Ciência Animal, 27 (3): 40-47, 2017.

ALTERAÇÕES MORFOLÓGICAS INDUZIDAS PELO VENENO DA SERPENTE *PhilodryaS nattereri* steindachner EM RATOS

(Morphological changes induced by the snake venom from Philodryas nattereri Steindachner in rats)

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ABSTRACT

Philodryas nattereri snake is commonly distributed in arid and semiarid regions of northeast of Brazil. There is a shortage in the literature on the systemic effects of this venom in animals and humans. After 2h of intraperitoneally injection with different doses in rats, hemorrhagic zones in skin and the abdominal muscles were observed as well as presence of abundant erytrocytes in alveolar spaces and pulmonary congestion, hypereosinophilic cytoplasm in cells of cardiac muscle. Kidney and liver showed Histopathological changes. The snake venom of *Philodryas nattereri* was capable of causing bleedind in different organs and morphological changes after poisoning.

Keywords: bleeding, opisthoglyphous, Dipsadidae

RESUMO

A serpente *Philodryas nattereri* é comumente distribuída em regiões áridas e semi-áridas do nordeste do Brasil. Há escassez na literatura sobre os efeitos sistêmicos deste veneno em animais domésticos e humanos. Após 2h de injeção intraperitoneal com doses diferentes em ratos, foram observadas zonas hemorrágicas na pele e nos músculos abdominais, bem como a presença de eritrócitos abundantes em espaços alveolares, congestão pulmonar e citoplasma hipereosinofilico em células do músculo estriado cardiaco. O rim e o figado apresentaram alterações histopatológicas. O veneno da serpente *Philodryas nattereri* foi capaz de causar sangramento em diferentes órgãos e mudanças morfológicas após o envenenamento.

Palavras-chave: Sangramento, opistóglifa, Dipsadidae.

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INTRODUCTION

In Brazil, aproximately 26,244 snakebites were reported only in the year of 2016, including venomous and non-venomous snakes (SINAN, 2017). About 20 to 40% of ophidian accidents in Brazil are caused by colubrid snakes. The Colubridae family includes opisthoglyphous and aglyphous snakes (WEINSTEIN *et al.*, 2013). Opisthoglyphous snakes are capable of causing accidents in animals and humans. These snakes possess a Duvernoy gland, responsible for the production of toxic secretion or poison (HESS and BAPTISTÃO, 2012).

Philodryas nattereri Steindachner, 1870 is commonly called "brown racer," reaching up to 180 mm in total length and it exhibits opistoglyph dentition. *P. nattereri* is distributed in arid and semiarid regions of Brazil and also in other countries of South America (NERY *et al.*, 2014).

There are no reports in the literature on the effects of venom of this species on humans. Factors such as the low amount of venom produced by the great majority of opisthoglyphous snakes and the inefficiency of extraction methods represent some of the difficulties of researches in this area (HESS and BAPTISTÃO, 2012).

The objectives of this work were to evaluate morphological changes induced after the inoculation of venom of *P. nattereri* snake in rats and to verify the histopathological changes in several organs after administration of venom in different doses.

METHODOLOGY

To the experiments, we had used adult male Wistar rats that were supplied by central animal house, State University of Ceará – Brazil. The rats weighed between 200-250g. Five rats per group were used. Before each experiment, the animals were maintained fasting for 8h, but with free water. All experiments followed the ethical standards for animal experiments in toxicological research recommended by the international society of toxinology and approved by Ethical Committee (number: 1571522/2016) from Ceará State University (UECE). Adult snakes of both sexes of *P. nattereri* species were used to extract the venom. Each animal remained in individual enclosure at the serpentarium of the Regional Centre of Ofiology (NUROF) – Federal University of Ceará, Brazil. To perform the extraction, the snakes were contained manually and the venom were collected directly from opistoglyphous fangs with capillary tubes. Pooled venom was obtained after several extractions. Venom was lyophilized and when required, was dissolved in phosphate buffered saline solution (PBS), pH 7,4.

