ARTÍCULO ORIGINAL

Oxidative stress and changes in hematological and biochemical biomarkers in *Crotalus durissus terrificus* (South American rattlesnake) venom-induced acute kidney injury in rats

Estrés oxidativo y alteraciones en biomarcadores hematológicos y bioquímicos en lesión renal aguda inducida por el veneno de Crotalus durissus terrificus (cascabel de America del Sur) en ratones

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Abstract. Acute kidney injury (AKI) is the major cause of mortality following bites by the South American rattlesnake *Crotalus durissus terrificus*. We investigated the early onset of *Crotalus durissus terrificus* venom-induced AKI in rats within 2 h of venom injection and its attenuation by antivenom. Several biomarkers were used to monitor AKI in the absence or presence of antivenom. Male Wistar rats were divided into five groups (n=5 each): G1, rats injected with saline (control); G2, rats injected with venom (6 mg kg^{-1}, intraperitoneally) and euthanized after 2 h to evaluate AKI; G3 and G4, rats injected with 0.9% sterile saline or antivenom 2 h after venom, respectively, and monitored until death or up to 24 h post-venom, and G5, rats injected with antivenom alone and monitored for 24 h. Blood, urine and renal tissue samples were collected immediately after death to assess oxidative stress, hematological and biochemical alterations, and renal histological damage. Venom caused AKI within 2 h (G2) that persisted for up to 8.2 ± 1.6 h (G3), as confirmed by increases in blood urea, creatinine, and renal proteinuria; these increases were attenuated by antivenom. There were no changes in blood protein concentrations in G2 and G3, whereas there were increases in blood reduced glutathione, glutathione peroxidase, and plasma TBARS (but not in catalase) that were attenuated to varying extents by antivenom. There were no marked changes in platelets or leukocytes, but an increase in erythrocytes after 8.2 h with venom alone was attenuated by antivenom. Renal glomerular and tubular damage was greatest after 2 h post-venom groups alone was attenuated by antivenom. Renal glomerular and tubular damage was greatest after 2 h post-venom and declined thereafter. Venom caused early-onset AKI, with variable effects on lipid peroxidation and oxidative stress. Antivenom attenuated the AKI, as shown by the decrease in blood urea and the normalization of proteinuria, without protecting against lipid peroxidation.

Keywords: Acute kidney injury; Antivenom; Biomarkers; *Crotalus durissus terrificus*; Oxidative stress.

Resumen. La injuria o lesión renal aguda (LRA) es la mayor causa de mortalidad debido a las mordeduras por cascabeles *Crotalus durissus terrificus*. Se estudió la instalación precoz de LRA, en ratas, inducida por el veneno de *Crotalus durissus terrificus* después de 2 h de su inoculación y la atenuación por el antiveneno. Se utilizaron diversos biomarcadores para monitorear LRA en ausencia o presencia del antiveneno. Ratas Wistar machos fueron divididos en 5 grupos (n=5 por grupo): G1, ratas inoculadas con solución salina (control); G2, ratas inoculadas con veneno (6 mg kg^{-1} dosis, vía intraperitoneal), y sacrificadas después de 2 h para evaluar LRA; G3 y G4, ratas inoculadas con 0.9% de solución salina esterilizada o antiveneno luego de 2 h después de inoculado el veneno, respectivamente, y monitoreadas hasta su muerte o hasta 24 h después de inoculado el veneno; y G5, ratas inoculadas con antiveneno solo y monitoreadas durante 24 h. Las muestras de sangre, orina, y tejido renal fueron colectadas inmediatamente después de la muerte de los animales para evaluar estrés oxidativo, alteraciones hematológicas y bioquímicas, y daño histológico renal. El veneno causó LRA dentro de las 2 h (G2) persistiendo durante más de 8,2 ± 1,6 h (G3), estando esto confirmado por el incremento de urea sanguínea, creatinina, y proteinuria renal; estos aumentos disminuyeron con la aplicación del antiveneno. No se

observaron alteraciones en las concentraciones de proteínas sanguíneas en G2 y G3, mientras que se encontraron incrementos en glutatión reducido sanguíneo, glutatión peroxidasa y TBARS plasmática (pero no en catalasa), que disminuyeron con la aplicación del antiveneno aunque en diferente grado. No ocurrieron alteraciones marcadas de plaquetas o leucocitos, mientras que el aumento de glóbulos rojos observado luego de 8,2 h de la inoculación con veneno, disminuyó con el antiveneno. El daño renal glomerular y tubular fue más importante luego de 2 h de la inoculación con veneno y posteriormente disminuyó. El veneno causó LRA precoz a las 2 h, con efectos variables sobre la peroxidación lipídica y el estrés oxidativo. El antiveneno redujo el daño renal, conforme lo demostrado por la disminución en la urea sanguínea y por la normalización de la proteinuria, aunque no se observó protección contra la peroxidación lipídica.

Palabras clave: Antiveneno; Biomarcadores; *Crotalus durissus terrificus*; Estrés oxidativo; Lesión renal aguda.

Background

Nephrotoxicity is an important clinical complication of snakebite by crotalid and viperid snakes (Warrell 2004; Sitprija 2006; Albuquerque *et al.* 2013, Gutiérrez *et al.* 2017; Albuquerque *et al.* 2020) and is frequently characterized by a combination of acute tubular necrosis, acute tubulointerstitial nephritis, renal cortical necrosis, mesangiolysis, vasculitis, glomerulonephritis, proteinuria, hematuria, and myoglobinuria (Sitprija 2006, 2008), as well as alterations in renal ion transport (Sitprija and Sitprija, 2016). The high vascularization of the kidneys and their involvement in blood filtration and the excretion of endogenous and xenobiotic substances makes these organs particularly prone to the actions of circulating snake venoms and their toxins (Sitprija 2006, 2008; Sanhajariya *et al.* 2018).

In Brazil, most cases of snakebite-induced acute kidney injury (AKI) result from envenomation by lanceheads (*Bothrops* spp.) and the South American rattlesnake (*Crotalus durissus terrificus* = *C. d. terrificus*) (Pinho *et al.* 2008; Rodrigues Sgrignolli *et al.* 2011; Berger *et al.* 2012; Albuquerque *et al.* 2013). Although *Bothrops* spp. are responsible for 87% of the bites by venomous snakes in this country compared to 9% by *C. d. terrificus* (data for the period 2001-2014), the overall mortality associated with the latter species is 2.6-fold greater than with the former genus (0.97% vs. 0.37%) (SINAN 2019). The greater lethality associated with envenomation by *C. d. terrificus* has been attributed primarily to its high content of crotoxin, a phospholipase A_2 (PLA_2) β-neurotoxin that accounts for ~50% of the venom dry weight (Hadler and Vital Brazil 1966; Sampaio *et al.* 2010).

Snake venom PLA_2 display a variety of activities, such as presynaptic neurotoxicity, myonecrosis, edema formation, cardiotoxicity, anticoagulant activity, activation/inhibition of platelet aggregation, hemorrhage, hemolysis and cytotoxicity (Kini 2003; Harris and Scott-Davery 2013). Most of these effects reflect the PLA_2-mediated hydrolysis of cell membrane phospholipids to release arachidonic acid that can be oxidized to generate reactive oxygen species (ROS) that in turn produce lipid peroxides, leading to cellular injury (Spiteller *et al.* 2001; Kini 2003; Teixeira *et al.* 2003).

Human envenomation by *C. d. terrificus* is characterized by peripheral neurotoxicity (palpebral ptosis, external ophthalmoplegia and facial weakness that produce 'myasthenic facies', generalized muscle weakness, mydriasis and, rarely, respiratory failure requiring mechanical ventilation), systemic myonecrosis (rhabdomyolysis, with a marked increase in serum myoglobin and creatine kinase, and myoglobinuria, as well as generalized myalgia), coagulopathy (afibrinogenemia leading to prolonged clotting time with no thrombocytopenia), and AKI, with little or no local manifestations such as hemorrhage, edema or necrosis at the bite site (pain related to traumatism caused by the bite may be present but is soon followed by paresthesia and anesthesia) (Amorim and Mello 1954; Rosenfeld 1971; Azevedo-Marques *et al.* 1985, 1987; Silveira and Nishioka 1992; Sano-Martins *et al.* 2001; Bucaretchi *et al.* 2002; Warrell 2004; Pinho *et al.* 2005; Berger *et al.* 2012; Frare *et al.* 2019; Medeiros *et al.* 2020). AKI is the major clinical complication and the principal cause of death in individuals who survive the severe initial phase of envenomation (Pinho *et al.* 2005; Frare *et al.* 2019).

Although the clinical profile of AKI induced by

C. d. terrificus venom in humans has been extensively studied, with an important mechanism of kidney damage being the renal deposition of myoglobin released by the rhabdomyolytic activity of the venom (Rosenfeld 1971; Azevedo-Marques *et al.* 1985, 1987; Silveira and Nishioka 1992; Bucaretchi *et al.* 2002; Warrell 2004; Pinho *et al.* 2005; Albuquerque *et al.* 2013; Frare *et al.* 2019), relatively few experimental studies have investigated the mechanisms involved in this phenomenon. The renal lesions caused by this venom in humans can be reproduced to varying extents in dogs (Sangiorgio *et al.* 2008) and mice (Frezzatti and Silveira 2011). In mice, *C. d. terrificus* venom causes renal dysfunction and oxidative stress (Yamasaki *et al.* 2008) that can be attenuated to varying extents by compounds such as lipoic acid (Alegre *et al.* 2010), allopurinol (Frezzatti and Silveira 2011), and N-acetyl-L-cysteine (Barone *et al.* 2014). The renal functional alterations seen after envenomation may be mediated directly by venom components such as crotoxin/PLA_2 (Monteiro *et al.* 2001; Amora *et al.* 2008) or indirectly by mediators released by cells such as macrophages exposed to the venom (Martins *et al.* 2003; Cruz *et al.* 2005).

