NOTES ABOUT ULTRASTRUCTURAL TESTICULAR CHANGE IN MICE INDUCED BY THE VENOM OF SOUTHAMERICAN MAPANARE SNAKE (*Bothrops colombiensis* Hallowell, 1845)

Notas acerca de los cambios ultraestructurales de testículo en ratones inducidos por el veneno de la serpiente mapanare suramericana (*Bothrops colombiensis* Hallowell, 1845)

Alexis Zárraga, Luisa Velásquez, Miriam Strauss and Alexis Rodríguez-Acosta

Immunochemistry Section and Cellular Biology Section, Tropical Medicine Institute, Universidad Central de Venezuela, Apartado 47423, Caracas 1041, Venezuela. E-mail: rodriguf@camelot.rect.ucv.ve

ABSTRACT

In Venezuela, *Bothrops colombiensis* is responsible for about 80% of the bites by venomous snakes. The aim of this study was to determine the testicular ultrastructural changes produced by the toxic aggression of *Bothrops colombiensis* venom. Ten mice were inoculated intramuscularly with crude venom (2.5 mg /kg weight). Groups of 5 mice were sacrificed at 1 and 48 hours after venom inoculation (the control group was inoculated with saline solution). Samples of testicular tissue were extracted and immediately fixed by routine procedures and then processed for observation in a Hitachi H-300 transmission electron microscope. Ultrastructural changes in testicular tissue showed mitochondria changes of Sertoli cells, acrosomal membranes of the spermatids, and spermatozoids in their different stages. Comparing mitochondria from exposed animals versus control group oedematous mitochondria with lysis of the cristae, presenting a poor electronics density were evidenced. Mitochondria were inserted in a cellular debris environment. Lesions of the acrosomes were characterized by alterations of the external acrosomal membrane, intense separation of the internal acrosomal membrane and nuclear band, severe alterations at sperm tail level, characterized by a large submembranal space in contrast with controls, where the close vicinity between the membrane and mitochondria of the tail of spermatide was evident. The mitochondrial swelling in other biological systems is related to an ionic deregulation (Ca^{2+}), which in this particular case could be assumed that the venom interact with the membrane, altering architecture and losing function as barrier.

Key words: *Bothrops colombiensis*, mapanare, testicle, ultrastructure, venom.

RESUMEN

El *Bothrops colombiensis* se responsabiliza por el 80% de las mordeduras de serpientes venenosas en Venezuela. El propósito de este estudio fue el determinar los cambios ultrastructurales en testículo de ratón producidos por la agresión tóxica del veneno de *Bothrops colombiensis*. Dos grupos de 5 ratones se inocularon intramuscularmente con el veneno crudo (2.5 mg/kg de peso) y se sacrificaron a las 1 y 48 horas después de la inoculación del veneno (el grupo control se inoculó con solución salina). Las muestras de tejido testicular fueron inmediatamente fijadas y procesadas por procedimientos de rutina para su observación en un microscopio electrónico de transmisión Hitachi H-300. Los cambios ultraestructurales del testículo inoculado incluían alteraciones mitocondriales de las células de Sertoli y de las membranas acrosomales de las espermatides. Se observaron mitocondrias edematosas con lisis de las crestas y una pobre densidad electrónica. Las lesiones de los acrosomas se caracterizaron por cambios de la membrana acrosomal externa, evidenciado por separación intensa de la membrana acrosomal interna y la banda nuclear; las alteraciones severas en el espermatozoide representadas por un aumento del espacio submembranal, donde la vecindad cercana entre la membrana y las mitocondrias de la cola de la espermátide era evidente. La tumefacción mitocondrial en otros sistemas biológicos se ha relacionado a una deregulación iónica (Ca^{2+}), pensándose que el veneno obra sobre la membrana, modificando su arquitectura y perdiendo su función como barrera.

Palabras clave: *Bothrops colombiensis*, testículo, ultraestructura, veneno.

Recibido: 11/ 11 / 02. Aceptado: 12 / 07 / 03.

