PRÓ-REITORIA DE PESQUISA E PÓS-GRADUAÇÃO
MESTRADO EM CIÊNCIA ANIMAL

DIEGO ROBERTO PEREIRA

AÇÃO PROTETORA DO VARESPLADIB (LY-315929) ISOLADO OU ASSOCIADO AO ANTIVENENO SOBRE OS EFEITOS LOCAIS E SISTÊMICOS INDUZIDOSPELO VENENO DE *Lachesis muta rhombeata* (SURUCUCU SUL-AMERICANA)EM RATO

Presidente Prudente - SP
2023

PRÓ-REITORIA DE PESQUISA E PÓS-GRADUAÇÃO
MESTRADO EM CIÊNCIA ANIMAL

DIEGO ROBERTO PEREIRA

AÇÃO PROTETORA DO VARESPLADIB (LY-315929) ISOLADO OU ASSOCIADO AO ANTIVENENO SOBRE OS EFEITOS LOCAIS E SISTÊMICOS INDUZIDOSPELO VENENO DE *Lachesis muta rhombeata* (SURUCUCU SUL-AMERICANA)EM RATO

Dissertação apresentada Pró-Reitoria de Pesquisa e Pós-Graduação, Universidade do Oeste Paulista, como parte dos requisitos para obtenção do título de Mestre em Ciência Animal – Área de concentração: Fisiopatologia Animal.

Orientador: Dra. Rosa Maria Barilli Nogueira

Presidente Prudente - SP
2023

636.089 Pereira, Diego Roberto.
P436a Ação protetora do varespladib (ly-315929) isolado ou associado ao antiveneno sobre os efeitos locais e sistêmicos induzidos pelo veneno de *Lachesis muta rhombeata* (surucucu sul-americana) em rato/ Diego Roberto Pereira. – Presidente Prudente, 2023.
86f.: il.

Dissertação (Mestrado em Ciência Animal) - Universidade do Oeste Paulista – Unoeste, Presidente Prudente, SP, 2023.
Bibliografia.
Orientador: Rosa Maria Barilli Nogueira.

1. Viperíodos 2. Inibidor fosfolipase A2 3. *Lachesis muta rhombeata* 5. Soro antiofídico. I. Título.
.

Catalogação na fonte: Michele Mologni – CRB 8 - 6204

DIEGO ROBERTO PEREIRA

AÇÃO PROTETORA DO VARESPLADIB (LY-315929) ISOLADO OU ASSOCIADO AO ANTIVENENO SOBRE OS EFEITOS LOCAIS E SISTÊMICOS INDUZIDOSPELO VENENO DE *Lachesis muta rhombeata* (SURUCUCU SUL-AMERICANA) EM RATO

Dissertação apresentada à Pró-Reitoria de Pesquisa e Pós-Graduação, Universidade do Oeste Paulista, como parte dos requisitos para obtenção do título de Mestre em Ciência Animal — Área de concentração: Fisiopatologia Animal.

Presidente Prudente, 09 de Abril de 2023.

BANCA EXAMINADORA

_____
Profa. Dra Rosa Maria Barilli Nogueira
Universidade do Oeste Paulista – Unoeste
Presidente Prudente – SP

_____
Prof. Dr. Rafael Stuani Floriano
Universidade do Oeste Paulista – Unoeste
Presidente Prudente – SP

_____
Prof. Dra. Karina Basso
Centro Universitário Filadélfia – Unifil
Londrina – PR

DEDICATÓRIA

Dedico este trabalho aos meus pais Carlito e Tânia (in memoriam), quem sempre fizeram infinitas renúncias para serem presentes, exemplos, e referência de amor. Proveram toda minha educação com infinito orgulho e satisfação de quem realiza o próprio sonho através das conquistas dos filhos. Ao meu irmão Danillo, que unido aos meus pais, sempre foi um amigo, inspiração e espelho. E também à minha amiga e madrasta Marcinha, fonte infinita de ternura.

Obrigado por existirem na minha vida. Amo vocês.

AGRADECIMENTOS

Gostaria de expressar os meus sinceros agradecimentos a todos aqueles quem contribuíram na realização do projeto que gerou esta dissertação. Ao Dr. Rafael Stuani Floriano e toda equipe do laboratório de pesquisa em cardiologia e toxicologia da UNOESTE, por todo apoio, tempo, confiança e experiência dividida durante os ensaios experimentais e publicações. À minha, pela segunda vez orientadora, Dra. Rosa Maria Barilli Nogueira, quem concedeu esta oportunidade, ensinando e apoiando, sempre estabelecendo uma relação de confiança e acolhimento. Minha eterna gratidão!

Agradeço à toda equipe do laboratório de patologia clínica e anatomia patológica do Hospital Veterinário da Unoeste por todo carinho e comprometimento no processamento das amostras colhidas. E não poderia faltar um especial agradecimento às acadêmicas Pamella Gutierez e Nataly Vieira, por todo empenho, amizade e dedicação. Meu muito obrigado!

“Pouco conhecimento faz com que as pessoas se sintam orgulhosas. Muitoconhecimento, que se sintam humildes. É assim que as espigas sem grãos erguemdesdenhosamente a cabeça para o céu, enquanto que as cheias as baixam para a terra, sua mãe.”

Leonardo da Vinci

RESUMO

AÇÃO protetora do varespladib (LY-315929) isolado ou associado ao antiveneno sobre os efeitos locais e sistemicos induzidos pelo veneno de *Lachesis muta rhombeata* (Surucucu Sul-Americana) em rato

Nos últimos anos, diversos estudos relatam a eficácia do varespladib (VPL) como potencial agente antiveneno frente aos venenos de serpente dependentes da fosfolipase A2, presentes nos grupos *Viperidae* e *Elapidae*. O objetivo foi avaliar a ação do varespladib (inibidor de phospholipase A2) associado ou não ao antiveneno nas alterações locais e sistêmicas induzidas pelo veneno de *Lachesis muta rhombeata* (Surucucu Sul-Americana) em ratos. Metodologia: Ratos machos Wistar (250-300 g) foram distribuídos em cinco grupos coms seis animais cada um sendo: G1-controle; G2-veneno de Lmr (1500 ug/kg); G3-veneno de Lmr/antiveneno; G4-veneno Lmr/VPL; G5-veneno Lmr/antiveneno/. Animais foram monitorados durante 120 minutos após injeção do veneno, seguidos de anestesia e eutanásia para coleta de amostras de sangue para realização de hemograma, análises bioquímicas de alanina aminotransferase (ALT), creatinina, ureia, fosfatase alcalina (FA), Creatinoquinase (CK) e lactato desidrogenase (LDH), e histologia do músculo gastrocnêmio que recebeu injeção e seu contralateral, fígado, pulmão, coração, rim. Resultados: o veneno causou lesão local e sistêmica; houve significativo aumento ($P<0,05$) de CK, LDH e uréia que foi reduzida em todos os grupos tratados; no G4 e G5 foi observado efeito protetivo do VPL e o sinergismo dos tratamentos associados (ATV/VPL). O G2 apresentou significante infiltrado neutrofílico inflamatório e necrose difusa, sendo nos demais grupos tratados observado atenuação destas alterações com diferença em relação ao G2 ($P<0.05$).Houve injúria em fígado, pulmão, coração, rim principalmente no G2 sendo no G3, G4 observado redução do escore lesional em relação ao G2. No G5 o escore lesional não diferiu do controle mostrando a efocicácia dos tratamentos associados. Conclusão: Em conclusão, o veneno de *L. muta rhombeata* induziu importante dano tecidual local e sistêmico em ratos após 2 horas da sua injeção, com VPL combinado com antiveneno houve maior eficiência em prevenir esta alteração. O envenenamento experimental induziu alterações sistêmicas que foram mais pronunciadas pelo aumento de CK, LDH e ureia, apenas com este último sendo prevenido pelo VPL isolado ou combinado com antiveneno. Não houve alteração significativa no hemograma, ALT, FA e creatinina;

Palavras-chave: Viperídeos; Inibidor de fospholipase A_2; *Lachesis muta rhombeata*; soro antiofídico.

ABSTRACT

Protective action of varespladib (LY-315929) isolated or associated with antivenon on the local ans sistemic effects induced by the venom of Lachesis muta rhombeata (Surucucu South American) in rats

In recent years, several studies have reported the efficacy of varespladib (VPL) as a potential antivenom agent against snake venoms dependent on phospholipase A2, present in the Viperidae and Elapidae groups. The objective was to evaluate the action of varespladib (phospholipase A2 inhibitor) associated or not with antivenom on local and systemic alterations induced by the venom of Lachesis muta rhombeata (Surucucu Sul-Americana) in rats. Methodology: Male Wistar rats (250-300 g) were divided into five groups with six animals each: G1-control; G2-Lmr venom (1500 µg/kg); G3-Lmr poison/antivenom; G4-venom Lmr/VPL; G5-venom Lmr/antivenom/. Animals were monitored for 120 minutes after venom injection, followed by anesthesia and euthanasia to collect blood samples for hemogram, biochemical analysis of alanine aminotransferase (ALT), creatinine, urea, creatine kinase (CK), alkaline phosphatase (FA), and lactate dehydrogenase (LDH), and histology of the injected gastrocnemius muscle and its contralateral liver, lung, heart, kidney. Results: the venom caused local and systemic damage; there was no significant change in blood count, ALT, FA and creatinine; there was a significant increase ($P<0.05$) in LDH and urea which was reduced in all treated groups; in G4 and G5, a protective effect of NPV and the synergism of associated treatments (ATV/NPV) was observed. G2 showed significant inflammatory neutrophilic infiltrate and diffuse necrosis, and in the other treated groups, attenuation of these changes was observed, with difference in relation to G2 ($P<0.05$). a reduction in the lesion score was observed in relation to G2. In G5, the lesion score did not differ from the control, showing the effectiveness of the associated treatments. Conclusion: In conclusion, *L. muta rhombeata* venom induced significant local and systemic tissue damage in rats 2 hours after its injection, with VPL combined with antivenom there was greater efficiency in preventing this change. Experimental poisoning induced systemic changes that were more pronounced by increasing LDH and urea, with only the latter being prevented by VPL alone or combined with antivenom.

Keywords: Viperids; Phospholipase A2 inhibitor; *Lachesis muta rhombeata*; Snake antivenom.

SUMÁRIO

o rafael@unoeste.br

Specialty section:This article was submitted toTranslational Pharmacology, a section of the journal Frontiers in Pharmacology

Received: 10 November 2021
Accepted: 09 December 2021
Published: 12 January 2022

Citation:
Gutierres PG, Pereira DR, Vieira NL, Arantes LF, Silva Jr. NJ,Torres-Bonilla KA, Hyslop S, Morais-Zani K, Nogueira RMB, Rowan EG and Floriano RS (2022)
Action of Varespladib (LY-315920), aPhospholipase A_2 Inhibitor, on the Enzymatic, Coagulant and Haemorrhagic Activities of Lachesismuta rhombeata (South-American Bushmaster) Venom.Front. Pharmacol. 12:812295. doi: 10.3389/fphar.2021.812295

OPEN ACCESS

Edited by:
Delavar Shahbazzadeh, Pasteur Institute of Iran (PII), Iran

Reviewed by:
Manuel a Berto Pucca, Federal University of Roraima, Brazil
Kamran Pooshang Bagheri, Pasteur Institute of Iran (PII), Iran
Roghi eh Mirzazadeh, Pasteur Institute of Iran (PII), Iran

*Correspondence: Rafael S. Floriano

ORIGINAL RESEARCH

(LY-315920), aPhospholipase A_2 Inhibitor, on the Enzymatic, Coagulant and Haemorrhagic Activities of *Lachesismuta rhombeata* (South-American Bushmaster) Venom

*Pamella G. Gutierres[1], Diego R. Pereira[1], Nataly L. Vieira[1], Lilian F. Arantes[2], Nelson J. Silva Jr.[3], Kristian A. Torres-Bonilla[4], Stephen Hyslop[4], Karen Morais-Zani[5],Rosa M. B. Nogueira[1], Edward G. Rowan[6] and Rafael S. Floriano[1]**

[1]Laboratory of Toxinology and Cardiovascular Research, University of Western São Paulo, Presidente Prudente, Brazil, [2]Graduate Program in Zootechnics, Rural Federal University of Pernambuco, Recife, Brazil, [3]Graduate Program in Environmental Sciencesand Health, School of Medical, Pharmaceutical and Biomedical Sciences, Pontifical Catholic University of Goiás, Goiânia, Brazil,[4]Department of Pharmacology, Faculty of Medical Sciences, State University of Campinas, Campinas, Brazil, [5]Laboratory of Herpetology, Butantan Institute, São Paulo, Brazil, [6]Strathclyde Institute of Pharmacy and Biomedical Sciences, University ofStrathclyde, Glasgow, United Kingdom*

published: 13 January 2022
doi: 10.3389/fphar.2021.812295

Varespladib (VPL) was primarily developed to treat inflammatory disturbances associated with high levels of serum phospholipase A_2 (PLA_2). VPL has also demonstrated to be a potential antivenom support agent to prevent PLA_2-dependent effects produced by snake venoms. In this study, we examined the action of VPL on the coagulant, haemorrhagic and enzymatic activities of *Lachesis muta rhombeata* (South-American bushmaster) venom. Conventional colorimetric enzymatic assays were performed for PLA_2, caseinolytic and esterasic activities; *in vitro* coagulant activities for prothrombin time (PT) and activated partial thromboplastin time (aPTT) were performed in rat citrated plasma through a quick timer coagulometer, whereas the dimensions of haemorrhagic haloes obtained after i.d. injections of venom in Wistar rats were determined using ImageJ software. Venom (1 mg/ml) exhibited accentuated enzymatic activities for proteases and PLA_2 *in vitro*, with VPL abolishing the PLA_2 activity from 0.01 mM; VPL did not affect caseinolytic and esterasic activities at any tested concentrations (0.001–1 mM). In rat citrated plasma *in vitro*, VPL (1 mM) alone efficiently prevented the venom (1 mg/ml)-induced procoagulant disorder associated to extrinsic (PT) pathway, whereas its association with a commercial antivenom successfully prevented changes in both intrinsic (aPTT) and extrinsic (PT) pathways; commercial antivenom by itself failed to avoid the procoagulant disorders by this venom. Venom (0.5 mg/kg)-induced hemorrhagic activity was slightly reduced by VPL (1 mM) alone or combined with antivenom (antivenom:venom ratio 1:3 'v/w') in rats, with antivenom alone producing no protective action on this parameter. In

Action of Varespladib

conclusion, VPL does not inhibit other major enzymatic groups of *L. m. rhombeata* venom, with its high PLA_2 antagonize activity efficaciously preventing the venom- induced coagulation disturbances.

Keywords: Viperidae snake, phospholipase A2 (PLA2), coagulating activity, haemorrhage, varespladib, antivenom, neutralization

INTRODUCTION

Snakes of *Lachesis* genus are represented by three species found in Central America (*L. stenophrys*, *L. melanocephala*, and *L. acrochorda*) and one in South America (*L. muta*), with the latter being recognized as two subspecies distributed in the Amazon river basin (*L. muta muta*) and Atlantic rainforest (*L. muta rhombeata*) in Brazil (Costa and Bérnils, 2018; Nogueira et al., 2019; Diniz-Sousa et al., 2020). Together, these snakes are responsible by the second most frequent cases of snakebites in Americas, being exceeded only by *Bothrops* snakes (Magalhães et al., 2019; Diniz-Sousa et al., 2020).

Envenomation by *Lachesis* spp. is characterized by intense local pain, oedema and necrosis (Damico et al., 2006; Ferreira et al., 2009; Damico et al., 2012), systemic myotoxicity (Fuly et al., 2000; Fuly et al., 2003; Damico et al., 2006), renal failure (Damico et al., 2007), haemorrhage and coagulopathy (Sánchez et al., 1987; Sánchez et al., 1991; Sánchez et al., 1995; Fuly et al., 1997; Rucavado et al., 1999; Estêvão-Costa et al., 2000; Torres-Huaco et al., 2013), including severe cardiovascular disorders (Diniz and Oliveira, 1992; Giovanni-De-Simone et al., 1997; Dias et al., 2016a; Dias et al., 2016b). Such effects have been associated predominantly with the presence of phospholipases A_2 (PLA_2) (Cordeiro et al., 2015; Diniz- Sousa et al., 2018), metalloproteases (Cordeiro et al., 2018) and serine proteases (Wiezel et al., 2019), including biologically active peptides (Graham et al., 2005; Soares et al., 2005; Sanz et al., 2008; Pla et al., 2013; Pinheiro-Júnior et al., 2018), in these venoms.