To the experiments, the rats were divided in 4 groups of 5 animals each. The rats were previously anesthetized by ketamine (75mg/Kg, I.M.) and xylazine (10mg/Kg, I.M.) according to Brazilian regulations and the injection was applied in the right caudal gracilis muscle. After anesthesia, the venom was applied intraperitoneally in the lower quadrant of the left abdomen. Five animals of each group received doses of 0.50, 0.70 and 0.9 mg dissolved in 0.3mL of PBS. The control group received 0.3 mL of PBS only. After 2h of venom injection, rats were euthanized by thiopental overdose, decapitated and a tricotomy was realized in the abdominal region. To assess the intensity of the hemorrhagic action of the

poison, it was traced lines that divided the abdomen of the animal in 4 quadrants: 2 upper quadrants (right and left) and 2 lower quadrants (right and left). We classified the hemorrhagic effects as: 1 - absent - no hemorrhagic area evidenced; 2- slight intensity - when hemorrhage was restricted to the lower left quadrant, site of venom inoculation; 3 - moderate intensity - when the hemorrhagic area was restricted to 2 quadrants and 4 - great intensity - when the hemorrhagic area exceeded 2 quadrants of the abdomen of the animal. After dissection, samples of heart, lung, kidney and liver were taken and fixed with 10% buffered formaldehyde solution for 24-48h. The tissue samples were dehydrated in several alcohol solutions, diaphanyzed in xylol and embedded in histological paraffin. Sections of 5µm were cut in a microtome and stained by hematoxylin and eosin (H.E.). A polarized trinocular microscope with fluorescence (NIKON Eclipse N*i*, Japan; Software Nis 4.0) was used for histological analysis and characterization.

Protein concentration was assayed in triplicate according to Bradford (1976). For the sample, 2 mg of the *P. nattereri* snake venom was used.

RESULTS AND DISCUSSION

The value of total protein from the venom was 719,0±121 µg/mg. Nery et al. (2014) verified a total protein from the venom of *P. nattereri* of 863,9 µg/mg. Zelanis et al. (2010) showed that the protein content was similar among the venoms: *Philodryas olfersii* (923±113 µg/mg), *Philodryas patagoniensis* (814±12 µg/mg) and *Philodryas nattereri* (847±91 µg/mg).

Hemorrhagic zones in skin and in abdominal muscles were observed after 2h of intraperitoneally injection with different doses of *P. nattereri* venom in rats. It was observed extensive hemorrhagic zones in the peritoneum and abdominal region of all animals that received 0,9 mg. There were classified as being of great intensity (Fig. 1: G-H). No animal of the control group demonstrated evidence of hemorrhage. Were classified as being absent (Fig. 1: A-B). However, the groups of animals that received 0.5 mg and 0.7 mg of venom showed small hemorrhagic zones at the injection site (lower left quadrant) were classified as having mild intensity (Fig. 1: C-D-E-F).

Urra *et al*. (2015) verified hemorrhagic zones in the peritoneum of mice after 1,5h after of an intraperitoneal injection of *Philodryas chamissonis* venom. Vasconcelos *et al*. (2017) observed the formation of hemorrhage in the skin of three groups of mice that were inoculated by venom of *P. nattereri*, being more evident in the group that received subcutaneous route at the concentration of 40 µg / mL.

Microscopic examination of lungs sections showed the prominent hemorrhage in the pulmonary tissue with presence of abundant erytrocytes in alveolar spaces and also congestion on pulmonary parenchyma (Fig. 2: asterisk). After administration of three different doses, liver sections showed congestion of blood vessels and sinusoidal dilatation (Fig. 2: arrows), and clear spaces in centrilobular region (Fig. 2). Kidney sections showed congestion blood in vessels of cortex zone (Fig. 2). Nery *et al*. (2014) observed significant increase of pressure perfusion and renal vascular resistance at 60 and 90 min after injecting dose of 1 mg/ml of *P. nattereri* venom.

