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CYTOSKELETON, ENDOPLASMIC RETICULUM AND NUCLEUS ALTERATIONS IN CHO-K1 CELL LINE AFTER *Crotalus durissus terrificus* (SOUTH AMERICAN RATTLESNAKE) VENOM TREATMENT

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ABSTRACT: Snake venoms are toxic to a variety of cell types. However, the intracellular damages and the cell death fate induced by venom are unclear. In the present work, the action of the South American rattlesnake *Crotalus durissus terrificus* venom on CHO-K1 cell line was analyzed. The cells CHO-K1 were incubated with *C. d. terrificus* venom (10, 50 and 100g/ml) for 1 and 24 hours, and structural alterations of actin filaments, endoplasmic reticulum and nucleus were assessed using specific fluorescent probes and agarose gel electrophoresis for DNA fragmentation. Significant structural changes were observed in all analyzed structures. DNA fragmentation was detected suggesting that, at the concentrations used, the venom induced apoptosis.

KEY WORDS: cell culture, actin filaments, nucleus, endoplasmic reticulum, *Crotalus*, rattlesnake venom, apoptosis.

CONFLICTS OF INTEREST: There is no conflict.

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INTRODUCTION

While envenomation by snakes of the *Bothrops* genus produce marked and local effects such as edema, hemorrhage, necrosis and pain (12, 18), envenomation by the South American rattlesnake, *Crotalus durissus terrificus*, mainly causes neurotoxicity, followed by renal damage and myoglobinuria, as well as relatively mild local effects – pain and some swelling (20). *Crotalus durissus terrificus* venom and its components can affect a variety of cell types, including macrophages (9), neutrophils (34), mast cells (8), platelets (7) and cells in culture (14), as well as the function of organs such as the heart (19) and the kidney (22).

Many of the local actions of snake venoms are mediated by a direct action on skeletal and vascular smooth muscle cells, endothelial cells, macrophages, neutrophils, mast cells, and platelets (17). The venom components responsible for cytotoxicity include disintegrins (27, 28) as well as enzymes such as L-amino acid oxidase (6), metalloproteinases (29) and phospholipases A_2 (PLA_2) (20, 21). The systematic fractionation of *C. d. terrificus* venom has revealed the presence of various toxins (convulxin, crotamine, crotoxin and gyroxin) and enzymes (5, 11), some of which may exert a potentially damaging effect on cells. Some of these components can induce in treated cells a type of cell death known as apoptosis (5, 23, 26, 40). Apoptosis is a programmed cell death with known morphological changes in the nucleus (24), actin filaments (16, 27) and endoplasmic reticulum (17, 32). The aim of the present work was, therefore, to determine the effect of crotalid venom on CHO-K1 cells, particularly its action on actin filaments, endoplasmic reticulum and nucleus.

MATERIALS AND METHODS

Snakes and venom

Snakes were maintained in the serpentarium at the Center of Nature Studies of UNIVAP according to standard procedures. Venom from 25 adult snakes of both sexes was collected, desiccated, separated into aliquots and stored at -20°C until used. Venom stock solutions (2.5mg/ml) were prepared in phosphate buffered saline (0.1M sodium phosphate, 0.15M NaCl), pH 7.2, sterilized using a 0.22μm filter (Millipore) and kept at 4°C. Crude venom solutions were diluted in cell culture medium before use.

Cell culture

CHO-K1 cell line derived from Chinese hamster ovary (ATCC CCL-61; American Type Culture Collection, Rockville, MD) was routinely cultivated using Ham-F12 medium® containing 10% (v/v) fetal bovine serum (FBS), 100IU/ml penicillin, 100mM/ml streptomycin, and 0.25µg/ml fungizone® (all from Gibco-Invitrogen Detection Technologies) at 37°C in a humidified atmosphere with 5% CO_2. When cells became confluent, medium was removed, the cell layer was washed with phosphate-buffered saline (PBS), and cells were detached with 0.025% trypsin® (Gibco Invitrogen Detection Technologies) - EDTA (Carlos Erba, ABC Lab, São Paulo). Cells ($2.5X10^4$) were seeded over coverslips in 24-well tissue culture plates (NUNC, Denmark).