Physiological and pathophysiological studies have shown that rats respond more like humans than do mice and has led to the extensive use of these rodents as a model for investigating normal and diseased states (National Human Genome Research Institute 2004), but no reports have examined the response to *C. d. terrificus* venom in this species. Hence, in this work, we examined the ability of *C. d. terrificus* venom to induce AKI after intraperitoneal (i.p.) injection in rats as a potentially useful model for the experimental evaluation of new therapeutic strategies to treat systemic envenomation.

We assessed AKI 2 h after venom injection to simulate the time interval between envenomation and seeking medical assistance in the clinical setting, including antivenom administration [mean interval of 3 h, according to Pinho *et al.* (2005)]. The occurrence of AKI was considered a positive confirmation of envenomation, and several other biochemical, hematological, and histological parameters were monitored to assess the extent of renal damage. We also examined the ability of antivenom given i.p. 2 h post-venom to rescue rats from the lethal effects of the venom and to attenuate the renal functional alterations associated with AKI.

Methods

Animals

Standard laboratory practices for animal care were followed according to the *Guide for the Care and Use of Laboratory Animals* (National Research Council of the National Academies 2011) and the Animal Research: Reporting of *In vivo* Experiments (ARRIVE) guidelines (Kilkenny *et al.* 2010). The experimental protocols were approved by the institutional Committee for the Care and Use of Experimental Animals at the University of Sorocaba (protocol no. 031/2014). Male Wistar rats (*Rattus norvegicus*) (290-400 g; 3-6 months old) purchased from the Central Animal Facility of the Institute of Biomedical Sciences of the University of São Paulo (São Paulo, SP, Brazil) were housed (3 rats/plastic cage on a wood shavings substrate) at the University of Sorocaba animal facility at 22 ± 3 oC and 50 ± 5% humidity on a 12 h light/dark cycle (lights on at 6 a.m.), with access to food and water *ad libitum*.

Venom

Crotalus d. terrificus venom was collected manually from 20 male and female young adult snakes (200-400 g, ~2 years-old) captured in the Paraiba Valley close to the city of São José dos Campos (23º 11' 0" S; 45º 53' 0" W) and kept in the serpentarium of the Center for Nature Studies (under Environmental Secretariat License SMA 15.380/2012) at the University of Vale do Paraiba (UNIVAP, São José dos Campos, SP, Brazil). The use of the venom was registered with the Brazilian National System for the Management of Genetic Patrimony and Associated Traditional Knowledge (SISGEN, registration no. ACB5FCO). The venom was lyophilized and stored at 4-8 ºC until used.

Antivenom

Therapeutic crotalic antivenom raised against *C. d. terrificus* venom (lot 135202/I, expiry date for human use: October 2016) produced by the Instituto Vital Brazil (Rio de Janeiro, RJ, Brazil) was kindly donated by the Escritório Regional de Saúde (ERSA) in Piracicaba, SP, Brazil. Although the antivenom was used beyond the expiry date for human use, the solution nevertheless conserved the macroscopic properties generally considered to be indicative of quality maintenance, i.e., lack of turbidity and no formation of precipitates. Indeed, previous studies have demonstrated the usefulness of antivenoms beyond their formal expiry dates

(O´Leary *et al.* 2009; Sanchez *et al.* 2019; Tan *et al.* 2019). The dose of antivenom was calculated based on the manufacturer´s stated potency in which 1 mL of antivenom neutralizes the lethality of 1.5 mg of reference *C. durissus* ssp. venom in mice.

In vivo protocols

All rats were anesthetized i.p. (always on the left side) using a schedule based on the *Guide for the Care and Use of Laboratory Animals*. The anesthetic mixture consisted of xylazine (10 mg kg^{-1}) and ketamine (10 mg kg^{-1}) (both purchased from Ceva®, Paulínia, SP, Brazil), with midazolam (1 mg kg^{-1}) being used for initial sedation (before venom administration) and tramadol (5 mg kg^{-1}) as an analgesic (both from Medley®, Campinas, SP, Brazil) administered soon after venom injection.

Urine, blood, and renal tissue samples were collected at death (with venom alone, ~8.2 h post-venom; see later) or 2 h or 24 h post-venom when the rats were killed with an overdose of isoflurane (Cristália®, Itapira, SP, Brazil). Hematological (leukocytes, platelets, and erythrocytes) and biochemical (protein, creatinine, and urea) parameters were determined in blood and urine, whereas blood and renal tissue samples were used to evaluate oxidative stress markers (glutathione – GSH, glutathione peroxidase – GPx, catalase – CAT, thiobarbituric acid reactive substances – TBARS). Alterations in renal morphology were analyzed by light microscopy.

In a preliminary set of experiments, rats were injected with different doses of *C. d. terrificus* venom (5, 6, 7.5, and 10 mg kg^{-1}, i.p.; one dose per rat, n=3 rats/dose, a total of 12 animals) to determine an appropriate minimal venom dose able to induce AKI. The venom was injected in a volume of 0.5 mL, which is considerably less than the volume of 10 mL kg^{-1} recommended elsewhere (Morton *et al.* 2001), and the venom dose of 6 mg kg^{-1} was selected based on a marked increase in blood urea and creatinine concentrations. These biomarkers are frequently used as indicators of renal dysfunction because they reflect a decrease in the glomerular filtration rate in response to renal alterations (Sato 2012). The rats were randomly distributed into five groups (n=5/group; total N = 25 rats), as follows: Group 1 (G1) – Saline (sham) control (n=5): Rats received 0.5 mL of sterile phosphate-buffered saline (PBS, i.p.) and were killed 2 h later with an overdose of isoflurane for blood and tissue sample collection; Group 2 (G2; n=5) – rats were injected with *C. d. terrificus* venom (6 mg kg-1, i.p.) and killed 2 h later with an overdose of isoflurane for blood and tissue sample collection. Pharmacokinetically, this 2 h interval provided sufficient time for the absorption and diffusion of the venom and its main toxin, crotoxin, from the site of application (Barral-Netto and von Sohsten 1991; Cura *et al.* 2002), with subsequent binding to the presynaptic terminations of striated muscle (Cura *et al.* 2002); Group 3 (G3) – Assessment of survival time (n=5): Rats were injected with venom (6 mg kg^{-1}, i.p.) and 2 h later received PBS (0.5 mL, i.p., n=5) after which they were monitored until imminent death when blood and tissue samples were collected; the rats in this group survived for 8.2 ± 1.6 h. Group 4 (G4) – *C. d. terrificus* venom followed by antivenom (n=5): Rats were injected with venom (6 mg kg^{-1}, i.p.) and 2 h later they received crotalic antivenom (from 1.3 to 1.9 mL, i.p.; 1 mL of antivenom neutralizes 1.5 mg of *C. d. terrificus* reference venom, according to the manufacturer) in a rescue model conceptualized by Baudou *et al.* (2017); blood and tissue samples were collected 24 h after venom injection (22 h after antivenom). Group 5 (G5) – PBS followed by antivenom (n=5): Rats were injected with PBS (0.5 mL, i.p.) and 2 h later they received crotalic antivenom i.p. (the same volume as in group G4); blood and tissue samples were collected 24 h after PBS injection (22 h after antivenom). The rats in groups G4 and G5 were killed with an overdose of isoflurane before blood and tissue sample collection.

Groups G1-G5 were all injected with PBS, venom, or antivenom on the right side. *Figure 1* shows the experimental schedule described above.

Hematological parameters

Blood samples were collected in polypropylene tubes (Injex® Indústria Cirúrgica Ltda., Ourinhos, SP, Brazil) containing ethylenediaminetetraacetic acid tripotassium salt (EDTA). Total leukocyte, platelet and erythrocyte counts were determined in triplicate per sample using a Sysmex XS 1000i™ Hematology Analyzer (Roche, Basel, Switzerland). Whole blood samples (~20 µL) were injected into the analyzer and platelets and erythrocytes were counted using an impedance detection method combined with hydrodynamic focusing technology (Yoshida *et al.* 2020). Leukocytes were counted using fluorescent flow cytometry.

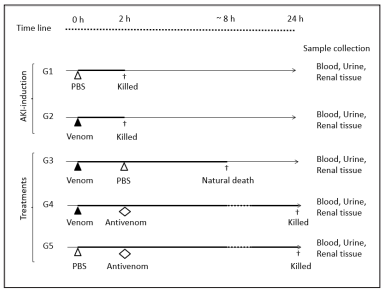


Figure 1. Experimental timeline for induction of AKI with *C. d. terrificus* venom (6 mg kg^{-1}, i.p.) and subsequent treatments. AKI, acute kidney injury; Antivenom, crotalic antivenom; PBS, phosphate-buffered saline.

Assessment of oxidative stress

Glutathione (GSH) was determined by sulfhydryl (SH) quantification using the Ellman method (Ellman 1959). Briefly, 100 µL of Triton X-100 was added to 150 µL of blood followed by 100 µL of 10% trichloroacetic acid (TCA). After centrifugation (5,000 rpm, 10 min, 4 °C) the supernatant was diluted in 1 M phosphate buffer (pH 7.4) and an aliquot was subsequently mixed with 10 mM 5,5´-dithiobis(2-nitrobenzoic acid) (DTNB) to yield a yellow complex, the absorbance of which was read at 412 nm. A similar procedure was used for renal tissue homogenates in which 250 mg of tissue was suspended in 5 mL of 0.15 mM KCl and homogenized with an ultrasonic tissue homogenizer in an ice bath. Thereafter, 600 µL of homogenate was mixed with 100 µL of TCA, centrifuged, and an aliquot of 250 µL was diluted in PBS and mixed with 10 mM DTNB. The GSH concentration was expressed in mM for blood or mmol g^{-1} for tissue.

