HISTOLOGIC AND FUNCTIONAL RENAL ALTERATIONS CAUSED BY BOTHROPS MOOJENI SNAKE VENOM IN RATS

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Abstract. Acute renal failure (ARF) is the main cause of death following snake bites by Bothrops species. In this study, we investigated the morphologic and functional renal disturbances caused by Bothrops moojeni venom in rats. Renal function was assessed based on creatinine and lithium clearances and on histologic examination of renal tissue 5 hr after the intravenous administration of 0.2 mg of venom/kg and 5 hr, 16 hr, and 48 hr after 0.4 mg of venom/kg. A venom dose of 0.4 mg/kg produced renal tubule disturbances, including acute impairment of proximal and post-proximal tubule sodium handling associated with acute tubule necrosis. The glomerular filtration rate (GFR) decreased significantly and was accompanied by severe morphologic disturbances in the renal glomeruli. These functional and morphologic findings were observed in the absence of any change in mean arterial blood pressure. The decrease in GFR was not related to the presence of fibrin deposits in the glomerular capillary loops. These results suggest an early nephrotoxic action of B. moojeni venom involving significant morphologic and functional changes similar to those observed in snakebite-induced ARF in humans.

Envenomation following snakebite is a health problem in tropical regions of the world. The genus Bothrops contains many species distributed from Mexico to Argentina.1 Of the 20,000 snakebite accidents per year in Brazil, about 90% are attributed to the genus Bothrops.2 Bothrops moojeni is the most common cause of snakebite in central Brazil (Cardoso JLC, unpublished data). In common with other Bothrops venoms,3,4,7 B. moojeni venom contains blood-clotting, hemorrhagic, phospholipase A2, and proteolytic activities.8

Envenomation by Bothrops species leads to local and systemic effects that develop simultaneously. The local lesions include edema, pain, erythema, ecchymosis, bullae, cyanosis, necrosis, and cellulitis. The most serious systemic change and primary cause of death is acute renal failure (ARF) secondary to acute tubular necrosis and, occasionally, glomerulonephritis.3,4,7,9-14 Thus, elucidation of the mechanisms involved in this nephrotoxicity is important. Experimental models used to investigate the pathogenesis of renal lesions caused by bothropic venoms have shown hemodynamic changes and hemolysis prior to renal ischemia.4,6,15 Ischemia caused by massive fibrin deposition in the glomerular capillaries as well as intravascular hemolysis appear to have a crucial role in the physiopathology of renal failure.16 However, a direct nephrotoxic action of the venom cannot be excluded.

In this work, we studied the pathogenesis of the alterations in renal function and morphology in rats after a single intravenous injection of B. moojeni venom.

MATERIALS AND METHODS

Animals and venom. Male Wistar rats (200–300 g) were obtained from an established colony maintained by the Central Animal House Service of the Universidade Estadual de Campinas. Lyophilized B. moojeni venom was donated by the Instituto Butantan (São Paulo, Brazil) and stored at −20°C. The rats were anesthetized with ether and received an intravenous injection of either 0.2 mg or 0.4 mg of B. moojeni venom per kg. The dose of 0.4 mg/kg was chosen based on an estimate of the volume of venom inoculated in a human snakebite.16 The venom was dissolved in sterile 0.15 M NaCl immediately before use.

Experimental protocol. One group of rats was injected with venom (V1) and another, which served as the control group, received 0.15 M NaCl solution (S1). The renal function of both groups was evaluated 5 hr after injection (V1, n = 5; S1, n = 5), 16 hr (V1, n = 6; S1, n = 4), and 48 hr (V1, n = 5; S1, n = 5) postinjection. Two other groups of rats that received venom (V2; 0.2 mg/kg; n = 5) or saline (S2; n = 5) were also studied 5 hr after injection. Fourteen hours before the renal function tests, LiCl (60 μmol/100 g of body weight) was given by gavage. Each rat received a tap water load by gavage (5% of body weight) 3.2 hr before killing, followed by a second load of equal volume 1 hr later. Twenty minutes after the second load, the rats were housed individually in metabolic cages and spontaneously voided urine was collected over a 2-hr period. At the end of the experiment, the

