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Short communication

Differences between renal effects of venom from two *Bothrops jararaca* populations from southeastern and southern Brazil

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ABSTRACT

Components from animal venoms may vary according to the snake's age, gender and region of origin. Recently, we performed a proteomic analysis of *Bothrops jararaca* venom from southern (*BjSv*) and southeastern (*BjSEv*) Brazil, showing differences in the venom composition, as well as its biological activity. To continue the study, we report in this short communication the different effects induced by the *BjSEv* and *BjSv* on isolated kidney and MDCK renal cells. *BjSEv* decreased perfusion pressure (PP) and renal vascular resistance (RVR) and increased urinary flow (UF) and glomerular filtration rate (GFR), while *BjSv* did not alter PP and RVR and reduced UF and GFR. Both types of venom, more expressively *BjSEv*, reduced %TNa^+, %TK^+ and %Cl^-. In MDCK cells, the two types of venom showed cytotoxicity with IC_{50} of 1.22 μg/mL for *BjSv* and 1.18 μg/mL for *BjSEv* and caused different profiles of cell death, with *BjSv* being more necrotic. In conclusion, we suggest that *BjSv* is more nephrotoxic than *BjSEv*.

1. Introduction

Snake venoms are a complex mixture of proteins, peptides and small inorganic molecules. Clinical manifestations of bothropic accidents consist in various local and systemic effects, mainly characterized by severe coagulopathy, necrosis, edema, spontaneous bleeding and, in some cases, compartment syndrome (França et al., 2003; Santoro et al., 2008). Systemic complications occur in the absence of antivenom administration, leading to more severe cases associated with intense bleeding, shock and acute kidney injury (AKI), which are closely related to death (Ribeiro and Jorge, 1997; De Castro et al., 2004; Sgrignolli et al., 2011).

There are many cases of snake envenomation annually in Brazil, most of them associated with the *Bothrops* genus (Queiroz et al., 2008). *Bothrops jararaca* (Wied-Neuwied, 1824) is a medically-relevant, semi-arboreal pit viper, found from the state of Bahia to Rio Grande do Sul in Brazil and in parts of Paraguay and Argentina. This wide geographical distribution range is associated with the most populated areas in Brazil (Sazima, 1992).

Recently, a combined proteomic study was conducted using venom pools from the outermost points of *B. jararaca* geographical distribution in Brazil. These representative venom pools of the southeastern and southern populations of *B. jararaca* revealed distinct quantitative and some qualitative overall toxin compositions of proteomes and transcriptomes. The different compositions of the venoms of *B. jararaca* from the south and southeast have demonstrated different biological actions, such as coagulant, proteolytic and of phospholipase A_2 actions (Gonçalves-Machado et al.,

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2016). To continue this study, we report in this short communication the differences in renal effects induced by both venoms on an isolated kidney perfusion model and cytotoxicity on MDCK renal epithelial cells.

2. Material and methods

B. jararaca venom samples from the southeastern snake population were collected from a number (n = 22) of adult specimens captured in the following states of Brazil: Rio de Janeiro, Espirito Santo and Minas Gerais. The specimens were maintained at the serpentarium of Vital Brazil Institute (Niterói, Rio de Janeiro, Brazil) under IBAMA registration n. 26354-9, while the venom samples of adult specimens of *B. jararaca* (n = 22) from the southern populations were captured in localities of the state of Rio Grande do Sul (RS) and kept in captivity in the serpentarium of Center of Ophiology of Porto Alegre, Rio Grande do Sul, Brazil, under IBAMA registration n. 1/43/1999/000764-9.

We performed kidney perfusion tests to evaluate the renal effects with no interference of systemic factors. In this assay, adult male Wistar rats (260–320 g) were fasted for 24 h with free access to water. The rats were anesthetized with sodium pentobarbitone (50 mg/kg, i.p) followed by careful dissection of the right kidney. The right renal artery was cannulated via the mesenteric artery without interrupting blood flow, as described by Bowman (1970). The perfusate consisted of modified Krebs-Henseleit solution (MKHS) consisting of (in mmol/L): 114.00 NaCl, 4.96 KCl, 1.24 KH_2PO_4, 0.5 $MgSO_4.7H_2O$, 2.10 $CaCl_2$ and 24.99 $NaHCO_3$. Bovine serum albumin (BSA 6 g%; fraction V), urea (0.075 g), inulin (0.075 g) and glucose (0.15 g) were added to the solution, resulting in a final perfusate volume of 100 mL. The pH was adjusted to 7.4. In each experiment, 100 mL of MKHS were recirculated for 120 min. The perfusion pressure (PP) was measured at the tip of the stainless steel cannula in the renal artery. Urine and perfusate samples were collected at 10 min-intervals for analysis of sodium, potassium and chloride levels by ion-selective electrodes (RapidChem 744, Bayer Diagnostic, UK); inulin, as described by Walser et al. (1955) and modified by Fonteles et al. (1983); and osmolality, which was measured in a vapor pressure osmometer (Wescor 5100C, USA). *BjSv* or *BjSEv* (10 μg/mL) was added to the system 30 min after the beginning of each perfusion. The perfusion pressure (PP), renal vascular resistance (RVR), urinary flow (UF), glomerular filtration rate (GFR), the percentage of sodium (%TNa+), potassium (%TK+) and chloride (%TCl-) tubular transport were determined (Martinez-Maldonado and Opava-Stitzer, 1978). The results were compared to the internal control group, at early 30 min in each experiment (n = 6). In this experiment, both right and left kidneys were fixed with formaldehyde until subsequent histological analysis. The fragments were subjected to dehydration, diaphanization and then cut into 3 μm thick slices. Then, hematoxylin-eosin (HE) staining was performed and the slides analyzed using an optical microscope.