Glutathione peroxidase (GPx) activity was assayed according to Paglia and Valentine (1967). Briefly, 10 µL of diluted blood was mixed with 390 µL of 100 mM potassium phosphate buffer, pH 7, containing 1 mM GSH, 0.1 U of GSH-reductase/mL, 0.15 mM nicotinamide adenine dinucleotide phosphate (NADPH), 1.25 mM sodium azide and 100 µL of 0.4 mM hydrogen peroxide (H_2O_2). Tissue samples were processed as described in the preceding paragraph. GPx activity was monitored as the decay in NADPH absorbance (greater activity = greater decay) for 2 min at 340 nm and was expressed in µmol NADPH min^{-1} g^{-1} of hemoglobin (Hb) for blood, or nmol NADPH min^{-1} g^{-1} of tissue.

Catalase (CAT) activity was assayed according to Aebi (1984). Briefly, 10 µL of blood was diluted in 590 µL of 50 mM potassium phosphate buffer, pH 7. A 20 µL aliquot was mixed with 70 µL of H_2O_2 to start the reaction and the enzymatic decomposition of H_2O_2 by CAT was monitored for 3 min at 240 nm. The reaction was run at 25 °C. A similar procedure was used for tissue homogenates prepared as described above. Enzyme activity was expressed as k min^{-1} g^{-1} of Hb for blood or k min^{-1} g^{-1} of tissue, where k is a rate constant.

Lipid peroxidation was assessed by quantifying thiobarbituric acid reactive substances (TBARS) according to Ohkawa *et al.* (1979). Briefly, 150 µL of plasma was mixed with 50 µL of 3 M NaOH and incubated at 60 °C for 30 min. This step was followed by the addition of 250 µL of 6% H_3PO_4, 250 µL of 0.8% thiobarbituric acid (TBA), and 100 µL of 10% sodium dodecyl sulfate (SDS) followed by incubation at 80 °C for 60 min. A similar procedure was used for tissue homogenates prepared as described above. Thereafter, 250 µL of the homogenate was mixed with 1.5 mL of 6% H_3PO_4, 500 µL of 0.8% TBA and 100 µL of 10% SDS. The reaction between lipid peroxidation products and TBA results in a pink/rose-colored compound detected spectrophotometrically at 532 nm. The results were expressed as TBARS concentration in µmol mL^{-1} of plasma or µmol g^{-1} of tissue.

Biochemical parameters

The biochemical parameters of renal function were assessed using commercial ready-to-use kits for spectrophotometric assays purchased from Bioclin® (Belo Horizonte, MG, Brazil) or Labtest® (Lagoa Santa, MG, Brazil). Total protein (biuret method; 545 nm), urea (urease method; 600 nm) and creatinine (alkaline picrate method; 510 nm) were evaluated in blood samples; urinary protein was quantified by the pyrogallol red method (600 nm), while urinary urea and creatinine were assayed with the same kits as used for blood samples. Control serum (Biocontrol N, normal) was used as an internal control and yielded the following values: protein = 5.3 g dL^{-1} (normal range values (NRV) provided by the manufacturer: 4.7-5.9 g dL^{-1}), urea = 16.0 mg dL^{-1} (NRV: 12.0-20.0 mg dL^{-1}) and creatinine =1.43 mg dL^{-1} (NRV: 1.14-1.72 mg dL^{-1}).

Histological analysis of renal tissue

At the end of the experiments, the kidneys from groups G1-G5 (n=5/group) were excised, washed in physiological solution, and imme-

diately fixed in 10% buffered formalin solution for 24 h at room temperature. The kidneys were then sectioned sagittally and immersed in 70% ethanol until histological processing using standard procedures for dehydration and embedding in paraffin. Sections (4 µm thick) were mounted on slides, dewaxed, cleared in xylene, and hydrated in running tap water, after which they were double-stained with hematoxylin-eosin (H&E) and examined with a light microscope (Olympus CBA, Tokyo, Japan). The slides from each group were examined for venom-induced alterations to the following structures: the glomeruli (hypercellularity due to mesangial proliferation and/or invasion, dilation of glomerular capillaries to form microaneurysms), tubules (loss of brush border, occurrence of cell swelling, hydropic alterations, presence of luminal cell casts), interstitium (medullary or cortical proliferation and/or loss of interstitium) and vessels (congestion, particularly in the arterioles). The potential protective effects of crotalic antivenom on renal morphology were also assessed. Representative histological fields from each group were captured using a digital 16-megapixel camera, with a 10x objective for a panoramic overview (1 cm = 20 µm) and a 40X objective (1 cm = 40 µm) for a detailed view.

The observational frequency of such alterations ranged arbitrarily from 0 to 6, with 0-1 representing the absence or presence of few alterations (the latter mainly due to tissue manipulation), 2-3 indicating a moderate number of alterations, and 5-6 representing a high frequency of morphological alterations.

Data analysis

All numerical parameters were expressed as the mean ± SD. Statistical comparisons among the experimental groups were done using one-way ANOVA followed by the Tukey-Kramer multiple comparisons test, with $p<0.05$ indicating significance in all cases. The normality of the data was assessed using the Shapiro-Wilk test prior to the statistical comparisons. All data analyses were done using Origin© v.8.0 (OriginLab Corporation, Northampton, MA, USA) or Statistica v.8.0 (Dell, Round Rock, TX, USA).

Results and discussion

Crotalus d. terrificus venom induces AKI in humans (Warrell 2004; Pinho *et al.* 2005, 2008; Albuquerque *et al.* 2013) and experimental animals such as mice (Yamasaki *et al.* 2008); venom-induced renal lesions have also been reported in dogs (Frezzatti and Silveira 2011), although this species is resistant to AKI (Sousa-e-Silva *et al.* 2003). Whereas the venom of *C. d. terrificus* and related subspecies causes functional and histological alterations in rat isolated perfused kidneys (Monteiro *et al.* 2001; Amora *et al.* 2008), the renal effects of *C. d. terrificus* venom *in vivo* in rats have not been investigated, in contrast to *Bothrops* venoms (Boer-Lima *et al.* 1999, 2002; Linardi *et al.* 2011; Gois *et al.* 2017; Yoshida *et al.* 2020). In this work, we examined the ability of *C. d. terrificus* venom injected i.p. to induce AKI in rats and the ability of antivenom to protect against this damage. We also assessed the potential changes in several renal biochemical, hematological, and histological parameters in response to the venom. Although not the normal or usual route of venom inoculation during a 'natural' bite, at least in humans, i.p. injection of venom could potentially occur in rodent prey bitten by snakes with long fangs. Since there have been no experimental studies of AKI induced by *C. d. terrificus* venom in rats we chose to use this route of administration because we had used it in an earlier investigation of AKI caused by *Bothrops jararaca* venom (Yoshida *et al.* 2020). The results of this study show that venom injected i.p. was able to cause AKI and that this could be a suitable model for studying venom-induced renal damage.

Biochemical parameters

Rats injected with venom (groups G2 and G3, but especially G3) showed an increase in the blood (*Fig. 2A,C*) and urine (*Fig. 2B,D*) concentrations of urea and creatinine compared to rats injected with saline alone (control, G1), a finding compatible with the development of AKI. The persistent elevation in blood urea and creatinine concentrations for up to 7.2 h indicated sustained and worsening renal damage after venom injection. Increases in urea and creatinine are standard criteria for defining and monitoring the progression of AKI (Mehta *et al.* 2007; Cerdá *et al.* 2008) but are considered by some to be insensitive, nonspecific, and of limited usefulness for the early detection of AKI (Vaidya *et al.* 2008). Although blood protein concentrations were generally unaltered (*Fig. 2E*), proteinuria indicative of defective glomerular filtration was observed (*Fig. 2F*). Renal proteinuria is a common indicator of AKI and is frequently seen after snakebites (Sitprija 2006),

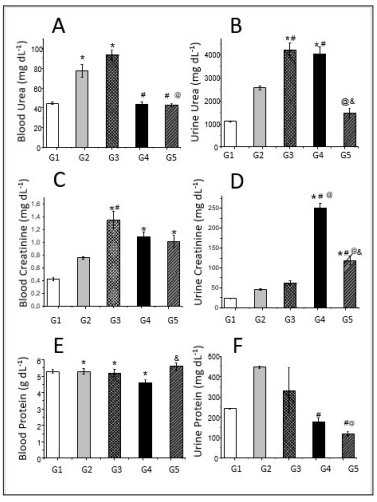


Figure 2. Urea, creatinine, and protein concentrations in blood (A, C, and E, respectively) and urine (B, D, and F, respectively) in rats injected with phosphate-buffered saline (PBS; G1), *C. d. terrificus venom* (6 mg kg^{-1}, i.p.; G2 – analyzed 2 h post-venom; G3 – analyzed 7.2 h post-venom), venom + *C. d. terrificus* antivenom (G4, analyzed 24 h post-venom) and antivenom alone (G5 – 24 h after antivenom). In groups G4 and G5, antivenom was administered 2 h after the injection of venom (G4) or PBS (G5). The antivenom:venom ratio used was based on the manufacturer's recommended ratio in which 1 mL of antivenom neutralizes the lethality of 1.5 mg of reference *C. durissus* ssp. venom in mice. The columns represent the mean ± SD (n=3-5/group). $^{*,\#,@,\&}$ $p<0.05$ compared to G1(*), G2($^{\#}$), G3($^{@}$), and G4($^{\&}$).

including by *C. d. terrificus* in humans (Pinho *et al.* 2005), dogs (Sousa-e-Silva *et al.* 2003), as well as rats (as shown here; *Fig. 2F*).