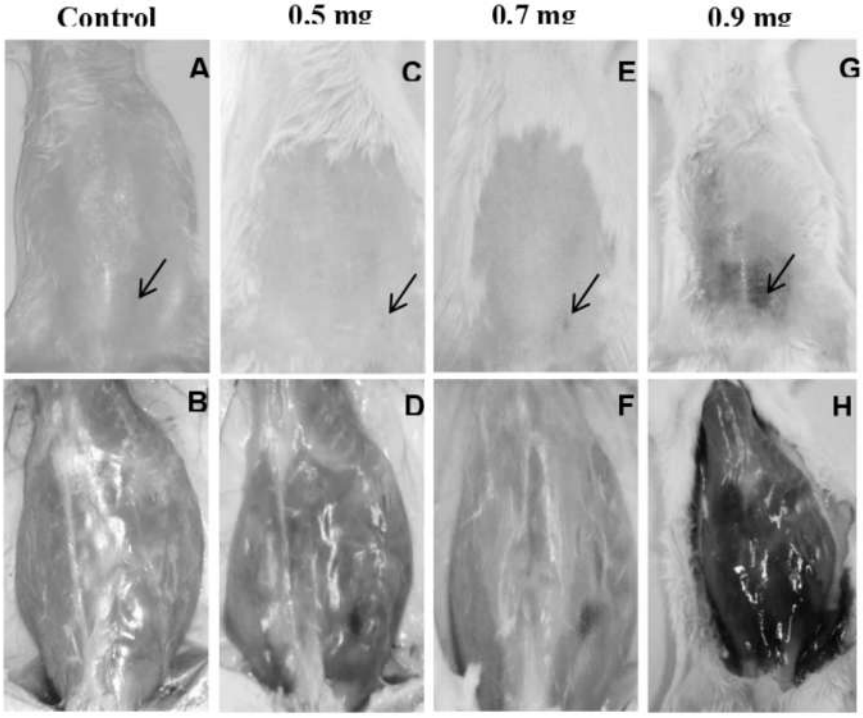

Figure 1: Macroscopic images after 2h of intraperitoneal inoculation (arrows) of three different doses of *Philodryas nattereri* snake venom.

A-**B**: Control group. Received 0.3 mL of PBS. No evidence hemorrhage. **C**-**D**: 0.5 mg and **E**-**F**: 0.7 mg of venom dissolved in 0.3 mL of PBS (1.66 mg/mL and 2.33 mg/mL respectively). Note the small hemorrhagic areas at the site of venom inoculation. It was observed an extensive hemorrhagic zone in the peritoneum of animals that received 0.9 mg (**G**-**H**) dissolved in 0.3 mL of PBS (3mg/mL).

The hypereosinophilic cytoplasm in cardiac muscle cells and erythrocytes presence between the fibers in longitudinal cut could be observed in (Fig. 2: 0.9mg-arrows). The reperfused acute myocardial infarction are usually hemorrhagic as a consequence of vascular injury and blood extravasation. To microscopic examination, myocytes with irreversible lesion that undergo reperfusion exhibit necrosis with bands of contraction; in this pathology process, the entry of calcium through the plasma membrane of myocytes intensifies the actin-myosin interactions, resulting in the formation of intensely eosinophilic bands of sarcomeres hypercontracted (KUMAR, ABBAS and ASTER, 2013). However, crushing, cutting and handling could result in the formation of scattered groups of hypereosinophilic fibers, which may mimic contraction bands (SCUDAMORE, 2014).

All animals that received 0.9mg of venom showed hypereosinophilic fibers in some regions of the heart and extravasation of erythrocytes between the longitudinal cuts. Thus, it is necessary to carry out further studies on the subject to know if these findings are relate to the association of venom with thiopental overdose at the time of euthanasia or whether it was due to handling during the experiments.

