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Short communication

Bothrops erythromelas (Amaral, 1923) venom induces apoptosis on renal tubular epithelial cells

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ABSTRACT

Bothrops erythromelas is responsible for a large number of snakebite incidents in Northeastern Brazil. Previously, we showed the effects of whole *B. erythromelas* venom in an isolated kidney model. To continue the study with *B. erythromelas* venom, the present work aims to study the effects of this venom on MDCK tubular epithelial cells and assess gene expression involved in kidney injury, aiming at elucidating the mechanisms responsible for renal toxicity. Cytotoxicity in MDCK cells showed an IC50 of 93 μg/mL and predominant apoptotic involvement demonstrated by flow cytometry assays and expression of caspase-3 and caspase-8. In conclusion, we suggest that *Bothropoides erythromelas* venom causes apoptosis with involvement of the caspases, probably through the extrinsic pathway.

1. Introduction

Ophidic accidents are an important public health problem in Brazil, with high morbidity and mortality (Warrell, 2004; Albuquerque et al., 2013). The genus Bothrops and Bothropoides are responsible for 70% of ophidic accidents in Brazil (Oliveira et al., 2010). Venom from species of Bothrops genus show variable composition and biological effects (Queiroz et al., 2008).

Bothrops erythromelas (Amaral, 1923) commonly known in Brazil as "Jararaca-da-seca" is responsible for a great deal of snakebites in Northeastern Brazil (Rocha, 2008). The envenomation causes hemorrhage, edema, pain, myonecrosis, hypotension and induces acute renal failure (Sanchez et al., 1992; Sgrignolli et al., 2011).

Acute kidney injury is one of the main causes of death in snakebite cases (Sgrignolli et al., 2011; Albuquerque et al., 2013; Martines et al., 2014) and its pathogenesis is not well understood. Previously, we showed the effects of whole *B. erythromelas* venom in an isolated kidney model (Martins et al., 2005). To continue the study with *B. erythromelas* venom (BeV), this short communication aims to study the effects of this venom on MDCK distal tubular epithelial renal cells and assess gene expression involved in kidney injury, aiming at elucidating the mechanisms responsible for renal toxicity.

2. Materials and methods

2.1. Cell culture, venom and chemical compounds

Madin–Darby Canine Kidney (MDCK) epithelial cells were cultivated in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) (Sigma) at 37 °C and 5% CO2. BeV was collected from adult specimens maintained in the Regional Nucleus of Ophiology of Federal University of Ceara (NUROF-UFC), lyophilized and maintained at −20 °C until further use. RPMI medium and (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay were obtained from Sigma Chemical Co. (St. Louis, MO, USA), AnnexinV/FITC Apoptosis Detection Kit was obtained from BD Pharmigen (CA, USA).

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2.2. Cytotoxicity assay

Mitochondrial functionality was measured by MTT colorimetric assay. MDCK cells were cultured in sterile 96-well microtiter plates and were left to adhere to the plate overnight. After that, they were treated with different concentrations of *Bothrops erythromelas* venom (100; 50; 25 and 12.5 μg/mL) and incubated at 37 °C for 24 h. MTT reagent (Sigma-Aldrich, St. Louis, MO, USA; 5 mg/mL in PBS) was added to each well and incubated for 4 h at 37 °C. Finally, the medium was removed and the precipitated formazan crystals were dissolved in 10% Sodium dodecyl sulphate (Vetec, Rio de Janeiro-RJ, Brazil) in HCl 0.01 N. After 17 h, absorbance at 570 nm was performed in a microplate reader (Biochrom® Asys Expert Plus). Cell viability was calculated in comparison with the control group. The IC50 (venom concentration capable of inhibiting 50% of cell growth) was determined by non-linear regression (Mosmann, 1983).

2.3. Annexin V-FITC and 7AAD staining

Cells treated with IC50 of BeV (93.3 μg/mL) incubated during 24 h were stained with fluorescein isothiocyanate (FITC)-conjugated to annexin V/7AAD according to the manufacturer's instructions (BD Pharmingen, CA, USA). The population of annexin V-7AAD viable cells was evaluated by flow-cytometry. Data were collected in a FACSCalibur (Becton-Dickinson, Mountain View, Calif.) and analyzed using Cell Quest software (Becton-Dickinson).

2.4. Gene expression pro-apoptotics and anti-apoptotic

The expression of pro-apoptotic (Caspase-3 and Caspase-8) and anti-apoptotic (*Mcl-1* and *Bcl-XL*) genes were analyzed using iQ5 Real Time PCR Detection System (Bio-Rad). The mRNA sequence of pro-apoptotic and anti-apoptotic genes was obtained from the website of the National Center for Biotechnology Information (NCBI). Primer design was performed using the Oligo Designer Perfect™ available on the Invitrogen® web site (http://www.invitrogen.com). To assess gene expression, MDCK cells were grown in 24-well culture plates at a concentration of 1×10^5 cells/mL. After 24 h of cultivation in an incubator with 5% CO_2 at 37 °C, BeV was added to each well at concentrations of 46.65 and 23.32 μg/mL (respectively 1/2 and 1/4 of IC_{50} of venom) in hexaplicate and the cells were incubated with 5% CO_2 at 37 °C. Doxorubicin (3.12 μg/mL) was used as a positive control. After 24 h, the cells were removed from the wells through exposure to trypsin-EDTA (0.25/0.02% v/v) and centrifuged. Six precipitates was obtained for each concentration of the test substance, which were mixed to obtain a single sample of each concentration. The following experiment was the isolation of total RNA using the RNeasy Mini kit (Qiagen) and Qiacube automation equipment.