Antivenom administered 2 h after venom was effective in preventing the increase in blood urea and renal proteinuria (*Fig. 2A,F*), but failed to prevent the changes in the other blood and urinary biochemical parameters (*Fig. 2B-E*). Most of these alterations are likely mediated by crotoxin that is rapidly cleared from the circulation in mice (Barral-Netto and von Sohsten, 1991) and Amaral *et al.* (1997) reported that *C. d. terrificus* venom and crotoxin were undetectable in most of their patients who were treated >8 h post-bite, and that crotoxin was undetectable in most patients by 4-8 h after being bitten. A kinetic analysis has shown that in humans crotoxin has a distribution half-life of 22 min and a terminal elimination half-life of 5.2 h (Cura *et al.* 2002). As shown here, by 2 h post-venom, when a considerable proportion of the crotoxin would have been distributed and bound to tissues, antivenom still offered some protection against the deleterious effects of the venom. The time interval of 2 h after venom injection used here was chosen to simulate the delay seen clinically between envenomation and treatment since in humans the mean interval between these events is ~3 h and antivenom is frequently given at post-envenomation intervals that are considerably greater than that used here (Bucaretchi *et al.* 2002; Pinho *et al.* 2005).

The lack of protection by antivenom against certain venom-induced alterations may reflect the availability of a less than optimal amount of antivenom to neutralize that particular effect, even though the antivenom:venom ratio used was that recommended by the manufacturer; poor neutralization because of a suboptimal amount of antivenom has also been noted by others (Baudou *et al.* 2017).

Crotalic antivenom did not prevent the venom-induced increase in urinary creatinine, but instead markedly enhanced the level of this marker (~10-fold increase compared to saline control in G1 and ~5-fold increase compared to G3; *Fig. 2D*). The marked elevation in creatinine in this group 22 h after antivenom administration was possibly related to venom-induced muscle damage (rhabdomyolysis), with the resulting myoglobinuria contributing to the development of AKI (Azevedo-Marques *et al.* 1985, 1987; Nogueira and Sakate 2006).

Creatinine, an uncharged endogenous substance (113 kDa) that is not bound to serum proteins, is generated from the nonenzymatic conversion of creatine and creatine phosphate, 95% of which is found in muscle (Thongprayoon *et al.* 2016). As shown here, antivenom alone increased the creatinine concentration in blood and urine after 22 h (*Fig. 2C,D*), although the increase in urine was less than in G4 rats. One possible explanation for the antivenom-induced increase in serum creatinine could be related to changes in creatinine kinetics (in this case enhanced elimination) resulting from alterations to the glomerular filtration rate (GFR), as occurs in paraquat poisoning (Mohamed *et al.* 2015). This finding suggests that the increase in creatinine concentrations in G4 rats treated with antivenom should not be attributed entirely to *C. d. terrificus* venom. In contrast to this effect, antivenom alone (G5) reduced the proteinuria to below the baseline concentration in G1 (*Fig. 2F*). This change had minimal effect on the creatinine concentration because

creatinine shows little tendency to bind other proteins.

Oxidative stress parameters

The antioxidant system of tissues, which consists of a series of enzymes and small molecules, provides an effective defense mechanism against renal damage by xenobiotics by delaying or inhibiting the oxidation of substrates and the generation of damaging reactive oxygen species (ROS) (Ferreira and Matsubara 1997). An increase in antioxidant enzyme activity generally has a protective effect whereas a decrease can lead to renal damage. Given the physiological importance of antioxidant mechanisms in tissue protection and the fact that lipid peroxidation, one of the first events in oxidative damage initiated by ROS, has been implicated in snake venom toxicity (Santosh *et al.* 2013), we examined the changes in lipid peroxidation (TBARS) and oxidative stress (GSH, GPx and CAT activity) in blood and renal tissue of rats injected with *C. d. terrificus* venom and the ability of crotalic antivenom to protect against these alterations.

Lipid peroxidation is a free radical-mediated chain of reactions that results in the oxidation of polyunsaturated lipids (Hampel *et al.* 2016), leading to the damage of membrane lipids and the attenuation of cell and tissue viability (Mylonas and Kouretas 1999). *Figure 3A,B* shows that 2 h post-venom (G2) there was no relevant increase in blood TBARS, whereas shortly before death (~8 h post-venom) TBARS had increased by approximately two-fold; there was no significant change in the TBARS levels in urine. This finding that TBARS increased in G3 but not in G2 indicated that lipid peroxidation was a late event and agrees with previous studies showing that *C. d. terrificus* venom and some of its components cause lipid peroxidation in other tissues (Silva *et al.* 2011; Gonçalves *et al.* 2014; Toyama *et al.* 2019). Treatment with antivenom 2 h post-venom did not attenuate the venom-induced increase in TBARS, possibly because antivenom alone also caused some lipid peroxidation.

Glutathione, which exists in reduced (GSH) or oxidized (GSSG) states, is one of the most important ROS scavengers (Halliwell and Gutteridge 1999) and increases in GSH levels have been observed in patients with chronic renal failure, possibly as a compensatory protective mechanism to deal with the oxidative imbalance produced by kidney disease (Lucchi *et al.* 2005). As shown here, there was only a small increase in blood GSH in response to venom alone after 2 h and ~8 h (*Fig. 3C,D*) but a marked increase in renal GSH after 2 h (G2) that returned to normal after ~8 h (G3). Treatment with antivenom (G4) attenuated the small increase in blood GSH seen with venom alone after ~8 h but did not markedly affect the renal levels of GSH. Antivenom alone (G5) slightly reduced the concentration of circulating (blood) GSH but increased the renal content of GSH.

Glutathione peroxidase (GPx) is a selenium-containing antioxidant enzyme that reduces H_2O_2 to water and lipid peroxides to lipid alcohols. In the absence of adequate GPx activity or GSH levels, hydrogen and lipid peroxides are not detoxified and are converted to OH- and lipid peroxyl radicals, respectively, by transition metals (Fe^{2+}) (Tabet and Touyz 2007). GPx oxidizes glutathione to glutathione disulfide in

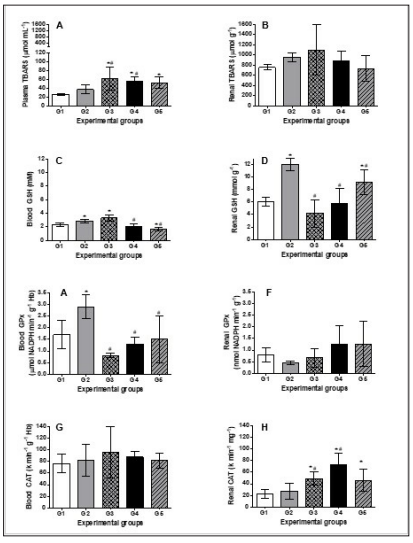


Figure 3. Oxidative stress biomarkers (TBARS, GSH, GPx, and CAT) in blood (A, C, E, and G, respectively) and urine (B, D, F and H, respectively) of rats injected with phosphate-buffered saline (PBS; G1), *C. d. terrificus* venom (6 mg kg^{-1}, i.p.; G2 – analyzed 2 h post-venom, G3 – analyzed 7.2 h post-venom), venom + *C. d. terrificus* antivenom (G4, analyzed 24 h post-venom) and antivenom alone (G5 – 24 h after antivenom). In groups G4 and G5, antivenom was administered 2 h after the injection of venom (G4) or PBS (G5). The antivenom:venom ratio used was based on the manufacturer´s recommended ratio as defined in the legend of Figure 2. The columns represent the mean ± SD (n=5/group). $^{*,\#}p<0.05$ indicate differences compared to G1(*) or G2(#). CAT, catalase; GPx, glutathione peroxidase; GSH, glutathione; TBARS, thiobarbituric acid reactive substances.

the cytosol, mitochondria, and a small percentage of the endoplasmic reticulum of mammalian tissues (Lu 2013). Glutathione is released as reduced GSH to extracellular spaces, including blood plasma, epithelial lining fluids, and exocrine secretions (Ballatori *et al.* 2009) in response to physiological disturbances. The GSH/GPx system thus acts as a buffer to protect crucial proteins against modifications induced by ROS (Chen *et al.* 2015).

Venom caused an increase in blood GPx activity after 2 h but this returned to below saline (control) levels after ~8 h (*Fig. 3E*). Antivenom did not markedly alter the response to venom (G4) compared to that seen in G3, nor did antivenom alone have a relevant effect on GPx activity. There were no marked venom-induced alterations in renal GPx activity after 2 h and ~8 h with venom alone, or after treatment with antivenom (*Fig. 3F*).

CAT is an enzyme located in peroxisomes that converts H_2O_2 to water and oxygen (Weydert and Cullen 2010). Neither venom nor antivenom (in the absence or presence of venom) markedly affected the blood CAT activity (*Fig. 3G*), but there was a venom-induced increase in the activity of this enzyme in renal tissue after ~8 h that was not attenuated by antivenom; antivenom alone increased the renal CAT activity to a level like venom alone (*Fig. 3H*). Indeed, antivenom alone (G5) enhanced the levels of several parameters, particularly blood and urinary creatinine, and to a lesser extent, renal GSH, blood TBARS and renal CAT in relation to saline-treated (control) rats but reduced the level of blood GSH and renal proteinuria.

These findings suggest that antivenom alone can alter the redox status in blood and renal tissue by mechanisms that could involve the activation of defense pathways against horse immunoglobulins (Bahrami *et al.* 2016) and an adverse reaction to antivenom (Silva *et al.* 2015). Adverse reactions to snake antivenom include both acute (anaphylatic or pyrogenic) and delayed (serum sickness type) reactions (León *et al.* 2013; de Silva *et al.* 2016; Negrin *et al.* 2021), but no reports have described such reactions in response to the i.p. injection of antivenom in rats. Intraperitoneal injection is considered a safe route of administration (Guarnieri 2016; Al Shoyaib *et al.* 2019), but no studies have reported on the renal responses to crotalic antivenom alone, such as done here. The i.p. administration of bothropic antivenom in rats resulted in proteinuria (Yoshida *et al.* 2020), one of the earliest signs of renal diseases and indicative of glomerular damage (Gowda *et al.* 2010). Thus, it is possible the potential effect of antivenom itself on a variety of parameters may have been masked by the concomitant administration of venom in studies that did not include an experimental group with antivenom alone. An additional source of interference could be the phenol used as a preservative in antivenom solutions since this chemical can impair leukocyte-endothelial interactions *in vivo* (Zychar *et al.* 2008), and high phenol inhalation for several weeks paralysis and severe injury to the heart, liver, kidneys, and lungs, and in some cases, death in experimental animais (Agency for Toxic Substances and Disease Registry 2008). The use of lyophilized antivenom (Mendonça-da-Silva *et al.* 2017) could potentially avoid phenol-related problems.