Polyvalent anti-venom (anti-*Bothrops*/*Lachesis* serum) therapies comprise the main therapeutic options to treat the systemic envenomation by *Lachesis* snakes (Madrigal et al., 2017; Solano et al., 2018). Recently, several studies have demonstrated the value of varespladib, a PLA_2 inhibitor drug (Lewin et al., 2016; Salvador et al., 2019), concerning its suppressive action on the biological effects of Elapidae and Viperidae venoms (Bittenbinder et al., 2018; Lewin et al., 2018; Wang et al., 2018; Zinenko et al., 2020; Gutiérrez et al., 2020a), including their toxins (Bryan- Quirós et al., 2019; Salvador et al., 2021). However, there are not reports about the action of varespladib, as a stand-alone therapy and/or combined with antivenoms, on the toxic effects caused by *Lachesis* venoms. In the present study, we have investigated the efficiency of this drug on some aspects of the envenomation by *Lachesis muta rhombeata* venom using *in vitro* and *in vivo* approaches for enzymatic, coagulant and haemorrhagic activities of this venom. We have also assessed an eventual synergic mechanism of action by varespladib when combined with a commercial antivenom used to treat envenomations by *Lachesis* in Brazil.

MATERIALS AND METHODS

Reagents

Varespladib (LY-315920) was obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, United States) and anti- *Bothrops*/*Lachesis* serum was from Butantan Institute (São Paulo, SP, Brazil); varespladib was dissolved in DMSO prior to use, whereas the antivenom was provided ready for injection
and maintained under refrigeration. Azocasein (A2765), Nα- Benzoyl-DL-arginine 4-nitroanilide hydrochloride (B4875) and
4-nitro-3-octanoyloxy-benzoic acid (N1646) substrates were also from Sigma-Aldrich Chemical Co. (St. Louis, MO, United States). *Lachesis m. rhombeata* venom was provided by Center for Biological Studies and Research of the Pontifical Catholic University of Goiás (PUC Goiás, Goiânia, GO, Brazil) through Dr Nelson J. Silva Jr. A lyophilized pool of venom obtained from one female adult snake was stored at −20°C and dissolved in ultrapure water prior to use.

Animals

Wistar rats (300–350 g; 2–3 months old) obtained from Central Bioterium of the University of Western São Paulo (UNOESTE, Presidente Prudente, SP, Brazil) were housed in plastic cages (3 animals/cage) with a wood-shaving substrate, at 23 ± 1°C on a 12-h light/dark cycle with lights on at 6 a.m. The animals had free access to food and water. The experimental procedures were approved by an institutional Committee for Ethics in Animal Use (CEUA/UNOESTE, Protocol No. 6713/2021) and were done according to the general ethical guidelines for animal use established by the Brazilian Society of Laboratory Animal Science (SBCAL) and Brazilian Federal Law No. 11.794 of October 8, 2008, in conjunction with the guidelines for animal experiments established by the Brazilian National Council for Animal Experimentation (CONCEA).

Phospholipase A_2 (PLA_2) Activity

PLA_2 activity was assayed essentially as described elsewhere (Carregari et al., 2013). The standard assay mixture contained
200 µl of buffer (10 mM Tris-HCl, 10 mM $CaCl_2$ and 100 mM NaCl, pH 8.0), 20 µl of substrate (3 mM 4-nitro-3-octanoyloxy- benzoic acid) and 20 µl of sample [venom alone (1 mg/ml) or venom (1 mg/ml) pre-incubated (for 30 min at 37°C) with varespladib (0.001–1 mM)] in a final volume of 240 µl. After adding sample, the mixture was incubated for 30 min at 37°C,
with one unit of enzymatic activity being defined as an increase in absorbance of 0.001/min at 425 nm. All assays were done in triplicate with readings at 60-s intervals using a SpectraMax 340

multiwell plate reader (Molecular Devices, San Jose, CA, United States).

Caseinolytic Activity

Caseinolytic activity was determined through colorimetric assay in a SpectraMax 340 multiwell plate reader (Molecular Devices, San Jose, CA, United States) using Azocasein as substrate, essentially as described elsewhere (Torres-Bonilla et al., 2020).

The standard assay mixture contained 90 µl of substrate (212 mM Azocasein), 10 µl of reaction buffer (0.05 M Tris-HCl, 1 mM $CaCl_2$, pH 8.0) and 10 µl of sample [venom alone (1 mg/ml) or venom (1 mg/ml) pre-incubated (for 30 min at 37°C) with varespladib (0.001–1 mM)] in a final volume of 110 µl. The mixture was incubated for 90 min at 37°C and then the reaction was terminated adding 200 µl of TCA 5% for 5 min at room temperature; the mixture was centrifuged (5 min at 8.000 g) and 150 µl of supernatant was transferred to the multiwell plate containing the same volume of NaOH (0.5 M). Finally, the absorbance was read at 440 nm via endpoint mode, with one activity unit being defined as an increase of absorbance of0.001/min.

Esterase Activity

Esterase activity was assayed essentially as described by (Erlanger et al., 1961) and adapted by (Torres-Bonilla et al., 2020). The standard assay mixture contained 200 µl of substrate (100 mM Nα-Benzoyl-DL-arginine 4-nitroanilide hydrochloride), 50 µl of reaction buffer (10 mM Tris-HCl, 10 mM $CaCl_2$, 100 mM NaCl, pH 8.0), 15 µl of ultrapure water and 5 µl of sample [venom alone (1 mg/ml) or venom (1 mg/ml) pre-incubated (for 30 min at 37°C) with varespladib (0.001–1 mM)] in a final volume of 270 µl. The mixture was incubated for 30 min at 37°C in a multiwell plate and then read under an absorbance at λ 410 nm via endpoint mode, with one activity unit being defined as an increase of absorbance of 0.001/min.

Coagulant Activity

Coagulant activity was performed using Labtest® commercial kits (Labtest Diagnóstica S.A., Lagoa Santa, MG, Brasil) performed in a quick timer Coagmaster 4.0 (Wama Diagnóstica Produtos para Laboratórios, São Carlos, SP, Brazil). Wistar rats were anesthetized by a non-lethal dose (1.8 mg/kg, i.p.) of thiopental (Cristália®, São Paulo, SP, Brazil) and, subsequently, subjected to intracardiac puncture in order to obtain blood samples using BD Vacutainer® Citrate Tubes with 3.2% buffered sodium citrate solution at an anticoagulant:blood ratio of 1:10 (v/v); after this procedure, the animals were euthanased in saturated atmosphere with CO_2. Blood samples were centrifuged (2,500 g, 4°C, 15 min) in order to obtain citrated platelet-poor plasma used in the prothrombin time (PT) and activated partial thromboplastin clotting time (aPTT) assays at 37°C; for each assay, 190 µl of platelet-poor plasma was incubated at 37°C with 10 µl of sample [1 – saline solution, 2 – varespladib (1 mM) or 3 – antivenom (antivenom: venom ratio 1:3 'v/w'), 4 – *L. m. rhombeata* venom (1 mg/ml), 5 – *L. m. rhombeata* venom + VPL, 6 – *L. m. rhombeata* venom +

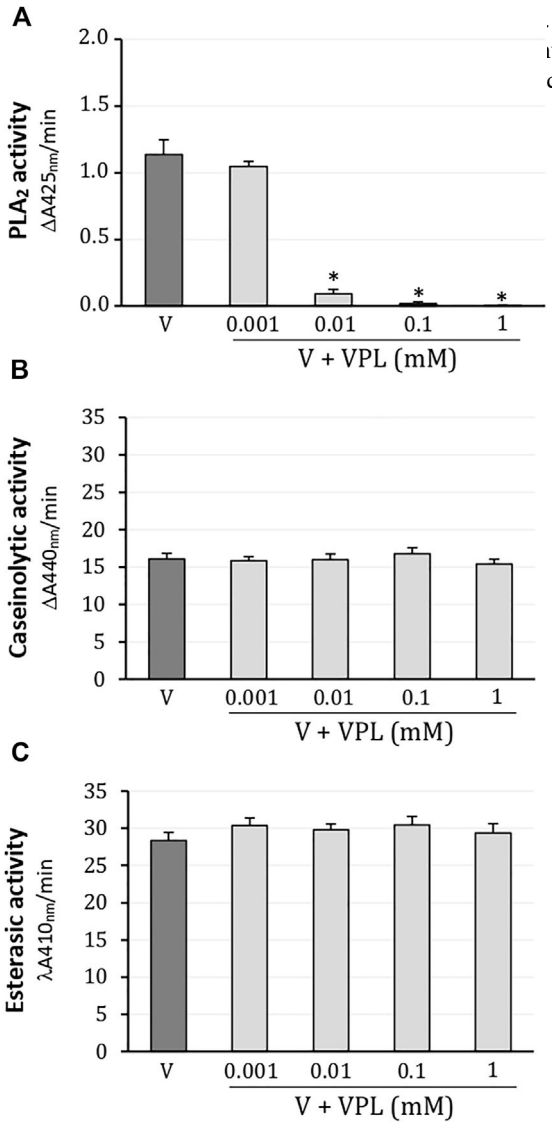


FIGURE 1 | Action of varespladib on the major enzymatic activities of *L. m. rhombeata* venom. (A) Varespladib abolished the PLA_2 activity at low concentrations. Unaffected (B) caseinolytic and (C) esterasic activities by varespladib at any tested concentration. Bars represent the mean ± SDM ($n = 5$). *$p < 0.05$ compared to venom alone.

PT- and aPTT-required clot formation recording; 2 − *L. . rhombeata* venom was directly exposed to VPL and/or ıtivenom and followed by immediate PT- and aPTT-quired clot formation recording.

antivenom, 7 − and *L. m. rhombeata* venom + VPL + antivenom]. Antivenom:venom ratio was based on the manufacturer's stated neutralizing capacity for the antivenom, where 1 ml of antivenom neutralizes 3 mg of *L. muta* venom (Instituto Butantan, São Paulo, SP, Brazil). The minimum effective dose of varespladib (1 mM) was confirmed in pilot experiments. Protocols: 1 − *L. m. rhombeata* venom was pre-incubated with VPL and/or antivenom for 30 min at 37°C before

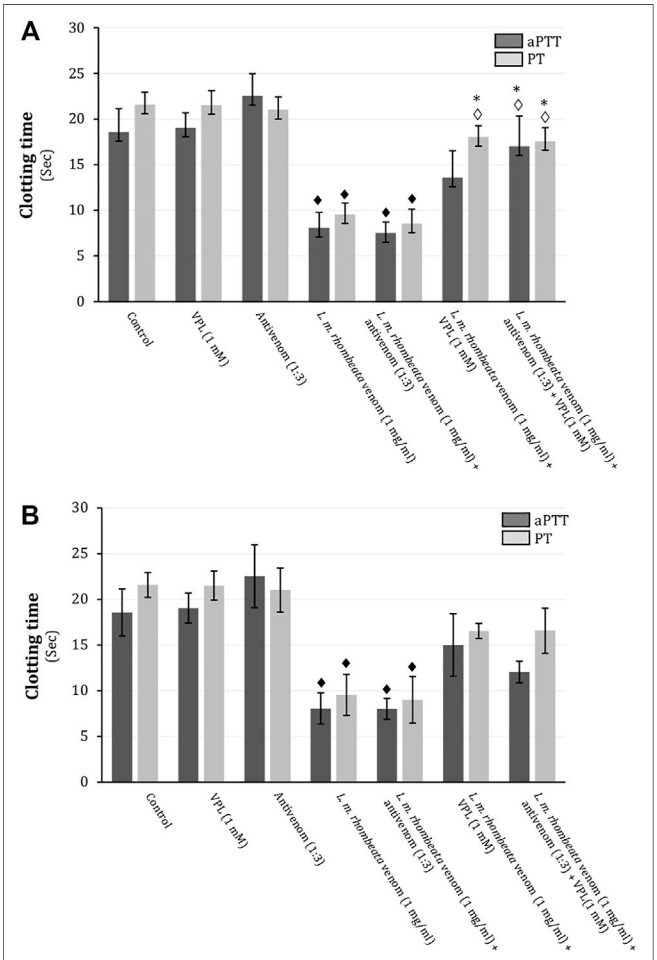


FIGURE 2 | Action of varespladib on the procoagulant activity of *L. m. rhombeata* venom in rat citrated plasma *in vitro*. (A) Antivenom did not prevent the venom-induced procoagulant action applying the pre-incubation protocol, whereas varespladib alone avoided the procoagulant action for PT and its association with antivenom successfully prevented both intrinsic and extrinsic disorders. (B) Antivenom did not prevent the venom-induced procoagulant action applying the direct exposure protocol, whereas varespladib alone or combined with antivenom produced minor protective action on both coagulant pathways even combined with antivenom. Bars represent the mean ± SDM (n = 6). •p < 0.05 compared to control, ◊p < 0.05 compared to venom alone and *p < 0.05 compared to antivenom alone.

Haemorrhagic Activity

Hemorrhagic activity was performed according to (Theakston and Reid, 1983). Male Wistar rats were anesthetized with sodium thiopental (1.8 mg/kg, i.p.) and their dorsal region was trichotomized in order to set the injection sites. Initially, some doses of *L. m. rhombeata* venom (0.01, 0.05, 0.1, 0.5 and 1 mg/kg) were tested in order to find the minimum haemorrhagic dose (0.5 mg/kg), which was used to investigate the neutralizing action of antivenom (antivenom:venom ratio of 1:3 'v/w') and varespladib (1 mM). Protocol: after injection of venom (i.d.), the animals were subsequently treated with antivenom and/or varespladib via an intraperitoneal injection, followed by monitoring period of 24 h. Control sites were injected with 0.9% NaCl, DMSO (varespladib solvent) or antivenom. After 24 h, the rats were euthanized in saturated atmosphere with CO_2, the dorsal skin was removed and the subcutaneous hemorrhagic halos in the inner surface were measured through ImageJ software (National Institute of Health, Bethesda, Maryland, United States).

Statistical Analysis

All results (enzymatic, coagulant and haemorrhagic) were expressed as the mean ± SDM and statistical comparisons were done using Student's t test or ANOVA followed by the Tukey–Kramer test, with $p < 0.05$ indicating significance. Data were analyzed using SAS University Edition software (SAS Institute Inc., Cary, NC, United States).

RESULTS

Inhibitory Action of Varespladib on the MainEnzymatic Groups of *L. m. rhombeata* Venom

In colorimetric assays, *L. m. rhombeata* (1 mg/ml) exhibited moderate enzymatic activity for PLA_2 which was promptly abolished from 0.01 mM of varespladib; the lowest concentration of varespladib (0.001 mM) did not produce inhibitory effect on the PLA_2 activity of this venom (Figure 1A). Caseinolytic (Figure 1B) and esterasic (Figure 1C) activities were not affected by any of these concentrations of varespladib tested on artificial substrates (0.001–1 mM).

