ORIGINAL PAPER

Renal- and calcium-dependent vascular effects of *Polybia paulista* wasp venom

Vinhote JFC (1), Torres AFC (2), Dantas RT (1), Praciano TP (2), Menezes RRPPB (1), Sousa DF (1), Brito TS (1), Lima FJB (1), Toyama MH (3), Magalhães PJ (1), Monteiro HSA (1), Martins-Nunes AMC (2)

(1) Department of Physiology and Pharmacology, Federal University of Ceará, Fortaleza, Ceará State, Brazil; (2) Department of Clinical and Toxicological Analyses, Federal University of Ceará, Fortaleza, Ceará State, Brasil; (3) São Paulo Experimental Coast Campus, São Paulo State University (UNESP – Univ Estadual Paulista), São Vicente, São Paulo State, Brazil.

Abstract: In the present study, the effects of *Polybia paulista* venom (PPV) on renal and vascular tissues were investigated. Isolated kidneys perfused with PPV (1 and 3 μg/mL) had increased perfusion pressure, renal vascular resistance, urinary flow, and glomerular filtration rate; and reduced sodium tubular transport. Histological evaluation demonstrated deposits of proteins in Bowman's space and tubular lumen, and focal areas of necrosis. The venom promoted a cytotoxic effect on Madin-Darby canine kidney (MDCK) cells. A significant increase in lactic dehydrogenase levels was observed in response to venom exposure. In isolated mesenteric vascular beds, pressure and vascular resistance augmented in a dose-dependent manner. PPV increased the contractility of aortic rings maintained under basal tension. This contractile response was inhibited when preparations were maintained in Ca^{2+}-free medium. Likewise, verapamil, a voltage-gated calcium channel blocker, also inhibited the contractile response. In this study, phentolamine, a blocker of α-adrenergic receptor blocker, significantly reduced the contractile effect of PPV in the aortic ring. In conclusion, PPV produced nephrotoxicity, which suggests a direct effect on necrotic cellular death in renal tubule cells. The vascular contractile effect of PPV appears to involve calcium influx through voltage-gated calcium channels via adrenergic regulation.

Key words: *Polybia paulista,* venom*,* kidney, aorta, MDCK.

INTRODUCTION

In Brazil, there are several species of social wasps and little is known about the toxicological properties of their venoms. Many of these species, such as the neotropical wasp *Polybia paulista*, are involved in stinging incidents in urban areas in southeastern Brazil (1). The victims commonly describe severe pain and local inflammation, symptoms that reveal intense biological activity of *P. paulista* (PPV). Recently, a study reported hyperalgesic and edematogenic effects of peptides isolated from PPV (2). The constituents of PPV – such as melittin, apamin, phospholipases, hyaluronidases, acid phosphatases, proteases,

nucleases, histamine and degranulating peptide mastoparan – are also reported to be components of Vespinae venoms, and have direct and indirect cytotoxic, hemolytic, neurotoxic and vasoactive properties (3-6).

Incidents involving these insects can be harmful, with a large number of patients, mainly allergic individuals, developing severe complications, including acute renal failure (ARF) (7, 8). It has been postulated that the underlying mechanism of ARF involves a direct nephrotoxic effect following wasp poisoning, which may then result in tubulointerstitial nephritis and acute tubular necrosis, possibly in association with hemolysis or rhabdomyolysis (9-14). In fact,

mastoparans, polycationic peptides isolated from PPV, cause apoptosis involving caspase-signaling pathways with mitochondrial damage, and cytokine activation (15).

Despite the demonstration of the myotoxic effects of its constituents, the deleterious effects of PPV on kidneys have not yet been defined. Thus, the present study was designed to characterize the effects of PPV on renal and vascular tissues.

MATERIAL AND METHODS

Animals

Male Wistar rats (250-350 g) were maintained under standard conditions of temperature and humidity in 12-hour light-dark cycles. The animals were fasted for 24 hours before any experimental procedure and water was provided *ad libitum*. All experiments were in accordance with the guidelines for the ethical use of experimental animals published by the Brazilian College on Experimental Animal Care (COBEA), and experimental procedures were approved by the Institutional Animal Care Committee at Federal University of Ceará (protocol number 17/09).

Venom and Reagents

Polybia paulista wasp venom was kindly provided by Prof. M. H. Toyama of São Paulo State University (UNESP). All chemicals and analytical grade solvents were purchased from commercially available suppliers (Bayer, Sigma-Aldrich, Labtest Diagnostic and Applied Biosystems).