2.5. Statistical analysis

All results were expressed as mean ± standard error of mean (SEM). The results of the experiments (n = 3) were submitted to analysis of variance (ANOVA) and Bonferroni post-test with a level of significance of $^*p < 0.05$.

3. Results and discussion

Previous studies showed that cell death plays an important role in the nephrotoxicity caused by snake venom from the Bothrops and Bothropoides genus (Morais et al., 2013; Mello et al., 2014; Marinho et al., 2015).

Previously, we demonstrated the effects of whole BeV on an isolated kidney model and it was be able to decreased percentage tubular transport of sodium and potassium (Martins et al., 2005). Tubular epithelial cells are the main targets of acute injury kidney caused by venoms (Sitprija and Sitprija, 2012). The loss of tubular epithelial cell function induced by BeV could cause decrease on percentage tubular transport of electrolytes. To continue these studies, we investigated the effects of BeV on the renal tubular epithelial cells.

MDCK cells constitute a very-well established cell line and have been extensively employed in the investigation of several cell processes, including epithelial transport and cell response to toxic agents and venoms (Chan et al., 1989; Collares-Buzato et al., 1994, 1998; 2002; Schwerdt et al., 2004; Peixoto and Collares-Buzato, 2005; Chen et al., 2006; Nascimento et al., 2007; Kusma et al., 2008).

In this study, we evaluated the cytotoxicity of whole venom on epithelial distal tubular MDCK renal cells using MTT assay and observed an $IC_{50} = 93$ μg/mL (Fig. 1A). The results indicated that the venom of *B. erythromelas* is cytotoxic to the renal cells, being similar the results of Collares-Buzato et al. (2002) who investigated the *in vitro* effects of whole venom of *Bothrops moojeni* using MDCK cell culture.

Apoptotic and necrotic cells can be detected by analyzing their light-scattering properties in flow cytometry (Krysko et al., 2011). Annexin-V and 7AAD were used for cell staining to identify the cell death pathway. In MDCK cells, flow cytometric analysis of BeV showed that the apoptotic cell and late apoptotic cell populations are significantly increased after 24 h-exposure to the IC50, with a small percentage of necrotic cells (Fig. 1B). Taken together, these results show that apoptosis is predominant.

The major compound of BeV is metalloproteinases (Jorge et al., 2015), a class of apoptosis-inducing snake venom protein, such as described by Brenes et al. (2010), Masuda et al. (1998, 2000, 2001). Furthermore, the *Bothropoides pauloensis* venom, of which main compound is metalloproteinases (Rodrigues et al., 2012) was also described as an inductor of apoptosis (Marinho et al., 2015).

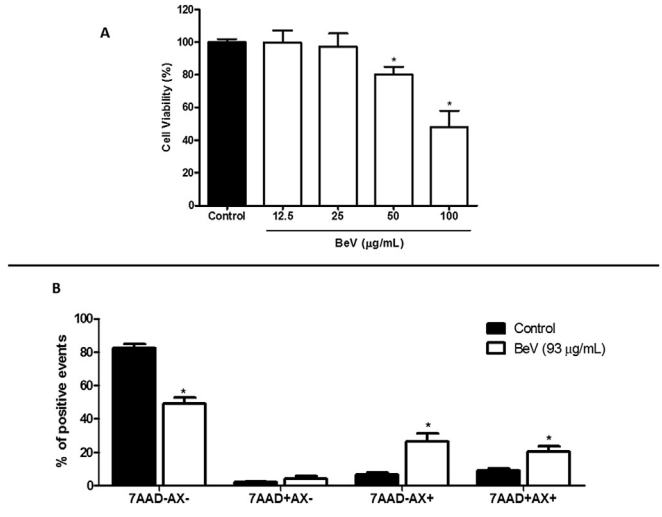

Fig. 1. Cytotoxic effect of *Bothropoides erythromelas* venom on MDCK cells. (A) MDCK cells were treated with different concentrations of *B. erythromelas* venom for 24 h and were evaluated by the reduction in the 3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide (MTT) salt in cells under BeV concentration-curve after 24 h of treatment. **(B)** Cell death was measured by annexin V and 7AAD staining and detected by flow cytometry. MDCK cells were treated with IC50 (93.31 μg/mL) of BeV after 24 h. All data are expressed as mean ± SEM of three independent experiments with three replicates and compared with negative control (One-way analysis of variance and Bonferroni post-test, $^*p < 0.05$).