Inhibitory Action of Varespladib on the Coagulant Effect of *L. m. rhombeata* Venomin Rat Citrated Plasma

In rat citrated plasma, *L. m. rhombeata* venom (1 mg/ml) exhibited procoagulant action on the aPPT (intrinsic pathway) and PT (extrinsic pathway), decreasing in approximately 56.5 and 55.7% these times, respectively (p < 0.05 compared to basal values for both, n 6) (Figures 2A,B). *L. m. rhombeata* venom (1 mg/ml)- induced procoagulant action (aPTT and PT) was not prevented by pre-incubating venom with antivenom (antivenom:venom ratio of 1:3 'v/w') alone for 30 min at 37°C before clotting assay; however, varespladib (1 mM) alone significantly prevented the venom-induced procoagulant action for PT, with approximately 16.4% of decreasing being verified (p < 0.05 compared to venom alone, n 6), and it partially avoided the venom-induced procoagulant action for aPTT, being observed approximately 26.9% of decreasing; the combination of both agents successfully contributed to avoid both intrinsic and extrinsic disorders in rat citrated plasma, resulting in 8.4 and 18.6% of decreasing for PT and aPTT, respectively (p < 0.05 compared to venom alone, n = 6) (Figure 2A). In assays

=

=

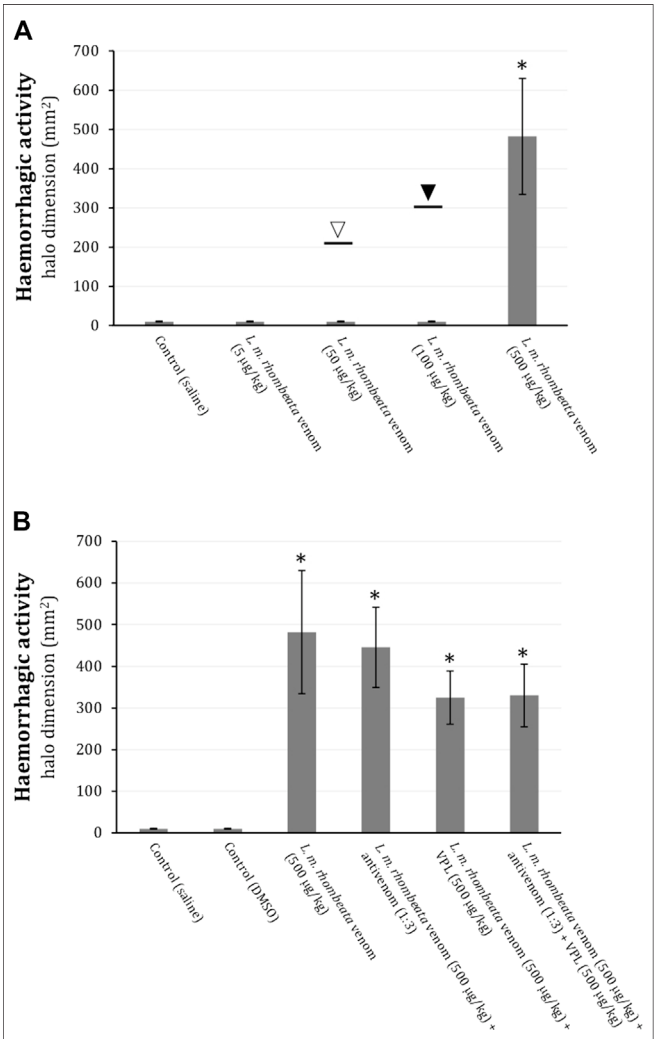


FIGURE 3 | Action of varespladib on the haemorrhagic activity of *L. m. rhombeata* venom in rats. (A) *L. m rhombeata* venom produced intense subcutaneous haemorrhage at a dose of 500 µg of venom/kg, with the lowest doses (5, 50, and 100 µg/kg) being ineffective to produce haemorrhage; the opened and filled arrows indicate the haemorrhagic dimension caused in single animals envenomed with 50 and 100 µg of venom/kg, respectively. (B) Antivenom did not prevent the venom-induced subcutaneous haemorrhage, with varespladib alone or combined with antivenom producing discreet reduction of this alteration. Bars represent the mean ± SDM (n = 6). *p < 0.05 compared to control (saline).

Inhibitory Action of Varespladib on the *L. m. rhombeata* Venom-Induced Subcutaneous Haemorrhage in Rat

L. m. rhombeata venom at low doses (5, 50 and 100 µg/kg) did not produce subcutaneous haemorrhagic action in rats, however, with exceptionally one animal exhibiting ~213 mm^2 of haemorrhagic halo for 50 µg of venom/kg (opened arrow) and another one ~301 mm^2 for 100 µg of venom/kg (filled arrow); the higher dose of this venom (500 µg/kg) induced pronounced haemorrhagic halo formation (p < 0.05 compared to control = saline, n 6) (Figure 3A). Antivenom (antivenom:venom ratio of 1:3 'v/w') administered (i.p) immediately after intradermic injections of *L. m. rhombeata* venom (500 µg/kg) was not able to prevent the venom-induced subcutaneous haemorrhage;

varespladib (1 mM) alone or associated with antivenom (antivenom:venom ratio of 1:3 'v/w') exhibited a mild protective effect on the venom-induced subcutaneous haemorrhage (Figure 3B).

DISCUSSION

performed immediately after the exposure *L. m. rhombeata* venom (1 mg/ml) to antivenom (antivenom:venom ratio of 1: 3 'v/w') and/or varespladib (1 mM), both of agents slightly delayed the *L. m. rhombeata* venom (1 mg/ml)-induced procoagulant action for PT and aPTT; under this experimental condition, varespladib alone or combined with antivenom were more effective in avoiding only the

venom-induced procoagulant action for PT, with approximately 23.3% of decreasing being verified using both agents (Figure 2B).

Envenomations by Viperidae snakes comprise a serious public health problem in Latin America (Chippaux, 2017; Ochoa-Avilés et al., 2020; Gutiérrez et al., 2020b). In Brazil, these snakes are represented by three main genera, i.e., *Bothrops*, *Crotalus* and *Lachesis* (Viperidae–Crotalinae), being responsible for more than 20,000 cases of snakebites per year in this country, as reported by Notifiable Diseases Information System of the Brazilian Ministry of Health (SINAN, Brasília, DF, Brazil). *Lachesis* snakes found in South America (*L. muta muta* and *L. m. rhombeata*) occasionally cause severe human envenomations (Magalhães et al., 2019; Diniz-Sousa et al., 2020), which are characterized by pronounced local and systemic disorders, e.g., necrosis (Damico et al., 2006; Ferreira et al., 2009; Damico et al., 2012), haemorrhage, coagulopathy (Sánchez et al., 1987; Sánchez et al., 1991; Sánchez et al., 1995; Fuly et al., 1997; Rucavado et al., 1999; Estêvão-Costa et al., 2000; Torres-Huaco et al., 2013) and hypotension (Dias et al., 2016a; Dias et al., 2016b), strongly associated with a variety of enzymatically active proteins such as snake venom metalloproteases, serine proteases, PLA_2, C-type lectins and L-amino acid oxidases (Weinberg et al., 2004; Junqueira-de-Azevedo et al., 2006; Bregge-Silva et al., 2012; Madrigal et al., 2012; Cordeiro et al., 2018; Diniz-Sousa et al., 2018; Wiezel et al., 2019) present in these venoms.

In recent years, the PLA_2 antagonistic activity of varespladib (Lewin et al., 2016; Salvador et al., 2019; Salvador et al., 2021), a synthetic drug developed to treat disturbances of inflammatory cascades associated with high levels of secreted PLA_2 (Varespladib, 2011), has been experimentally explored as an useful therapeutic alternative to complement antivenom therapies applied in envenomations by Elapidae and Viperidae snakes, with potential even to replace them in special situations in which these antivenoms are not available. Varespladib has high efficacy to suppress the systemic effects caused by several venoms from Elapidae (Lewin et al., 2016; Bittenbinder et al., 2018; Lewin et al., 2018; Wang et al.,

2018; Oliveira et al., 2020; Gutiérrez et al., 2020a; Dashevsky et al.,2021; Kazandjian et al., 2021; Silva-Carvalho et al., 2021) andViperidae (Lewin et al., 2016; Wang et al., 2018; Youngmanet al., 2020; Zinenko et al., 2020; Gutiérrez et al., 2020a; Liuet al., 2021) snakes. However, there is no report associating theefficacy of varespladib with toxicological aspects of *Lachesis* venoms.Based on this premise, we have unprecedentedly investigated the action of varespladib on the enzymatic, coagulant and haemorrhagicactivities of *Lachesis muta rhombeata* venom to determine itsefficiency as a single pharmacological tool or combined with a commercial antivenom used in Brazil. In summary, we havedemonstrated that varespladib used as a single pharmacologicaltool abolishes the PLA_2 activity of *L. m. rhombeata* venom at low concentrations, without affecting the catalytic activity for proteases(metalloprotease and serino protease) of the venom, indicating avery specific inhibitory activity; its high PLA_2 antagonistic activitywas reflected on the venom-induced procoagulant action, mostlyinterfering on the extrinsic pathway disorders produced by venomin rat citrated serum (pre-incubation protocol), whereas the drug didnot prevent the haemorrhagic activity induced by *L. m. rhombeata*venom in rats. The association of varespladib with a commercialantivenom used in Brazil to treat envenomations by *Lachesis* spp.did not produce important synergic actions on the procoagulant(direct incubation protocol) and haemorrhagic effects induced by *L. m. rhombeata* venom; such interaction resulted in a major prevention of the venom-induced intrinsic and extrinsic coagulant disorders seen under pre-incubation protocol.

Although the procoagulant action of Viperidae venoms has been mostly associated with the presence of serine proteases in these venoms (Gutiérrez et al., 2021), varespladib can partially prevent the procoagulant action of *L. m. rhombeata* venom, indicating an eventual role of PLA_2 in these processes. Accordingly, varespladib also contributes to prevent coagulating disorders induced by other groups of snake venoms, e.g., *Bothrops* (Viperidae-Crotalinae), *Daboia*, *Echis*, *Oxyuranus*, *Naja*, *Pseudechis* and *Bitis* spp., which exhibit high PLA_2 activity (Bittenbinder et al., 2018; Xie et al., 2020; Youngman et al., 2020; Zdenek et al., 2020). On the other hand, varespladib does not affect the *L. m. rhombeata* venom-induced subcutaneous haemorrhage in rats, reflecting its disability in antagonizing the metalloproteases of this venom, since local and systemic haemorrhagic actions produced by Viperidae venoms are mainly mediated by this family of toxins (Escalante et al., 2011; Seo et al., 2017). In addition, although an Asp49 PLA_2 (LmrTX) with anticoagulant activity has been already isolated from *L. m. rhombeata* venom (Damico et al., 2012), it does not appear to contribute expressively with the subcutaneous haemorrhage induced in rats, as reported in this study.

L. m. rhombeata venom has been an important object of study for structural characterization of toxins such as metalloproteases (Cordeiro et al., 2018), serine proteases (Aguiar et al., 1996; Wiezel et al., 2019), C-type lectins (Wiezel et al., 2019), basic and acid PLA_2 (Damico et al., 2012; Cordeiro et al., 2015; Diniz- Sousa et al., 2018), phospholipase B and hialuronidase (Wiezel et al., 2015), including bradykinin-potentiating peptides (BPPs) (Pinheiro-Júnior et al., 2018). As previously commented, these toxins are responsible by developing the main toxicological

aspects of the envenomation by *Lachesis*. However, the factual involvement of PLA_2 toxins on the effects produced by *Lachesis* venoms have been poorly investigated, with a few reports describing their inhibitory action of platelet aggregation (Cordeiro et al., 2015), anticoagulant and antithrombotic activities (Damico et al., 2012), and cytotoxicity on C2C12 myotubes (Diniz-Sousa et al., 2018).

In Brazil, envenomations by *L. m. muta* and *L. m. rhombeata* are treated with anti-*Bothrops*/*Lachesis* serums, with their rescue action being dependent on early i.v. administration (Pla et al., 2013; Madrigal et al., 2017; Solano et al., 2018). There are some negative factors that resulting in deaths by accidents involving *Lachesis* snakes in Brazil: 1) limited availability of antivenoms, 2) difficulties in accessing health services in certain regions of the country, and 3) lack of a specific anti-*Lachesis* serum. Consequently, other types of antivenoms, e.g., anti-*Bothrops* serum and anti-*Bothrops*/*Crotalus* serum, have been inaccurately recommended to treat envenomations by *L. muta* in the absence of anti-*Bothrops*/*Lachesis* serum (Magalhães et al., 2019; Muniz et al., 2021). These challenges involving the treatment of envenomations by *Lachesis* snakes strengthen the search for therapeutically useful adjuncts, with varespladib rising as a plausible tool.

In conclusion, varespladib abolishes efficiently the PLA_2 activity of *L. m. rhombeata* venom at low concentrations and does not affect other majority enzymatic groups of this venom, e.g., metalloproteases and serine proteases. Varespladib alone might partially prevent the procoagulant effect of *L. m. rhombeata* venom, with its combination with antivenom avoiding alterations in both intrinsic and extrinsic pathways. Varespladib does not reduce the subcutaneous haemorrhage formation induced by *L. m. rhombeata* venom in rats due to the lack of inhibitory action on the proteases of the venom. The association of varespladib with a recommended polyvalent antivenom does not produce synergic action on the venom-induced haemorrhagic action but it helps to prevent the venom-induced coagulation disorders.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the Article/Supplementary Material, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by the Committee for Ethics in Animal Use of University of Western São Paulo (CEUA/UNOESTE, Protocol No. 6713/2021).

AUTHOR CONTRIBUTIONS

RMBN and RSF contributed to conception and design of the study. PGG, DRP, and NLV organized the database. LFA performed the statistical analysis. PGG, DRP, and KAT-B wrote the first draft of the manuscript. NJSJ and SH wrote

sections of the manuscript. KM-Z, EGR, and RSF contributed to manuscript revision, read, and approved the submitted version.

FUNDING

This work was funded by institutional resources from University of Western São Paulo (UNOESTE, Presidente Prudente, SP, Brazil) and Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP, São Paulo, SP, Brazil, Grant No. 2020/04287-6 and 2021/14375-2). PGG was also supported by a studentship from FAPESP (Grant No. 2020/ 11268-8). NJSJ and SH are supported by a research fellowship from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, Brasília, DF, Brazil, Grant Nos. 309320/2016-0 and 310547/2014-8, respectively).

REFERENCES

Aguiar, A. S., Alves, C. R., Melgarejo, A., and Giovanni-De-Simone, S. (1996). Purification and Partial Characterization of a Thrombin-Like/gyroxin Enzyme from Bushmaster (Lachesis muta Rhombeata) Venom. *Toxicon* 34 (5), 555–565. doi:10.1016/0041-0101(95)00159-X

Bittenbinder, M. A., Zdenek, C. N., Op den Brouw, B., Youngman, N. J., Dobson, J. S., Naude, A., et al. (2018). Coagulotoxic Cobras: Clinical Implications of Strong Anticoagulant Actions of African Spitting *Naja* Venoms that Are Not Neutralised by Antivenom but Are by LY315920 (Varespladib). *Toxins (Basel)* 10 (12), E516. doi:10.3390/toxins10120516

Bregge-Silva, C., Nonato, M. C., de Albuquerque, S., Ho, P. L., Junqueira de Azevedo, I. L., Vasconcelos Diniz, M. R., et al. (2012). Isolation and Biochemical, Functional and Structural Characterization of a Novel L-Amino Acid Oxidase from *Lachesis muta* Snake Venom. *Toxicon* 60 (7), 1263–1276. doi:10.1016/j.toxicon.2012.08.008

Bryan-Quirós, W., Fernández, J., Gutiérrez, J. M., Lewin, M. R., and Lomonte, B. (2019). Neutralizing Properties of LY315920 toward Snake Venom Group I and II Myotoxic Phospholipases A_2. *Toxicon* 157, 1–7. doi:10.1016/ j.toxicon.2018.11.292

Chippaux, J. P. (2017). Snakebite Envenomation Turns Again into a Neglected Tropical Disease!. *J. Venom. Anim. Toxins Incl. Trop. Dis.* 23, 38–42. doi:10.1186/s40409-017-0127-6

Carregari, V. C., Stuani Floriano, R., Rodrigues-Simioni, L., Winck, F. V., Baldasso, P. A., Ponce-Soto, L. A., et al. (2013). Biochemical, Pharmacological, and Structural Characterization of New Basic PLA2 Bbil-TX from Bothriopsis Bilineata Snake Venom. *Biomed. Res. Int.* 2013, 612649. doi:10.1155/2013/612649

Cordeiro, F. A., Coutinho, B. M., Wiezel, G. A., de Castro Figueiredo Bordon, K., Bregge-Silva, C., Rosa-Garzon, N. G., et al. (2018). Purification and Enzymatic Characterization of a Novel Metalloprotease from Lachesis muta Rhombeata Snake Venom. *J. Venom. Anim. Toxins Incl. Trop. Dis.* 24, 32. doi:10.1186/ s40409-018-0171-x

Cordeiro, F. A., Perini, T. G., Bregge-Silva, C., Cremonez, C. M., Rodrigues, R. S., Boldrini-França, J., et al. (2015). A New Phospholipase A_2 from Lachesis muta Rhombeata: Purification, Biochemical and Comparative Characterization with Crotoxin B. *Protein Pept. Lett.* 22 (9), 816–827. doi:10.2174/0929866522666150706112431

Costa, H. C., and Bérnils, R. S. (2018). Répteis Do Brasil e suas Unidades Federativas: Lista de espécies. *Herpetologia Brasileira* 8, 11–57.