Renal Effects Induced by PPV

Isolated rat kidney assay

Rat kidneys were isolated and perfused according to the method described by Bowman (16) and modified by Fonteles *et al.* (17). Briefly, rats were anesthetized with sodium pentobarbital (50 mg/kg, intraperitoneally). The abdomen was opened through a midline incision, and the right kidney was exposed. After careful dissection of the kidney, the renal artery was cannulated via the mesenteric artery without interrupting blood flow. The perfusate solution consisted of a modified Krebs-Henseleit solution (MKHS) with the following composition in mmol/L: 118.0 NaCl, 1.2 KCl, 1.18 KH_2PO_4, 1.18 $MgSO_4.7H_2O$, 2.50 $CaCl_2.7H_2O$ and 25.0 $NaHCO_3$. Six grams of bovine serum albumin (BSA) was added to 100 mL of MKHS and dialyzed for 48 hours at 4°C against 10 volumes of MKHS. Immediately before the beginning of each perfusion protocol, 100 mg of urea, 50 mg of inulin and 50 mg of glucose were added to every 100 mL of perfusate, and the pH was adjusted to 7.4.

In each experiment, 100 mL of MKHS was recirculated for 120 minutes. Perfusion pressure (PP) was measured at the tip of a stainless steel cannula in the renal artery. Samples of urine and perfusate were collected at 10-minute intervals for the determination of sodium, chloride and potassium levels by means of ion-selective electrodes (Rapid Chem 744®, Bayer Diagnostic, UK). Inulin was determined by direct hydrolysis, as described by Walser *et al.* (18) and modified by Fonteles *et al.* (17). Osmolality was measured using a vapor pressure osmometer (5100C, Wescor, USA). PPV (1 or 3 µg/mL) was added to the system just after the basal perfusion pressure had stabilized (30 minutes after the beginning of each experiment). Perfusion pressure (PP), renal vascular resistance (RVR), urinary flow (UF), and glomerular filtration rate (GFR) were evaluated. Urine and perfusate samples were collected at 30-minute intervals to evaluate the percentage tubular transport for Na^+ (% TNa^+), K^+ (% TK^+) and Cl^- (% TCl^-) as determined by Pitts (19) and Martinez-Maldonato and Opava-Stitzer (20).

Histological evaluation

After completion of the experiment, the kidney was removed from the experimental set-up and kept in 10% formaldehyde for histological evaluation. Hematoxylin and eosin staining were used for light microscopy analysis.

Cytotoxic effects induced by PPV

Epithelial Madin-Darby canine kidney (MDCK) cells were cultured in RPMI-1640 medium, supplemented with FBS (10%), penicillin (100 U/mL), and streptomycin (100 µg/mL). Cells were seeded at a concentration of 1×10^5 cells/mL in microplates, and incubated at 37°C with 5% CO_2 for two hours. After cells were washed with sterile phosphate buffered solution (PBS) at pH 7.4, they were treated with PPV (3.12, 6.25, 12.5, 25, 50 or 100 µg/mL) for 24 hours and then evaluated using an inverted microscope. The negative control was prepared with medium plus cells and PBS instead of venom. Before each

MTT assay

MDCK cells were cultured as previously described, treated with PPV or PBS (negative control) and subjected to the MTT assay in microtiter plates after 24 hours of incubation. The supernatant of the cells was removed and 3-(4,5-dimethythiazol-2-yl)-2,5 diphenyltetrazolium (MTT) (2.5 mg/mL in PBS; Sigma, Brazil) was added to each plate well (10 μL/well). The method is based on reduction of tetrazolium salt by active mitochondria in living cells to insoluble purple formazan crystals (22). After incubation for four hours at 37°C with 5% CO_2, the supernatant was removed, and 10% sodium dodecyl sulfate (SDS) in 0.01N HCL was added to solubilize the formazan crystals. The plates were incubated for 17 hours, and then absorbance was measured by spectrophotometry (570 nm). Cell viability was determined by evaluating the mean percentage of surviving cells at a given PPV concentration. Survival measured in the negative control was taken to be equal to 100%.

Lactate dehydrogenase (LDH) assay

MDCK cells were cultured as previously described, treated with PPV or PBS (negative control) and subjected to the LDH assay in 96-well plates after 24 hours of incubation. After treatment, 20 μL of the supernatant was incubated with 1 mL of a solution of NADH (2.5 mg/mL phosphate buffer) and pyruvate (2.5 mg/mL phosphate buffer) (Labtest Diagnostics AS, Brazil). The rate of NADH oxidation was determined by measuring the decrease in absorbance at 340 nm for three minutes at 30-second intervals at 25°C using a spectrophotometer.

Vascular Effects Induced by PPV

Isolated perfused mesenteric bed

Perfusion of the isolated mesenteric bed was carried out following the descriptions of McGregor (23). Briefly, Wistar rats were anesthetized with sodium pentobarbital (50 mg/kg, intraperitoneally). After opening the abdomen, pancreatic-duodenal, ileum-colic and colic branches of the superior mesenteric artery were tied. Then, the superior mesenteric artery was cleaned of surrounding tissue and cannulated with a polyethylene tube (PE20). The intestine was separated from the mesenteric bed by cutting close to the intestinal border of the mesentery. The mesenteric bed was perfused with Krebs solution containing: NaCl 114.0 mM; KCl 4.96 mM; KH_2PO_4 1.24 mM; $MgSO_4.7H_2O$ 0.5 mM; $NaHCO_3$ 24.99 mM; $CaCl_2.2H_2O$ 2.10 mM; and glucose 3.60 mM. The perfusion solution was kept warm at 37°C, and the mesenteric bed was perfused using a constant flow rate (4 mL/minute).