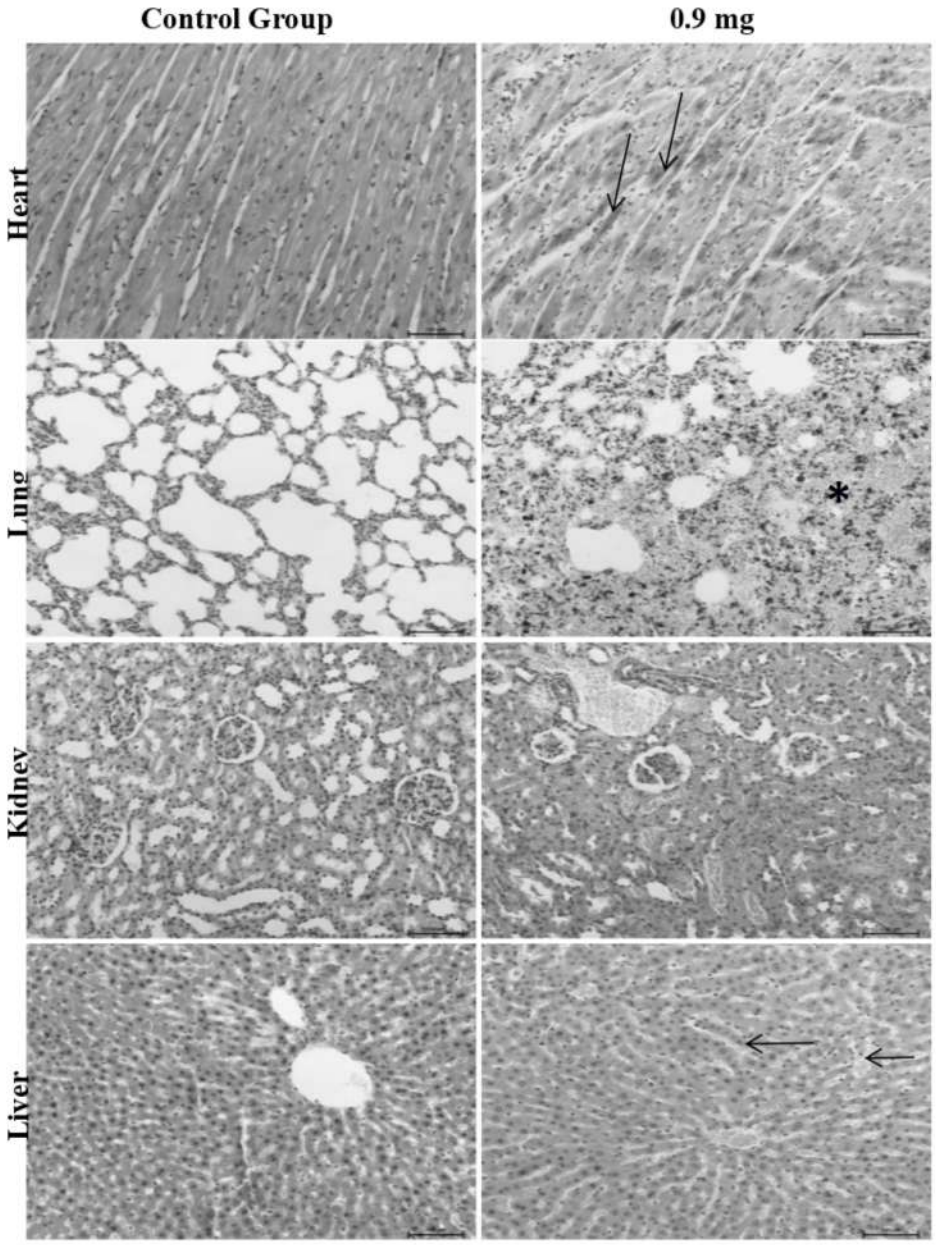

Figure 2: Light Photomicrographs. They showed the histopathological alterations after 2h of *Philodryas natterei* venom i.p. inoculation in rats (dose: 0.9 mg).

Heart: It showed the hypereosinophilic cytoplasm in cardiac muscle cells (arrows) and erythrocytes present between the fibers in longitudinal cut. **Lung**: It showed hemorrhage in pulmonar parenchyma with presence of abundant erytrocytes in alveolar spaces and vascular congestion. **Kidney**: These sections showed vascular congestion in cortex zone. **Liver**: These sections showed vascular congestion and sinusoidal dilatation (arrows), and clear spaces in centrilobular region. All these findings were observed in all animals that received 0.9 mg. Scale bar, 100 µm. 200x H.E. Trinocular Light Microscope (NIKON Eclipse N*i,* Japan; Software Nis 4.0).

After inoculation of 3 different doses of the venom it was possible to verify macroscopically that all the animals that received 0.9mg had hemorrhagic areas in the lung (Fig. 3-B: arrows). No bleeding was evidente in animals of control group.

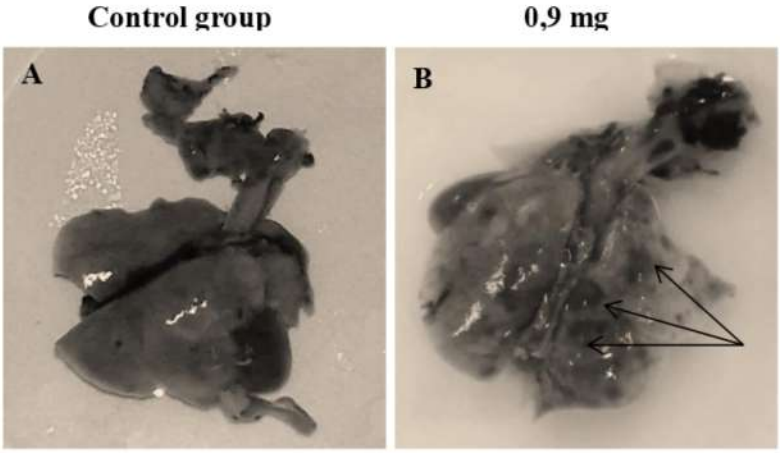

Figure 3: Macroscopic image of the lungs. **A**- Lung of the control group. No bleeding was evident. **B**- Lung of the group that received 0.9 mg of the venom. This image show hemorrhage areas (arrows).