Table 1

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>Time (hr)</th>
<th>Ccr (μl/min/100 g)</th>
<th>FENA (%)</th>
<th>FEK (%)</th>
<th>CLI (μl/min/100 g)</th>
<th>FEPNa (%)</th>
<th>FEPPNa (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>≥5 (n = 19)</td>
<td>270.9 ± 20</td>
<td>0.51 ± 0.15</td>
<td>0.31 ± 0.14</td>
<td>71.0 ± 8.4</td>
<td>31.8 ± 13.5</td>
<td>1.82 ± 0.47</td>
</tr>
<tr>
<td>0.2</td>
<td>5 (n = 5)</td>
<td>220.4 ± 26</td>
<td>1.32 ± 0.12†</td>
<td>0.95 ± 0.17†</td>
<td>140.2 ± 15.9†</td>
<td>77.5 ± 17.6</td>
<td>1.9 ± 0.11</td>
</tr>
<tr>
<td>0.4</td>
<td>5 (n = 7)</td>
<td>173.5 ± 11†</td>
<td>1.16 ± 0.12‡</td>
<td>0.34 ± 0.08</td>
<td>128.6 ± 5.9‡</td>
<td>88.9 ± 18.9‡</td>
<td>1.44 ± 0.62</td>
</tr>
<tr>
<td>0.4</td>
<td>16 (n = 6)</td>
<td>184.5 ± 27.6†</td>
<td>0.88 ± 0.36‡</td>
<td>0.67 ± 0.04‡</td>
<td>75.1 ± 2.5</td>
<td>48.5 ± 15.1</td>
<td>2.02 ± 0.39</td>
</tr>
<tr>
<td>0.4</td>
<td>48 (n = 5)</td>
<td>180.8 ± 25.5†</td>
<td>1.46 ± 0.44‡</td>
<td>0.52 ± 0.05‡</td>
<td>87.2 ± 2.3</td>
<td>29.7 ± 13</td>
<td>3.35 ± 0.75‡</td>
</tr>
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* Values are the mean ± SEM.
† P < 0.05 compared with the saline group by analysis of variance.
Figure 1. Morphologic appearance of kidney tubules stained with hematoxylin and eosin. Control (a), 5 hr (b, c, e, and f) and 16 hr (d) after injection of Bothrops moojeni venom. In c, the proximal tubules showed degenerative changes characterized by acidophilic coloration, cytoplasmic vacuolation (arrowheads) and pyknotic nuclei (N). Tubular necrosis was present 5 hr (b) and 16 hr (d) after envenomation. Hemorrhage (e) was present after 5 hr and red blood cells (arrows) were seen in the tubule lumen (f). (Magnification × 1,125, bar = 10 μm.)
Figure 2. Periodic acid–Schiff–stained renal tissue of saline-treated rats (a). Glomerular lobulations with nodular lesions, distal tubule (D) swelling, discontinuity of proximal tubule cells, thickening of the brushborder, and nuclear pyknosis (b and c) were present after 5 hr. After
rats were anesthetized and blood samples were drawn by cardiac puncture. The kidneys were removed and processed for light microscopy.

**Histopathologic analysis.** The kidneys were cut sagittally and fixed in situ with Bouin solution for 24 hr. The tissues were washed for 12 hr, dehydrated in a graded ethanol series, and embedded in Histosec (Merck, Rio de Janeiro, Brazil). Sections 5 μm thick were stained with hematoxylin and eosin, periodic acid-Schiff, and Mallory’s phosphotungstic hematoxylin for examination by light microscopy.

**Measurement of blood pressure.** Systemic arterial pressure was measured in conscious rats by an indirect tail-cuff method using an electrophysgmannometer (Narco Bio-Systems, Austin, TX) combined with a pneumatic pulse transducer/amplifier, which provides output signals proportional to cuff pressure and amplified Korotkoff sounds. The mean of 3 consecutive readings represented the blood pressure 5 hr, 16 hr, and 48 hr after venom injection.

**Biochemical determinations.** Plasma and urinary sodium, potassium, and lithium concentrations were measured by flame photometry (B262; Micronal, São Paulo, Brazil). Creatinine was determined spectrophotometrically (362; Micronal, São Paulo, Brazil).