INTRODUCTION

Venomous snakes in Venezuela are responsible for more than 5000 annually reported accidents. Approximately 80% are caused by *Bothrops colombiensis* [7]. The bothropic venom possesses mainly three activities: proteolysis, clotting and haemorrhage. Moreover, at distance it also can produce organ pathologies until now non described [3].

Testicle from the morphological and functional point of view is a organ with two functions: Leydig interstitial cells are responsible for the testosterone biosynthesis and secretion (secretory function) and the seminiferous tubules of the spermatogenesis (cytogenetic function) [2]. The purpose of this study was to determine the mice testicular ultrastructural changes caused by the toxic aggression of *Bothrops colombiensis* venom, which produce damage to testicular cells viability.

Because of deregulation central role in the mediation of several intracellular events, acute or chronic deregulation of Ca^{2+}, by a cell injury would be expected to result in events such as activation of proteases, nucleases and lipases enzymes, which may lead to cytoskeletal protein alterations [4,10]. Electron microscopic studies will provide details on the ultrastructural changes of the spermatic cells and those of support, including several of their organelles or subcellular compartments.

MATERIALS AND METHODS

Venom

B. colombiensis venom was obtained from a pool of snakes recently captured and maintained at Immunochemistry section of the Tropical Medicine Institute of the Universidad Central de Venezuela. The venom was lyophilized and kept at -70°C until used.

Animals

Twenty (ten for experimental group and ten for controls) C57/Bl strain male mice of 18-22 g maintained under laboratory conditions, and obtained from the National Institute of Hygiene “Rafael Rangel" were used. The investigation complies with the bioethical norms taken from the guide “Principles of laboratory animal care” [1].

Lethality assay

The lethal dose (LD50) values of the *B. colombiensis* crude venom by intramuscular injection of mice were calculated according to the Spearman-Kärber method [8]. Five mice per dose weighing 20-22 g were injected i.v. with 200 µL of serially diluted crude venom from 2 to 200 µg. Deaths were recorded during a 48 hr period.

Transmission electronic microscopy procedure

Testicles samples from mice injected intraperitoneally with crude venom were obtained from the experimental group and controls and selected according to the previously established times of 1 and 48 h. Samples were fixed immediately *in situ* with 3% glutaraldehyde and 1% OsO_4 (both fixatives diluted in pH 7.4, 320 mOsm phosphate buffer), dehydrated in ethanol and embedded in a LX-112 resin (Ladd Research Inc.). Ultrathin sections were stained with uranyl acetate and lead citrate and observed in Hitachi H-300 electron microscopy operated at 70 kV [6].

RESULTS

The testicular tissue of mice inoculated with *B. colombiensis* venom showed severe alterations in the support cells (Sertoli cells), as well as in the spermatic cells in their different stages. These alterations with the evolution of the envenoming were increased. The testicular response to the *B. colombiensis* venom toxic aggression and the sequential follow-up of controls versus mice envenomed allowed to evaluate from 1 h to 48 h, the early and later testicular ultrastructural changes. One hour after saline solution injection, normal control spermatic cells showed nucleus with healthy acrosomes and acrosomals membranes. Sections showed principal and median piece of the tail with normal mitochondria (FIG. 1). Forty eight hours after saline solution injection normal structures remained. Mitochondria showed an orthodox feature, with a regular cristae arrangement (FIG. 2).

One hour after *Bothrops colombiensis* crude venom mice injection, Sertoli cells from testicular tissue showed swelling mitochondria, some of them degenerated with destruction of mitochondrial matrix and lysis and/or tubular cristae presence.