Damico, D. C., Bueno, L. G., Rodrigues-Simioni, L., Marangoni, S., da Cruz- Höfling, M. A., and Novello, J. C. (2006). Functional Characterization of a Basic D49 Phospholipase A_2 (LmTX-I) from the Venom of the Snake Lachesis muta Muta (Bushmaster). *Toxicon* 47 (7), 759–765. doi:10.1016/ j.toxicon.2006.02.007

Damico, D. C., Nascimento, J. M., Lomonte, B., Ponce-Soto, L. A., Joazeiro, P. P., Novello, J. C., et al. (2007). Cytotoxicity of Lachesis muta Muta Snake (Bushmaster) Venom and its Purified Basic Phospholipase A_2 (LmTX-I) in Cultured Cells. *Toxicon* 49 (5), 678–692. doi:10.1016/j.toxicon.2006.11.014

Damico, D. C., Vassequi-Silva, T., Torres-Huaco, F. D., Nery-Diez, A. C., de Souza, R. C., Da Silva, S. L., et al. (2012). LmrTX, a Basic PLA_2 (D49) Purified from Lachesis muta Rhombeata Snake Venom with Enzymatic-Related Antithrombotic and Anticoagulant Activity. *Toxicon* 60 (5), 773–781. doi:10.1016/j.toxicon.2012.06.010

Dashevsky, D., Bénard-Valle, M., Neri-Castro, E., Youngman, N. J., Zdenek, C. N., Alagón, A., et al. (2021). Anticoagulant Micrurus Venoms: Targets

and Neutralization. *Toxicol. Lett.* 337, 91–97. doi:10.1016/j.toxlet.2020.11.010

Dias, L., Rodrigues, M. A., Inoue, B. R., Rodrigues, R. L., Rennó, A. L., de Souza, V. B., et al. (2016a). Pharmacological Analysis of Hemodynamic Responses to Lachesis muta (South American Bushmaster) Snake Venom in Anesthetized Rats. *Toxicon* 123, 25–44. doi:10.1016/j.toxicon.2016.10.002

Dias, L., Rodrigues, M. A., Rennó, A. L., Stroka, A., Inoue, B. R., Panunto, P. C., et al. (2016b). Hemodynamic Responses to Lachesis muta (South American Bushmaster) Snake Venom in Anesthetized Rats. *Toxicon* 123, 1–14. doi:10.1016/j.toxicon.2016.10.001

Diniz, M. R., and Oliveira, E. B. (1992). Purification and Properties of a Kininogenin from the Venom of Lachesis muta (Bushmaster). *Toxicon* 30 (3), 247–258. doi:10.1016/0041-0101(92)90867-5

Diniz-Sousa, R., Caldeira, C. A. S., Kayano, A. M., Paloschi, M. V., Pimenta, D. C., Simões-Silva, R., et al. (2018). Identification of the Molecular Determinants of the Antibacterial Activity of LmutTX, a Lys49 Phospholipase A_2 Homologue Isolated from Lachesis muta muta Snake Venom (Linnaeus, 1766). *Basic Clin. Pharmacol. Toxicol.* 122 (4), 413–423. doi:10.1111/bcpt.12921

Diniz-Sousa, R., Moraes, J. D. N., Rodrigues-da-Silva, T. M., Oliveira, C. S., and Caldeira, C. A. D. S. (2020). A Brief Review on the Natural History, Venomics and the Medical Importance of Bushmaster (Lachesis) Pit viper Snakes. *Toxicon X* 7 (7), 100053. doi:10.1016/j.toxcx.2020.100053

Erlanger, B. F., Kokowsky, N., and Cohen, W. (1961). The Preparation and Properties of Two New Chromogenic Substrates of Trypsin. *Arch. Biochem. Biophys.* 95 (2), 271–278. doi:10.1016/0003-9861(61)90145-x

Escalante, T., Rucavado, A., Fox, J. W., and Gutiérrez, J. M. (2011). Key Events in Microvascular Damage Induced by Snake Venom Hemorrhagic Metalloproteinases. *J. Proteomics* 74 (9), 1781–1794. doi:10.1016/j.jprot.2011.03.026

Estêvão-Costa, M. I., Diniz, C. R., Magalhães, A., Markland, F. S., and Sanchez, E. F. (2000). Action of Metalloproteinases Mutalysin I and II on Several Components of the Hemostatic and Fibrinolytic Systems. *Thromb. Res.* 99 (4), 363–376. doi:10.1016/s0049-3848(00)00259-0

Ferreira, T., Camargo, E. A., Ribela, M. T., Damico, D. C., Marangoni, S., Antunes, E., et al. (2009). Inflammatory Oedema Induced by Lachesis muta muta (Surucucu) Venom and LmTX-I in the Rat Paw and Dorsal Skin. *Toxicon* 53 (1), 69–77. doi:10.1016/j.toxicon.2008.10.016

Fuly, A. L., Calil-Elias, S., Martinez, A. M., Melo, P. A., and Guimarães, J. A. (2003). Myotoxicity Induced by an Acidic Asp-49 Phospholipase A(2) Isolated from Lachesis muta Snake Venom. Comparison with Lysophosphatidylcholine. *Int. J. Biochem. Cell Biol.* 35 (10), 1470–1481. doi:10.1016/s1357-2725(03)00129-8

Fuly, A. L., Calil-Elias, S., Zingali, R. B., Guimarães, J. A., and Melo, P. A. (2000). Myotoxic Activity of an Acidic Phospholipase A_2 Isolated from Lachesis muta (Bushmaster) Snake Venom. *Toxicon* 38 (7), 961–972. doi:10.1016/s0041-0101(99)00208-1

Fuly, A. L., Machado, O. L., Alves, E. W., and Carlini, C. R. (1997). Mechanism of Inhibitory Action on Platelet Activation of a Phospholipase A_2 Isolated from Lachesis muta (Bushmaster) Snake Venom. *Thromb. Haemost.* 78 (5), 1372–1380. doi:10.1055/s-0038-1665414

Giovanni-De-Simone, S., Aguiar, A. S., Gimenez, A. R., Novellino, K., and de Moura, R. S. (1997). Purification, Properties, and N-Terminal Amino Acid Sequence of a Kallikrein-like Enzyme from the Venom of Lachesis muta Rhombeata (Bushmaster). *J. Protein Chem.* 16 (8), 809–818. doi:10.1023/a:1026372018547

Graham, R. L., Graham, C., McClean, S., Chen, T., O'Rourke, M., Hirst, D., et al. (2005). Identification and Functional Analysis of a Novel Bradykinin Inhibitory Peptide in the Venoms of New World Crotalinae Pit Vipers. *Biochem. Biophys. Res. Commun.* 338 (3), 1587–1592. doi:10.1016/j.bbrc.2005.10.130

Gutiérrez, J. M., Albulescu, L-O., Clare, R. H., Casewell, N. R., Abd El-Aziz, T. M., Escalante, T., et al. (2021). The Search for Natural and Synthetic Inhibitors that Would Complement Antivenoms as Therapeutics for Snakebite Envenoming. *Toxins (Basel)* 13 (7), 451. doi:10.3390/toxins13070451

Gutiérrez, J. M., Lewin, M. R., Williams, D. J., and Lomonte, B. (2020a). Varespladib (LY315920) and Methyl Varespladib (LY333013) Abrogate or Delay Lethality Induced by Presynaptically Acting Neurotoxic Snake Venoms. *Toxins (Basel)* 12 (2), 131. doi:10.3390/toxins12020131

Gutiérrez, J. M., Castillo, L., de Naves, K. M. D., Masís, J., and Alape-Girón, A. (2020b). Epidemiology of Snakebites in El Salvador (2014-2019). *Toxicon* 186, 26–28. doi:10.1016/j.toxicon.2020.07.027

Junqueira-de-Azevedo, I. L., Ching, A. T., Carvalho, E., Faria, F., Nishiyama, M. Y., Jr, Ho, P. L., et al. (2006). Lachesis muta (Viperidae) cDNAs Reveal Diverging Pit viper Molecules and Scaffolds Typical of Cobra (Elapidae) Venoms: Implications for Snake Toxin Repertoire Evolution. *Genetics* 173, 877–889. doi:10.1534/genetics.106.056515

Kazandjian, T. D., Arrahman, A., Still, K. B. M., Somsen, G. W., Vonk, F. J., Casewell, N. R., et al. (2021). Anticoagulant Activity of *Naja nigricollis* Venom Is Mediated by Phospholipase A_2 Toxins and Inhibited by Varespladib. *Toxins (Basel)* 13, 302. doi:10.3390/toxins13050302

Lewin, M., Samuel, S., Merkel, J., and Bickler, P. (2016). Varespladib (LY315920) Appears to Be a Potent, Broad-Spectrum, Inhibitor of Snake Venom Phospholipase A_2 and a Possible Pre-referral Treatment for Envenomation. *Toxins (Basel)* 8 (9), 248. doi:10.3390/toxins8090248

Lewin, M. R., Gilliam, L. L., Gilliam, J., Samuel, S. P., Bulfone, T. C., Bickler, P. E., et al. (2018). Delayed LY333013 (Oral) and LY315920 (Intravenous) Reverse Severe Neurotoxicity and rescue Juvenile Pigs from Lethal Doses of Micrurus fulvius (Eastern Coral Snake) Venom. *Toxins (Basel)* 10 (11), E479. doi:10.3390/toxins10110479

Liu, C. C., Wu, C. J., Hsiao, Y. C., Yang, Y. H., Liu, K. L., Huang, G. J., et al. (2021).
Snake Venom Proteome of Protobothrops Mucrosquamatus in Taiwan: Delaying Venom-Induced Lethality in a Rodent Model by Inhibition of Phospholipase A_2 Activity with Varespladib. *J. Proteomics* 234, 104084. doi:10.1016/j.jprot.2020.104084

Madrigal, M., Pla, D., Sanz, L., Barboza, E., Arroyo-Portilla, C., Corrêa-Netto, C., et al. (2017). Cross-reactivity, Antivenomics, and Neutralization of Toxic Activities of Lachesis Venoms by Polyspecific and Monospecific Antivenoms. *Plos Negl. Trop. Dis.* 11 (8), e0005793. doi:10.1371/ journal.pntd.0005793

Madrigal, M., Sanz, L., Flores-Díaz, M., Sasa, M., Núñez, V., Alape-Girón, A., et al. (2012). Snake Venomics across Genus Lachesis. Ontogenetic Changes in the Venom Composition of Lachesis Stenophrys and Comparative Proteomics of the Venoms of Adult Lachesis Melanocephala and Lachesis Acrochorda. *J. Proteomics* 77, 280–297. doi:10.1016/j.jprot.2012.09.003

Magalhães, S. F. V., Peixoto, H. M., Moura, N., Monteiro, W. M., and de Oliveira,
M. R. F. (2019). Snakebite Envenomation in the Brazilian Amazon: a Descriptive Study. *Trans. R. Soc. Trop. Med. Hyg.* 113, 143–151. doi:10.1093/trstmh/try121

Muniz, E. G., Noronha, M. D. D. N., Saraiva, M. D. G. G., Monteiro, W. M., and Oliveira, S. S. (2021). Neutralization of Hemostatic Disorders Induced by Lachesis muta Venom using Brazilian Antivenoms. *Toxicon* 191, 44–47. doi:10.1016/j.toxicon.2020.12.013

Nogueira, C. C., Argôlo, A. J. S., Arzamendia, V., Azevedo, J. A., Barbo, F. E., Bérnils, R. S., et al. (2019). Atlas of Brazilian Snakes: Verified point-locality Maps to Mitigate the Wallacean Shortfall in a Megadiverse Snake Fauna. *South. Am. J. Herpetol.* 14 (1), 1–274. doi:10.2994/sajh-d-19-00120.1

Ochoa-Avilés, A., Heredia-Andino, O. S., Escandón, S. A., Celorio-Carvajal, C. A., Arias-Peláez, M. C., Zaruma-Torres, F., et al. (2020). Viperidae Snakebites in Ecuador: a Review of Epidemiological and Ecological Aspects. *Toxicon X* 7, 100051. doi:10.1016/j.toxcx.2020.100051

Oliveira, I. C. F., Gutiérrez, J. M., Lewin, M. R., and Oshima-Franco, Y. (2020). Varespladib (LY315920) Inhibits Neuromuscular Blockade Induced by Oxyuranus Scutellatus Venom in a Nerve-Muscle Preparation. *Toxicon* 187, 101–104. doi:10.1016/j.toxicon.2020.08.023

Pinheiro-Júnior, E. L., Boldrini-França, J., de Campos Araújo, L. M. P., Santos- Filho, N. A., Bendhack, L. M., Cilli, E. M., et al. (2018). LmrBPP9: A Synthetic Bradykinin-Potentiating Peptide from Lachesis muta Rhombeata Venom that Inhibits the Angiotensin-Converting Enzyme Activity *in vitro* and Reduces the

Blood Pressure of Hypertensive Rats. *Peptides* 102, 1–7. doi:10.1016/ j.peptides.2018.01.015

Pla, D., Sanz, L., Molina-Sánchez, P., Zorita, V., Madrigal, M., Flores-Díaz, M., et al. (2013). Snake Venomics of Lachesis muta Rhombeata and Genus-wide Antivenomics Assessment of the Paraspecific Immunoreactivity of Two Antivenoms Evidence the High Compositional and Immunological Conservation across Lachesis. *J. Proteomics* 89, 112–123. doi:10.1016/ j.jprot.2013.05.028

Rucavado, A., Flores-Sánchez, E., Franceschi, A., Magalhaes, A., and Gutiérrez,

J. M. (1999). Characterization of the Local Tissue Damage Induced by LHF-II, a Metalloproteinase with Weak Hemorrhagic Activity Isolated from Lachesis muta muta Snake Venom. *Toxicon* 37 (9), 1297–1312. doi:10.1016/s0041- 0101(98)00268-2

Salvador, G. H. M., Borges, R. J., Lomonte, B., Lewin, M. R., and Fontes, M. R. M. (2021). The Synthetic Varespladib Molecule Is a Multi-Functional Inhibitor for PLA2 and PLA2-like Ophidic Toxins. *Biochim. Biophys. Acta Gen. Subj* 1865 (7), 129913. doi:10.1016/j.bbagen.2021.129913

Salvador, G. H. M., Gomes, A. A. S., Bryan-Quirós, W., Fernández, J., Lewin, M. R., Gutiérrez, J. M., et al. (2019). Structural Basis for Phospholipase A2-like Toxin Inhibition by the Synthetic Compound Varespladib (LY315920). *Sci. Rep.* 9 (1), 17203. doi:10.1038/s41598-019-53755-5

Sánchez, E. F., Costa, M. I., Chavez-Olortegui, C., Assakura, M. T., Mandelbaum, F. R., and Diniz, C. R. (1995). Characterization of a Hemorrhagic Factor, LHF-I, Isolated from the Bushmaster Snake (Lachesis muta muta) Venom. *Toxicon* 33 (12), 1653–1667. doi:10.1016/0041-0101(95)00097-6

Sánchez, E. F., Magalhës, A., Mandelbaum, F. R., and Diniz, C. R. (1991). Purification and Characterization of the Hemorrhagic Factor II from the Venom of the Bushmaster Snake *(Lachesis muta muta)*. *Biochim. Biophys. Acta* 1074 (3), 347–356. doi:10.1016/0304-4165(91)90084-t

Sánchez, E. F., Magalhães, A., and Diniz, C. R. (1987). Purification of a Hemorrhagic Factor (LHF-I) from the Venom of the Bushmaster Snake, Lachesis muta muta. *Toxicon* 25 (6), 611–619. doi:10.1016/0041-0101(87) 90107-3

Sanz, L., Escolano, J., Ferretti, M., Biscoglio, M. J., Rivera, E., Crescenti, E. J., et al. (2008). Snake Venomics of the South and Central American Bushmasters. Comparison of the Toxin Composition of Lachesis muta Gathered from Proteomic versus Transcriptomic Analysis. *J. Proteomics* 71 (1), 46–60.doi:10.1016/j.jprot.2007.10.004

Seo, T., Sakon, T., Nakazawa, S., Nishioka, A., Watanabe, K., Matsumoto, K., et al. (2017). Haemorrhagic Snake Venom Metalloproteases and Human ADAMs Cleave LRP5/6, Which Disrupts Cell-Cell Adhesions *in vitro* and Induces Haemorrhage *in vivo*. *FEBS J.* 284 (11), 1657–1671. doi:10.1111/ febs.14066

Silva-Carvalho, R., Gaspar, M. Z., Quadros, L. H. B., Lobo, L. G. G., Rogério, L. M., Santos, N. T. S., et al. (2021). *In Vivo* treatment with Varespladib, a Phospholipase A_2 Inhibitor, Prevents the Peripheral Neurotoxicity and Systemic Disorders Induced by *Micrurus corallinus* (Coral Snake) Venom in Rats. *Toxicol. Lett.* 356, 54–63. doi:10.1016/j.toxlet.2021.11.003

Soares, M. R., Oliveira-Carvalho, A. L., Wermelinger, L. S., Zingali, R. B., Ho, P. L., Junqueira-de-Azevedo, I. L., et al. (2005). Identification of Novel Bradykinin- Potentiating Peptides and C-type Natriuretic Peptide from Lachesis muta Venom. *Toxicon* 46 (1), 31–38. doi:10.1016/j.toxicon.2005.03.006

Solano, G., Gómez, A., Corrales, G., Chacón, D., Estrada, R., and León, G. (2018).Contributions of the Snake Venoms of Bothrops asper, Crotalus Simus and Lachesis Stenophrys to the Paraspecificity of the Central American Polyspecific Antivenom (PoliVal-ICP). *Toxicon* 144, 1–6. doi:10.1016/j.toxicon.2018.01.016 Theakston, R. D., and Reid, H. A. (1983). Development of Simple Standard AssayProcedures for the Characterization of Snake Venom. *Bull World Health Organ.* 61 (6), 949–956.

