Nephrotoxicity induced by the venom of Hypnale hypnale from Sri Lanka: Studies on isolated perfused rat kidney and renal tubular cell lines

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**ABSTRACT**

The hump-nosed pit viper Hypnale hypnale is responsible for a high number of snakebite cases in southwestern India and Sri Lanka. Although most patients only develop local signs and symptoms of envenoming, there is a growing body of evidence indicating that these envenomings may be associated with systemic alterations, including acute kidney injury. In this study we evaluated the renal toxicity of *H. hypnale* venom by using a perfused isolated rat kidney system and by assessing cytotoxicity in two different renal tubular cell lines in culture. The venom caused alterations in several renal functional parameters, such as reduction on perfusion pressure, renal vascular resistance, and sodium and chloride tubular transport, whereas glomerular filtration rate and urinary flow initially decreased and then increased after venom perfusion. In addition, this venom was cytotoxic to proximal and distal renal tubular cells in culture, with predominance of necrosis over apoptosis. Moreover, the venom affected the mitochondrial membrane potential and induced an increment in reactive oxygen species in these cells. Taken together, our results demonstrate a nephrotoxic activity of *H. hypnale* venom in these experimental models, in agreement with clinical observations.

1. **Introduction**

Snakebite envenoming is a serious medical hazard in the Indian peninsula and Sri Lanka (Warrell, 1995). In addition to the traditional so-called ‘big four’ species (*Daboia russelii, Echis carinatus, Naja naja* and *Bungarus caeruleus*), which are responsible for the majority and most severe snakebite envenomings, the hump-nosed viper, *Hypnale hypnale*, causes a high number of snakebites in this region (Warrell, 1995; Ariaratnam et al., 2008, 2009; Shivanthan et al., 2014). In the past, *H. hypnale* was considered as incapable of inflicting severe envenomings in humans, but growing clinical evidence clearly shows that this snake is able to cause systemic manifestations.

The majority of envenomings by *H. hypnale* are characterized by only local effects, i.e. edema of variable extension and pain (Warrell, 1995; Ariaratnam et al., 2008; Kularatne et al., 2011). In addition, a number of cases also develop systemic effects of envenoming, such as mild coagulopathy, bleeding, thrombotic microangiopathy, acute kidney injury (AKI), and chronic kidney injury (CKI) (de Silva et al., 1994; Kularatne and Ratnatunga, 1999; Joseph et al., 2007; Ariaratnam et al., 2008, 2009; Kularatne et al., 2011; Herath et al., 2012; Maduwage et al., 2013; Karunarathne et al., 2013; Shivanthan et al., 2014; Rathnayaka et al., 2018, 2019a, 2019b). AKI is one of the main causes of morbidity and mortality in envenomings inflicted by many snake species (Albuquerque et al., 2014).

The pathogenesis of AKI induced by the venom of *H. hypnale* has remained elusive, although the effects of this venom in the kidney at experimental level include damage to proximal convoluted tubules (Gunatilake et al., 2003; Silva et al., 2012; Tan et al., 2012). As with other snake venoms that affect the kidney, it is likely that nephrotoxicity by *H. hypnale* is of multi-factorial nature, probably involving mechanisms such as (a) renal ischemia secondary to hemodynamic disturbances, (b) thrombotic microangiopathy and hemolytic
2. Materials and methods

2.1. Venom

A pool of venom from 35 adult specimens of *H. hypnale* was used in this study. Venom was obtained from specimens collected in the field in four provinces of Sri Lanka [Central Province (wet zone); North Western province (dry and intermediate zones); Southern province (wet zone); Sabaragamuwa province (wet zone)] following the granting of a collecting permit from the Department of Wildlife Conservation, Sri Lanka (WL/3/2/7), and kept at the serpentarium of Animal Venom Research International (AVRI) in Sri Lanka. The male: female ratio was 20 : 15. Upon collection, venom was frozen and then freeze-dried, and stored at −20 °C until used. The identification of the specimens as belonging to *H. hypnale* was based on the keys previously published by Maduwage et al. (2009).