Hematological parameters

Hematological alterations are a common manifestation of systemic envenoming after snakebite and the detection of abnormal blood parameters is an important step in the management of envenomed persons. *Figure 4* shows the hematological results for the various experimental groups. There was a slight reduction in leukocyte numbers in G2 and mild leukocytosis in G3; treatment with antivenom appeared to attenuate the leukocytosis in group G4. This attenuation may have been partly mediated by the antivenom itself, independently of its effect on venom, since antivenom alone caused a reduction in leukocyte numbers. Mild leukocytosis has been observed in humans (Sano-Martins *et al.* 2001) and dogs (Sousa-e-Silva *et al.* 2003; Nogueira and Sakate 2006; Nogueira *et al.* 2007) envenomed by *C. d. terrificus* and has been attributed to neutrophilia (Sousa-e-Silva *et al.* 2003). Nogueira *et al.* (2007) argued that this neutrophilia involved an acute-phase response with the release of catecholamines and cellular mediators; serum chemotactic factors have also been implicated in human envenomation by *C. d. terrificus* (Martins *et al.* 2002). Souza-e-Silva *et al.* (2003) reported that leukocytosis persisted for up to 48 h in dogs injected with a venom dose of 1 mg kg^{-1}, i.m., and was reversed by treatment with antivenom i.v. 2 h after envenomation, as also observed here in rats using antivenom (G4 in *Fig. 4A*).

Platelet number was unaffected by the venom (*Fig. 4B*), a finding in general agreement with studies in dogs (Sousa-e-Silva *et al.* 2003)

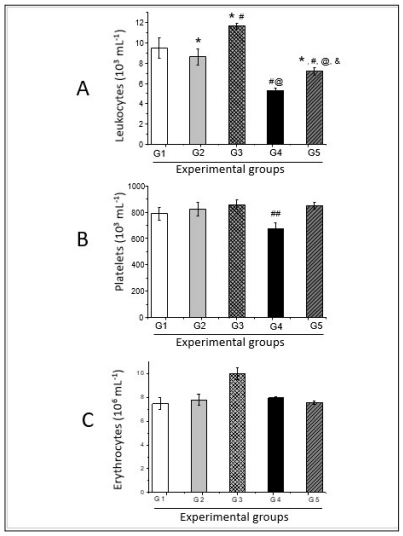


Figure 4. Hematological parameters (leukocyte, platelet and erythrocyte counts) in rats injected with phosphate-buffered saline (PBS; G1), *C. d. terrificus* venom (6 mg kg^{-1}, i.m.; G2 – analyzed 2 h post-venom, G3 – analyzed 7.2 h post-venom), venom + *C. d. terrificus* antivenom (G4, analyzed 24 h post-venom) and antivenom alone (G5 – 24 h after antivenom). In groups G4 and G5, antivenom was administered 2 h after the injection of venom (G4) or PBS (G5). The antivenom:venom ratio used was based on the manufacturer´s recommended ratio as defined in the legend of Figure 2. The columns represent the mean ± SD (n=3-5/group). $^{*,\#,@,\&,\#\#}p<0.05$ compared to G1 (*), G2 ($^{\#}$), G3 ($^{@}$), G4 ($^{\&}$), and G3 and G5 ($^{\#\#}$).

and humans (Sano-Martins *et al.* 2001) that also reported unaltered platelet numbers after envenomation by *C. d. terrificus*. Antivenom alone (G5) did not affect platelet number but decreased this number when associated with venom (G4). However, this decrease was not clinically relevant since there was no significant difference compared to saline-treated (control) rats (G1). The lack of effect on circulating platelet numbers could perhaps be the most useful parameter for distinguishing between clinical envenomation by *C. d. terrificus* (Sano-Martins *et al.* 2001) and *Bothrops* species (Sano-Martins *et al.* 1997; Yoshida *et al.* 2020) since venoms of the latter genus can cause marked thrombocytopenia. Such a distinction would be of clinical relevance in facilitating the choice of the appropriate antivenom, thereby ensuring better treatment of the patient, particularly in cases where the offending snake is not brought for identification.

There was no difference in the number of erythrocytes 2 h (G2 vs. G1) or 7.2 h (G3 vs. G1) after envenomation (*Fig. 4C*). The delayed administration of antivenom (G4) attenuated the slight increase in the number of erythrocytes in G3 to a level similar to that seen with saline and antivenom alone (*Fig. 4C*). Other reports have also observed no alterations in the number of erythrocytes, the hematocrit, or the hemoglobin concentration in dogs (Sousa-e-Silva *et al.* 2003) and humans (Sano-Martins *et al.* 2001) envenomed by *C. d. terrificus*.

Histological analysis

The kidneys regulate the body´s internal composition through a complex network of nephrons and collecting ducts that filter, absorb and secrete fluid and electrolytes and promote the elimination of metabolic waste products via the urine (Santoro *et al.* 2008). Glomerular damage leads to several pathologies (Greka and Mundel 2012) and circulating snake venom components can cause morphological abnormalities that result in functional disturbances.

Figure 5 shows representative panels and insets for the different groups that illustrate the main histological alterations seen in the renal parenchyma. Histological analysis of renal sections from the different experimental groups (G2-G5) showed that morphological changes were not evenly distributed throughout the renal parenchyma since there were areas with visible abnormalities juxtaposed to regions normal in appearance. This heterogeneity reflected the differential effects of venom components on the kidney in that all nephrons were not affected simultaneously or in the same manner.

Except for G1 (saline control) that showed the typical normal morphology of renal corpuscles, proximal and distal convoluted tubules, the loop of Henle and collecting ducts (*Fig. 5A,B*), all the other groups exhibited varying degrees of alterations in their structural segments (*Fig. 5C-J*). The changes included glomerular alterations (indicated by arrows in the panels and insets of *Fig. 5*). Major and more numerous glomerular alterations were seen in G2 and G3 (see insets); antivenom (G4) partially prevented the glomerular morphological alterations (inset in G4). Of note were the glomerular alterations in G5 (inset in G5) that received antivenom alone.

The greatest structural changes in the cortical tubular nephron segments and medullary collecting tubules were seen in G2: the changes included loss or central collapse of the brush

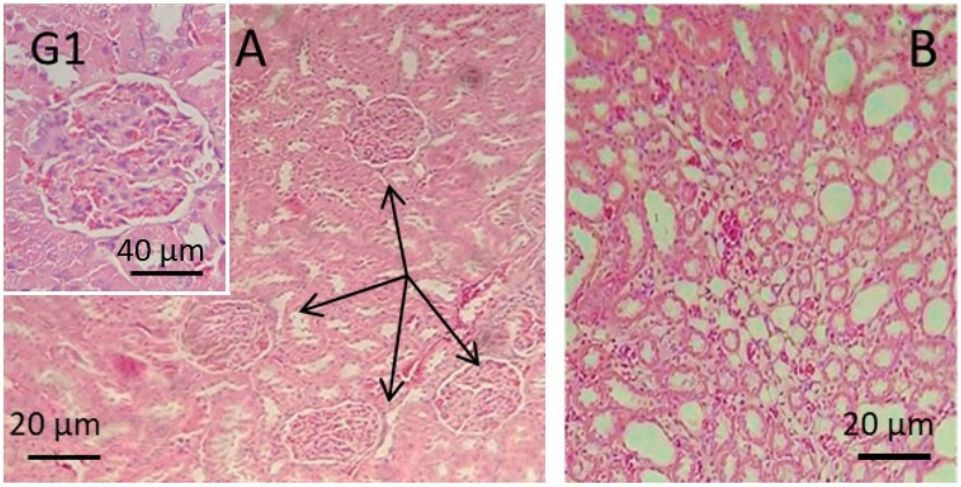
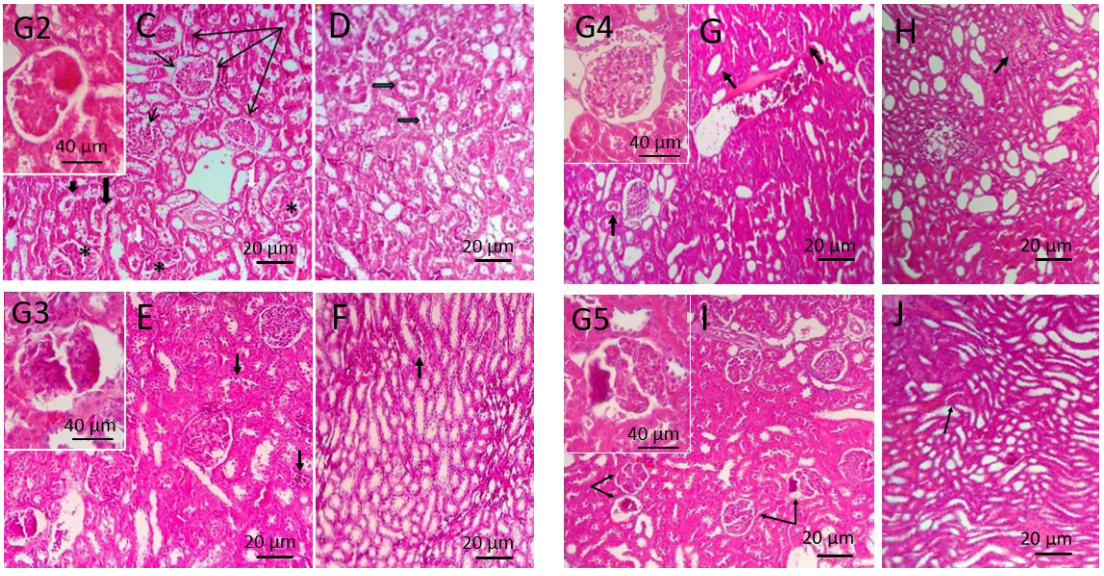


