 153(2): 133-164, 1973.

Klauber, L. M.: Rattlesnakes. Their habits, life histories, and influences on mankind. University of California Press, Berkeley, 829-950, 1972.

Knight, J. P.: A comaprative study on the effects of ouabain, rattlesnake (Crotalus viridis viridis) venom, and myotoxin a on the contractile response and ultrastructure of rat skeletal muscle. Masters thesis, Oklahoma State University, July, 1978.

Lin Shiau, S. Y., Huang, M. C. and Lee, C. Y.: Mechanism of action of cobra cardiotoxin in the skeletal muscle. Journal of Pharmacology and Experimental Theurapeutics 196(3): 758-770, 1976.

Maron, B. J., Ferrans, V. J. and Roberts, W. C.: Ultrastructural features of degenerated cardiac muscle cells in patients with cardiac hypertrophy. American Journal of Pathology 79(3): 387-434, 1975.

Maron, B. J. and Ferrans, V. J.: Ultrastructural features of hypertrophied human ventricular myocardium. Progress in Cardiovascular Diseases 21(3): 207-238, 1978.

Minton, S. A. and Minton, M. R.: Venomous Snakes. Charles Scribner's Sons, New York, 1969.

Ownby, C. L., Kainer, R. A. and Tu, A. T.: Pathogenesis of hemorrhage induced by rattlesnake venom. An electron microscopic study. American Journal of Pathology 76: 401-404, 1974.

Ownby, C. L.: Pathogenesis and chemical treatment of hemorrhage induced by rattlesnake venom. Doctoral thesis, Colorado State University, August, 1975.

Ownby, C. L., Cameron, D. and Tu, A. T.: Isolation of myotoxic component from rattlesnake (Crotalus viridis viridis) venom. Electron microscopic analysis of muscle damage. American Journal of Pathology 85: 145-166, 1976.

Palmer, J. W., Tandler, B. and Hoppel, C. L.: Biochemical properties of sarcolemmal and interfibrillar mitochondria isolated from rat cardiac muscle. Journal of Biological Chemistry 252(23): 8731-8739, 1977.

Publicover, S. J., Duncan, C. J. and Smith, J. L.: The use of A23187 to demonstrate the role of intracellular calcium in causing ultrastructural damage in mammalian muscle. Journal of Neuropathology and Experimental Neurology 37: 544-557, 1978.

Richardson, K. C., Jarrett, L. and Finke, E. H.: Embedding in epoxy resins for ultrathin sectioning in electron microscopy. Stain Technology 35: 313-323, 1960.

Somlyo, A. V.: Bridging structures spanning the junctional gap at the triad of skeletal muscle. Journal of Cell Biology 80: 743-750, 1979.

Sommer, J. R. and Johnson, E. A.: Cardiac Muscle. A comparative study of purkinje fibers and ventricular fibers. Journal of Cell Biology 36(3): 497-526, 1968.

Sommer, J. R., Steere, R. L., Johnson, E. A. and Jewett, P. H.: Ultrastructure of cardiac muscle. A comparative review with emphasis on the muscle fibers of the ventricles. IN: Hibernation and hypothermia, perspectives and challenges. Elsevier, New York, 291-355, 1972.

Statham, H. E., Duncan, C. J. and Smith, J. L.: The effects of the ionophore A23187 on the ultrastructure and electrophysiological properties of frog skeletal muscle. Cell Tissue Research 173: 193-209, 1976.

Stemmer, E. A., McCart, P., Stanton, W. W., Thibault, W., Dearden, L. S. and Connolly, J. E.: Functional and structural alterations in the myocardium during aortic cross-clamping. Journal of Thoracic and Cardiovascular Surgery 66(5): 754-770, 1973.

Stemmer, E. A., Joy, I., Aronow, W. S., Thibault, W., McCart, P. and Connolly, J. E.: Preservation of myocardial ultrastructure. Journal of Thoracic and Cardiovascular Surgery 70(4): 666-676, 1975.

Stirnger, J. M., Kainer, R. A. and Tu, A. T.: Myonecrosis induced by rattlesnake venom. An electron microscopic study. American Journal of Pathology 67: 127-140, 1972.

Venable, J. H. and Coggeshall, R.: A simplified lead citrate stain for use in electron microscopy. Journal of Cell Biology 25: 407-408, 1965.

Walker, M. J.: The cardiac actions of a toxin-containing material from the jellyfish Cyanea capillata. Toxicon 15(1): 15-27, 1977.

Walker, M. J., Martinez, T. T. and Godin, D. V.: Investigations into the cardiotoxicity of a toxin from the nematocysts of the jellyfish, Cyanea capillata. Toxicon 15(4): 339-346, 1977.

VITA

LaVeta Carole Bennett

Candidate of the Degree of

Master of Science

Thesis: AN ELECTRON MICROSCOPIC ANALYSIS OF CARDIAC MUSCLE MYONECROSIS INDUCED BY TWO MYOTOXIC COMPONENTS ISOLATED FROM RATTLESNAKE (*CROTALUS VIRIDIS VIRIDIS*) VENOM

Major Field: Physiological Sciences

Biographical:

Personal Data: Born in Oklahoma City, Oklahoma, December 30, 1953, the daughter of Exie C. and Tony Bennett.

Education: Graduated from Putnam City High School in May, 1972; received a Bachelor of Science degree from Central State University in May, 1977, with a double major in Chemistry and Biology; completed requirements for the Master of Science degree at Oklahoma State University in July, 1979.

Professional Experience: Central State University: Laboratory Assistant, September, 1975 to May, 1977; Oklahoma State University: Research Assistant, June, 1978 to August 1978; Teaching Assistant, September, 1978 to December, 1978; Research Assistant, January, 1979 to May, 1979.