2.2. Kidney perfusion experiments

Adult male Wistar rats (260–320 g) were fasted for 24 h with free water access. They were anesthetized with sodium pento-barbitone (50 mg/kg, i.p.) and, after dissection of the right kidney, the renal artery was cannulated through the mesenteric artery. The kidney was perfused with a modified Krebs-Henseleit solution (MKHS) with the following composition (in mmol/L): 114 NaCl, 4.96 KCl, 1.24 KH2PO4, 0.5 MgSO4.7 H2O, 2.10 CaCl2, 25 NaHCO3. Bovine serum albumin (BSA, 6% v/v), urea (0.75 mg/mL), and glucose (1.5 mg/mL) were added to the solution, and the pH was adjusted to 7.4. Thereafter, inulin (0.75 mg/mL) was added. In each experiment, 100 mL of MKHS were recirculated during 120 min through the system. The perfusion pressure was kept between 120 and 140 mmHg. Initially, perfusion was performed without venom for 30 min in order to have basal levels of each determination. After this, *H. hypnale* venom (10 μg/mL) was added to the system and changes were observed. Samples of urine and perfusate were collected every 10 min and perfusion pressure (mmHg) and flow (L/min) were recorded every 5 min. A control group perfused with MKHS only was followed during 120 min. Five rats were used for each treatment.

Perfusion pressure (PP) was determined at the tip of the stainless steel cannula in the renal artery. Osmolarity was measured by using a PZL-1000 osmometer (Wescor 5100C, USA), and concentrations of sodium, potassium and chloride were determined by using a 9190 Electrolyte Analyzer (Rapidchem 744, Bayer Diagnostic, UK). Inulin concentration was quantified according to Walse et al. (1995) as modified by Fontes et al. (1983). The following renal functional parameters were determined: perfusion pressure (PP), renal vascular resistance (RVR), urinary flow (UF), glomerular filtration rate (GFR), and the percentages of sodium (%TNa+), potassium (%TK+), and chloride (%TCl−) total tubular transport and proximal tubular transport (Martinez-Maldonado and Opava-Stitzer, 1978). Results were compared to the internal control group corresponding to the values at the 30 min perfusion period prior to venom introduction into the system. The protocol was approved the Ethics Committee on Animal Research (CEPA) of the Federal University of Ceará (Permit Number 79/08).

2.3. Cytotoxicity of venom on kidney-derived cells

Cytotoxicity of venom was tested in cell culture using the cell lines LLC-MK2 (Monkey Kidney epithelial cell line) and MDCK (Madin-Darby Canine Kidney epithelial cells), as previously described (Morais et al., 2013; Mello et al., 2014). These cells correspond to proximal and distal renal tubular cells, respectively. Cells were grown in DMEM supplemented with 10% bovine fetal serum, under 5% CO2 at 37 °C. Upon confluence, cells were grown in DMEM without fetal serum for 24 h. Then, the confluent monolayers were treated with 0.1% trypsin and cells were grown in 96 well plates at a seeding density of 104 cells/well. Cells were left to adhere to the plate overnight and were then treated with venom. For this, solutions of various venom concentrations were added to wells and incubated for 24 h. Control wells were treated with 0.12 M NaCl, 0.04 M phosphate, pH 7.2 (PBS) solution. Assays were run in triplicates. Cytotoxicity was assessed by reduction of 3-(4,5-di-methylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT, Sigma, St. Louis, MO, USA). Ten microliters of MTT solution (2.5 mg/mL in PBS) were added to each well, at the time intervals indicated, and incubated at 37 °C for 4 h. The medium was removed and the precipitated formazan crystals were dissolved in 10% sodium dodecyl sulphate (SDS) in 0.01 N HCl. After 17 h of incubation, absorbances at 570 nm were recorded in a microplate reader (Biochrom® Asys Expert Plus). Cell viability was estimated in comparison with the control group. The Median Cytotoxic Concentration (CC50), i.e. the venom concentration that induced 50% cytotoxicity, was estimated using the program GraphPad Prism.

2.4. Annexin V-FITC staining

Staining of cells with Annexin was used to assess apoptosis. LLC-MK2 or MDCK cells were seeded at 105 cells/well, as described, and incubated for 24 h at 37 °C. Then, solutions of two venom concentrations, corresponding to 1 and 2 CC50, were added. Controls included cells incubated with PBS. After an incubation period of 24 h, cells were trypsinized, centrifuged at 500 g for 5 min, and the supernatant was discarded. This step was repeated twice using PBS. The cellular pellet was labeled with the Annexin/7AAD using the kit BD Pharmingen PE Annexin Apoptosis Kit I (BD Pharmingen, CA, USA). Controls included cells incubated with either Annexin/7AAD alone. Samples were incubated for 30 min at room temperature in the dark. Then, samples were analyzed by flow cytometry in a FACS Calibur (Becton-Dickinson, Mountain View, CA, USA) and analyzed using a Cell Quest software (Becton-Dickinson).