Changes in perfusion pressure were continuously measured with a pressure transducer (Statham P23, Gould, USA) and recorded using a four-channel physiograph (Narco BioSystems, USA). After the basal perfusion pressure had stabilized, the mesenteric vascular beds were perfused with Krebs solution containing PPV (3, 10 or 100 μg/mL; n = 6) for ten minutes. Changes in the perfusion pressure of the mesenteric bed induced by PPV were compared to perfusion pressure recorded when preparations were perfused with vehicle alone at the same rate. Perfusion of the mesenteric bed with a solution of phenylephrine (5 μM) for ten minutes was used as positive control.

Aortic Ring Assay

Male Wistar rats (250-300g) were sacrificed by stunning followed by cervical dislocation. Thoracic aortas were removed and immersed in perfusion medium at room temperature. After removal of adhering fat and connective tissue, aortas were cut transversally into cylindrical ring-like segments (1 × 5 mm) and attached to steel wire triangular pieces (0.3 mm diameter), which were suspended in 5-mL organ baths containing Tyrode solution (NaCl 136 mM; KCl 5.0 mM; $MgCl_2$ 0.98 mM; $CaCl_2$ 2.0 mM; NaH_2PO4 0.36 mM; $NaHCO_3$ 11.9 mM and glucose 5.5 mM; pH 7.4) and continuously aerated at 37°C. Endothelium-containing strips were stretched with a passive tension of 0.5 g – recorded using an isometric force transducer (Grass Model FTO3, USA) connected to a PC-based Dataq acquisition system (PM-1000 ,CWE Inc., Akron, USA).

After an equilibration period of at least 60 minutes, control contractions were induced by adding a submaximal concentration (60 mM) of potassium chloride (KCl) to the bath. When

two successive control contractions showed similar amplitudes, preparations were considered equilibrated. To assess the effects of PPV, preparations were exposed during five-minute periods to increasing concentrations (0.1; 0.3; 1.0; 3.0; 10.0; and 20.0 µg/mL, n = 4) of PPV, which were directly added to the buffer solution in a cumulative manner. Contraction data were expressed as percentages of the potassium-induced contraction. Concentration-effect curves were constructed by exposing the preparations to cumulatively increasing concentrations of PPV on basal tone in preparations maintained in Ca^{2+}-containing medium, in Ca^{2+}-free medium (containing 2×10^{-5} M EGTA), in the presence of verapamil [10 µM, a well-know L-type voltage-operated Ca^2+ channel (VOCC) blocker], and in presence of phentolamine (5 µM), an α-adrenergic blocker.

Statistical Analysis

Data are presented as means ± SEM. The means were evaluated by Student's unpaired t-test and ANOVA, followed by the Bonferroni or Holm-Sidak test when appropriate. The level of acceptance for statistically significant differences was set at 5% ($p < 0.05$). CI_{50} values were estimated by curve interpolation as venom concentrations that resulted in 50% of cell inhibition, which correlates the mean percentages of dead cells and the venom concentration examined by non-linear regression analysis using GraphPad Prism 5.

RESULTS

Effects of PPV on Isolated Perfused Kidney

After basal perfusion pressure stabilized, rat kidneys were perfused for two hours with Krebs Henseleit solution containing either 1 µg/mL (n = 6) or 3 µg/mL (n = 6) of PPV. Then, perfusion parameters were evaluated at 30-minute intervals (30, 60, 90 and 120 minutes). Treatment with PPV (3 µg/mL, but not with 1 µg/mL) significantly increased ($p < 0.05$; ANOVA-Bonferroni test) perfusion pressure (PP; Figure 1– A), renal vascular resistance (RVR; Figure 1 – B), urinary flow (UF; Figure 1 – C), and glomerular filtration rate (GFR; Figure 1 – D) when compared to control perfused kidneys. Table 1 shows the effect of PPV on electrolyte transport. PPV at 3 µg/mL was also able to decrease sodium tubular transport (%TNa+) ($p < 0.05$), but chloride

(%TCl−) and potassium (%TK+) tubular transport were not changed.