Upon microscopic examination, hemorrhage was evidenced in several organs of rats after inoculation of *P. nattereri* snake venom. These results are similar to the study realized by Peichoto *et al*. (2006) using venom of *Philodryas patagoniensis*, which verified bleeding in different organs. Bleeding may have been caused due to the action of snake venom metalloproteinases (SVMPs) present in the venom of *P. nattereri*. In the subfamily Dipsadinae, SVMPs are predominant components in transcriptomes and the proteomes (FOX and SERRANO, 2008).

SVMPs were classified into structural classes based on the presence of various domain structures and according to their domain organization. The first class, named P-I is simplest and small SVMPs comprised of only a metalloproteinase domain and the molecular weight is aproximately 25 kDa. The second class of SVMPs is P-II, characterized by the presence of pro domain, proteinase domain and desintegrin domain. The molecular weigth of P-II classes is between 25 – 50 kDa. The P-III class, the large SVMPs, has much more domains than P-II. Contain pro-domain, proteinase, desintegrin –like and cysteine - rich domain. This family of metalloproteinases has molecular weight of more than 50 kDa (FOX and SERRANO, 2008; JÚNIOR and SWENSON, 2013; GÂZ et al., 2016). All sequences described in Colubridae up to date belong to the P-III class of SVMPs (AZEVEDO *et al*., 2016). SDS-polyacrylamide gel electrophoresis showed multiple protein bands ranging 45 Kda to 100 Kda of *P. nattereri* venom (NERY *et al*., 2014). The study of Nery *et al*. (2014) showed that P. nattereri venom comprised molecular weight above 50 kDa, belonging to the P-III class of SVMPs.

P- III class of SVMPs contain two non-catalytic domains (disintegrin- like

and cysteine – rich domain)and has high hemorrhagic activity associated with other effects, such as a degradation of the components of the basement membrane.

There is a two-step model to explain the mechanism of action of hemorrhagic SVMPs in capillary vessel. In the first step, SVMPs bind to and hydrolyze critical structural components of the basement membrane of capillary vessels, particular type IV collagen and perlecan, and possibly other molecules that link the basement membrane to the fibrilar extracelular matrix (GUTIÉRREZ *et al.*, 2016). Each collagen is composed of three chains that form a trimer in the form of a triple helix. Glycoproteins (laminin and entactin) and proteoglycans (heparan sulfate and perlecan) adhere to the collagenous supra- structure (KUMAR *et al*., 2010). The cleavage of peptide bonds of basement membrane components results in the mechanical weakening of this structure. In the second step, the hydrostatic pressure, induce a distention of the vessel wall, until the capillary is eventually disrupted, with the consequent extravasation of blood (GUTIÉRREZ *et al*., 2016).

A recent study about local and hematological alterations induced by *Philodryas offersii* snake venom in mice showed that the venom caused thrombocytopenia, leukopenia, lymphopenia and neutrophilia after administration of the 3 different doses in gastrocnemius muscle (i.m.). Histological analysis of this study revealed the presence of edema, progressive myonecosis and an in inflammatory infiltrate (OLIVEIRA *et al*., 2017). According with authors, these various alterations are probabely mediated by metalloproteinases, serine proteinases, CRISPs and C- Type lectins (OLIVEIRA *et al*., 2017).

The continuity in studying this snake venom of our Northeast biodiversity region is necessary to investigate the biological effects of total venom and its fractions in different biological systems.

As a conclusion, the snake venom of *Philodryas nattereri* was capable of causing bleeding in different organs and morphological changes after poisoning. Animals that received 0.5 mg and 0.7 mg of venom showed small hemorrhagic zones at the injection site. All animals that received 0.9 mg showed hemorrhagic zones classified how great intensity. Liver sections showed congestion of blood vessels, hypereosinophilic cytoplasm in cardiac muscle cells and erythrocytes between longitudinal fibers were observed.

REFERENCES

AZEVEDO, I.L.M.J.; CAMPOS, P.F.; CHING, A.T.C.; MACKESSY, S.P. Colubrid Venom Compostion: An-Omics Perspective. Toxins, v.8, 230p., 2016.