2.5. Rhodamine staining

Staining of cells with Rhodamine 123 (Sigma) was used to assess the mitochondrial membrane potential (Baracca et al., 2003). Cells were incubated with venom solutions, as described above. Then, after centrifugation, the cellular pellet were resuspended in 100 μL PBS, and 100 μL of a Rhodamine 123, dissolved in PBS, were added. After an incubation of 30 min at room temperature in the dark, 1 mL of PBS was added and the preparation was centrifuged at 500 g for 5 min. The pellets were resuspended in 500 μL PBS and flow cytometry analysis was then carried out.

2.6. DCFH-DA staining

Staining of cells with 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA) (Sigma) was used to measure the cellular levels of reactive oxygen species, as described by Morais et al. (2015). Cells were incubated with venom, as described above. After incubation, cells were trypsinized and centrifuged at 500 g for 5 min. After several washing steps, cells were resuspended in PBS containing 5 μM DCFH-DA, and incubated for 30 min. Controls included cell suspensions in which
DCFH-DA was not added. After incubation, cells were analyzed by flow cytometry. The data based on the FL1 channel were analyzed with the Cell Quest program.

2.7. Statistical analyses

Results were expressed as mean ± S.E.M. of three or five replicates, in the case of cell culture and renal perfusion experiments, respectively. The significance of the differences between mean values was assessed by ANOVA, using a Dunnett post-hoc test to compare pairs of means, using the program GraphPad Prism® 5.0. Values of p < 0.05 were considered statistically significant.

3. Results

3.1. Effects of venom on kidney functional parameters

Control experiments carried out on kidneys perfused with Krebs-Henseleit solution only did not show any significant changes in the parameters evaluated along the 120 min observation time. The perfusion of a venom solution (10 μg/mL) in the isolated kidney preparation induced changes, as compared to controls, in almost all functional parameters evaluated. There was a significant reduction in perfusion pressure at 90 and 120 min (PP; Fig. 1A), renal vascular resistance at 90 and 120 min (RVR; Fig. 1B), percentage of transported sodium at 90 and 120 min (%pNa⁺; Fig. 1E), percentage of proximal tubule transported sodium at 60, 90 and 120 min (%pNa⁺; Fig. 1F), percentage of transported chloride at 120 min (%Cl⁻; Fig. 1H), and percentage of proximal tubule transported chloride at 120 min (%pCl⁻; Fig. 1J). Regarding urinary flow, there was a slight initial reduction at 60 and 90 min (UF; Fig. 1C), followed by an increment at 120 min, whereas the glomerular filtration rate had a reduction at 60 and 90 min (GFR; Fig. 1D), followed by recovery at 120 min. Of the evaluated parameters, only the percentage of transported potassium was not altered upon infusion of the venoms (Fig. 1G and H).

3.2. Cytotoxicity on kidney-derived cells

When cytotoxicity of venom on two kidney-derived cell lines was assessed by the MTT assay, a dose- and time-dependent cytotoxic effect was observed in both cell lines. The dose-dependency was more evident when incubation was carried out for 24 h and, therefore, the estimation of the Median Cytotoxic Concentration (CC50) was made at this time. CC50s corresponded to 9.0 ± 2.9 μg/mL and 19.7 ± 12 μg/mL for LLC-MK2 and MDCK cell lines, respectively. Thus, venom was cytotoxic for cell lines derived from proximal and distal renal tubular cells.

3.3. Evaluation of cell death mechanisms

Cells of both lines were exposed to a concentration of venom corresponding to one CC50 for 24 h, after which cells were stained with annexin (to assess apoptosis) and with 7AAD (to assess plasma membrane disruption, i.e., necrosis). As depicted in Fig. 2, necrosis was the predominant effect at the venom concentration tested.

Cells of both lines exposed to a concentration of venom corresponding to one CC50 were stained with Rhodamine, in order to assess the effects on the mitochondrial membrane potential. Venom induced a drastic reduction in Rhodamine fluorescence at both concentrations (Fig. 3), hence evidencing a drop in the mitochondrial membrane potential. Moreover, venom induced an increment in the concentration of reactive oxygen species in the two kidney-derived cells lines, at concentrations corresponding to one and two CC50, as revealed by an increment in the relative fluorescence of DCFH-DA (Fig. 4).