We also performed assays in cells to evaluate renal cytotoxicity. Epithelial Madin–Darby Canine Kidney (MDCK) cells were cultured in RPMI 1640 medium (RPMI, Sigma-Aldrich; St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich, St. Louis, MO, USA), 1% penicillin/streptomycin solution at 37 °C, 5% CO_2. MDCK cells were cultured and treated with different concentrations of *BjSv* or *BjSEv* (3.12; 1.56; 0.78; 0.39 μg/mL), incubated at 37 °C for 24 h and mitochondrial function was measured by MTT colorimetric assay. MTT reagent (Sigma-Aldrich, St. Louis, MO, USA; 5 mg/mL in PBS) was added and incubated for 4 h at 37 °C. Finally, the medium was removed and the precipitated formazan crystals were dissolved in 10% Sodium dodecyl sulfate (Vetec, Rio de Janeiro, RJ, Brazil). After 17 h, absorbance was read at 570 nm in a microplate reader (Biochrom® Asys Expert Plus). Cell viability was calculated in comparison with the control group. The IC_{50} was determined by non-linear regression (Mosmann, 1983).

Finally, the cell death profile was evaluated with Annexin V-FITC (AX) and 7AAD staining. Cells treated with IC_{50} and 2 × IC_{50} of *BjSv* (1.22 and 2.44 μg/mL) and *BjSEv* (1.18 and 2.36 μg/mL) incubated during 12 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 AX-7AAD-viable cells was evaluated by flow cytometry. Data were collected in a FACSCalibur flow cytometer and analyzed using Cell Quest software (Becton-Dickinson, Mountain View, CA, USA).

Statistical analysis shows values as mean ± S.E.M of triplicate values for each cell experiment and hexaplicate values for perfused renal experiments. The significance of the difference between the experimental and control groups was assessed by one-way analysis of variance (ANOVA). Significance was defined as $p < 0.05$.

3. Results and discussion

Bothrops jararaca venom consists of a mixture of molecules, such as metalloproteinases, disintegrin, vasoactive peptides (VAP; including BPPs, bradykinin-potentiating peptides), phospholipase A_2 (PLA_2), serine proteinases (Serprot) and L-amino-acid oxidases (LAAO). The relative occurrence (in percentage of total venom proteins) of toxins from venom pools of *B. jararaca* populations from the Southeastern and Southern Atlantic forest regions disclose many differences. *BjSEv* has 35% of metalloproteinases, 7% of disintegrin, 16% of VAP, 4% of PLA_2, 14% of Serprot; while *BjSv* has 10% of metalloproteinases, 0.2% of disintegrin, 22% of VAP, 20.2% of PLA_2, 28% of Serprot, and (Gonçalves-Machado et al., 2016).

The intrinsic toxicity of toxins and their abundance in venom are relevant when evaluating their potential medical impact (Laustsen et al., 2015). Phospholipases and metalloproteinases, among several enzymes, are believed to contribute to renal toxicity (Sitprija and Sitprija, 2012). The *BjSEv* has more metalloproteinases, while the *BjSv* has a higher amount of venom PLA_2 (Gonçalves-Machado et al., 2016).

The effects of Bothrops venoms on isolated kidney have been previously demonstrated. The *Bothrops leucurus* (Morais et al., 2013), *Bothrops marajoensis* (Evangelista et al., 2010) and *Bothropoides pauloensis* (Marinho et al., 2015) cause reduction in PP, RVR, UF, GFR and tubular transport of electrolytes. Furthermore, *Bothrops moojeni* (Barbosa et al., 2002), *Bothropoides erythromelas* (Martins et al., 2005) and *Bothrops jararacussu* venoms (Havt et al., 2001) decrease PP, RVR, tubular transport of electrolytes and increase UF and GFR.

Our results showed different profiles of renal alterations on isolated kidney. *BjSEv* decreased PP (Fig. 1A) and RVR (Fig. 1B) at 60 min, increased UF (Fig. 1C) at 90 and 120 min and GFR (Fig. 1D) at 120 min. *BjSv*, in turn, did not alter PP, RVR (Fig. 1A and B) and reduced UF and GFR (Fig. 1C and D) at 60, 90 and 120 min. Both venoms caused renal tubular dysfunction by altering the percentage of tubular transport of electrolytes. They reduced $\%TNa^+$ (Fig. 1E), $\%TK^+$ (Fig. 1F) and $\%TCl^-$ (Fig. 1G) at 60, 90 and 120 min, with the reduction of $\%TNa^+$ and $\%TCl^-$ induced by *BjSEv* being more significant.

Bothrops erythromelas venom has shown alterations in the perfused kidney (Martins et al., 2005), similar to alterations observed in *BjSEv.* Metalloproteinases are the major component in *B. erythromelas* venom (Jorge et al., 2015) and *BjSEv,* suggesting this fraction may be responsible for part of the renal alterations. *BjSEv* showed a diuretic effect, in turn, *BjSv* showed a sharp decline of glomerular filtration rate together with tubular dysfunction.

Renal pathological changes in patients with AKI caused by snakebites include tubular necrosis, cortical necrosis,