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Cytotoxicity of *Bothrops pauloensis* Venom on Madin-Darby Canine Kidney Cells: Cell Death Mechanism

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Abstract

Bothrops is a genus of pit vipers (snakes) endemic to Central and South America. Snake envenomation *(Bothrops genus)* specifically *Bothropoides pauloensis,* a venomous snake popularly known as *"Jararaca pintada"*, which is found in the Brazilian territory or more commonly in the southwest of the state of Sao Paulois. Acute kidney injury is one of the complications observed in *Bothrops* snakebite with relevant morbidity and mortality. Here, we showed the cytotoxic effect of *Bothropoides pauloensis* venom on MDCK cells, intracellular mitochondrial membrane potentiality (ΔΨm), BpV regulated Reactive Oxygen Species (ROS) and activation of caspases 3 and 7 in MDCK cells. MDCK cells were treated with different concentrations of *B. pauloensis* venom for 24 hrs and cell death were measured with annexin V and PI staining and detected by flow cytometry. MDCK cells were treated with two different concentrations (IC50 and 2 × IC50) of BpV after 24 hrs. All data are expressed as mean ± SEM of three independent experiments with six replicates (ANOVA and Dunnett test, *p<0.05). Cytotoxicity was assessed by MTT assay and the treatment with BpV caused decrease in cell viability, with an IC50 of 7.5 µg/mL. After the treatment, amounts of ROS in the BpV-treated MDCK-cells (7.5 µg/mL) showed significant right dislocation of the fluorescence peak in the histogram, when compared with the untreated control group. Caspase 3 and 7 activity was determined in presence of the fluorogenic Ac-DEVDafc substrate after treatment with *Bothropoides pauloensis* venom (12 hrs) then the result indicated apoptotic involvement in BpV-induced cell death. Similar results were also found for venoms of other *Bothrops* and *Bothropoides* genus snakes such as *B. alternatus, B. insularis* and *B. leucurus*.

Keywords: Cytotoxicity; Venom; Kidney cells; Flow cytometry; Apoptosis; Nephrotoxicity

Introduction

Bothropoides pauloensis, a venomous snake popularly known as *"Jararaca pintada",* which is found in the Brazilian territory more commonly in the southwest of the state of Sao Paulo [1]. Experimental studies made with *Bothrops* venom suggest a multifactorial pathogenesis for ARF which includes a variety of mechanisms [2]. In order to elucidate the mechanism of direct nephrotoxicity the aim of the present work was to investigate the effect of *B. pauloensis* venom (BpV) on renal epithelial cells.

Materials and Methods

BpV was kindly donated by Dr. Marcos H Toyama, Paulista State University (UNESP). Mitochondrial functionality was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay. Madin-Darby Canine Kidney (MDCK) epithelial cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (Sigma) at 37°C and 5%. After seeding, cells were treated with different concentrations of BpV (100, 50, 25, 12.5, 6.25 and 3.12 µg/mL) and incubated at 37°C for 24 hrs. Cell viability was calculated in comparison with the control group. The IC_{50} (venom concentration able to inhibit 50% of cell growth) was determined by non-linear regression [3].

Cytotoxicity induced by BpV was assessed by lactate dehydrogenase (LDH) leakage into the culture medium (12 hrs of treatment) using the Promega kit (6179A). Furthermore, cells treated with different concentrations of BpV (15 and 7.5 µg/mL) were stained with fluorescein isothiocyanate (FITC)-conjugated to annexin V/propidium iodide (PI) (Becton-Dickinson) according to the manufacturer's instructions. The populations of annexin V-PI viable cells and annexin V+ apoptotic cells were evaluated by flow cytometry. Data were collected in a FACS Calibur (Becton-Dickinson) and analyzed using Cell Quest software (Becton-Dickinson). For ΔΨm measurements, MDCK cells (105) were incubated with BpV (7.5 µg/mL) for 12 hrs. Then, cell culture was incubated with 25 nM tetramethylrhodamine, ethyl ester (TMRE) (Molecular Probes) for 30 min at 37°C in the dark (analyzed on by flow cytometry). For measurement of Cytosolic Reactive Oxygen Species (ROS), 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA) (Sigma). Cells were treated for the recommended period of time (BpV 7.5 µg/mL for 12 hrs). After that, they were submitted to flow cytometric analysis using a FACScan flow cytometer (Becton-Dickinson). The data based on the FL1 channel were analyzed with the Cell Quest software. For caspase 3/7 (#96615