4. Discussion

The majority of envenomings caused by H. hypnale are characterized only by local effects, i.e. pain and edema, but a growing body of published clinical evidence demonstrates that this vipersid species is also capable of inducing severe envenomings characterized by systemic manifestations, such as mild coagulopathy, hemorrhage, thrombotic microangiopathy, and AKI (de Silva et al., 1994; Ariaratnam et al., 2008, 2009; Kularatne et al., 2011; Herath et al., 2012; Maduwage et al., 2013; Shivanthan et al., 2014; Rathnayaka et al., 2018, 2019a, 2019b). The pathogenesis of AKI in envenomings by this and other snake species is likely to be multifactorial (Sitprija and Sitprija, 2012). The clinical expression of AKI is an oliguric or anuric state associated with acute tubular necrosis after envenoming (Albuquerque et al., 2014).

Previous studies have assessed the effects of venoms of various species of snakes of the genus Bothrops in the isolated kidney model used in the present investigation (Evangelista et al., 2010; Morais et al., 2013; Marinho et al., 2015). Bothrops sp venoms induced a drop in perfusion pressure, renal vascular resistance, urinary flow, glomerular filtration rate, and sodium tubular transport. Bothrops moojeni and B. jararacussu venoms showed similar effects, but caused an increase in urinary flow (Havt et al., 2001; Barbosa et al., 2002). In the present work, H. hypnale venom promoted a reduction of perfusion pressure, renal vascular resistance, sodium and chloride tubular transport, and an initial drop of glomerular filtration rate and urinary flow, followed by an increment in these two parameters. Our findings underscore the ability of H. hypnale venom to induce renal damage. Although the mechanisms behind these alterations remain to be elucidated, our study provides experimental evidence that this venom alters functional parameters, such as reduction on RVR and PP. These alterations could lead to renal ischemia secondary to hemodynamic disturbances. The pathogenesis of AKI in envenomings by H. hypnale may be also mediated through the formation of microthrombi, i.e. thrombotic microangiopathy, which contribute to renal ischemia (Rathnayaka et al., 2019b).

The infusion of H. hypnale venom in the isolated rat kidney caused alterations in practically all the parameters investigated, hence underscoring a widespread effect of the venom on renal function. Moreover, our observations suggest that this venom affects the function of both the tubular cells and the glomeruli, as evidenced by electrolyte transport and glomerular filtration rate. Previously, H. hypnale venom had been shown to induce AKI in experimental models, as revealed by increments in serum urea and creatinine concentrations, hematuria, proteinuria, and by histopathological changes in the kidney (Gunatilake et al., 2003; Silva et al., 2012; Tan et al., 2012). Our observations on the effect of this venom on sodium tubular transport is in agreement with a recent clinical observation of a patient who developed urinary sodium loss associated with hyponatremia (de Silva et al., 2018).

H. hypnale venom was cytotoxic to both renal tubular cell types tested in culture (MDCK and LLC-MK2), as judged by the MTT assay. Cytotoxicity involving necrosis and apoptosis has been described in the same cell lines with the venom of Bothrops sp (Morais et al., 2013; Mello et al., 2014; Marinho et al., 2015). It was previously shown that the venoms of H. hypnale, H. zara and H. nepa inhibited cell proliferation of rat aorta smooth muscle cells at a relatively low concentration (Maduwage et al., 2011). In our observations, when cells were incubated with a venom concentration corresponding to one CC50 for 24 h, more cells were positively stained with 7AAD than with annexin, thus indicating that at this dose and time of incubation the majority of cells was affected by necrosis instead of apoptosis. It has to be considered, however, that the balance between necrosis and apoptosis in this model may be shifted when using other venom concentrations, as shown for a lymphoblastoid cell line incubated with a myotoxic PLA2 homologue from the venom of Bothrops asper (Mora et al., 2005). The direct toxicity on tubular epithelial cell lines by necrosis can lead to a
Effects of *H. hypnale* venom (10 μg/mL), on perfusion pressure (A), renal vascular resistance (B), urinary flow (C), glomerular filtration rate (D), percent of sodium, potassium and chloride tubular transport (E, G, I) and percent of proximal tubule sodium, potassium and chloride transport (F, H, J), in the isolated perfused rat kidneys. The columns and bars represent the mean ± S.E.M. for five rats. The results in samples treated with venom were compared to the control corresponding to the first 30 min of perfusion with Krebs-Henseleit solution before venom was added (black bar) (*p < 0.05 as compared to control).
Fig. 2. Effects on cell death of the *H. hypnale* venom measured by Annexin V-PE and 7-AAD staining and detected by flow cytometry. MDCK (B, C) and LLC-MK2 (E, F) cells were treated with 1 CC50 of *H. hypnale* venom for 24 h. The control group corresponds to cells without venom treatment (A: MDCK, D: LLC-MK2). On the flow cytometric scatter graphs, the left lower quadrant represents the live cells, the right lower quadrant represents the population of early apoptotic cells, the right upper quadrant represents the accumulation of cells in terminal stage of cell death, and the left upper quadrant corresponds to the population of necrotic cells. Results in C and F correspond to means ± S.E.M. of three replicates from three independent experiments (*p < 0.05 as compared to controls).