Venom treatment

The CHO-K1 cells were incubated with *C. d. terrificus* venom at 10, 50 and 100µg/ml, diluted in medium supplemented with 5% FBS. After incubation (1 and 24 hours), cells were carefully washed with PBS and incubated with the fluorescent probes. The cytotoxicity assay was carried out through direct counting of the cells using the trypan blue exclusion method (13).

Fluorescence staining procedures

Just before imaging, cells were stained with the fluorescent dyes (Invitrogen Detection Technologies) by incubating in dye-containing medium at 37°C in the following conditions: rhodamine-phalloidin (1:100, 10 min), DioC6(3) 3,3' dihexylocarbocyanine iodide (10µg/ml, 15 min), and Hoechst 33342 (2.5µg/ml, 5 min) for actin filaments, endoplasmic reticulum and nucleus staining, respectively. After that, cells were washed with PBS and fixed for 30 min with 4% recently prepared formaldehyde in PHEM buffer, rinsed with PHEM and mounted in slides with n-propyl gallate® (Sigma, St. Louis, MO). Microscopy observations and photographs were performed using a Leica photomicroscope (DMLB), equipped with a HBO 100W mercury lamp and the corresponding filters sets for fluorescence microscopy: UV, blue and green.

DNA isolation and gel electrophoresis

Apoptotic DNA fragments were analyzed using the procedure described by Ahmad *et al*. (1), with modifications. After 1, 6 and 12 hours of *C. d. terrificus* crude venom treatment, CHO-K1 cells ($1X10^7$) were washed twice with PBS (pH 7.2), suspended in 500μl lysis buffer (10mM Tris, pH 7.5; 400mM NaCl; 1mM EDTA; 1% Triton X-100), incubated on ice for 15 min and made into pellets by centrifugation at 13600X*g* for 15 min at 4°C. The supernatant was incubated with Rnase (0.2mg/ml) and proteinase K (0.1mg/ml) for 2 hours at 37°C. DNA was extracted using phenol/chloroform (1:1, v/v) and precipitated with 96% ethanol overnight at -80°C. The DNA precipitate was centrifuged at 13600X*g* for 15 min at 4°C and the pellet was air dried and dissolved in 20μl of Tris-EDTA buffer (10mM Tris-HCl, pH 8.0; 1mM EDTA). The DNA obtained was analyzed using electrophoresis in 1.5% agarose gel containing 0.3μg/ml ethidium bromide in TRIS-borate-EDTA buffer for 1.5 hour at 75V. Bands were visualized on a UV transilluminator and photographed using Kodak Gel Logic 100 Imaging System.

RESULTS

Control group presented intact actin filaments, with no membrane retraction (Figure 1A). Depolymerization of actin filaments were observed after 24 hours of incubation with 10 or 50μg/ml venom. The filaments were completely depolymerized leading to plasma membrane retraction (compare Figure 1A with Figure 1B). Increasing the venom concentration to 100μg/ml resulted in intense depolymerization of actin filaments and large spacing between cells due to membrane retraction (Figure 1C).

Another important observation in comparison with control (Figure 2A) was the increase of vesicle formation in the endoplasmic reticulum after treatment with 10μg/ml venom (Figure 2B). Vesiculation of this organelle was more evident when 100μg/ml venom was used (Figure 2C).

Evaluation of the venom effect on the nucleus through Hoechst labeling demonstrated that control cells exhibited normal chromatin and no nuclear fragmentation (Figure 3A). After incubation with 10 or 100μg/ml for 1 hour, cells presented chromatin condensation and nucleus fragmentation (Figure 3B), and after 24 hours, nuclei fragmentation increased (Figure 3C).

To ascertain the induction of cell death by *Crotalus* venom, DNA fragmentation, reflecting the endonuclease activity, characteristic of apoptosis, was analyzed. Figure 4 showed that treatment with venom at a concentration of 10, 50 and 100μg/ml for 6 hours resulted in the formation of more fragments than treatment for 1 and 12 hours, which could be seen via electrophoretic examination as a characteristic ladder pattern.