Torres-Bonilla, K. A., Panunto, P. C., Pereira, B. B., Zambrano, D. F., Herrán- Medina, J., Bernal, M. H., et al. (2020). Toxinological Characterization of Venom from Leptodeira annulata (Banded Cat-Eyed Snake; Dipsadidae, Imantodini). *Biochimie* 174, 171–188. doi:10.1016/j.biochi.2020.04.006

Torres-Huaco, F. D., Werneck, C. C., Vicente, C. P., Vassequi-Silva, T., Nery-Diez,

A. C., Mendes, C. B., et al. (2013). Rapid Purification and Procoagulant and Platelet Aggregating Activities of Rhombeobin: a Thrombin-like/gyroxin-like Enzyme from Lachesis muta Rhombeata Snake Venom. *Biomed. Res. Int.* 2013, 903292. doi:10.1155/2013/903292

Varespladib (2011). Varespladib. *Am. J. Cardiovasc. Drugs* 11:137–43. doi:10.2165/11533650-000000000-00000

Wang, Y., Zhang, J., Zhang, D., Xiao, H., Xiong, S., and Huang, C. (2018). Exploration of the Inhibitory Potential of Varespladib for Snakebite Envenomation. *Molecules* 23 (2), E391. doi:10.3390/molecules23020391

Weinberg, M. L., Felicori, L. F., Bello, C. A., Magalhães, H. P., Almeida, A. P., Magalhães, A., et al. (2004). Biochemical Properties of a Bushmaster Snake Venom Serine Proteinase (LV-Ka), and its Kinin Releasing Activity Evaluated in Rat Mesenteric Arterial Rings. *J. Pharmacol. Sci.* 96, 333–342. doi:10.1254/ jphs.fpj04005x

Wiezel, G. A., Bordon, K. C., Silva, R. R., Gomes, M. S., Cabral, H., Rodrigues, V. M., et al. (2019). Subproteome of Lachesis muta Rhombeata Venom and Preliminary Studies on LmrSP-4, a Novel Snake Venom Serine Proteinase. *J. Venom. Anim. Toxins Incl. Trop. Dis.* 25, e147018. doi:10.1590/1678-9199-JVATITD-1470-18

Wiezel, G. A., dos Santos, P. K., Cordeiro, F. A., Bordon, K. C., Selistre-de-Araújo, H. S., Ueberheide, B., et al. (2015). Identification of Hyaluronidase and Phospholipase B in Lachesis muta Rhombeata Venom. *Toxicon* 107 (Pt B), 359–368. doi:10.1016/j.toxicon.2015.08.029

Xie, C., Albulescu, L. O., Still, K. B. M., Slagboom, J., Zhao, Y., Jiang, Z., et al. (2020). Varespladib Inhibits the Phospholipase A_2 and Coagulopathic Activities of Venom Components from Hemotoxic Snakes. *Biomedicines* 8 (6), 165. doi:10.3390/biomedicines8060165

Youngman, N. J., Walker, A., Naude, A., Coster, K., Sundman, E., and Fry, B. G. (2020). Varespladib (LY315920) Neutralises Phospholipase A_2 Mediated Prothrombinase- Inhibition Induced by Bitis Snake Venoms. *Comp. Biochem. Physiol. C Toxicol. Pharmacol.* 236, 108818. doi:10.1016/j.cbpc.2020.108818

Zdenek, C. N., Youngman, N. J., Hay, C., Dobson, J., Dunstan, N., Allen, L., et al. (2020). Anticoagulant Activity of Black Snake (Elapidae: Pseudechis) Venoms: Mechanisms, Potency, and Antivenom Efficacy. *Toxicol. Lett.* 330, 176–184. doi:10.1016/j.toxlet.2020.05.014

Zinenko, O., Tovstukha, I., and Korniyenko, Y. (2020). PLA_2 Inhibitor Varespladib as an Alternative to the Antivenom Treatment for Bites from Nikolsky's viper Vipera berus Nikolskii. *Toxins (Basel)* 12 (6), 356. doi:10.3390/toxins12060356

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as apotential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

ARTIGO CIENTÍFICO 2

Protective action of Varespladib (LY-315929) associated or not with antivenomon the hematological, biochemical and histological effects induced by Lachesis muta rhombeata (South American Surucucu) venom in rats.

Diego R Pereira[1], Pamella G Gutierres[1] , Nataly L Vieira[1] , Nelson J Silva Jr[2], Juliana

R. Gerez[3], Elisangela O. Silva[4], Cecília L Santarém[5], Rafael S Floriano[1], Rosa MBNogueira[1*]

[1]Laboratory of Toxinology and Cardiovascular Research, University of Western São Paulo (UNOESTE),Presidente Prudente, SP, Brazil

[2]Graduate Program in Environmental Sciences and Health, School of Medical, Pharmaceutical andBiomedical Sciences, Pontifical Catholic University of Goiás (PUC Goiás), Goiânia, GO, Brazil [3]Department of Histology, State University of Londrina, Londrina, PR, Brazil

[4]Laboratory of Pathological Anatomy, Veterinary Hospital, University of Western São Paulo(UNOESTE), Presidente Prudente, SP, Brazil

[5]Laboratory of Clinical Pathology, Veterinary Hospital, University of Western São Paulo (UNOESTE),Presidente Prudente, SP, Brazil

Highlights

- L. muta rhombeata venom induced local and systemic tissue damage in rats
- There was an increase in CK, urea, LDH, local tissue damage in muscle and systemictissue damage in liver, lung, heart, and kidney
- All treatments reduced venom-induced changes
- There was synergism in the VPL/antivenom association, preventing local andsystemic alterations with greater efficiency

Corresponding author. E-mail: rosa@unoeste.br

[1] Paper written according to Toxicon magazine standards. Impact Factor: 3.033

S U M M A R Y

The objective was to evaluate the action of varespladib (phospholipase A2 inhibitor) and antivenom on local and systemic changes induced by the venom of Lachesis muta rhombeata (South American Surucucu) in rats, since studies have demonstrated the efficacy of varespladib (VPL) when used in snake venoms dependent on Phospholipase A2, present in the Viperidae and Elapidae groups.

Through hemogram, serum biochemistry and histological analysis, we evaluated the protective action of 500 ug/kg of VPL and commercial polyvalent antivenom (ATV) used alone or associated in the treatment of Wistar rats experimentally poisoned with Lachesis muta rhombeata (Lmr) venom. Methodology: Male Wistar rats (250-300 g) were divided into five groups of six animals each: G1-control; G2-poison L. m. rhombeata (1500 ug/kg), G3-poison L. m. rhombeata/antivenom; G4-poison L. m. rhombeata/VPL; G5-poison L. m. rhombeata/antivenom/VPL. The animals were monitored for 120 minutes after venom application, followed by anesthesia and euthanasia for collection of blood samples for hemogram, biochemical analysis of alanine aminotransferase (ALT), creatinine, creatine kinase (CK), urea, alkaline phosphatase (AP), lactate dehydrogenase (LDH) and fragments of the gastrocnemius muscle that received venom and the contralateral one, in addition to liver, lung, heart, and kidney samples for histological analysis. Results: the venom caused local and systemic damage; there was no significant change in blood count, ALT, FA and creatinine; there was a significant increase ($p<0.05$) in LDH and urea that were reduced in all treated groups; in G4 and G5, the protective effect of VPL and the synergism of associated treatments (ATV/VPL) were observed. G2 showed a significant inflammatory

neutrophilic infiltrate and diffuse necrosis, with the other treated groups showing attenuation of these alterations with a difference in relation to G2 ($P<0.05$). There was injury in the liver, lung, heart, kidney, mainly in G2, while in G3, G4, a reduction in the lesion score was observed in relation to G2. In G5, the lesion score did not differ from the control, showing the effectiveness of the associated treatments. Conclusion: In conclusion, the venom of L. muta rhombeata induced significant local and systemic tissue damage in rats after 2 hours of its injection, with VPL combined with antivenom had greater efficiency in preventing this alteration. Experimental poisoning induced systemic changes that were most highlighted by increases in LDH and urea, with only the last one being prevented by VPL alone or combined with antivenom.

Keywords: Viperids; phospholipase A2 inhibitor; Lachesis muta rhombeata; Snake antivenom.

1 Introduction

Accidents caused by snakes are considered by the World Health Organization (WHO) to be neglected tropical and subtropical diseases, due to the significant number of accidents worldwide that affect mainly the poorest populations in rural regions, settlements and native communities. In Brazil, the numbers are also alarming, with more than 20,000 snakebites reported annually (Croda, Haraki, 2020).

According to the most recent data published by the Ministry of Health, in 2021, 31,354 accidents were reported by venomous snakes of the genera Bothrops 'jararacas' (22,223 accidents = 70.89%), Crotalus 'rattlesnakes' (2,522 accidents = 8.04%) , Lachesis 'surucucus' (345 accidents = 1.10%) and Micrurus 'corals' (281 accidents = 0.90%) (SINAN, 2021). This low occurrence of accidents with Lachesis muta (a representative species of the Brazilian fauna) reflects the low population density of these snakes and their distribution limited to the Amazon river basin ('L. muta muta') and the Atlantic forest ('L. muta rhombeata'). in the north and coastal regions of the country, respectively (Zamudio, Greene, 1997; Campbell, Lamar, 2004; Costa, Bérnils, 2018;), however, they are considerably serious due to the severe systemic alterations that characterize lachetic poisoning.

Snakes of the genus Lachesis correspond to the largest viperids (Viperidae) in the Americas, reaching up to 350 cm in length (Campbell, Lamar, 2004; Melgarejo, 2009) and are represented by four terrestrial species widely distributed throughout Central America and South America, namely: Lachesis stenophrys (found along the Caribbean coast), Lachesis melanocephala (distributed on the Pacific coast of Costa Rica), Lachesis acrochorda (distributed on the Pacific and Atlantic coasts of Panama and northwest Colombia and Ecuador), and Lachesis muta (exclusively South American species found in the Amazon river basin 'L. m. muta' and Atlantic forest 'L.
m. rhombeata') (Zamudio, Greene, 1997; Campbell, Lamar, 2004; Costa, Bérnils, 2018). In Brazil, Lachesis m. rhombeata is distributed exclusively

in the coastal forests of the northeast and southeast regions, and can be found from the southern region of the state of Rio Grande do Norte down to the state of Rio de Janeiro (Diniz-Sousa et al. 2020).

In Brazil, poisoning by L. muta muta and L. m. rhombeata are treated with the bivalent antivenom Bothrops/Lachesis, and its action depends on early intravenous administration (Pla et al., 2013; Madrigal et al., 2017; Solano et al., 2018). There are some negative factors that result in deaths from accidents involving Lachesis snakes in Brazil: 1) limited availability of antivenoms, 2) difficulties in accessing health services in certain regions of the country and 3) lack of specific anti-Lachesis serum. Consequently, other types of antivenoms, e.g. anti-Bothrops serum and anti- Bothrops/Crotalus serum, have been incorrectly recommended to treat L. muta spp. poisoning in the absence of anti-Bothrops/Lachesis serum (Magalhães et al., 2019; Muniz et al., 2021). These challenges involving the treatment of envenomation by Lachesis snakes strengthen the search for therapeutically useful adjuncts, with varespladib emerging as a plausible tool.

In recent years, several investigations into new additional therapeutic strategies and/or alternatives to serum therapy, especially with plant extracts (Floriano et al., 2009; Collaço et al., 2012; Ferraz et al., 2014; Tribuiani et al., 2014 ; Cremonez et al., 2016; Ferreira-Rodrigues et al., 2016; Harder et al., 2017; Tribuiani et al., 2017) or their isolated components (Silva et al., 2016), also including antioxidant drugs with N-acetyl-L-cysteine (Sunitha et al., 2011; Sunitha et al., 2013; Barone et al., 2014, Aline
G. Leão Torres et al., 2021), have shown to be potentially promising with regard to their contributions to the treatment of envenomation by viperids and elapids. In this perspective, the PLA2 inhibitor varespladib (Lewin et al., 2016; Salvador et al., 2019) has been presented as a potential therapeutic tool due to its suppressive action of the systemic effects induced by venoms and toxins from Elapidae and Viperidae snakes (Bittenbinder et al., 2018; Bryan-Quirós et al., 2018; Lewin et al., 2018; Wang et al., 2018; Gutiérrez et al., 2020; Zinenko et al., 2020).

Several researches have demonstrated the efficiency of the drug

varespladib (LY315920), a synthetic molecule clinically tested to block inflammatory cascades of several diseases associated with high levels of secreted PLA2 (Varespladib, 2011), regarding its suppressive action of the systemic effects induced by venoms and toxins from Elapidae and Viperidae snakes (Lewin et al., 2016; Bittenbinder et al., 2018; Lewin et al., 2018; Bryan-Quirós et al., 2018; Wang et al., 2018; Salvador et al., 2019; Gutiérrez et al., 2020; Zinenko et al., 2020). Among these, Lewin et al. (2016) demonstrated that varespladib and its orally bioavailable prodrug (methyl-varespladib) produced significant PLA2 inhibition at low concentrations against twenty-eight types of venoms obtained from snakes of medical interest (Elapidae and Viperidae) distributed in six continents. Later, the same authors reported that varespladib proved to be potentially effective in attenuating the clinical manifestations of envenomation by the North American coral snake M. fulvius (Elapidae) in swine, significantly reducing its hemostatic, coagulant and myotoxic action (Lewin et al., 2018). In addition, Wang et al. (2018) also evaluated the PLA2 inhibitory activity of varespladib on the hemorrhagic, myotoxic and hepatotoxic aspects of the venoms of two Asian elapid snakes (Deinagkistrodon acutus and Agkistrodon halys); the inhibitor significantly reduced local and systemic muscle damage (plasma release of creatine kinase-CK), the release of markers of liver damage (LDH1, AST and ALT) and the occurrence of subcutaneous hemorrhage. Varespladib also efficiently inhibited the myotoxic and cytotoxic effects of the MjTX-II toxin (PLA2 Lys49) from the venom Bothrops moojeni (Viperidae, Crotalinae), a South American pit viper of significant medical importance (Salvador et al., 2019). Bryan-Quirós et al. (2018) evaluated the PLA2 inhibitory effect of varespladib on Pseudechis colletti (Elapidae), Bothrops asper and Crotalus vegrandis (Viperidae, Crotalinae) venoms, including their respective major PLA2 myotoxins, e.g., pseudoxin, Mt-I and Crotoxin B; the inhibitor proved to be very effective in reducing the enzymatic activity of both crude venoms and toxins, reflected prevention of cytotoxicity in C2C12 myotube culture and systemic myotoxicity (plasma CK release) in mice. More recently, Gutiérrez et al. (2020) and Zinenko et al. (2020) demonstrated the efficiency of varespladib in preventing the death of mice

exposed to the venom of Vipera berus nikolskii (Nicolsky viper – 'Viperinae') and decreasing the lethality of snake venoms with presynaptic neurotoxic action (Notechis scutatus, Bungarus multicinctus , Oxyuranus scutellatus 'Elapidae' and Crotalus durissus terrificus 'Viperidae, Crotalinae'), respectively.