and #9492, respectively-Cell Signaling) activity measurements, cells were treated with BpV (25, 15 and 7.5 µg/mL) for 12 hrs. Quantification of the total protein concentration was performed using the BCA protein assay (Thermo Scientific). For immunoblotting, protein concentration was determined by the BCA protein assay. Cell lysates were incubated overnight with a specific primary antibody. Membranes were washed and probed with the appropriate secondary antibody conjugated to horseradish peroxidase for enhanced chemiluminescence detection (Amersham Pharmacia Biotech). The data of experiments were ere expressed as mean ± SEM and the statistical analysis was performed using ANOVA with Bonferroni or Dunnett ($p<0.05$).

Result

Cytotoxicity was first assessed by MTT assay and the treatment with BpV caused decrease in cell viability, with an IC_{50} of 7.5 µg/mL (Figure 1A). Flow cytometry with annexin V and PI showed cell death (7.5 and 15 µg/mL), suggesting the participation of apoptosis and late apoptosis (Figure 1C). The results indicated an apparent membrane rupture in MDCK cells at the highest concentrations studied (Figure 1B), showing an increase in LDH release. Apoptosis caused by snake venoms is mediated by various different components, such as metalloprotease, phospholipase A2 and L-amino acid oxidase [4-8]. Several studies have confirmed the involvement of *Bothrops* and *Bothropoides* venom components in apoptosis [9,10].

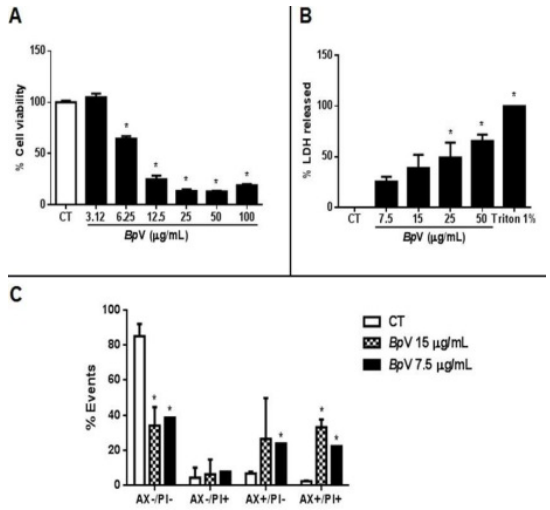

Figure 1: Cytotoxic effect of *Bothropoides pauloensis* venom on MDCK cells. **(A)** MDCK cells were treated with different concentrations of *B. pauloensis* venom for 24 hrs and were evaluated by the reduction in the 3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide (MTT) salt in cells under BpV concentration curve after 24 hrs of treatment. **(B)** Percentage release of the lactate dehydrogenase enzyme of MDCK cells treated under a BpV concentration curve treatment. **(C)** Cell death was measured by annexin V and PI staining and detected by flow cytometry. MDCK cells were treated with two different concentrations (IC_{50} and 2 x IC_{50}) of BpV after 24 hrs. All data are expressed as mean ± SEM of three independent experiments with six replicates (ANOVA and Dunnett test, $^{*}p<0.05$).

BpV (7.5 µg/mL) caused a left dislocation in TMRE fluorescence after 12 hrs of treatment (Figure 2A). It is believed that the loss of mitochondrial transmembrane potential is due to the opening of the permeability transition pore, a mitochondrial mega channel, which is strongly affected by oxidative stress conditions [10].

Results showed that the amounts of ROS in the BpV-treated MDCK-cells (7.5 µg/mL) showed significant right dislocation of the fluorescence peak in the histogram, when compared with the untreated control group (Figure 2B). We measured the changes in caspases 3 and 7, key proteases required for apoptosis [11], in MDCK cells.