Histological evaluation revealed moderate amounts of proteinaceous substances in the urinary spaces of kidney glomeruli and tubules
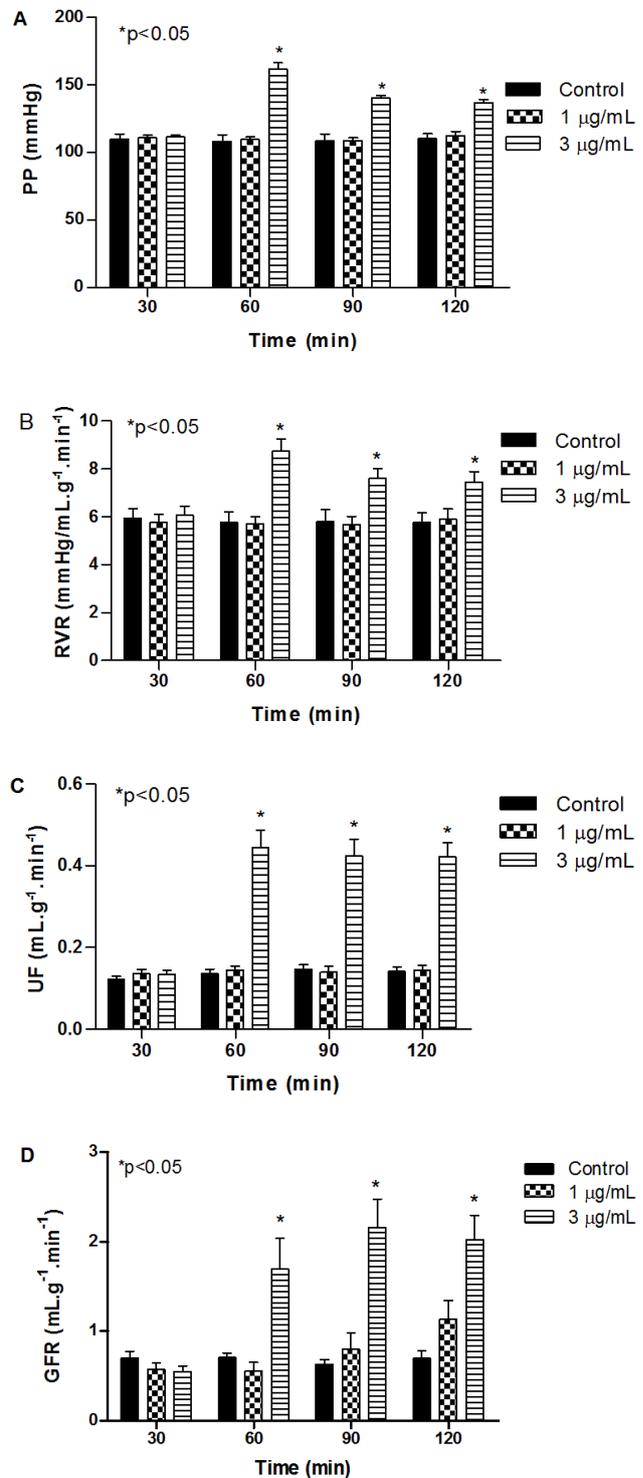

Figure 1. Effects of *Polybia paulista* venom (PPV; 1 and 3 µg/mL) on: (**A**) perfusion pressure (PP), (**B**) renal vascular resistance (RVR), (**C**) urinary flow (UF) and (**D**) glomerular filtration rate (GFR) at various times. Values represent means ± SEM (n = 6).

Table1. Effects of *Polybia paulista* venom (3 µg/mL) on electrolytes transport in isolated kidney

Renal parameters	30 minutes	60 minutes	90 minutes	120 minutes
%TNa⁺				
Control	80.66± 1.09	80.52 ± 1.15	80.52 ± 1.06	81.15 ± 0.56
Venom	79.02± 3.08	68.00 ± 4.00	74.19 ± 4.60	72.35 ± 5.10*
%TK⁺				
Control	69.13 ± 4.14	69.04 ± 5.68	71.84 ± 4.21	69.94 ± 6.86
Venom	62.62 ± 3.62	58.54 ± 5.00	71.48 ± 5.00	70.81 ± 5.40
%TCl⁻				
Control	78.81 ± 1.25	80.18 ± 2.90	81.25 ± 1.20	80.49 ± 2.47
Venom	71.84 ± 3.66	70.10 ± 5.25	73.35 ± 5.39	67.31 ± 6.01