BRADFORD, M.M. A rapid sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein –dye binding. Analytical biochemistry. v.72, p.248-254, 1976.

FOX, J.W; SERRANO, S.M.T. Insights into and speculations about snake venom metalloproteinase (SVMP) synthesis, folding and disulfide bond formation and their contribution to venom complexity. FEBS Jounal, 275, p.3016-3030, 2008.

GÂZ, F.S.A.; GÂZ, F.A.; KELEMEN, H.; MUNTEAN, D.L. Snake Venom Metalloproteinases. Acta Medica Marisiensis, v.62, p.106-111, 2016.

GUTIÉRREZ, J.M.; ESCALANTE, T.; RUCAVADO, A.; HERRERA, C.

Hemorrhage Caused by Snake Venom Metalloproteinases: A Journey of Discovery and Understanding. Toxins, v.8, p.93-99, 2016.

HESS, P.L.; BAPTISTÃO, C.C.S. Estudos de Biologia Ambiente e Diversidade, v.34, n.83, p.135-142, 2012.

JÚNIOR, F. S. M.; SWENSON, S. Snake venom metalloproteinases. Toxicon, v.62, p. 3-18, 2013.

KUMAR, V.; ABBAS, A.K.; ASTER, J.C. Robbins Patologia Básica. 9ª ed. São Paulo: Elsevier, 2013.

KUMAR, V.; ABBAS, A. K.; FAUSTO, N.; ASTER, J.C. Robbins & Cotran / Patologia. Bases Patológicas das Doenças. 8ª ed. São Paulo: Elsevier, 2010.

NERY, M.D.A.; ALVES, N.T.Q.; ALVES, R.S.; SOUSA, D.F.; MENEZES, D.B.; NERY, E.A.; AQUINO, H.D.; RIBEIRO, R.T.M.; MONTEIRO, H.S.A. The renal effects and initial characterizationof venom from *Philodryas nattereri* Steindachner, 1870. Toxicology Reports, v.1, p.812-819, 2014.

OLIVEIRA, J.S.; ANNA, L.B.S.; JUNIOR, M.C.O.; SOUZA, P.R.M.; SOUZA, A.S.A.; RIBEIRO, W.; VIEIRA, R.P.; HYSLOP, S.; COGO, J.C. Local and hematological alterations induced by Philodryas olfersii snake venom in mice. Toxicon, v.132, p.9-17, 2017.

PEICHOTO, M.E.; TEIBLER, P.; RUÍZ, R.; LEIVA, L.; ACOSTA, O. Systemic pathological alterations caused by *Philodryas patagoniensis* snake venom in rats. Toxicon, v.48, p.520-528, 2006.

SCUDAMORE, C.L. A practical guide to histology of mouse. Chichester : John Wiley and sons, 2014.

SINAN. Sistema de Informação de Agravos de Notificação. Casos de ofidismo de 2000 a 2016. Acesso em 08 de maio de 2017. Available from: http://portalarquivos.saude.gov.br/images/pdf/2017/abril/28/1Casos_Ofidismo_2000_2016.pdf.

URRA, F.A.; PULGAR, R.; GUTIÉRREZ, R.; HODAR, C.; CAMBIAZO, V.; LABRA, A. Identification and molecular characterization of five putative toxins from the venom gland of the snake *Philodryas chamissonis* (Serpentes: Dipsadidae). Toxicon, v. 108, p. 19-31, 2015.

VASCONCELOS, R.H.; SILVEIRA, J.A.M.; MORAIS, G.B.; NETO, J.O.; JUNIOR, F.A.F.X.; MACAMBIRA, K.D.S. ALVES, N.T.Q.; BRAGA, R.R.; NOJOSA, D.M.B.; SAMPAIO, C.M.S.; MONTEIRO, H.S.A.; EVANGELISTA, J.S.A.M. Alterações Morfológicas de Pele de Camundongos Inoculados com Veneno Total da Serpente *Philodryas nattereri*. Veterinária e Zootecnia, v.24, n.2, p.323-335, 2017.

ZELANIS, A.; ROCHA, M.M.T.; FURTADO, M.F.D. Preliminary biochemical characterization of the venom of five Colubridae species from Brazil. Toxicon, v.55, p.666-669, 2010.

WEINSTEIN, S.A.; WHITE, J.; KEYLER, D.E.; WARRELL, D.A. Non-front-fanged snakes: A current evidence- based analysis of medical significance. Toxicon, v.69, p.103-113, 2013.