**Renal function.** The urine eliminated by venom (0.4 mg/kg)-treated animals 5 hr and 16 hr after venom injection was red, indicative of strong hematuria. This dose of venom caused a dramatic and significant decrease in the GFR compared with the control rats (Table 1). This decrease was seen ≥ 5 hr after venom injection. After a venom dose of 0.2 mg/kg, this decrease was not significantly different from that in saline-treated rats, although the venom-treated rats showed a significant increase in FENa⁺. An increase in urinary sodium excretion was seen in both venom-treated groups (V₁ and V₂), although Ccr was unaltered after a venom dose of 0.2 mg/kg (Table 1). There was a significant increase in FEK⁺ 16 hr after the injection of 0.4 mg of venom/kg. After 48 hr, FEK⁺ had decreased, but was still higher than control levels (Table 1). A venom dose of 0.2 mg/kg also significantly increased the excretion of this ion. Proximal tubular sodium excretion increased significantly 5 hr after both doses of venom and coincided with the increase in CLi (Table 1). After the venom dose of 0.4 mg/kg, this increase was followed by a compensatory increase in fractional post-proximal sodium reabsorption (Table 1). The proximal and post-proximal tubular sodium excretion remained elevated 16 hr after venom injection, even though the natriuresis was significant when compared with the saline-treated group (Table 1). Forty-eight hours after envenomation, the CLi and FEPNa⁺ did not differ significantly from control values. The sustained increase in FENa⁺ observed at this time was associated with an increase in post-proximal sodium reabsorption (Table 1).

**Blood pressure.** Mean ± SEM arterial pressure did not change significantly after a venom dose of 0.4 mg/kg. The basal blood pressure (108 ± 8.7 mm of Hg) was not significantly altered after 5 hr (101 ± 10.3 mm of Hg), 16 hr (119 ± 18.6 mm of Hg), and 48 hr (117.9 ± 16.9 mm of Hg).

**Histology.** Light microscopy showed that kidneys from rats injected with 0.15 M NaCl solution had a normal morphology (Figures 1a and 2a). There were no changes in the renal parenchyma 5 hr after 0.2 mg of venom/kg. In contrast, a venom dose of 0.4 mg/kg increased tubular cell acidophilia, indicating cell damage and peritubular capillary congestion (Figure 1e and f).

Marked changes were observed in the glomeruli (Figure 2b) 5 hr after venom injection (0.4 mg/kg). The glomeruli showed either withdrawal and lobulation of the capillary tufts or partially destroyed glomerular capillaries with dilated Bowman’s space. Nodular lesions and neutrophils were observed in the glomerular capillaries (Figure 3a). The glomerular changes seen 16 hr after envenomation resembled those seen after 5 hr (Figure 3b). The severity of the lesions varied with some showing capillary tufts with a diffuse distribution of the mesangial matrix. The latter was responsible for the poor staining with hematoxylin and eosin and low reactivity to periodic acid–Schiff (Figure 3c). Hypertrophied nuclei were frequently observed in these glomeruli (Figure 3c). Staining with Mallory’s phosphotungstic hematoxylin showed no fibrin in the capillaries 5 hr or 16 hr after venom (Figure 4b and c). After 48 hr, most of the glomeruli were normal in appearance (Figure 3d).

Degenerative changes were seen in the proximal tubules 5 hr after envenomation. These changes consisted of a loss of proximal brush border, cytoplasmic vacuolation, and in some tubules, degeneration and desquamation of necrotic

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16 hr, proximal tubules containing nuclei and acidophilic cellular debris and a discontinuous epithelium were present (e). Mitosis was also seen (arrow). There was cellular debris in the lumen of the collecting ducts (C) and the brushborder (arrowheads) was discontinuous (d) (a and b, magnification × 225, bar = 50 μm; c and d, magnification × 450, bar = 25 μm; e, magnification × 1,125, bar = 10 μm).
FIGURE 3.  a, a damaged glomerulus 5 hr after venom injection showing glomerular lobular lesions and nodules that form a densely clumped, strongly stained mesangial matrix (arrows). Note also the widened capsular space (*) and scattered neutrophils (arrowheads). b, severely damaged glomeruli with indistinguishable glomerular-like structure after 16 hr. U = urinary pole with hematic cast. c, glomeruli with a heterogeneously distributed, diffuse mesangial matrix containing neutrophils and hyperthrophied nuclei 16 hr after envenomation. d, reversal of the structural changes after 48 hr. (Periodic acid-Schiff stained, magnification × 1,125, bar = 10 μm.)
cells. The nuclei of the various proximal tubular cells often showed pyknosis with clumping of chromatin material (Figures 1c, 2b, and c). In some tubules, the renal epithelium was completely necrotic whereas the basement membrane was either intact (Figure 1b) or disrupted by tubular necrosis. The distal tubules, loops of Henle, and collecting ducts had swollen lumens (Figure 2b). In some areas, there was disruption of both the peritubular capillaries and tubular walls, indicating hemorrhage (Figure 1e). Hyaline casts, red blood cells (intact or hemolyzed) and sloughed cellular debris filled the lumens (Figure 1f).