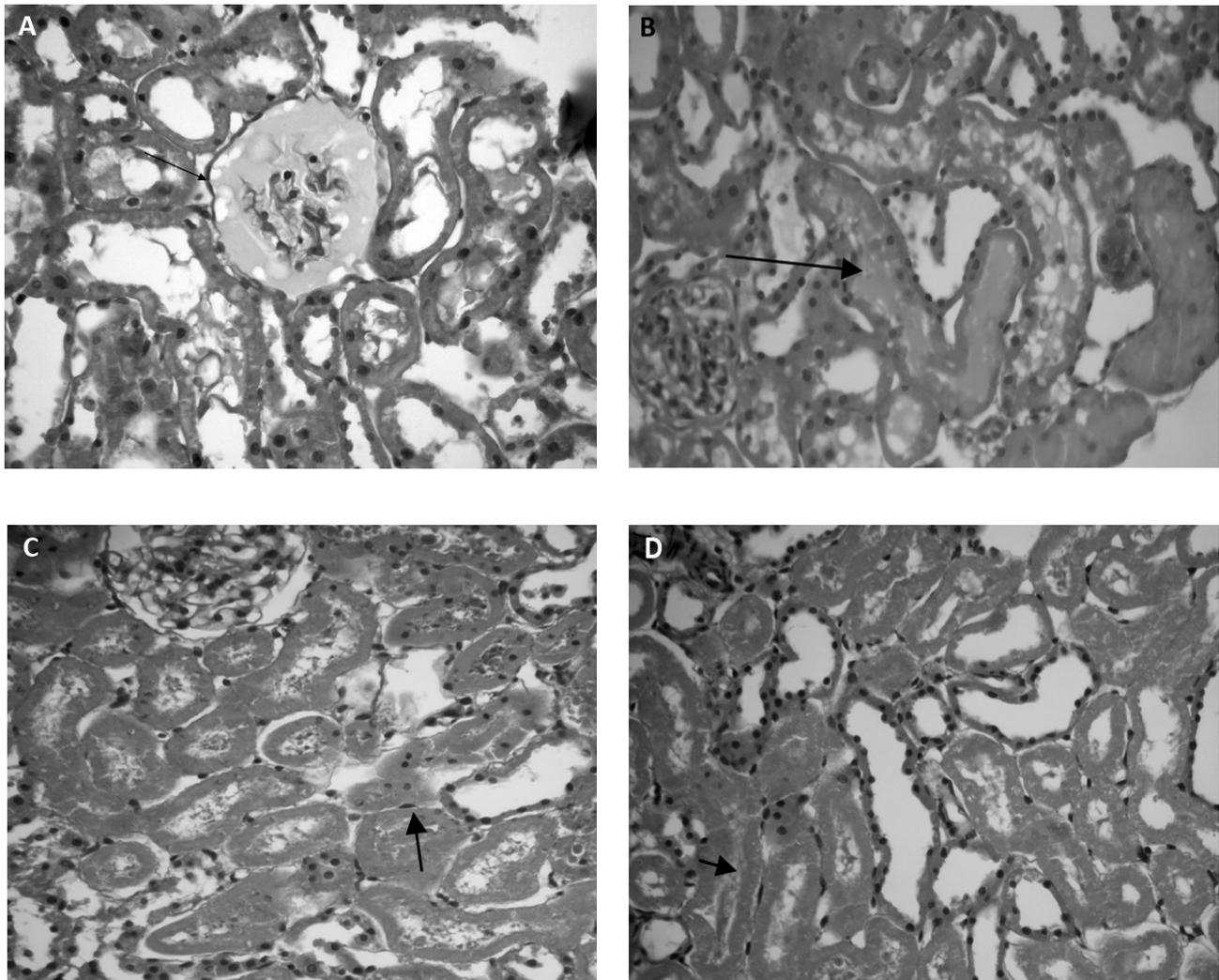The results are expressed as the mean ± SEM, $p < 0.05$

Figure 2. Photomicrograph of kidneys perfused with *Polybia paulista* venom (PPV) at a concentration of 3 µg/mL, which indicates (**A**) proteinaceous material in the urinary spaces and (**B**) tubules. Foci of necrosis, (**C**) nuclei and (**D**) cells without nuclei (hematoxylin-eosin, 400x).

following treatment with PPV. Mild focal acute tubular necrosis was also observed in kidneys after PPV exposure (Figure 2). No changes were observed in control kidneys.

Cytotoxic Effects of PPV on Renal Tubular Cells

The cytotoxic effects of PPV in MDCK cells were analyzed by MTT and LDH assay. The venom promoted a concentration-dependent cytotoxic effect with an CI_{50} value of 25.81 µg/mL. Figure 3 shows the dose-response curve for MDCK cell viability after 24 hours of venom exposure and indicates significant decrease in cell viability for doses ≥ 25 µg/mL ($p < 0.05$), as well as loss of membrane integrity accompanied by an increase in LDH activity.

Effects of PPV on Isolated Perfused Mesenteric Bed

Isolated perfused mesenteric beds had a perfusion pressure of 39.76 ± 0.60 mmHg after the stabilization period. When mesenteric preparations were subjected to 3 µg/mL of PPV perfusion for ten minutes, perfusion pressure was 39.87 ± 0.80 mmHg, a value without statistical difference ($p > 0.05$, paired Student's t-test) compared to values before PPV treatment. Increases in perfusion pressure were observed when mesenteric preparations were perfused with PPV at concentrations higher than 3 µg/mL. Perfusion pressure was significantly increased from 37.73 ± 0.40 mmHg before perfusion with

PPV to 43.10 ± 1.77 mmHg ($p < 0.05$, paired Student's t-test) after perfusion with 10 µg/mL of PPV and to 52.15 ± 2.37 mmHg ($p < 0.05$, paired Student's t-test) at 100 µg/mL. As a positive control, phenylephrine (5 µM) increased perfusion pressure values to 123.40 ± 9.08 mmHg (Figure 4).