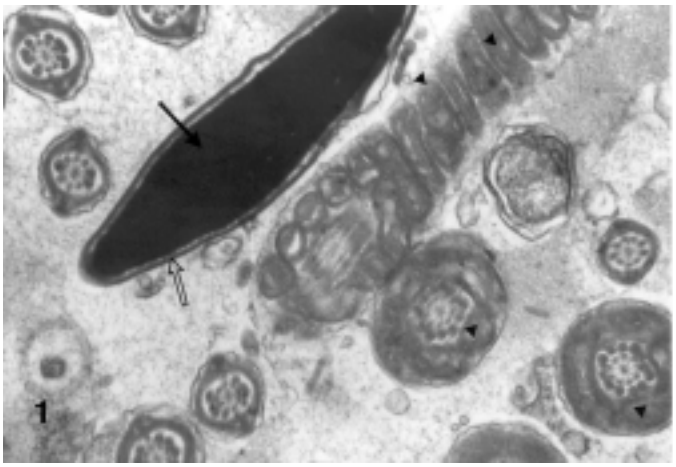

FIGURE 1: ELECTRON MICROGRAPH 1 H AFTER SALINE SOLUTION INJECTION OF MICE, NORMAL TESTICULAR ARCHITECTURE WAS OBSERVED. LONGITUDINAL SECTIONS FROM SPERMATIC CELLS SHOWED NUCLEUS (ARROW) WITH HEALTHY ACROSOMES AND ACROSOMALS MEMBRANES (EMPTY ARROW). THE TRANSVERSE AND LONGITUDINAL SECTIONS SHOWED PRINCIPAL AND MEDIAN PIECE OF THE TAIL WITH NORMAL MITOCHONDRIA (ARROWHEADS). X 30.000.

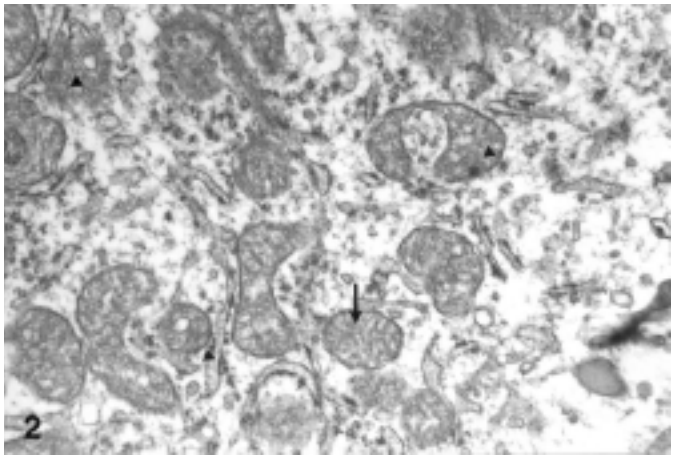

FIGURE 2: ELECTRON MICROGRAPH 48 H AFTER SALINE SOLUTION INJECTION OF MICE, SERTOLI CELLS NORMAL STRUCTURES REMAINED (TRIANGLE). MITOCHONDRIA SHOWED AN ORTHODOX FEATURE, WITH A REGULAR CRISTAE ARRANGEMENT (ARROW). X 30.000.

The damaged mitochondria are inserted in a cellular debris environment; most of them exhibited electron dense particles (FIG. 3).

Forty eight hours after *B. colombiensis* crude venom mice injection, cristae previously oedematous, are refolded to the periphery, looking destroyed and appreciated as condensed images. Spermatids (development germinal cells) showed damages in the external acrosomal membrane (plasmalemma) and separation of the internal acrosomal membrane of the nuclear band, giving a ballooning aspect of the plasmalemma, which showed a more intense electron density in the vicinity of the swelled plasmalemmal space. Vacuoles were also observed (FIG. 4).

DISCUSSION

The mitochondrial oedema with lysis and retraction of the cristae of Sertoli cells could be related to an alteration in the calcium deposition as reported by other authors [4,9]. In different biological systems from works carried out by other workers, the mitochondrial oedema has been explained as an alteration of the ionic deregulation, related to external mitochondrial membrane permeability lost, something which can be associated to unspecific enzymatic activation, such as lipase and protease activities [10]. As the internal compartment swell, mitochondria metrical enzymes have more access to and also possibly leak into the contiguous cytosol [10]. On the other hand, mitochondrial condensation is typical of an inhibited oxidative phosphorilation and respiration; therefore it can produce an intense decrease in the ATP production and cellular energy. The increased mitochondria electron density may be related to Ca++ and proteins precipitation. Mitochondria membrane alteration is caused by an oxidative stress with ionic balance modification [10].