By 16 hr after envenomation, a significant proportion of the renal tubules showed increased acidophilia and dilatation. The brush border occasionally showed thickening, cytoplasmic vacuolation, and nuclear pyknosis. The mitosis seen in proximal cells was suggestive of regeneration. Cell debris and casts resulting from necrosis were observed in the lumen of proximal and distal tubules and in the collecting ducts (Figure 2d and e). Necrotic areas were present in tubules of the cortico-medullary region (Figure 1d). Peritubular capillary congestion was seen in the damaged areas.

After 48 hr, these alterations were much less marked and the proximal and post-proximal tubular epithelia assumed a normal appearance.

**DISCUSSION**

The renal function results obtained with unanesthetized, unrestrained rats demonstrated that *B. moojeni* venom (0.4 mg/kg given intravenously) caused acute tubular and glomerular changes compatible with ARF; i.e., a decrease in the GFR and a sustained increase in tubular sodium rejection. This picture remained unchanged up to 48 hr after venom injection. These findings were compatible with the morphologic changes seen in the glomeruli and nephron tubules and explain the direct nephrotoxic effect of *B. moojeni* venom.

In contrast to the present observations, a decrease in blood pressure and in fractional sodium excretion was reported for rats treated with *B. jararaca* venom, suggesting indirect renal injury. 6 Burdmann and others 16 observed no significant changes in arterial blood pressure following renal lesions. Acute tubular necrosis (ATN) has not been observed after higher doses of bothropic venoms in experimental models. 6,15,18 In contrast, ATN has been described in humans following snakebite by various species, 4, 10, 12, 19–23 including *Bothrops* spp. 3, 4, 9, 11, 24 In this study, a dose of 0.4 mg of *B. moojeni* venom/kg produced ATN and functional abnormalities that peaked 5 hr and 16 hr after envenomation.

The use of lithium clearance as a marker to evaluate renal tubule sodium handling 25 showed that after 5 hr the natriuretic responses resulted from a functional impairment in the proximal nephron. The histopathologic findings for post-proximal nephron segments corroborated compensatory sodium reabsorption as shown by the prominent enlargement of both distal tubules and collecting ducts. This dilatation probably occurred in response to a rise in distal sodium and water delivery provoked by the rejection of this ion by the proximal nephron. After 16 hr, sodium reabsorption had improved, although the fractional sodium excretion had not returned to baseline values, i.e., there was incomplete recovery of the basal sodium excretion rate. The proximal nephron thus excreted less sodium after 16 hr than after 5 hr, and there was no compensatory reabsorption by the post-proximal nephron. The presence of mitotic tubule cells showed that epithelial regeneration was occurring. The morphologic preservation of the cortical nephrons provided unequivocal evidence that functional recovery was in progress. Indeed, mitosis has been reported to coexist with areas of necrotic renal parenchyma. 26

Despite the recovery in sodium absorption at 16 hr post-venom, the kidneys still showed regions of tubular necrosis throughout the cortico-medullary region. This finding suggests that the severity of the alterations was directly related to the higher flow in the tubule segments of the nephrons. The increase in sodium and potassium excretion after 48 hr was caused by the decrease in post-proximal nephron function. Histologic observations indicated that morphologic abnormalities, i.e., cell vacuolation, brushborder discontinuities, tubular swelling and glomerular damage, were largely resolved after 48 hr. This evidence supports the functional recovery of the proximal nephrons shown by the lithium clearance, which did not differ significantly from that of saline-treated rats. The discrepancy between the morphologic recovery observed here and that reported in the literature 6, 15 may be attributed to differences in experimental models, the type and quality of venom, route of envenomation, and dose used. The morphologic changes induced by *B. moojeni* venom were evident only with a dose of 0.4 mg/kg, although functional changes were seen with 0.2 mg/kg, thus indicating an asynchronous onset of morphologic and functional changes. Surprisingly, no significant decrease in the GFR was observed, despite an increase in the urinary excretion of Na⁺ and K⁺ after a venom dose of 0.2 mg/kg. This finding may reflect differences in the response of nephron constituents to the nephrotoxic components of the venom, but may also indicate the existence of a threshold of damage beyond which glomerular filtration and tubule Na⁺ and K⁺ handling are independent of each other. The lithium clearance with a venom dose of 0.4 mg/kg showed that this dysfunction was predominantly proximal. We believe that subcellular lesions are sufficient to provoke these alterations. Solez and others 27 reported that the morphologic alterations in ATN in humans were relatively subtle when compared with the intense reduction observed in the GFR. Solez and Finck 28 showed that tubular necrosis in one area may provoke a substantial back flow of the glomerular filtrate, whereas in another, a single cast obstructing an entire nephron may be sufficient to produce a similar effect. Thus, from a functional point of view the severity of ATN may be correlated more with the number of damaged and/or obstructed nephrons than with the total mass of renal tissue affected. Subtle but important morphologic alterations may go unnoticed because of the size of the tissue sample examined.