Effects of PPV on Isolated Aorta

In endothelium-containing aorta preparations, PPV (0.1-20 µg/mL) induced contractile responses in a concentration-dependent manner ($p < 0.001$; ANOVA-Holm-Sidak). The magnitude of the contraction induced by 20 µg/mL of PPV corresponded to 135.7 ± 14.6% (n = 4) of a reference contraction induced by 60 mM K^+ (K60). In aortic rings maintained in Ca^{2+}-free medium, the contractile effects induced by PPV were almost abolished and the magnitude of the maximal contraction was 7.5 ± 1.2% of the K60 value (n = 4; $p < 0.05$; Figure 5 – A), a value significantly lower than that observed in Ca^{2+}-containing medium. Pretreatment of aortic rings with phentolamine (5 µM) also significantly reduced PPV -induced contraction (20 µg/mL) to 48.9 ± 16.0% of the K60 value (n = 4; $p < 0.05$) (Figure 5 – B). In order to evaluate the effect of a voltage-gated calcium channel blocker on PPV-induced contraction, preparations were treated with verapamil (10 µM) and contractions elicited by PPV (20 µg/mL) significantly diminished to 24.6 ± 10.6% of the K60 value (n = 4; $p < 0.05$, ANOVA-Holm-Sidak) (Figure 5 – C).

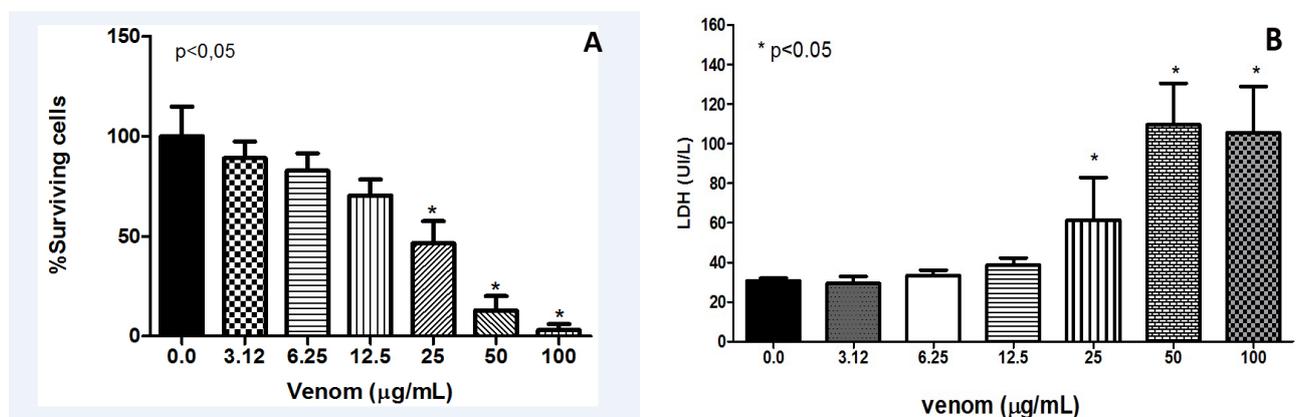**Figure 3.** Cytotoxic effects of *Polybia paulista* venom (PPV; 3.12-100 µg/mL) on MDCK cells by (**A**) MTT and (**B**) lactic dehydrogenase assays. Data are expressed as means ± SEM, ANOVA, Bonferroni post-test, *p < 0.05.
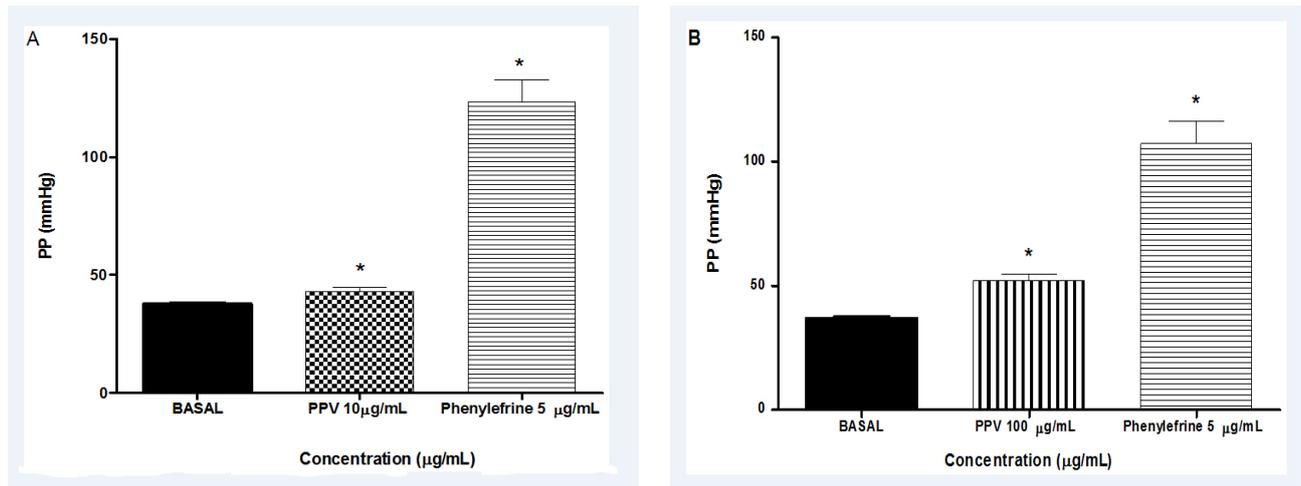
Figure 4. Effects of *Polybia paulista* venom (PPV) at concentrations of (**A**) 10 μg/mL and (**B**) 100 μg/mL on perfusion pressure (PP) of arteriolar mesenteric beds. *p < 0.05 compared with basal pressure.
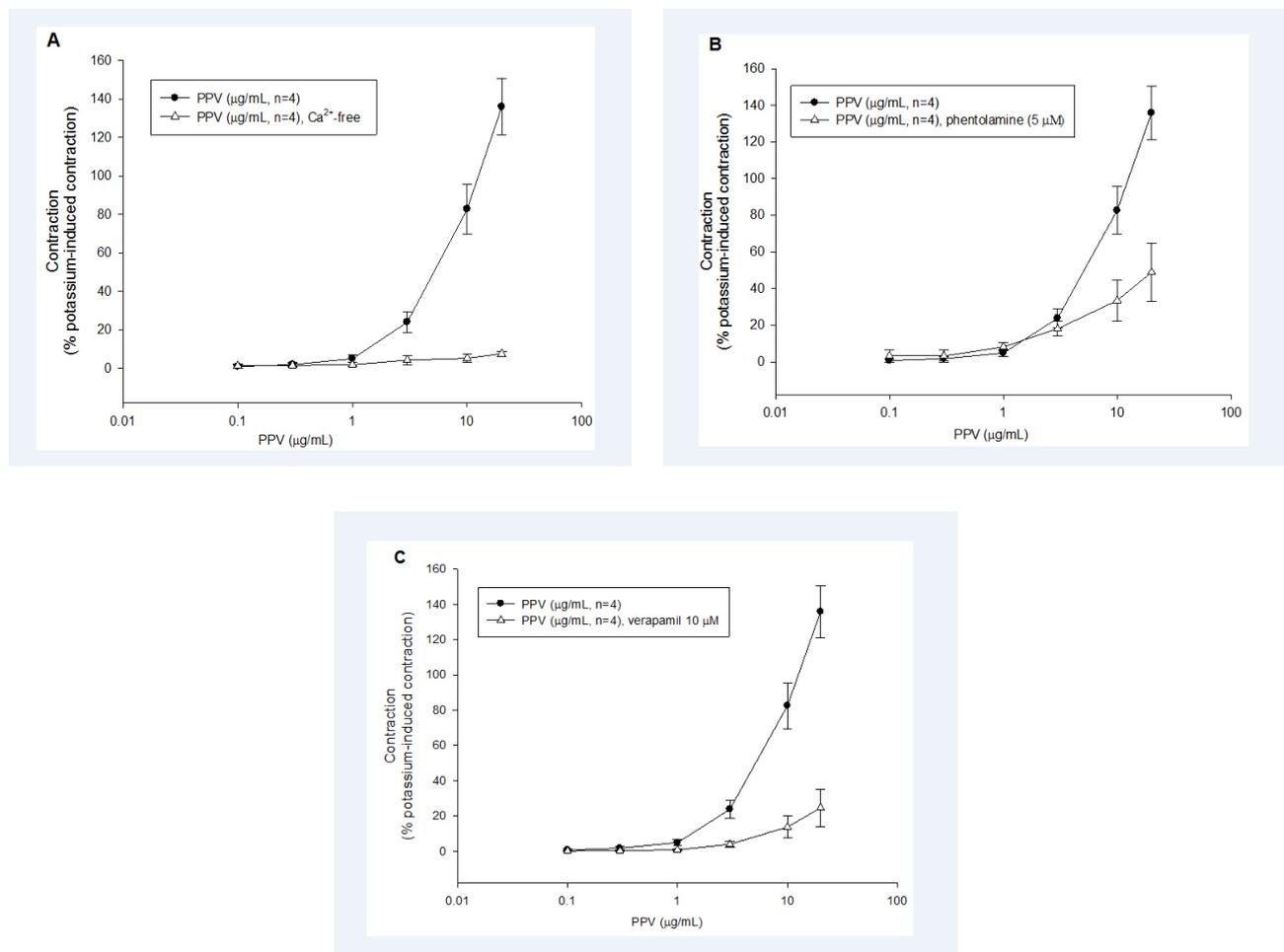