Fig. 3. *H. hypnale* venom effects on mitochondrial transmembrane potential. MDCK (A, B) and LLC-MK2 (C, D) cells were treated with 1 CC50 and 2 CC50 of *H. hypnale* venom for 24 h. Then, cells were stained with rhodamine 123 (Rho123) and analyzed in the flow cytometer to measure the decrease in Rho123 accumulation in mitochondria. A and C show a histogram of representative mitochondrial transmembrane potential analysis in cell populations treated with venom. B and D present the fluorescence ratio relative to control ± SEM, of three replicates from three independent experiments (*p < 0.05 as compared to control).
rapid cell loss in vivo, with a consequent electrolyte dysfunction, as a contributory factor to the development of AKI.

Experiments were also performed with Rhodamine 123 in order to assess the effect on the mitochondrial membrane potential and DCF to assess the ROS production. There was a drastic reduction in this potential and an increase on ROS generation, underscoring a process of cell damage. The loss of mitochondrial membrane potential has been associated with the opening of the permeability transition pore in mitochondrial membranes (Bernardi and Rasola, 2007), a mega-channel that can assemble as a consequence of mitochondrial calcium overload, owing to increments in cytosolic calcium concentration following plasma membrane damage, and also following an increment in reactive oxygen species in the cell (Rottenberg and Hoek, 2017). It is therefore suggested that cytotoxicity induced by H. hypnale venom in these cell lines is likely to depend on a direct action of venom cytotoxic components on the plasma membrane, with a disruption in its integrity. This is followed by a calcium influx which directly affects mitochondria and contributes to the generation of reactive oxygen species, ending in irreversible cell damage. Owing to the high proportion of phospholipases A2 in the proteome of this venom (Ali et al., 2013; Tan et al., 2015; Vanuopadath et al., 2018), it is proposed that venom cytotoxicity is due to the action of this hydrolytic enzymes on plasma membrane phospholipids, a hypothesis that awaits the isolation of these components.

The antivenoms used in Sri Lanka and the Indian peninsula do not include Hypnale sp venom in their immunizing mixture, they are ineffective in envenoming by H. hypnale (Sellahewa et al., 1995; Keyler et al., 2013). Recently, a new polyspecific antivenom was developed which included the venom of H. hypnale in the immunizing scheme. This antivenom proved effective in the neutralization of lethal, hemorrhagic, in vitro coagulant, proteinase and phospholipase A2 activities of H. hypnale venom (Villalta et al., 2016). It would be relevant to assess whether this antivenom is effective at abrogating the renal effects of the venom.

In conclusion, our findings provide evidence of a nephrotoxic action of the venom of H. hypnale, as demonstrated by perturbation of several renal functions in an isolated kidney preparation and by a direct cytotoxic effect on proximal and distal tubular epithelial cell lines. These experimental observations are in line with previous experimental studies describing nephrotoxicity by this venom, and by clinical reports highlighting the potential of H. hypnale venom to induce AKI. The mechanisms of this pathophysiological effect and its neutralization by antivenoms deserve further investigations.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Ethical statement

The experimental protocol used with rats was approved the Ethics Committee on Animal Research (CEPA) of the Federal University of Ceará (Permit Number 79/08).

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