Figure 1: Visualization of actin filaments of CHO-K1 cells after *C. d. terrificus* crude venom treatment. (A) Control cells. Note the normal appearance of actin filaments and spreading of cells. (B) Cells treated with 10μg/ml of venom for 24 hours. Some actin filaments can be seen (arrow). (C) Cells treated with 100μg/ml of venom for 24 hours. Depolymerization of actin filaments (arrows) and compromised cell adhesion resulting in membrane retraction (arrowheads) can be seen. X 2,500.

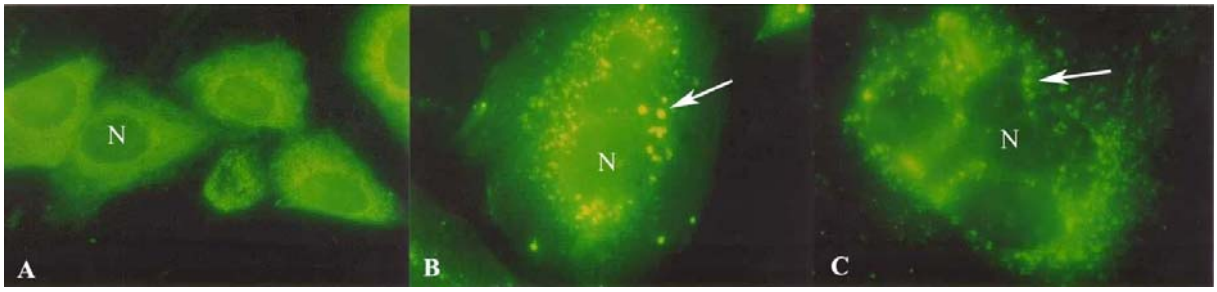

Figure 2: Visualization of endoplasmic reticulum of CHO-K1 cells after *C. d. terrificus* crude venom treatment. (A) Control cells. (B) Cells treated with 10μg/ml of venom for 24 hours. Fluorescent dots can be seen around the nucleus, probably due to venom collection (arrow). (C) Cells treated with 100μg/ml of venom for 24 hours. Presence of vesicles and venom collection (arrow). N= nucleus. X 2,500.

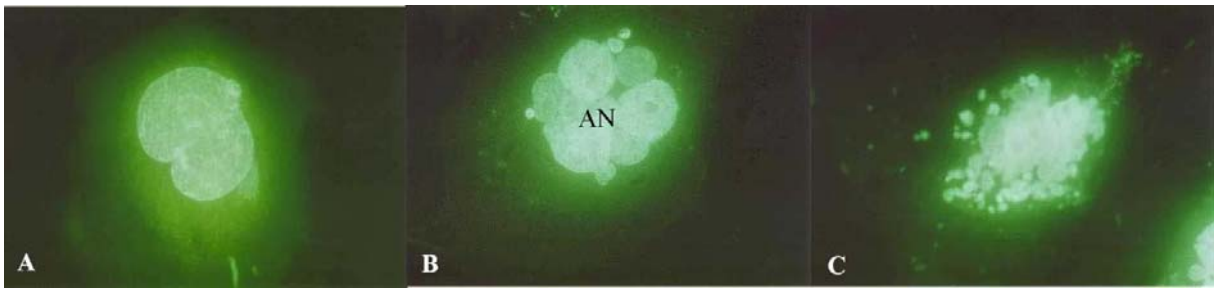

Figure 3: Visualization of nucleus of CHO-K1 cells after *C. d. terrificus* crude venom treatment. (A) Control cells. (B) Cells treated with 10µg/ml of *C. d. terrificus* crude venom for 1 hour. Apoptotic nucleus (AN). (C) Cells treated with 100µg/ml of crude venom for 24 hours. Chromatin condensation and nucleus fragmentation. X 2,500.