In the present study, the protective action of varespladib alone and associated with a commercial antivenom on systemic and local alterations present in tissue samples and serum from Wistar rats experimentally poisoned by L. m rhombeata venom was evaluated in an unprecedented way, in order to determine a possible protective and synergistic effect for local and systemic injuries, seeking one more tool for the therapeutic arsenal in this type of accident.

2 Material and Methods

2.1. Reagents and Venom

Varespladib (LY-315920) was obtained from Sigma-AldrichChemical Co. (St. Louis, MO, United States). varespladib was dissolved in DMSO prior to use, whereas the antivenom was provided ready for injection and maintained under refrigeration and anti-Bothrops/Lachesis serum was a commercial polyvalent antivenom (anti-Bothrops- Crotalus Vencofarma®, Brazil); Lachesis m. rhombeata venom was provided by Center for Biological Studies and Research of the Pontifical Catholic University of Goiás (PUC Goiás, Goiânia, GO, Brazil) through Dr Nelson J. Silva Jr. A lyophilized pool of venom obtained from one female adult snake was stored at −20°C and dissolved in ultrapure water prior to use.

2.2 Animals

Wistar rats (300–350 g; 2–3 months old) obtained from Central Bioterium of the University of Western São Paulo (UNOESTE, Presidente Prudente, SP, Brazil) were placed in plastic cages (3animals/cage) with a

wood-shaving substrate, at 23 ± 1°C on a 12-h light/dark cycle with lights on at 6 a.m. The animals had free access to food and water. The experimental procedures were approved by an institutional Committee for Ethics in Animal Use (CEUA/UNOESTE, Protocol No. 6713/2021) and were done according to the general ethical guidelines for animal use established by the Brazilian Society of Laboratory Animal Science (SBCAL) and Brazilian Federal Law No. 11.794 of October 8, 2008, in conjunction with the guidelines for animal experiments established by the Brazilian National Council for Animal Experimentation(CONCEA).

2.3 Experimental groups 5 groups (G1- control, G2- Lmr Venom, G3- Lmr V + antivenom, G4-Lmr V + VPL, G5-Lmr V + VPL + antivenom) of male Wistar rats (285- 320 g) anesthetized recieved injection in right gastrocnemius of L. m. rhombeata venom, were euthanized after two hours followed by serum ALT, Creatinine, Urea, Alkaline Phosphatase, lactate dehydrogenase isoenzyme 1 measurement, CK and gastrocnemius, renal, hepatic histopathological analysis. The results were expressed as the mean ± SEM and statistical comparisons were done using Bartlett and Shapiro- Wilk test followed by the ANOVA and Tukey, with $p < 0.05$ indicating significance. Data analyses were performed using the softwares Microcal Origin 8 SR4 versão 8.0951 (Microcal Software Inc., Northampton, MA, EUA) or GraphPad Prism v.4.03 (GraphPad Software Inc., La Jolla, CA, EUA) and R-project.

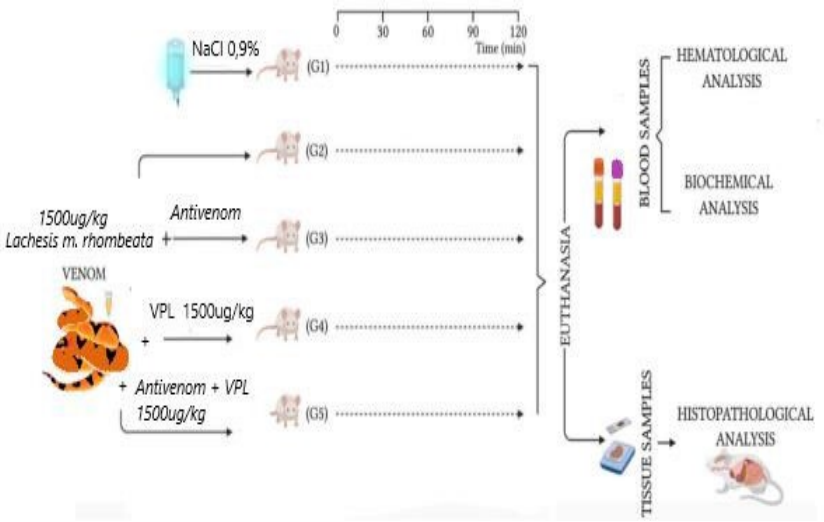


Figure 1. Representation of the experimental design for this investigation. In groups G3, G4 and G5, "+" indicates the combined action. VPL: Varespladib.

2.4 Varespladib and Antivenom

The dose of L. m. rhombeata was confirmed in a pilot experiment based on the dose of 1.5 mg of venom/kg described by Dias et al. (2016 ab) for L. m. mutate; the dose of varespladib was estimated based on the optimal inhibitory concentration of PLA2 activity for the venom of L. m. rhombeata and confirmed in a pilot experiment. The antivenom ratio used in this study was that recommended by the manufacturer (Vencofarma®) in which 1 ml of anti-Bothrops/Lachesis serum neutralizes 1.5 mg of L. m. mutate.

2.5 Material collection

The animals were anesthetized with Ketamine 200 mg⁄kg associated with Xylazine at a dose of 30 mg/kg intraperitoneally, according to the Brazilian resolution. Concea (2018) and four minutes after consciousness loss and interdigital reflexes, an intracardiac puncture was performed to collect blood in a vacuum tube (BD Vacutainer®) without anticoagulant for the analysis of serum biochemistry (alanine aminostranferase-ALT, creatinine, urea , creatine kinase-CK, alkaline phosphatase- FA, lactate

dehydrogenase-LDH) following the rules of the Clinical Analysis Laboratory of the Veterinary Hospital of UNOESTE. Then, the animals were euthanized by exsanguination and tissue samples from the right and contralateral gastrocnemius muscle, liver, lung, heart and kidney were collected for histopathological analysis and processed according to the standard method of paraffin embedding and hematoxylin/eosin staining for histological analysis. The scoring criteria for the histopathological analysis of lesions eventually caused by toxins were based on those described by Mann et al. (2012) and Gibson-Corley et al. (2013) and analyzed at the Laboratory of Pathological Anatomy of the Veterinary Hospital of UNOESTE.

2.6 Serum biochemical analyses and hematologic essays

The serum biomarkers for systemic myotoxic, cardiotoxic, hepatotoxic and nephrotoxic activities were determined using Cobas C111 commercial kits (Roche Holding AG, Basel, Switzerland) for creatine kinase 'CK' (code 07442017-190), creatine kinase myocardial band 'CKMB' (code 05401763-190), alanine aminotransferase 'ALT' (code 04718569-190) and creatinine 'Cr' (code 05401755- 190), respectively. The assays were performed using a Cobas C111 analyzer (RocheHolding AG, Basel, Switzerland).

2.7. Hematological analysis

Erythrocytes, hemoglobin, hematocrit, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), red blood cell distribution width (RDW) and total leukocyte count were determined using the POCH-100 iV DIFF hematology analyzer (Sysmex do Brasil Indústria e Comercio Ltda., Sao José dos Pinhais, PR, Brazil); the relative values for reticulocytes were determined as a percentage of total red blood cells (Bessman, 1990; Brandow, 2018). For differential counting of leukocytes,

blood smears were stained with Diff-Quick (Panotico ® – Laborclin Produtos para Laboratorios Ltda., Pinhais, PR, Brazil) and then analyzed under an E– 200 Nikon light microscope (Nikon Inc., Tokyo, Japan) at 100× magnification The total plasma protein (TPP) concentration was quantified by the ATC-ITREF-200 refractometer (Instrutemp Instrumentos de Medição Ltda., São Paulo, SP, Brazil) and the results were expressed as g/dL.

2.8 Histopathological analysis

After collecting the blood samples, the animals were subsequently euthanized than subjected to dissection in order to collect tissue samples from the gastrocnemius muscle, heart, lungs, liver and kidney. The samples were immediately fixed in 10% formaldehyde overnight and then washed for 30 min in 0.1 M phosphate-buffered saline and 30 min in distilled water prior to storage in 70% ethanol overnight. The samples were dehydrated in graded ethanol (80%, 95% and 100%), cleared in xylene (1:1 ethanol:xylene, 1:1 xylene:paraffin) and finally embedded in paraplast. Three to five serial sections per sample (5 µm thick), separated from each other by 25 µm, were cut and mounted on plain glass slides for hematoxylin–eosin (HE) staining. The slides were examined with a Leica ICC50HD camera coupled to a Leica DM750 light microscope (Leica Microsystems, Wetzlar, Germany) and the images were then captured and analyzed semi qualitatively using a LAS 4.2 software (Leica Microsystems, Wetzlar, Germany). The morphological changes and frequency of lesions were compared among the treatments based on a lesion score, as essentially described elsewhere (Gerez et al., 2015; Grenier et al., 2011).

3 Statistical analysis

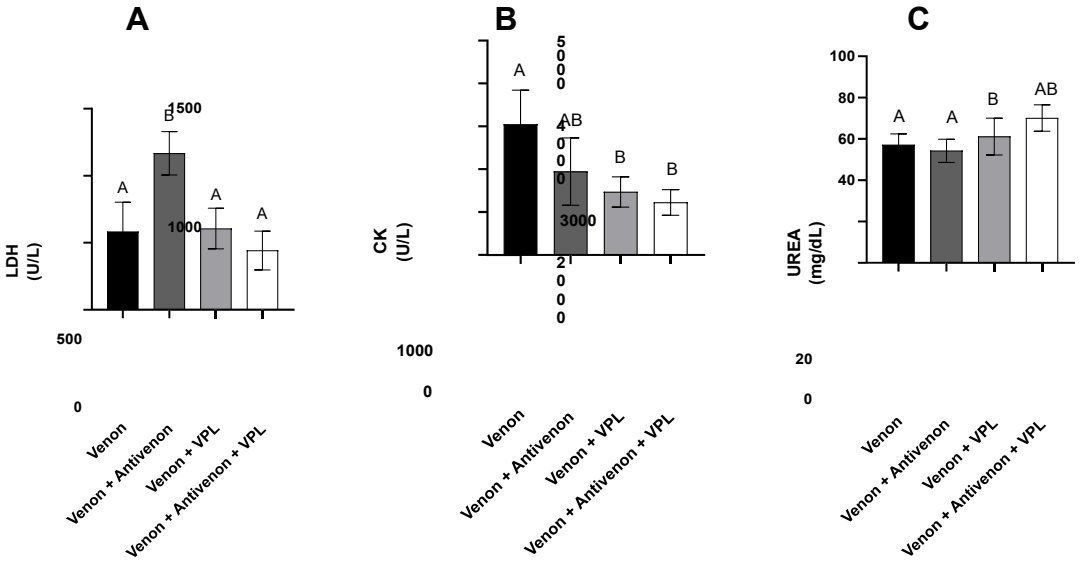
Bartlett's test was applied to verify the homoscedasticity of the variances of the treatment groups studied. Afterwards, the Shapiro-Wilk test was performed to analyze the normality between the data. Therefore,

the Analysis of Variance (ANOVA) test was used to verify whether there was a statistical difference between the treatment groups in relation to the injury score of the right gastrocnemius, liver, lung, kidney and heart muscles, as well as whether there was a statistical difference between the groups. of treatment verifying the difference in relation to each analyzed variable (ALT, creatinine, CK, urea, alkaline phosphatase, LDH). As there was a difference between the treatment groups, the Tukey test was chosen to analyze which groups showed statistical difference between them, in relation to the lesion score of the right gastrocnemius muscle, and the Tukey-Kramer test to analyze which groups were different in relation to the group control and between groups according to each variable considered (ALT, creatinine, CK, urea, alkaline phosphatase, LDH). All analyzes assumed significance of 5% ($p<0.05$).The data analyzes were performed using Microcal Origin 8 SR4 software version 8.0951 (Microcal Software Inc., Northampton, MA, USA) or GraphPad Prism v.4.03 (GraphPad Software Inc., La Jolla, CA, USA) and the software R-project.

4 Results

In the current study, there was a statistical difference ($P<0.05$) in the biochemical analyzes of CK, urea and LDH, muscle histological analysis, liver, lung, heart, and kidney. In the biochemical tests ALT, creatinine, FA and blood count, no significant differences were observed ($P>0.05$).

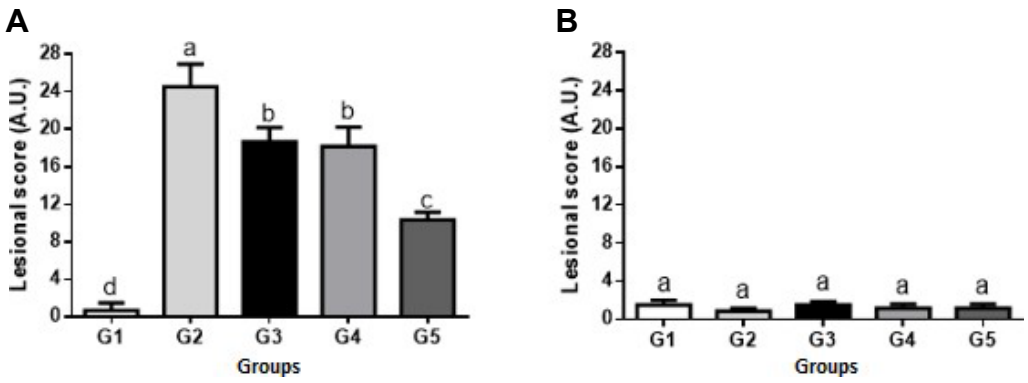
Picture 2 shows the difference and significance between the treatments applied, with the variables CK, urea and LDH showing a smaller increase in the groups that received the treatments (G3, G4, G5). Only G2 showed an increase ($P<0.05$) in the concentration of urea in the bloodstream, in relation to the control group.



Picture 2. Difference between treatments applied for serum biochemistry variables Lactatedehydrogenase (LDH U/l), creatine kinase (CK U/l), Urea mg/dl.