The high proteolytic and phospholipase A₂ activities of *B. moojeni* venom 6 may be responsible for most of the deleterious effects on kidney epithelia. The proteolytic activity of the venom may have major cytotoxic effects in many cell types, 29, 30 and may contribute directly or indirectly to the nephrotoxicity seen in this study. In a hypothetical mechanism of action on cultured cells, liposomes and skeletal muscle cells in vivo, *Bothrops* myotoxins would behave more like cytotoxins than as myotoxins. 30 These investigators sug-
gested that myotoxins would bind to a still unidentified site in the cell plasma membrane. Subsequently, the myotoxins would penetrate the bilayer through hydrophobic interactions. Penetration into the hydrophobic core of the bilayer would be responsible for membrane destabilization, with a consequent impairment of the permeability to ions and macromolecules. A prominent calcium influx would probably be the most important consequence of membrane disturbance and would be responsible for the onset of a variety of destructive mechanisms such as cytoskeletal alterations, mitochondrial damage, and the activation of calcium-dependent proteases and endogenous phospholipases which, in turn, would cause further cellular damage. The alterations in brushborder organization and in sodium handling by proximal tubular cells described above may result initially from cytoskeletal alterations, which subsequently would be summed to the effects of endogenous Ca\(^{2+}\)-dependent proteases. The extensive surface of the microvilli in the proximal epithelium has a pivotal functional and structural role. These microvilli are maintained by cytoskeletal elements including actin filaments which interact with intercellular junctions, desmosomes, and transmembrane proteins such as Na\(^{+}\)-K\(^{-}\)-ATPase. A direct action of *B. moojeni* venom proteases on tubule cell membranes could result in cytoskeletal disarrangement that would lead to morphologic and functional disturbances. The disruption of actin filaments could result in the loss of important interactions with the components of intercellular junctions, leading to desquamation of the tubule cells. Interference with the functioning and/or location of Na\(^{+}\)-K\(^{-}\)-ATPase could explain the altered sodium and potassium handling by proximal cells. In addition to high proteolytic and phospholipase A\(_2\) activities, *B. moojeni* venom also has high thrombin-like activity, and is hemolytic and hemorrhagic. The red urine observed suggests hematuria and/or hemoglobinuria. Several studies have demonstrated that ischemic changes lead to ATP depletion and cause cytoskeletal alterations. Thus, both a direct action of the venom and an indirect ischemic mechanism may induce morphologic and functional renal alterations. Ischemia has been associated with changes in arterial pressure and the deposition of fibrin thrombi in renal capillaries, neither of which was observed with *B. moojeni* venom.

Glomerular injuries following bothropic envenomation have been associated with massive fibrin deposition in glomerular capillaries. Since *B. moojeni* venom has a potent coagulant action, we expected to find fibrin in and around the glomerular capillaries, but did not. Electron microscopic studies are presently being designed to confirm or not these findings and elucidate about ultrastructural changes responsible for glomerular dysfunction. Glomerular injury is likely to be related more to structural disorganization of the glomerular capillary tuft following a direct action of the venom on the mesangial matrix, glomerular basement membrane, and podocytes than to fibrin deposition in the capillaries.

In conclusion, the present findings indicate that a direct nephrotoxic action of *B. moojeni* venom on tubule cells and glomerular structures is the most important physiopathologic factor in *B. moojeni* venom-induced renal failure.

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