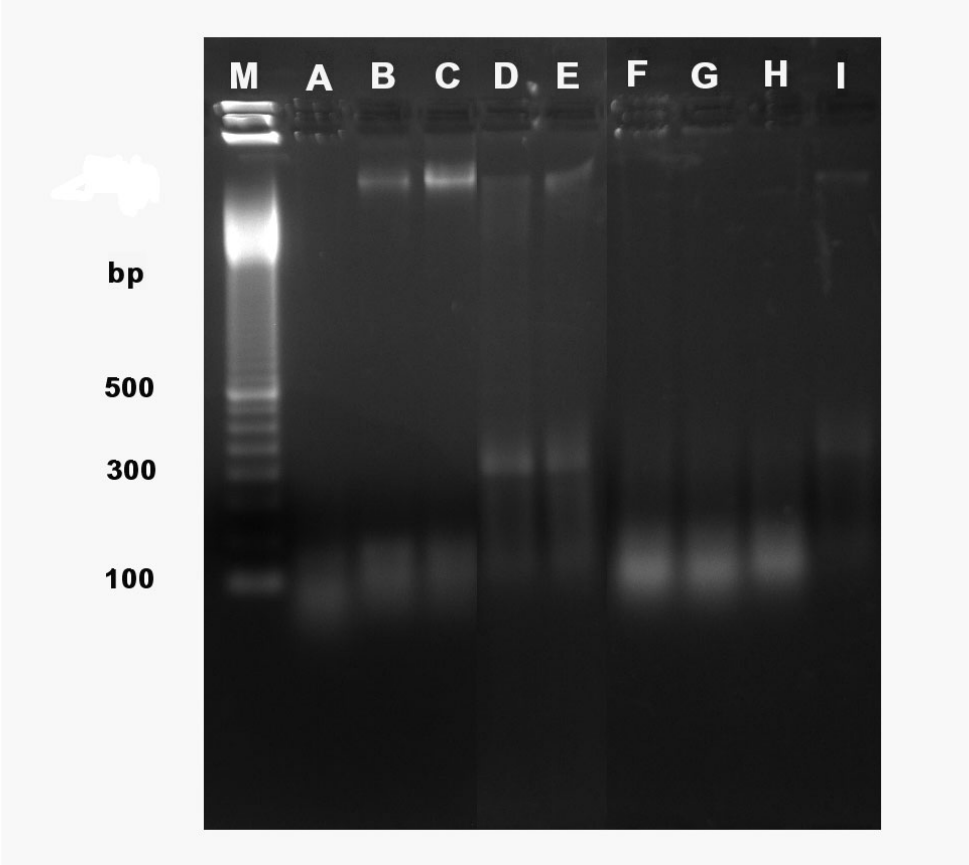

Figure 4: Electrophoretic analysis of genomic DNA of CHO-K1 cells after *C. d. terrificus* crude venom treatment. Cells were incubated for 1, 6 and 12h with 10, 50 and 100µg/ml venom. Genomic DNA was extracted and analyzed by electrophoresis on a 1.5% agarose gel containing ethidium bromide. CHO-K1 cells treated with *C. d. terrificus* crude venom exhibited the ladder pattern characteristic of apoptosis. M, Marker; A, 10µg/ml; B, 50µg/ml; C, 100µg/ml – after 1 h; D, 10µg/ml; E, 50µg/ml; F, 100µg/ml – after 6 h; G, 10µg/ml; H, 50µg/ml; I, 100µg/ml – after 12 h.

DISCUSSION

Venoms exert their effects on cells through the action of their various biologically active components (35). Nephrotoxicity is a complication observed after most snakebites (36). The effects of snake venoms can be directly or indirectly promoted by the release of active pharmacological substances (4). Giron *et al*. (14) described that, in mice, cytoskeletal proteins and basal membrane components suffered substantial alterations after the action of crude venom and the hemorrhagic fraction (Uracoina-1) of *Crotalus vegrandis* venom.

Apoptosis is a type of cell death with typical morphological alterations (2) and has been described in cells after venom treatment (3, 10). This type of cell death may be correlated to the physiological complications caused by snakebites. Snake venom treatment has induced apoptosis in different cell types. Rattlesnake venom is a very potent promoter of apoptosis in vascular endothelial cells (VEC) (29), but the molecular mechanism of apoptosis induced by the venom remained undefined. The term apoptosis is used to describe sometimes only the morphological changes, and sometimes the underlying molecular mechanism. At some stages, apoptosis will probably affect all the components and organelles in a dying cell (12, 23). Three mechanisms are involved in the apoptotic process: a receptor-ligand mediated mechanism, a mitochondrial pathway, and a mechanism in which the endoplasmic reticulum plays a central role (12).