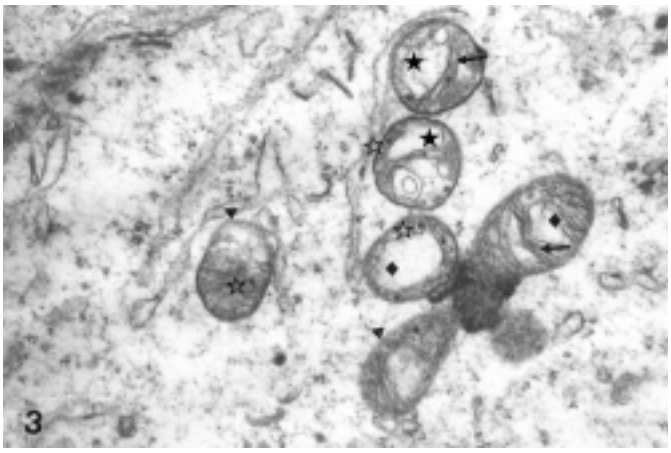

FIGURE 3: ELECTRON MICROGRAPH 1 HOUR AFTER *B. COLOMBIENSIS* CRUDE VENOM INJECTION, NOTE SERTOLI TESTICULAR CELLS SHOWING MITOCHONDRIA SWELLING (STAR), SOME OF THEM DEGENERATED WITH DESTRUCTION OF MITOCHONDRIAL MATRIX AND LYSIS OF THEIR CRISTAE (RHOMBUS). TUBULAR CRISTAE WERE PRESENT (ARROWS). ELECTRON PARTICLES INTO THE MITOCHONDRIA WERE NOTICED (EMPTY STARS). SOME MITOCHONDRIA SHOWED MEMBRANE LOST (TRIANGLE). X 30.000.

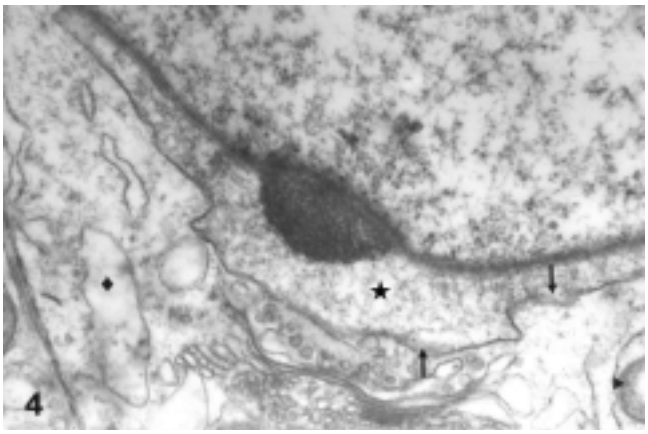

FIGURE 4: ELECTRON MICROGRAPH 48 H AFTER *B. COLOMBIENSIS* CRUDE VENOM INJECTION, CRISTAE PREVIOUSLY OEDEMATOUS, IS REFOLDED TO THE PERIPHERY, LOOKING DESTROYED AND APPRECIATED AS CONDENSED IMAGES (TRIANGLE). SPERMATIDS (IN DEVELOPMENT GERMINAL CELLS) SHOWED DAMAGES IN THE EXTERNAL ACROSOMAL MEMBRANE (PLASMALEMMA) (ARROW) AND SEPARATION OF THE INTERNAL ACROSOMAL MEMBRANE OF THE NUCLEAR BAND, GIVING A BALLOONING ASPECT OF THE PLASMALEMMA (STAR). VACUOLES AS SIGN OF CELLULAR DEGENERATION WERE OBSERVED (RHOMBUS). X 30.000.

The change of the acrosomal membrane, where acrosomal enzymes (necessary for the interaction with the pellucid zone of the oocyte) could be altered, in addition the damage of the tail mitochondria could reduce the fertile life of the sperm, as well as to jeopardize the necessary energy to its mobilization until the feminine gamete [2].