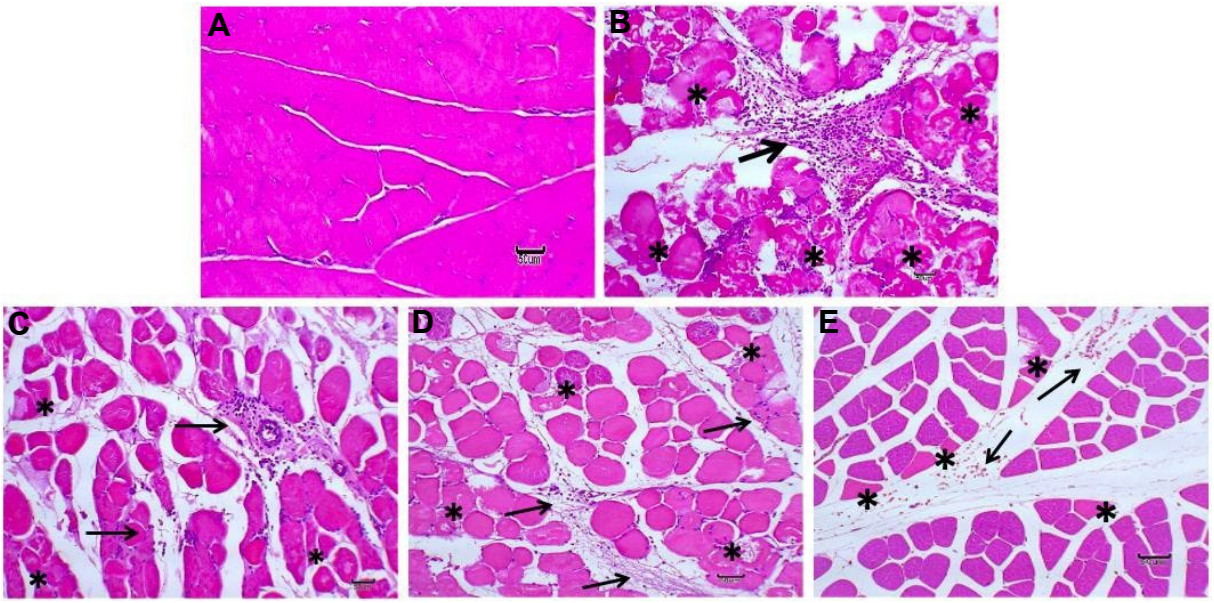
3.1. Efcts of L. m. rhombeata venom on muscle, kidney and liver morphology, followedby treatment with and *varespladib.*

To compare the injury score of the muscles of the right posterior limb, the left gastrocnemius muscle (contralateral) was used (Picture 3 A and B).
All treatments performed in relation to the control group showed difference ($P<0.05$) in relation to the lesion score of the right gastrocnemius muscle, with G5 having the lowest lesion score in relation to the other treated groups (G2, G3, G4). In the collateral limb, left muscle, there was no statistical difference ($p>0.05$).



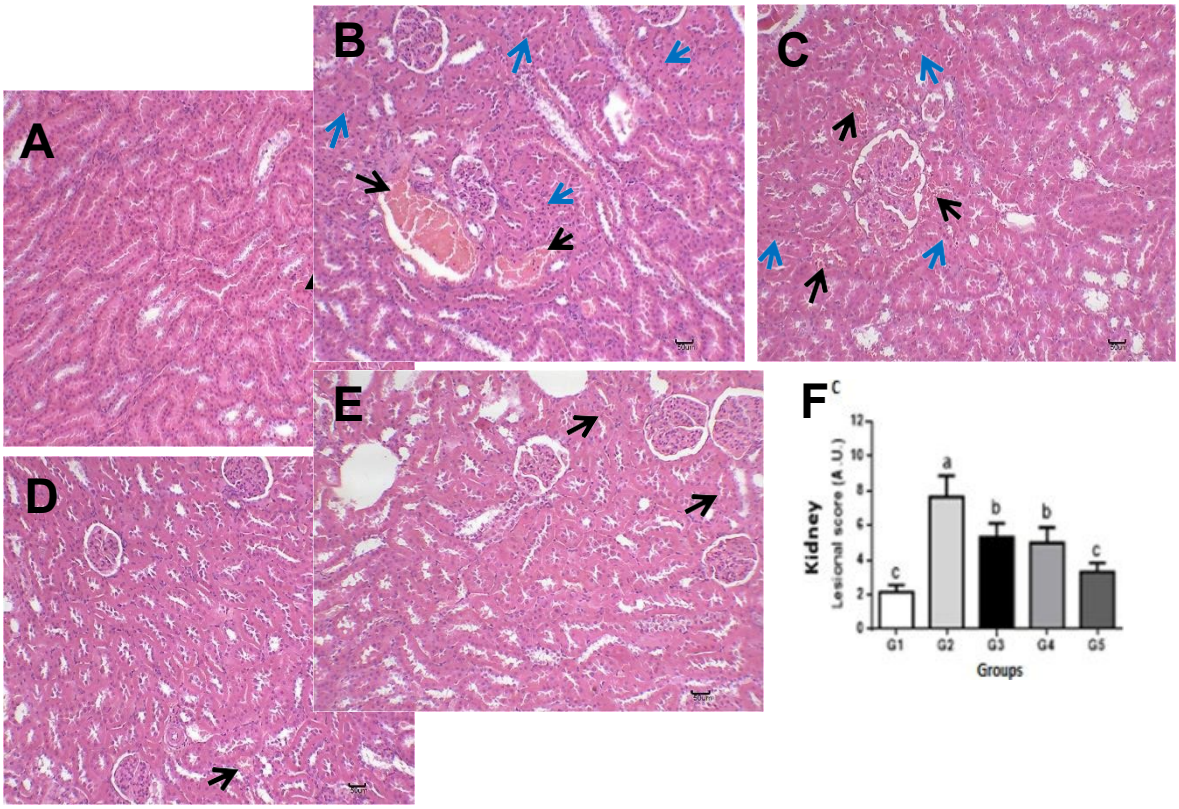
Picture 3 – Lesion score in right hind limb muscle (a) and left hind limb muscle (b) of animals in control (G1), venom (G2), venom/antivenom (G3), venom/varespladib (G4) groups , venom/antivenom/varespladib (G5). Tukey test $p<0.0001$.
The columns represent the mean ± SD (n = 6), with $p < 0.05$ indicating significance.

Using the right gastrocnemius of the control group (Picture 4A) to compare the histological findings of the same muscle in the other groups, a marked neutrophilic inflammatory infiltrate and diffuse myocyte necrosis were observed in G2 (Picture 4B), which may explain the statistical difference found between the use of venom isolated in group 2 and the control group.
In group 3, this neutrophilic infiltrate was moderate with the presence of necrosis and myocyte degeneration (Picture 4C); in group 4, there was a mild neutrophilic inflammatory infiltration associated with edema, moderate degeneration and myocyte necrosis (Picture 4D); and in G5, the findings were the mildest, with a mild neutrophilic inflammatory infiltration and mild myocyte degeneration (Picture 4E).

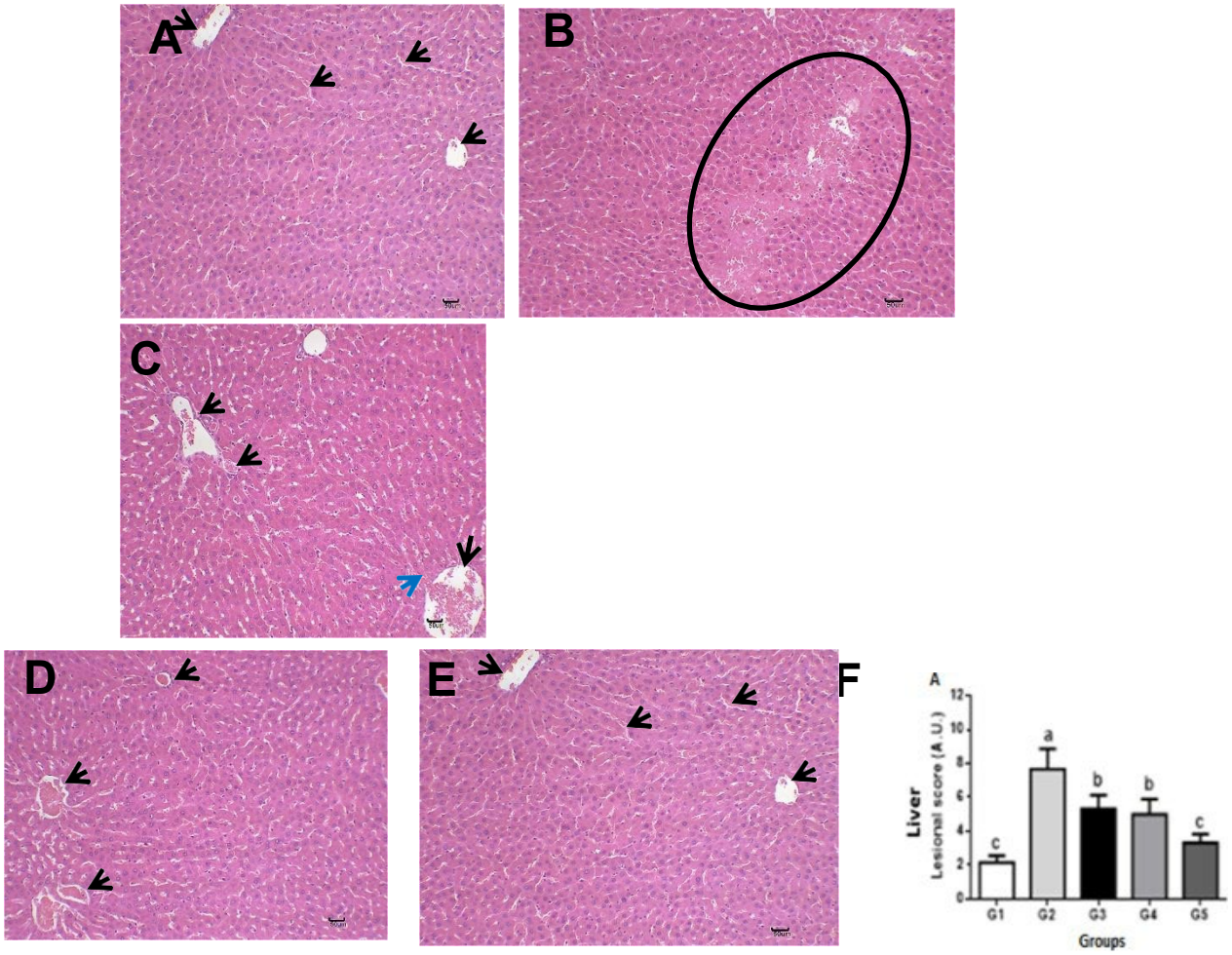


Picture 4.- Photomicroscopy of the right gastrocnemius muscle A- Animal group control. B- Animal from the venom group showing marked neutrophilic inflammatory infiltration (arrow) and diffuse myocyte necrosis (*); C- Animal from the venom/antivenom group showing moderate neutrophilic inflammatory infiltration (arrows) and myocyte necrosis and degeneration (*); D- Animal from the venom/varespladib group showing a mild neutrophilic inflammatory infiltration and edema (arrows), moderate degeneration and myocyte necrosis (*); E- Animal from the venom/antivenom/varespladib group showing mild neutrophilic inflammatory infiltration (arrow) and mild myocyte degeneration (*). All images stained withHematoxylin-eosin (HE), bar 50µm.

The same pattern found in the treated groups can be seen in the liver andkidney histology, figures 5 and 6 respectively.



Picture 5.- Renal Photomicroscopy A- Animal from the control group with mild acute tubular necrosis (arrow).; B- Animal from the severe congestion group (black arrow) and acute tubular necrosis (blue arrow).; C- Animal from the venom/antivenom group: moderate congestion (black arrow) and discrete acute tubular necrosis (blue arrow); D- Animal from the venom/varespladib group discreet congestion (black arrow) and acute tubular necrosis (blue arrow).; E- Animal from the venom/antivenom/varespladib group showing: discreet acute tubular necrosis (arrow). All images stained with Hematoxylin-eosin (HE), bar 50µm. . (F)- Lesion score in kidney of animals in control (G1), venom (G2), venom/antivenom (G3), venom/varespladib (G4), venom/antivenom/varespladib (G5). (Tukey test $p \leq 0.05$).



Picture 6 – Hepatic Photomicroscopy (A)- Animal from the control group with mild congestion (arrow).; (B)- Animal from the severe necrosis of hepatocytes group associated with hemorrhage (highlight).; (C)- Animal from the poison/antivenom group: moderate congestion (black arrow) and discreet local hemorrhage (blue arrow); (D)- Animal from the venom/varespladib group with severe congestion (arrow).; (E)- Animal from the venom/antivenom/varespladib group showing: mild vascular congestion (arrow). All images stained with Hematoxylin-eosin (HE), bar 50µm. (F)- Lesion score in liver of animals in control (G1), venom (G2), venom/antivenom (G3), venom/varespladib (G4), venom/antivenom/varespladib (G5). (Tukey test p≤0.05).

5. Discussion

The synergism of the verespladib/antivenom association was able to prevent systemic damage and decrease the lesion score values of the different organs evaluated without difference ($p > 0,05$) in relation to the control group, however, varespladib and antivenom alone did not prevent the increase of the lesion scores, which is due to the inhibition action of only PLA_2 by varespladib and although antivenom therapy was chosen, the antivenom used was polyvalent, in general antivenom serum are produced by a “pool of venom” and not regionalized , which can affect its neutralizing capacity, in addition to having a low immunogenic capacity when compared to other venoms such as bothrops (Stephano et al., 2005).

Diniz-Sousa et al., (2018) report the ability of varespladib to reduce cytotoxicity in myotubes and myotoxicity mediated in part by the interaction of C-terminal amino acid residues of PLA2 with negative sites of cell membranes, which causes increased permeability to calcium ions and cell rupture (Guterrez and Lomonte, 1995; Lomonte and Calderón, 2003), in addition to the action of the Lys49 protein (Fernandes et al., 2014).

The effects of a marked diffuse inflammatory infiltration in samples from animals from G2 in this study are justified, as mentioned by the authors, by the capacity of the venom of L. m rhombeata to produce an intense systemic effect and subcutaneous hemorrhage from a dose of 500 µg/Kg , with the lowest doses (5, 50, and 100 µg/kg) being ineffective. As mentioned by some authors, the association antivenom/varespladib, isolated antivenom or isolated varespladib was able to produce a slight reduction of this alteration (Lewin et al., 2016; Wang et al., 2018; Youngman et al., 2020; Zinenko et al. ., 2020; Gutiérrez et al., 2020; Liu et al., 2020; Oliveira et al., 2020; Dashevsky et al., 2021; Kazandjian et al., 2021; Silva-Carvalho et al., 2021;). In the current study, the neutrophilic inflammatory infiltration and tissue lesions, including necrosis were reduced in the ascending order of the groups respectively, and in the group treated with the antivenom/varespladib-G5 association there was no

necrosis and only degeneration was observed, statistically they had similar lesion scores to the control-G1 group. Few studies report the action of PLA2 on the effects produced by lacquetic venom, such as the anticoagulant and antithrombotic effect. (Damico et al., 2012), inhibition of platelet aggregation (Cordeiro et al., 2015) and cytotoxicity in C2C12 myotubes (Diniz-Sousa et al., 2018). Most studies related to transcriptomic (Junqueira-de-Azevedo et al. 2006) and proteomic (Madrigal et al. 2012; Wiezel et al. 2019) analyzes of Lachesis spp. revealed the presence of biologically active peptides, e.g., bradykinin enhancers (BPPs) (Soares et al. 2005; Pla et al. 2013; Sanz et al. 2008; Pinheiro Junior et al. 2018) and bradykinin receptor antagonists (Graham et al. al. 2005), in addition to a wide variety of enzymes and toxins previously mentioned (Damico et al., 2012; Cordeiro et al., 2015; Wiezel et al., 2015; Cordeiro et al., 2018; Diniz-Sousa et al. al., 2018; Pinheiro-Júnior et al., 2018; Wiezel et al., 2019). Even so, the biological actions of these toxins in the poisoning process by Lachesis spp. are still little known, with many of their effects being characterized in vitro experimental models, e.g., proteolytic, fibrinogenolytic, anticoagulant and plateletinhibitory activities.

The serum urea concentration increased significantly in the animals when the isolated venom was used (G2) and the LHD concentration only did not increase significantly when treated with the antivenom/varespladib association (G5). Thus, this association reduced the cellular inflammatory process, also confirmed by histological findings, that is, it demonstrated an inhibitory effect on markers of tissue and kidney injury in the treated groups, with greater evidence of a synergistic effect in the use of antivenom and VPL.

Elevation in CK, urea and LDH levels are associated with acute muscle and kidney injury usually following elevation of urea followed by increased creatinine concentrations as an indication of decreased glomerular filtration rate (Cremonez et al., 2016), however, creatinine values remained within normal limits, probably due to the acute intoxication model, a similar condition reported by Pardal et al. (2004) which a slight increase in creatinine normalized one day after the snakebite.