Depolymerization of actin filaments of the cytoskeleton could be observed in cells incubated with a higher concentration of *C. d. terrificus* venom. The venom concentrations used caused the cells to lose their projections, leading to spaces between cells. One of the most striking changes during the observation of apoptosis under the microscope was the reorganization of the outer limits of the cell and the changes in its shape (38). Cells undergoing apoptosis round up and sever contacts with neighboring cells in tissues. Protrusions from the cell surface become visible both by light and electron microscopy. The basis of these changes is probably the concerted reorganization of cytoskeleton structures. Several recent studies have reported induction of apoptosis in mammalian cells associated with changes in the actin cytoskeleton (25, 30). A decrease of actin dynamics might lead to pores or channels that open for a prolonged time, thus reducing membrane potential and increasing the release of reactive oxigen species (ROS) into the cytoplasm (15). The

metalloproteinases present in *C. d. terrificus* venom may cleave some key components that are part of the hemidesmosomes or some other components of the anchoring filaments, which would help to explain the rounding up and detachment of cells (21).

In the present work, venom treatment also induced changes in the endoplasmic reticulum and nucleus. The neurotoxic mechanism of several PLA_2-related snake venoms is probably based on the inhibition of the net uptake of Ca^{2+} into sarcoplasmic reticulum vesicles and brain mitochondria (4, 37). Undoubtedly, the death of the cell will at some stage concern all organelles. Dilatation of the endoplasmic reticulum during apoptosis can be seen ultrastructurally (28). Changes in the endoplasmic reticulum or in enzymes localized in it can cause apoptosis (42). Endoplasmic reticulum stress has signaled apoptosis through a mitochondrial-dependent pathway by activating Bcl-2/Bcl-X_L-associated death promoter (BAD), perhaps through the dephosphorylation of serine by a Ca^{2+}-dependent phosphatase, calcineurin, which is activated by an increase in cytoplasmic Ca^{2+} concentration (31, 39). The alterations observed in the endoplasmic reticulum of cells incubated with venom probably result from similar events.

Cells incubated with a higher concentration of venom (100μg/ml) presented nuclear fragmentation and apoptotic bodies. Nuclear change is one of the features most frequently described during the apoptotic process. Detectable morphological changes in the nucleus are chromatin condensation and, at a later stage, fragmentation of the nucleus into several bodies. Changes in the nuclear appearance can be detected by electron microscopy or also very clearly by light microscopy using DNA-intercalating dyes such as acridine orange or Hoechst dyes to visualize the nucleus (33), as used in our experiments.

In addition, DNA fragmentation was observed using agarose gel electrophoresis. Apoptosis is linked to the activation of endonucleases and results in the fragmentation of DNA into well-defined fragments, which could be seen during electrophoretic examination as a characteristic ladder pattern (41).

The action of *Crotalus durissus* venom *in vitro* demonstrated the need for more studies of the proteins present in the venom and their participation in the apoptosis process. Our results indicated that a factor present in *C. d. terrificus* venom also produced notable changes in the cells morphology. These changes meet the

stringent morphological criteria for apoptosis as confirmed by DAPI staining. Apoptotic bodies were characteristically present in venom-treated cells stained with DAPI. Cells undergoing apoptosis also exhibited cytoplasmic blebbing, nuclear shrinkage, chromatin condensation, irregularity in shape and retraction of processes (38).

The data presented here showed the effects of *C. d. terrificus* venom on CHO-K1 cells, including its capability to induce apoptosis, to promote changes in the cell shape and rearrangement of actin network as well as severe alterations in the endoplasmic reticulum and nucleus. Our results suggested that the venom promoted a disruption of actin filaments, followed by reticulum vesiculation, chromatin condensation, nucleus fragmentation, and consequent apoptosis.

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