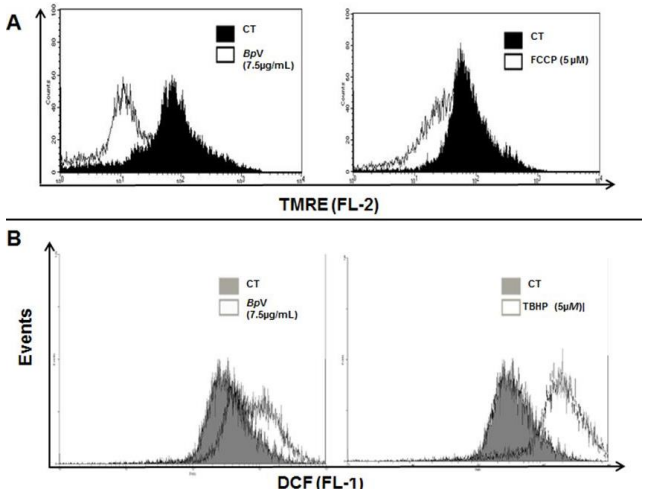

Figure 2: Intracellular mitochondrial membrane potentiality (ΔΨm) and BpV regulated Reactive Oxygen Species (ROS). **(A)** Alterations in ΔΨm was measured by TMRE staining (20 nM, 20 min at 37˚C) and detected by flow cytometry. MDCK cells were treated with (IC_{50}, 7.5 µg/mL) of BpV for 12 hrs. Displacement of the TMRE Fluorescence to the left, when compared with negative control, shows the decrease in ΔΨm. FCCP (5 µM) was used as positive control. **(B)** Intracellular BpV-induced ROS generation (7.5 µg/mL) in MDCK cells. After treatment with the venom for 12 hrs, cells were incubated with 5 µM DCFH-DA for 30 min and then immediately submitted to flow cytometric analysis. ROS generation was expressed as a ratio of relative fluorescent intensity compared to the control group. One representative histogram of three independent experiments for each sample is presented.

In Figure 3A and 3B, BpV (15 and 25 µg/mL) treatment induced activation of caspase 3 and caspase 7. These data indicate apoptotic involvement in BpV-induced cell death in these cells.

Similar results were found for venoms of other *Bothrops* and *Bothropoides* genus snakes. *B. alternatus* [12], *B. insularis* [10] and *B. leucurus* [9] also showed caspases 3/7 activation in MDCK cells.

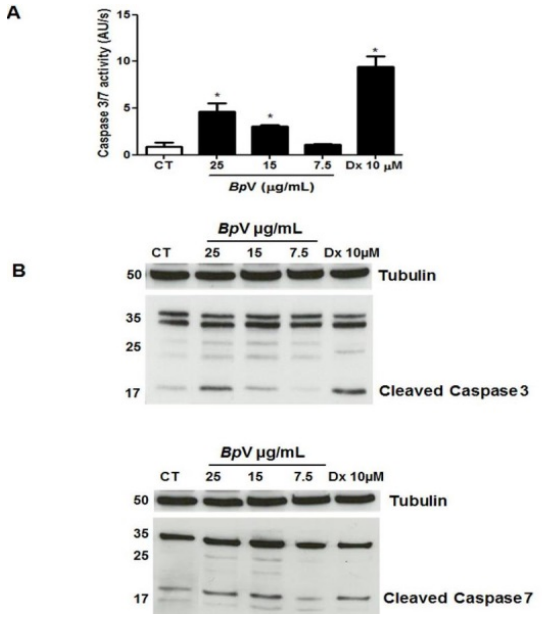

Figure 3: Activation of caspases 3 and 7 in MDCK cells. **(A)** Caspase 3/7 activity determined in presence of the fluorogenic Ac-DEVD-afc substrate after treatment with *Bothropoides pauloensis* venom (12 hrs). **(B)** Caspase-3 and Caspase-7 activity: Immunoblotting analysis of activated caspase-3 and Caspase- 7 in MDCK cells after 24 hrs of treatment with BpV in different concentrations, Doxorubicin was used as a positive control.

Discussion and Conclusion

The characterization of these effects in the renal tubular cells gives strong evidences that the acute renal failure induced by this venom is a result of the direct nephrotoxicity, which may involve the cell death mechanism. The elucidation of these cell death mechanisms will allow interfering with their signaling molecular pathways at some point and help develop drugs and more effective therapeutic strategies.

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