Figure 5. (**A**) Concentration response curve of *Polybia paulista* venom (PPV) in isolated aortic rings (●, n = 4; 0.1-20 μg/mL), and venom with Ca^{2+}-free medium (Δ, n = 4; 1μM). (**B**) Concentration-response curve of PPV in isolated aortic rings (●, n = 4; 0.1-20 μg/mL) and venom with phentolamine (Δ, n = 4; 5 μM). (**C**) Concentration-response curve of PPV on potassium-induced (K^+, 60 mM) contraction of isolated aortic rings (●, n = 4; 0.1-20 μg/mL) and venom with verapamil (Δ, n = 4; 10 μM). Data are expressed as means ± SEM, one-way ANOVA, post-test Holm-Sidak, *p < 0.001, compared with corresponding baseline values.

DISCUSSION

The study of the biological effects of wasp venoms has gained scientific attention due to the involvement of these arthropods in stinging incidents with important clinical outcomes. In this regard, the major findings of the present study are that PPV markedly changed kidney function and promoted vasoconstrictor effects. Changes in kidney function include increase in renal parameters such as perfusion pressure, vascular resistance, glomerular filtration rate and urinary flow. Renal changes observed in this study corroborate earlier reports with other animal venoms which registered similar changes in renal function (24, 25).

Despite the yet unknown profile of toxicological actions elicited by PPV on kidney tissue, there is a general assumption that it may exert a direct nephrotoxic effect, which is involved in the development of acute renal failure eventually observed in patients attacked by wasps (9). Herein, we demonstrated that PPV induced mild focal acute tubular necrosis. In fact, tubulointerstitial nephritis and acute tubular necrosis are often observed in patients envenomed by wasps (8, 11-13). Moreover, cell viability and membrane integrity decreased when the survival of untreated cells was compared with the mean percentage of surviving MDCK cells under a given PPV concentration using the LDH assay. This fact indicates that such changes are involved in the underlying mechanisms for development of PPV-induced toxic actions. Since the loss of membrane integrity was followed by an increase in LDH activity, it was suggested that cell death is the result of necrosis, confirming the histological findings in kidneys perfused with venom. Although toxic effects of PPV on cultured cells were observed in the concentration ranging from 25 to 100 μg/mL, the perfusion of isolated kidneys with PPV at 3 μg/mL resulted in slight changes in tubular function by means of decrease in the Na^+ tubular transport rate.

The ability of PPV to induce deposition of proteinaceous material in urinary and tubular spaces is noteworthy and may be due to its toxic effect on the barrier function of glomeruli. This hypothesis is corroborated by Grisotto *et al.* (26), who reported that protein leakage by kidney tubules was induced by bee venom. Another implication of the finding is a putative myotoxic

effect induced by PPV. It was previously reported that rhabdomyolysis could occur following wasp envenomation by deleterious effects of mastoparans on muscle cells involving caspase signaling pathways and mitochondrial injury (15). Notwithstanding, whether or not such actions due to these polycationic peptides are involved in PPV-induced renal effects, further studies will tell.

A hallmark of PPV-elicited actions was the vasoconstrictor effect induced by the venom in vascular preparations. PPV increased perfusion pressure and vascular resistance in isolated mesenteric beds and contracted aortic rings *in vitro* in a concentration-dependent manner, effects that were comparable to classically known vasoactive agents such as phenylephrine and potassium. Vasoconstrictor actions of PPV are probably involved in the major functional changes observed after perfusion of isolated kidneys with PPV. The venom increased kidney perfusion pressure, renal vascular resistance, urinary flow and glomerular filtration rate. It is interesting to note that the renal effects of PPV were established 60 minutes after perfusion, but were sustained for up to 120 minutes. Such effects agree with the results observed in vascular tissues, because contractions induced by PPV in vessel preparations were tonic and reached a sustained plateau, which was then maintained over the course of tissue perfusion or in the presence of PPV in the bath chamber.

In order to investigate the underlying mechanisms involved in the contraction of vascular smooth muscle, aortic preparations were kept in Ca^{2+}-free medium. Since under Ca^{2+}-free conditions PPV did not induce contractile effects, we concluded that its vasoactive properties are dependent of a transmembrane Ca^{2+} influx from the extracellular milieu. It is well known that smooth muscle cells have a straight dependence on extracellular Ca^{+2} for contraction (27). Indeed, following the activation of specific receptors, excitatory substances such as neurotransmitters promote Ca^{2+} influx through membrane channels, increasing ionic intracellular levels to induce cellular events including force development in vascular smooth muscle (28). Likewise, verapamil, a voltage-gated Ca^{2+} channel blocker, reduced the magnitude of the contractile response provoked by PPV, confirming that Ca^{2+} influx is stimulated by PPV and at least part of the

contractile response is mediated by the passage of Ca^{2+} ions through voltage-gated Ca^{2+} channels. Additionally, phentolamine decreased the contractile behavior of aortic rings, indicating the involvement of α-adrenergic receptors in PPV-induced contractile effects, an issue that deserves further investigation.

In conclusion, *Polybia paulista* wasp venom produced nephrotoxicity, which suggests a direct effect on cellular death through necrosis in renal tubular cells. The contractile vascular effect, which involves calcium influx through voltage-gated channels, is probably regulated by adrenergic pathways.

CONFLICTS OF INTEREST

There is no conflict.

ETHICS COMMITTEE APPROVAL

All experiments were in accordance with the guidelines for the ethical use of experimental animals published by the Brazilian College on Experimental Animal Care (COBEA). Moreover, the present study was approved by the Institutional Animal Care Committee at Federal University of Ceará (protocol number 17/09).

CORRESPONDENCE TO

ALICE MARIA COSTA MARTINS NUNES, Departamento de Análises Clínicas e Toxicológicas, Universidade Federal do Ceará, Fortaleza, CE, 60.420-970, Brasil. Phone: +55 85 3366 8263. Fax: +55 85 3366 8292. Email: martinsalice@gmail.com.

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