Figure 5. Histological renal tissue assessment in groups G1 (A and B), G2 (C and D), G3 (E and F), G4 (G and H), and G5 (I and J) (scale bar = 20 µm in all major panels). Insets in all groups show renal corpuscles (scale bar = 40 µm in all inserts). G1 (control rats injected with phosphate-buffered saline; arrows indicate the normal appearance of renal corpuscles). The effects of severe damage induced by *C. d. terrificus* venom are seen in G2 (venom group): panel C, long arrows = widened filtration space and hypercellularity; short arrows = microaneurysms; panel D = luminal cellular casts resulting from detachment of tubular epithelial lining; G3 (rats treated with venom and saline): panel E = hyalinization of both cortical segments and glomeruli; panel F, medullary segments showing few alterations. G4 (rats treated with venom and antivenom) and G5 (rats treated with antivenom): all panels show tissue hyalinization leading to poorly defined cell limits; panels G and I = hemorrhagic foci and glomerular microaneurysms (arrows); panels H and J = medullary sections showing the flattened epithelial lining of tubular segments with a distended lumenal space. A description of the alterations is provided in the text.



border in the proximal tubules, and luminal widening with cellular casts in the proximal and distal tubules and collecting ducts (*Fig. 5C,D*). Groups G3, G4, and G5 had a general hyaline appearance in the nephron segments located in the renal cortex that obscured visualization of their cellular constituents (*Fig. 5E,G,I*). The renal medulla showed less damage in envenomed rats treated with physiological saline solution (G3) (*Fig. 5F*), whereas greater damage was seen in envenomed rats treated with antivenom (G4) and in rats treated with antivenom

alone (G5) (*Fig. 5H,J*) compared to G3 (*Fig. 5F*); similar renal alterations in response to antivenom have been described elsewhere (Oliveira *et al.* 2021), but the reasons remain to be investigated. All the changes observed here are characteristic of renal disease (Cardoso *et al.* 1993; Haraldsson *et al.* 2008).

A comparative analysis of the frequency of histological alterations in groups G1-G5 yielded the following scores: G2 = a score of 5-6 representing a very high-frequency of alterations; G3 = 2-3, a relatively low-to-moderate frequency of alterations; G4 and G5 = 4 indicating a high frequency, and G1 = 0-1 indicating few or no alterations (most of the alterations were attributable to technical artifacts).

The results of this study allow us to address two specific issues: (a) The early onset of AKI: Pinho *et al.* (2005) have shown that approximately two-thirds of patients who develop AKI following envenomation by *C. d. terrificus* do so within 24 h of being bitten and that the mean time between being bitten and receiving antivenom (among patients with and without AKI) was 3 h (range: 1-48 h). Risk factors for the development of AKI included age (greater for children <12 years old), the time until antivenom administration (the greater the interval, the greater the risk), and CK >2000 IU/mL; a diuresis >90 mL/h was considered a protective factor. The 2 h time frame used to assess AKI and for antivenom administration in the present study was based on the mean interval until antivenom administration noted by Pinho *et al.* (2005). (b) The parameters used to assess AKI: Parameters commonly used to analyze AKI induced by *C. d. terrificus* include urinary output (diuresis) and glomerular filtration rate (based on creatinine clearance), fractional excretion of Na^+ and K^+, serum creatine kinase (CK), serum and urinary creatinine and proteinuria, although not all of these have the same usefulness (sensitivity or predictive value); additional ancillary parameters include hemoglobin, hematocrit, and platelet counts. The choice of parameters used here (creatinine, urea, and proteinuria) as markers for AKI was based on previous reports in the literature and also because these can be easily measured in serum and urine, samples of which can be obtained with relative ease in the clinical setting.

Conclusion

The findings of this study show that *C. d. terrificus* venom (6 mg kg^{-1}, i.p.) induced AKI in rats within 2 h, with an increase in blood and renal urea, creatinine, and GSH concentrations, as well as enhanced blood GPx activity and renal protein concentrations. These parameters, together with histological analysis, could be potentially useful biomarkers for assessing the severity of AKI. Antivenom was effective in attenuating the increase in blood urea, in normalizing the proteinuria, and in inactivating antioxidant defense through CAT activity, but did not protect against lipid peroxidation.

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Authors' contributions: EHY, MMJD, ICFO, and JAMF did the experiments under supervision by YOF; JCC milked the snakes and provided the venom; DG was responsible for interpreting the oxidative stress data; MACH was responsible for the histological analyses. VMHY and YOF designed the study, and YOF, DG, MACH, and SH wrote the manuscript.

References

Aebi H. 1984. Catalase in vitro. Methods in Enzymology. 105:121-126.

Agency for Toxic Substances and Disease Registry (ATSDR). 2008. Toxicological Profile for Phenol. Atlanta, GA: U.S. Department of Health and Human Services, Public Health Service. [Accessed July 2, 2020]. Available from: https://www.atsdr.cdc.gov/toxfaqs/tfacts115.pdf

Albuquerque PL, Jacinto CN, Silva Junior GB, Lima JB, Veras MS, Daher EF. 2013. Acute kidney injury caused by *Crotalus* and *Bothrops* snake venom: a review of epidemiology, clinical manifestations and treatment. Revista do Instituto de Medicina Tropical de São Paulo. 55(5):295-301.

Albuquerque PLMM, Paiva JHHGL, Martins AMC, Meneses GC, da Silva GB, Buckley

N, Daher EF. 2020. Clinical assessment and pathophysiology of *Bothrops* venom-related acute kidney injury: a scoping review. Journal of Venomous Animals and their Toxins Including Tropical Diseases. 26:e20190076.

Alegre VS, Barone JM, Yamasaki SC, Zambotti-Villela L, Silveira PF. 2010. Lipoic acid effects on renal function, aminopeptidase activities and oxidative stress in *Crotalus durissus terrificus* envenomation in mice. Toxicon. 56(3):402-410.

Al Shoyaib A, Archie SR, Karamyan VT. 2019. Intraperitoneal route of drug administration: should it be used in experimental animal studies? Pharmceutical Research. 37(1):12.

The missing reference Amaral et al. (1997) (cited at the bottom of the left-hand column of page 20 of the main text) into the reference list, placing it before this work by Amora et al. (2008). DN, Martins AMC, Roesner N, Senter R, Ostrowsky T, Weinberg JM, Monteiro HSA. 2008. Mitochondrial dysfunction induced by pancreatic and crotalic (*Crotalus durissus terrificus*) phospholipases A_2 on rabbit proximal tubules suspensions. Toxicon. 52(8):852-857.

Amorim MF, Mello RF. 1954. Intermediate nephrosis from snake poisoning in man. American Journal of Pathology. 30(3):479-499.

Azevedo-Marques MM, Cupo P, Coimbra TM, Hering SE, Rossi MA, Laure CJ. 1985. Myonecrosis, myoglobinuria and acute renal failure induced by South American rattlesnake (*Crotalus durissus terrificus*) envenomation in Brazil. Toxicon. 23(4):631-636.

Azevedo-Marques MM, Hering SE, Cupo P. 1987. Evidence that *Crotalus durissus terrificus* (South American rattlesnake) envenomation in humans causes myolysis rather than hemolysis. Toxicon. 25(11):1163-1168.

Bahrami S, Shahriari A, Tavalla M, Azadmanesh S, Hamidinejat H. 2016. Blood levels of oxidant/antioxidant parameters in rats infected with *Toxoplasma gondii*. Oxidative Medicine and Cellular Longevity. 2016:8045969.

Ballatori N, Krance SM, Marchan R, Hammond CL. 2009. Plasma membrane glutathione transporters and their roles in cell physiology and pathophysiology. Molecular Aspects of Medicine. 30(1-2):13-28.

Barone JM, Frezzatti R, Silveira PF. 2014. Effects of N-acetyl-L-cysteine on redox status and markers of renal function in mice inoculated with *Bothrops jararaca* and *Crotalus durissus terrificus* venoms. Toxicon. 79:1-10.

Barral-Netto M, von Sohsten RL. 1991. Serum kinetics of crotoxin from *Crotalus durissus terrificus* venom in mice: evidence for a rapid clearance. Toxicon. 29(4-5):527-531.

Baudou FG, Litwin S, Lanari LC, Laskowicz RD, Damin CF, Chippaux JP, de Roodt AR. 2017. Antivenom against *Crotalus durissus terrificus* venom: Immunochemical reactivity and experimental neutralizing capacity. Toxicon. 140:11-17.

Berger M, Vieira MAR, Guimarães JA. 2012. Acute kidney injury induced by snake and arthropod venoms. In: Polenakovic M, editor. Renal failure - The facts. London: InTech Open. p. 157-186. [Accessed April 30, 2022]; Available from: https://www.intechopen.com/books/renal-failure-the-facts/acute-kidney-injury-induced-by-snake-and-arthropod-venoms.

Boer-Lima PA, Gontijo JA, Cruz-Höfling MA. 1999. Histologic and functional renal alterations caused by *Bothrops moojeni* snake venom in rats. American Journal of Tropical Medicine and Hygiene. 61(5):698-706.

Boer-Lima PA, Gontijo JA, Cruz-Höfling MA. 2002. *Bothrops moojeni* snake venom-induced glomeruli changes in rat. American Journal of Tropical Medicine and Hygiene. 67(2):217-222.