The sperm membranes changes observed, as well as Sertoli cell organelles alterations could be explained by bothropic venom phospholipasic activity; these enzymes attach to a receptor of the cellular membrane (well studied in the skeletal muscle cells, capillaries and liposomes) [3,5], penetrate the lipidic bilayer, destabilize the membrane, with the consequent alteration in the ionic and macromolecular permeability regulation. The membrane damage produces a prominent calcium influx (probably the most important disorder) that generates an alteration of the cellular skeleton, with mitochondrial damages, and the activation of calcium dependent proteins, with enzymatic activity, which it could increase the cellular damage [10].

The germinal cells damage could also be related to indirect damage when the venom proteolytic fractions alter the hemato-testicular barrier, allowing the action of the immunologic system components on the spermatic proteins, mainly those which are not recognized as own [2].

CONCLUSIONS

Different types of studies yield information on toxic responses. Animal studies provide most of the information because anybody cannot ethically expose humans to dangerous materials. Target organs are the specific organs or tissues adversely affected by a particular venom. Organs may be more sensitive to certain toxins because of the way the venom is distributed in the body or because of the way the organ reacts with, responds to, or metabolizes the venom. Mechanism of action includes the biochemical, physiologic, and anatomic changes caused by a toxin that result in its characteristic toxic effects. *B. colombiensis* venom has an intense proteolytic activity on different testicular structures such as germinal cells and support Sertoli cells, which demonstrated the development of reproductive problems in patients bitten by this snake species, work on acute and chronic patients is in progress to establish this. In future, necropsy evaluations of the first group of offspring of each generation and half of the venom inoculated pregnant females after each mating detect embryologic malformations, the number of embryos, and abnormalities of implantation or fetal development. Detection of teratogenic snake venom effects or adverse effects on female or male reproductive functions or capacity may be further evaluated by more specialized studies

BIBLIOGRAPHIC REFERENCES

[1] National Institute of Health. **Principles of Laboratory Animal Care**. Maryland, USA: 1-96 pp. 1985.

[2] GONZÁLEZ, A**. Fisiología endocrina**. Federación Panamericana de Asociaciones de Facultades y Escuelas de Medicina y Fondo Educativo Interamericano, S.A, México: 1-250 pp. 1980.

[3] GUTIERREZ, J.M.; LOMONTE,B. Review article phospholipase A2 myotoxins from *Bothrops* snake venoms. **Toxicon**. 33: 1405-1424. 1995.

[4] HENNINGS, H.; KRUSZEWSKI, F.H.; YUSPA, S.H.; TUCKER, R.W. Intracellular calcium alterations in response to increased external calcium innormal and neoplastic keranocytes. **Carcinogenesis**. 10: 777-780. 1989.

[5] KINI, R.M.; EVANS, H.J. Structure-function relationships of phospholipases. The anticoagulant region of phospholipases A2 . **J. Biol. Chem**. 262: 14402-14407. 1987.

[6] OGURA, M.; URBINA, C.; GONZÁLEZ, M.; RODRÍGUEZ, P.; FINOL, H.J. **Introducción a la Microscopía Electrónica**. Centro de Microscopía Electrónica. Facultad de Ciencias, Universidad Central de Venezuela. Caracas, Venezuela. 1-168 pp. 1997.

[7] RODRIGUEZ-ACOSTA, A.; MONDOLFI, A, ORIHUELA, A **¿Qué hacer frente a un accidente ofídico?** Venediciones C.A. Caracas. 21-40 pp. 1995.

[8] SPEARMAN-KARBER, R. Alternative Methods of Analysis for Quantal Responses. In: **Statistical Method in Biological Assay**. Finney & Griffin, London. 1-89 pp. 1978.

[9] TRUMP, B.F.; BEREZESKY, I.K. The role of calcium in cell injury and repair. A hypothesis. **Surv. Synth. Pathol. Res**. 4: 434-454. 1985.

[10] TRUMP, B.F.; BEREZESKY, I.K. Cellular and Molecular Basis of Toxic cell Injury. In: **Cardiovascular Toxicology**. Raven Press Ltd, New York. 75-113 pp. 1992.