Bucaretchi F, Herrera SRF, Hyslop S, Baracat ECE, Vieira RJ. 2002. Snakebites by *Crotalus durissus* ssp. in children in Campinas, São Paulo, Brazil. Revista do Instituto de Medicina Tropical de São Paulo. 44(3):133-138.

Cardoso JLC, Fan HW, França FOS, Jorge MT, Leite RP, Nishioka SA, Avila A, Sano-Martins IS, Tomy SC, Santoro M, *et al.* 1993. Randomized comparative trial of three antivenoms in the treatment of envenoming by lance-headed vipers (*Bothrops jararaca*) in São Paulo, Brazil. Quarterly Journal of Medicine. 86(5):315-325.

Cerdá J, Lameire N, Eggers P, Pannu N, Uchino S, Wang H, Bagga A, Levin A. 1988. Epidemiology of acute kidney injury. Clinical Journal of the American Society of Nephrology. 3(3):881-886.

Chen J, Bhandar B, Kavdia M. 2015. Interaction of ROS and RNS with GSH and GSH/GPX systems. FASEB Journal. 29(Supplement):636.7 (Abstract).

Cruz AH, Mendonça RZ, Petricevich VL. 2005. *Crotalus durissus terrificus* venom interferes with morphological, functional, and biochemical changes in murine macrophages. Mediators of Inflammation. 2005(6):349-359.

Cura JE, Blanzaco DP, Brisson CB, Cura MA, Cabrol R, Larrateguy L, Mendez C, Sechi JC, Silveira JS, Theiller E, *et al.* 2002. Phase I and pharmocokinetic study of crotoxin (cytotoxic PLA_2, NSC-624244) in patients with advanced cancer. Clinical Cancer Research. 8:1033-1041.

de Silva HA, Ryan NM, de Silva HJ. 2016. Adverse reactions to snake antivenom, and their prevention and treatment. British Journal of Clinical Pharmacology 81(3):446-452.

Ellman GL. 1959. Tissue sulfhydryl groups. Archives of Biochemistry and Biophysics. 82(1):70-77.

Ferreira ALA, Matsubara LS. 1997. Radicais livres: conceitos, doenças relacionadas, sistema de defesa e estresse oxidativo. Revista da Associação Médica Brasileira. 43(1):61-68.

Frare BT, Silva Resende YK, Dornelas BC, Jorge MT, Souza Ricarte VA, Alves LM, Izidoro LFM. 2019. Clinical, laboratory, and therapeutic aspects of *Crotalus durissus* (South American rattlesnake) victims: a literature review. BioMedical Research International. 2019:1345923.

Frezzatti R, Silveira PF. 2011. Allopurinol reduces the lethality associated with acute renal failure induced by *Crotalus durissus terrificus* snake. PLoS Neglected Tropical Diseases. 5(9):e1312.

Gois PHF, Martines MS, Ferreira D, Volpini R, Canale D, Málaque C, Crajoinas R, Girardi ACC, Shimizu MHM, Seguro AC. 2017. Allopurinol attenuates acute kidney injury following *Bothrops jararaca* envenomation. PLoS Neglected Tropical Diseases. 11(11):e0006024.

Gonçalves R, Vargas LS, Lara MVS, Güllich A, Mandredini V, Ponce-Soto L, Marangoni S, Dal Belo CA, Mello-Carpes PB. 2014. Intrahippocampal infusion of crotamine isolated from *Crotalus durissus terrificus* alters plasma and brain biochemical parameters. International Journal of Environmental Research and Public Health. 11:11438-11449.

Gowda S, Desai PB, Kulkarni SS, Hull VV, Math AAK, Vernekar SN. 2010. Markers of renal function tests. North American Journal of Medical Science. 2(4):170–173.

Greka A, Mundel P. 2012. Cell biology and pathology of podocytes. Annual Review of Physiology. 74:299-323.

Guarnieri M. 2016. Considering the risks and safety of intraperitoneal injections. Laboratory Animals 45:131.

Gutiérrez JM, Calvete JJ, Habib AG, Harrison RA, Williams DJ, Warrell DA. 2017. Snakebite envenoming. Nature Reviews Disease Primers. 3:17063.

Hadler WA, Vital Brazil O. 1966. Pharmacology of crystalline crotoxin. IV. Nephrotoxicity. Memórias do Instituto Butantan. 33(3):1001-1008.

Halliwell B, Gutteridge JMC. 1999. Antioxidant defense enzymes: the glutathione peroxidase family. In: Halliwell B, Gutteridge JMC (eds.) Free radicals in biology and medicine. 3rd ed. Oxford: Clarendon Press. p. 140-146.

Hampel M, Blasco J, Martín Díaz ML. 2016. Biomarkers and effects. In: Blasco J, Chapman PM, Campana O, Hampel M (eds.) Marine ecotoxicology. New York: Academic Press. p. 121-165.

Haraldsson B, Nyström J, Deen WM. 2008. Properties of the glomerular barrier and mechanisms of proteinuria. Physiological Reviews. 88(2):451-487.

Harris JB, Scott-Davey T. 2013. Secreted phospholipases A_2 of snake venoms: effects

on the peripheral neuromuscular system with comments on the role of phospholipases A_2 in disorders of the CNS and their uses in industry. Toxins. 5(12):2533-2571.

Kilkenny C, Browne WJ, Cuthill IC, Emerson M, Altman DG. 2010. Improving bioscience research reporting: the ARRIVE guidelines for reporting animal research. PLoS Biology. 8(6):e1000412.

Kini RM. 2003. Excitement ahead: structure, function and mechanism of snake venom phospholipase A_2 enzymes. Toxicon. 42(8):827-840.

León G, Herrera M, Segura Á, Villalta M, Vargas M, Gutiérrez JM. 2013. Pathogenic mechanisms underlying adverse reactions induced by intravenous administration of snake antivenoms. Toxicon. 76:63-76.

Linardi A, Rocha e Silva TAA, Miyabara EH, Franco-Penteado CF, Cardoso KC, Boer PA, Moriscot AS, Gontijo JAR, Joazeiro PP, Collares-Buzato CB, *et al.* 2011. Histological and functional renal alterations caused by *Bothrops alternatus* snake venom: expression and activity of Na^+/K^+ ATPase. Biochimica Biophysica Acta - General Subjects. 1810(9):895-906.

Lu SC. 2013. Glutathione synthesis. Biochimica Biophysica Acta. 1830(5):3143-3153.

Lucchi L, Bergamini S, Iannone A, Perrone S, Stipo L, Olmeda F, Caruso F, Tomasi A, Albertazzi A. 2005. Erythrocyte susceptibility to oxidative stress in chronic renal failure patients under different substitutive treatments. Artificial Organs. 29(1):67-72.

Martins AMC, Toyama MH, Havt A, Novello JC, Marangoni S, Fonteles MC, Monteiro HS. 2002. Determination of *Crotalus durissus cascavella* venom components that induce renal toxicity in isolated rat kidneys. Toxicon. 40(8):1165-1171.

Martins AMC, Lima AAM, Toyama MH, Marangoni S, Fonteles MC, Monteiro HSA. 2003. Renal effects of supernatant from macrophages activated by *Crotalus durissus cascavella* venom: the role of phospholipase A_2 and cyclooxygenase. Pharmacology and Toxicology. 92(1):14-20.

Medeiros JM, Oliveira IS, Ferreira IG, Alexandre-Silva GM, Cerni FA, Zottich U, Pucca MB. 2020. Fatal rattlesnake envenomation in northernmost Brazilian Amazon: a case report and literature overview. Reports. 3(2):9.

Mehta RL, Kellum JA, Shah SV, Molitoris BA, Ronco C, Warnock DG, Levin A. 2007. Acute kidney injury network. Report of an initiative to improve outcomes in acute kidney injury. Critical Care. 11(2):R31.

Mendonça-da-Silva I, Tavares AM, Sachett J, Sardinha JF, Zaparolli L, Santos MFG, Lacerda M, Monteiro WM. 2017. Safety and efficacy of a freeze-dried trivalent antivenom for snakebites in the Brazilian Amazon: An open randomized controlled phase IIb clinical trial. PLoS Neglected Tropical Diseases. 11(11):e0006068.

Mohamed F, Endre Z, Jayamanne S, Pianta T, Peake P, Palangasinghe C. 2015. Mechanisms underlying early rapid increases in creatinine in paraquat poisoning. PLoS One. 10(3):e0122357.

Monteiro HSA, da Silva IMSC, Martins AMC, Fonteles MC. 2001. Actions of *Crotalus durissus terrificus* venom and crotoxin on the isolated rat kidney. Brazilian Journal of Medical and Biological Research. 34(10):1347-1352.

Morton DB, Jennings M, Buckwell A, Ewbank R, Godfrey C, Holgate B, Inglis I, James R, Page C, Sharman I, *et al.* 2001. Joint Working Group on Refinement. Refining procedures for the administration of substances. Report of the BVAAWF/FRAME/RSPCA/UFAW Joint Working Group on Refinement. British Veterinary Association Animal Welfare Foundation/ Fund for the Replacement of Animals in Medical Experiments/Royal Society for the Prevention of Cruelty to Animals/Universities Federation for Animal Welfare. Laboratory Animals. 35(1):1-41.

Mylonas C, Kouretas D. 1999. Lipid peroxidation and tissue damage. *In Vivo*. 13(3):295-309.

National Human Genome Research Institute. 2004. Rat genome compared with human, mouse. The Wellcome Trust. January 4, 2004. [Accessed July 2, 2020]. Available from: http://genome.wellcome.ac.uk/doc_WTD020720.htm.

National Research Council of the National Academies. 2011. Guide for the Care and Use of Laboratory Animals. 8th ed. Washington DC: National Academies Press. [accessed September 14, 2019]. Available from: https://grants.nih.gov/grants/olaw/Guide-for-the-Care-and-use-of-laboratory-animals.pdf

Negrin A, Battocletti MA, Juanena C, Morais V. 2021. Reports of doses administered and adverse reactions to snake antivenom used in Uruguay in 2018. Frontiers in Toxicology. 3:690964

Nogueira RMB, Sakate M. 2006. Clinical and hematological alterations in dogs during experimental envenomation with *Crotalus durissus terrificus* venom and treated with antiophidic serum. Journal of Venomous Animals and their Toxins including Tropical Diseases. 12(2):285-296.

Nogueira RMB, Sakate M, Sangiorgio F, Laposy CB, Melero M. 2007. Experimental envenomation with *Crotalus durissus terrificus* venom in dogs treated with antiophidic serum – part I: clinical evaluation, hematology and myelogram. Journal of Venomous Animals and their Toxins including Tropical Diseases. 13(4):800-810.

Ohkawa H, Ohishi N, Yagi K. 1979. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Analytical Biochemistry. 95(2):351-358.

O'Leary MA, Kornhauser RS, Hodgson WC, Isbister GK. 2009. An examination of the activity of expired and mistreated commercial Australian antivenoms. Transactions of the Royal Society of Tropical Medicine and Hygiene. 103(9):937-942.

The missing reference to Oliveira et al. (2021) (cited in the first paragraph of page 25 of the proofs) here in the list, before Paglia: Oliveira ICF, Yoshida EH, Dini MMJ, Paschoal ABO, Cogo JC, da Cruz-Höfling MA, Hyslop S, Oshima-Franco Y. 2021. Evaluation of protection by caffeic acid, chlorogenic acid, quercetin and tannic acid against the in vitro neurotoxicity and in vivo lethality of Crotalus durissus terrificus (South American rattlesnake) venom. Toxins. 13(11):801. DE, Valentine WN. 1967. Study on the quantitative and qualitative characterization of erythrocyte glutathione peroxide. Journal of Laboratory and Clinical Medicine. 70(1):158-169.

Pinho FMO, Zanetta DMT, Burdmann EA. 2005. Acute renal failure after *Crotalus durissus* snakebite: a prospective survey on 100 patients. Kidney International. 67(2):659-667.

Pinho FMO, Yu L, Burdmann EA. 2008. Snakebite-induced kidney injury in Latin America. Seminars in Nephrology. 28(4):354-362.

Rodrigues Sgrignolli L, Florido Mendes GE, Carlos CP, Burdmann EA. 2011. Acute kidney injury caused by *Bothrops snake* venom. Nephron Clinical Practice. 119(2):c131-136; discussion c137.

Rosenfeld G. 1971. Symptomatology, pathology and treatment of snake bites in South America. In: Bücherl W, Buckley EE, Deulofeu V, editors. Venomous animals and their venoms. Vol. 2. New York: Academic Press. p. 345-384.

Sampaio SC, Hyslop S, Fontes MR, Prado-Franceschi J, Zambelli VO, Magro AJ, Brigatte P, Gutierrez VP, Cury Y. 2010. Crotoxin: novel activities for a classic α-neurotoxin. Toxicon. 55(6):1045-1060.

Sánchez EE, Migl C, Suntravat M, Rodriguez-Acosta A, Galan JA, Salazar E. 2019. The neutralization efficacy of expired polyvalent antivenoms: An alternative option. Toxicon. 168:32-39.

Sangiorgio F, Sakate M, Nogueira RMB, Tostes RA. 2008. Histopathological evaluation in experimental envenomation of dogs with *Crotalus durissus terrificus* venom. Journal of Venomous Animals and their Toxins including Tropical Diseases. 14(1):83-87.

Sanhajariya S, Duffull SB, Isbister GK. 2018. Pharmacokinetics of snake venom. Toxins. 10(2):E73.

Sano-Martins IS, Santoro ML, Castro SC, Fan HW, Cardoso JL, Theakston RD. 1997. Platelet aggregation in patients bitten by the Brazilian snake *Bothrops jararaca*. Thrombosis Research. 87(2):183-195.

Sano-Martins IS, Tomy SC, Campolina D, Dias

MB, de Castro SCB, de Sousa-e-Silva MCC, Amaral CFS, Rezende NA, Kamiguti AS, Warrell DA, *et al.* 2001. Coagulopathy following lethal and non-lethal envenoming of humans by the South American rattlesnake (*Crotalus durissus*) in Brazil. Quarterly Journal of Medicine. 94(10):551-559.

Santoro ML, Sano-Martins IS, Fan HW, Cardoso JL, Theakston RD, Warrell DA. 2008. Butantan Institute Antivenom Study Group. Haematological evaluation of patients bitten by the jararaca, *Bothrops jararaca*, in Brazil. Toxicon. 51(8):1440-1448.

Santosh MS, Sundaram MS, Sunitha K, Kemparaju K, Girish KS. 2013. Viper venom-induced oxidative stress and activation of inflammatory cytokines: a therapeutic approach for overlooked issues of snakebite management. Inflammation Research. 62(7):721-731.

Sato N. 2012. Blood urea nitrogen as an integrated biomarker for hospitalized heart failure. Circulation Journal. 76(10):2329-2330.

Silva HA, Ryan NM, de Silva HJ. 2015. Adverse reactions to snake antivenom, and their prevention and treatment. British Journal of Clinical Pharmacology. 81(3):446-452.

Silva JG, Soley BS, Gris V, Pires ARA, Caderia SMSC, Eler GJ, Hermoso APM, Bracht A, Dalsenter PR, Acco A. 2011. Effects of the *Crotalus durissus terrificus* snake venom on hepatic metabolism and oxidative stress. Journal of Biochemistry and Molecular Toxicology. 25(3):195-203.

Silveira PVP, Nishioka SA. 1992. South American rattlesnake bite in a Brazilian teaching hospital. Clinical and epidemiological study of 87 cases with analysis of factors predictive of renal failure. Transactions of the Royal Society of Tropical Medicine and Hygiene. 86(5):562-564.

SINAN – Sistema de Informação de Agravos de Notificação [Brazilian Information System for Notifiable Diseases.] 2019. [Accessed September 4, 2019]; Available at http://www.portalsinan.saude.gov.br.

Sitprija V. 2006. Snakebite nephropathy. Nephrology (Carlton). 11(5):442-448.

Sitprija V. 2008. Animal toxins and the kidney. Nature Clinical Practice Nephrology. 4(11):616-627.

Sitprija V, Sitprija S. 2016. Animal toxins and renal ion transport: Another dimension in tropical nephrology. Nephrology (Carlton). 21(5):355-362.

Sousa-e-Silva MC, Tomy SC, Ravares FL, Navajas L, Larsson MH, Lucas SR, Kogika MM, Sano-Martins IS. 2003. Hematological, hemostatic and clinical chemistry disturbances induced by *Crotalus durissus terrificus* snake venom in dogs. Human and Experimental Toxicology. 22(9):491-500.

Spiteller P, Kern W, Reiner J, Spiteller G. 2001. Aldehydic lipid peroxidation products derived from linoleic acid. Biochimica Biophysica Acta. 1531(3):188-208.

Tabet F, Touyz RM. 2007. Reactive oxygen species, oxidative stress, and vascular biology in hypertension. In: Lip G, Hall J, editors. Comprehensive hypertension. St. Louis (MO): Mosby Elsevier. p. 337-347.

Tan KY, Liew ST, Tan QY, Abdul-Rahman FN, Azmi NI, Sim SM, Tan NH, Khomvilai S, Sitprija V, Tan CH. 2019. Evaluating the physicochemical properties and efficacy of recently expired and aged antivenom products from Thailand and Taiwan. Toxicon. 160:55-58.

Teixeira CF, Landucci EC, Antunes E, Chacur M, Cury Y. 2003. Inflammatory effects of snake venom myotoxic phospholipases A_2. Toxicon. 42(8):947-962.

Thongprayoon C, Cheungpasitporn W, Kashani K. 2016. Serum creatinine level, a surrogate of muscle mass, predicts mortality in critically ill patients. Journal of Thoracic Disease. 8(5):E305-E311.

Toyama MH, Costa CR, Belchor MN, Novaes DP, de Oliveira MA, Ie R, Gaeta HH, Toyama DO. 2019. Edema induced by $sPLA_2$ from *Crotalus durissus terrificus* involves PLC and PKC signaling, activation of $cPLA_2$, and oxidative stress. In: Kumar V, Salgado AA, Athari SS, editors. Inflammation in the 21st Century. London: IntechOpen. Available from: https://www.

intechopen.com/chapters/65249 doi: 10.5772/intechopen.80848

Vaidya VS, Ferguson MA, Bonventre JV. 2008. Biomarkers of acute kidney injury. Annual Review of Pharmacology and Toxicology. 48:463-493.

Warrell DA. 2004. Snakebites in Central and South America: epidemiology, clinical features, and clinical management. In: Campbell JA, Lamar WW, editors. Venomous reptiles of the Western hemisphere. Vol. 2. Ithaca (NY): Comstock Publishing Associates/Cornell University Press. p. 709-761.

Weydert CJ, Cullen JJ. 2010. Measurement of superoxide dismutase, catalase, and glutathione peroxidase in cultured cells and tissue. Nature Protocols. 5(1):51-66.

Yamasaki SC, Villarroel JS, Barone JM, Zambotti-Villela L, Silveira PF. 2008. Aminopeptidase activities, oxidative stress and renal function in *Crotalus durissus terrificus* envenomation in mice. Toxicon. 52(3):445-454.

Yoshida EH, Dini MMJ, Campanholi J, Cogo JC, Grotto D, Hyslop S, Hanai-Yoshida VM, Oshima-Franco Y. 2020. Acute kidney injury caused by the intraperitoneal injection of *Bothrops jararaca* venom in rats. Natural Products Research. 34(17):2533-2538.

Zychar BC, Castro NC Jr, Marcelino JR, Gonçalves LR. 2008. Phenol used as a preservative in *Bothrops* antivenom induces impairment in leukocyte-endothelial interactions. Toxicon. 51(7):1151-1157.