The systemic alterations of hepatotoxicity, cardiotoxicity and nephrotoxicity and lung injury were evidenced here by the increase of CK, urea and LDH, the latter enzyme being present in several organs and tissues, respectively after 120 min of poisoning ($P<0.05$ in relation to G1), corroborating with other authors who report pulmonary thrombosis, renal failure, bradycardia, hypotension, hematological disorders of generalized bleeding and coagulopathies (Giovanni et al., 1997; Torres et al., 2013; Dias et al., 2016; Leão et al. ., 2021; Gutierres et al., 2022).

Although Gutiérrez et al. (2021) has reported the presence of serine proteases in Viperidae venoms and these are responsible for the pro-coagulant action, the study by Gutierres et al. (2022) demonstrate the role of PLA2, since varespladib prevented the procoagulant action of L. m. rhombeata, even partially. This suggests the ability of varespladib to prevent coagulation disorders in accidents caused by Bothrops, Viperidae-Crotalinae, Daboia, Echis, Oxyuranus, Najah, Pseudechis and Bitis spp, but does not antagonize the local and systemic hemorrhagic effects, since which are mediated by the action of metallo and serine proteases (Escalante et al., 2011; Seo et al., 2017), even though it has already been isolated in the venom of L. m. rhombeata by Damico et al. (2012) an Asp49 PLA2 (LmrTX) with anticoagulant activity.

The isolated use of varespladib has shown, in some studies with other species of snakes, that it works by inhibiting PLA2, which would be a useful therapeutic alternative to complement antivenom therapies. In the lachetic venom, in addition to PLA2, there are other toxins such as serine proteases, metalloproteases, type C lectin, phospholipase B, hyaluronidase and bradykinin potentiating peptides (BPPs) and interfering with their action (Aguiar et al., 1996; Weinberg et al., 2004; Junqueira-De-Azevedo et al., 2006; Bregge-Silva et al., 2012; Madrigal et al., 2012; Cordeiro et al., 2018; Wiezel et al., 2019; Wiezel et al., 2015; Pinheiro-Júnior et al., 2018; Diniz-Sousa et al., 2018).

All these substances are involved with local clinical signs of pain, edema, necrosis, cytotoxicity (Damico et al., 2006; Ferreira et al., 2009; Damico et al., 2012; Stransky et al. 2018), in addition to systemic ones such as hemorrhage, coagulopathy and hypotension (Sánchez et al.,

1987; Sánchez et al., 1991; Sánchez et al., 1995; Fuly et al., 1997; Rucavado et al., 1999; Estêvão-Costa et al., 2000 ; Torres-Huaco et al., 2013; Dias et al., 2016ab), bradycardia (Sanz et al., 2008; Soares et al., 2005; Pla et al., 2013), renal failure (Damico et al., 2007; Alves, 2010) and neuromuscular blockade (Damico et al. 2005a; Damico et al. 2005b; Damico et al. 2006) which can cause death.

Although the blood count (leukogram and erythrogram) was within normal limits in this study, other authors report that Lachesies muta muta venom promoted a significant increase in the number of leukocytes, neutrophils, eosinophils and monocytes (Dias et al. 2016; Angel et al. al., 2020;).

6 Conclusion

Treatment with Varespladib associated with antivenom was more efficient than its use alone in controlling local and systemic injuries induced by Lachesis muta rhombeata venom. This synergistic effect could be confirmed through lower systemic and tissue scores in the blood and muscle, liver, lung, kidney and heart tissue of treatedanimals.

References

1. Aguiar, A.S., Alves C.R., Melgarejo, A., Giovanni-De-Simone, S., 1996. Purification and partial characterization of a thrombin-like/gyroxin enzyme from bushmaster *Lachesis muta rhombeata* venom. Toxicon 34, 555-65. https://doi.org/10.1016/0041-0101(95)00159-X.
2. Aline G. Leão-Torres, Carina V. Pires, Amanda C. Ribelato, Maria C. Zerbinatti, Cecília L. Santarém, Rosa M.B. Nogueira, Inês C. Giometti, Rogério Giuffrida, Elisangela O. Silva, Juliana R. Gerez, Nelson J. Silva, Edward G. Rowan, Rafael
   S. Floriano,Protective action of N-acetyl-L-cysteine associated with a

polyvalent antivenom on the envenomation induced by Lachesis muta muta (South American bushmaster) in rats,Toxicon,Volume 198,2021,Pages 36-47,ISSN 0041-0101, https://doi.org/10.1016/j.toxicon.2021.04.018.

3. Angel CKL, Guerrero VJA, De Carvalho EF, Lima SK, De Siqueira RJB, et al. (2020) Disturbances in cardiovascular parameters in rats and in human blood cells caused by Lachesis acrochorda snake venom. Toxicon 184: 180-191. https://doi.org/10.1016/j.toxicon.2020.06.009.
4. Alves, C.D., 2010. Estudo dos efeitos renais do veneno da serpente *Lachesis muta muta*. MSc [Dissertação]. Universidade Federal de Ceará, Fortaleza. Available from: www.repositorio.ufc.br/bitstream/riufc/2269/1/2010_dis_cdalves.pdf.
5. Barone, J.M., Frezzatti, R., Silveira, P.F., 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. https://doi.org/10.1016/j.toxicon.2013.12.010.
6. Bittenbinder, M.A., Zdenek, C.N., Brouw, B., Youngman, N.J., Dobson, J.S., Naude, A., Vonk, F.J., Fry, B.G., 2018. Coagulotoxic cobras: clinical implications of strong anticoagulant actions of african spitting *Naja* venoms that are not

neutralised by antivenom but are by LY315920 (Varespladib). Toxins 10, 12, 516.https://doi.org/10.3390/toxins10120516
7. Bregge-Silva, C., Nonato, M.C., Albuquerque, S., Ho, P.L., Junqueira de Azevedo, I.L., Vasconcelos Diniz, M.R., Lamonte, B., Rucavado, A., Díaz, C., Gutiérrez, J.M., Arantes, E.C., 2012. Isolation and biochemical, functional and structural characterization of a novel l-amino acid oxidase from *Lachesis muta* Snake Venom. Toxicon 60, 7, 1263-76. https://doi.org/10.1016/j.toxicon.2012.08.008.

Bessman, J.D., 1990. Reticulocytes. In: Walker, H.K., Hall, W.D., Hurst, J.W. (Eds.), Clinical Methods: the History, Physical, and Laboratory

Examinations, third ed. Butterworths, Boston, MA https://www.ncbi.nlm.nih.gov/books/NBK264.

Bryan-Quirós, W., Fernández, J., Gutiérrez, J.M., Lewin, M.R., Lomonte, B., 2018. Neutralizing properties of LY315920 toward snake venom group I and II myotoxic phospholipases A_2. Toxicon, 157, 1-7. https://doi.org/10.1016/j.toxicon.2018.11.292.

Campbell, J.A., Lamar, W.W., 2004. Venomous reptiles of the western hemisphere, 2 Vol. Comstock Publishing Associates/Cornell University Press, Ithaca.

Collaço, R.C., Cogo, J.C., Rodrigues-Simioni, L., Rocha, T., Oshima-Franco, Y., Randazzo-Moura, P., 2012. Protection by *Mikania laevigata* (guaco) extract against the toxicity of *Philodryas olfersii* snake venom. Toxicon 60**,** 4, 614-22. https://doi.org/10.1016/j.toxicon.2012.05.014.

Cordeiro, F.A., Coutinho, B.M., Wiezel, G.A., Bordon, K.C.F., Bregge-Silva, C., Rosa- Garzon, N.G., Cabral, H., Ueberheide, B., Arantes, E.C. 2018. Purification and enzymatic characterization of a novel metalloprotease from *Lachesis muta rhombeata* snake venom. J. Venom. Anim. Toxins Incl. Trop. Dis. 24, 32. https://doi.org/10.1186/s40409-018-0171-x.

Cordeiro, F.A., Perini, T.G.K., Bregge-Silva, C., Cremonez, C.M., Rodrigues, R.S., Boldrini-França, J., Bordon, K., Souza, C.F., Ache, D.L.N., Rodrigues, D.C., Santos, V.M., Santos, W.F., Rosa, J.C., Arantesa, E.C. 2015. A new phospholipase A_2 from *Lachesis muta rhombeata*: purification, biochemical and comparative characterization with crotoxin B. Protein Pept. Lett. 22, 9, 816-27. https://doi.org/10.2174/0929866522666150706112431.

Costa, H.C., Bérnils, R.S., 2018. Répteis do Brasil e suas Unidades Federativas: Listade espécies. Herpetologia Brasileira, 8, 11-57.

Cremonez, C.M., Leite, F.P., Bordon, K.C., Cerni, F.A., Cardoso, .IA., Gregório, Z.M., Souza, R.C., Souza, A.M., Arantes, E.C., 2016. Experimental *Lachesis muta rhombeata* envenomation and effects of soursop (*Annona muricata*) as natural antivenom. J. Venom Anim. Toxins Incl. Trop. Dis. 22, 12. 10.1186/s40409-016-0067-6.

Croda, J.R.,Haraki, C.A.C., 2020. Acidentes ofídicos no Brasil, 2018. Boletim Epidemiológico 51, 9, 35-41.

Damico, D.C.S., Bueno, L.G.F., Rodrigues-Simioni, L., Marangoni, S., Cruz-Höfling, M.A., Novello, J.C., 2005a. Neurotoxic and myotoxic actions of *Lachesis muta muta* (Surucucu) whole venom on the mouse and chick nerve-muscle preparations. Toxicon 46, 2, 222-29. https://doi.org/10.1016/j.toxicon.2005.04.011.

Damico, D.C.S., Lilla, S., Nucci, G., Ponce-Soto, L.A., Winck, F.V., Novello, J.C., Marangoni, S., 2005b. Biochemical and enzymatic characterization of two basic Asp49 phospholipase A_2 isoforms from *Lachesis muta muta* (Surucucu) venom. Biochim. Biophys. Acta 1726, 1, 75-86. https://doi.org/10.1016/j.bbagen.2005.05.022.

Damico, D.C.S., Bueno, L.G.F., Rodrigues-Simioni, L., Marangoni, S., Cruz-Höfling, M.A., Novello, J.C., 2006. Functional characterization of a basic D49 phospholipase A_2 (LmTX-I) from the venom of the snake *Lachesis muta muta* (bushmaster). Toxicon 47, 7, 759-65. https://doi.org/10.1016/j.toxicon.2006.02.007.

Damico, D.C.S., Nascimento, J.M., Lomonte, B., Ponce-Soto, L.A.,

Joazeiro, P.P., Novello, J.C., Marangoni, S., Collares-Buzato, C.B., 2007. Cytotoxicity of *Lachesis muta muta* snake (Bushmaster) venom and its purified basic phospholipase A_2 (LmTX-I) in cultured cells. Toxicon 4

Damico, D.C.S., Vassequi-Silva, T., Torres-Huaco, F.D., Nery-Diaz, A.C.C., Souza, R.C.G., Silva, S.L., Vicente, C.P., Mendes, C.B., Antunes, E., Werneck, C.C., Marangoni, S., 2012. LmrTX, a basic PLA_2 (D49) purified from *Lachesis muta rhombeata* snake venom with enzymatic-related antithrombotic and anticoagulant activity. Toxicon 60, 5, 773-81. https://doi.org/10.1016/j.toxicon.2012.06.010.

Dashevsky, D.; Bénard-Valle, M.; Neri-Castro, E.; Youngman, N. J.; Zdenek, C. N.; Alagón, A., Portes-Junior, J.A., Frank, N., Fry, B.G., 2021. Anticoagulant Micrurus Venoms: targets and neutralization. Toxicol. Lett. 337, 91-7. https://doi.org/10.1016/j.toxlet.2020.11.010.

De Luca, A. Minucci, J. Trias, D. Tripodi, G. Conti, C. Zuppi, E. Capoluongo. Varespladib inhibits secretory phospholipase A_2 in bronchoalveolar lavage of differenttypes of neonatal lung injury Sou. J. Cardiovasc. Drogas , 11 (2011) , pp. 137 – 143. https://doi.org/10.1177/0091270011405498.

Dias, L., Rodrigues, M.A.P., Inoue, B.R,. Rodrigues, R.L., Rennó, A.L., Souza, V.B., Torres-Huaco, F.D., Sousa, N.C., Stroka, A., Melgarejo, A.R., Hyslop, S., 2016a. Pharmacological analysis of hemodynamic responses to *Lachesis muta* (South American Bushmaster) snake venom in anesthetized rats. Toxicon 123, 25-44. https://doi.org/10.1016/j.toxicon.2016.10.002

Dias, L., Rodrigues, M.A.P., Rennó, A.L., Stroka, A., Inoue, B.R., Panunto, P.C.,Melgarejo, A.R., Hyslop, S., 2016b. Hemodynamic responses to *Lachesis muta* (South American bushmaster) snake venom in

anesthetized rats. Toxicon 123, 1–14. https://doi.org/10.1016/j.toxicon.2016.10.001

Diniz, M.R., Oliveira, E.B., 1992. Purification and properties of a kininogenin from the venom of *Lachesis muta* (bushmaster). Toxicon 30, 3, 247–58. 10.1016/0041- 0101(92)90867-5.

Diniz-Sousa, R., Caldeira, C.A.S., Kayano, A.M., Paloschi, M.V., Pimenta, D.C., Simões-Silva, R., Ferreira, A.S., Zanchi, F.B., Matos, N.B., Grabner, F.P., Calderon, L.A., Zuliani, J.P., Soares, A.M., 2018. Identification of the molecular determinants of the antibacterial activity of LmutTX, a Lys49 phospholipase A_2 homologue isolated from *Lachesis muta muta* snake venom (Linnaeus, 1766). Basic Clin. Pharmacol. Toxicol. 22, 4, 413–23. https://doi.org/10.1111/bcpt.12921.

Diniz-Sousa, R., Moraes, J.N., Rodrigues-da-Silva, T.M., Oliveira, C.S., Caldeira, A.S., 2020. A brief review on the natural history, venomics and the medical importance of bushmaster (*Lachesis*) pit viper snakes. Toxicon: X, 7, 100053. https://doi.org/10.1016/j.toxcx.2020.100053

Escalante, T., Rucavado, A., Fox, J.W., Gutiérrez, J.M. 2011. Key events in microvascular damage induced by snake venom hemorrhagic metalloproteinases. J. Proteomics, 74, 9, 1781-94. https://doi.org/10.1016/j.jprot.2011.03.026.

Estevão-Costa MI, Diniz CR, Magalhães A, Markland FS, Sanchez EF, (2000). Action of metalloproteinases mutalysin I and II on several components of the hemostatic and fibrinolytic systems. Thromb. Res. 99, 4, 363–76. https://doi.org/10.1016/s0049-3848(00)00259-0.

Fernandes, C.A.H.; Borges, R. J.; Lomonte, B.; Fontes, M.R.M. A structure-based proposal for a comprehensive myotoxic mechanism of phospholipase A2-like proteins from viperid snake venoms. Biochim.

Biophys. Acta, 2014, 1844, 2265-2276. https://doi.org/10.1016/j.bbapap.2014.09.015.

Ferraz, M.C., Yoshida, E.H., Tavares, R.V., Cogo, J.C., Cintra, A.C., Dal Belo, C.A,,
Franco, L.M., Santos, M.G., Resende, F.A., Varanda, E.A., Hyslop, S., Puebla, P., San Feliciano, A., Oshima-Franco, Y., 2014. An isoflavone from *Dipteryx alata* Vogel is active against the *in vitro* neuromuscular paralysis of *Bothrops jararacussu* snake venom and bothropstoxin I, and prevents venom-induced myonecrosis. Molecules 19, 5, 5790–5805. https://doi.org/10.3390%2Fmolecules19055790.

Ferreira, T., Camargo, E.A., Ribela, M.T.C.P., Damico, D.C., Marangoni, S., Antunes, E., De Nucci, G., Landucci, E.C.T., 2009. Inflammatory oedema induced by *Lachesis muta muta* (surucucu) venom and LmTX-I in the rat paw and dorsal skin. Toxicon 53, 1, 69–77. https://doi.org/10.1016/j.toxicon.2008.10.016.

Ferreira-Rodrigues, S.C., Rodrigues, C.M., Santos, M.G., Gautuz, J.A., Silva, M.G., Cogo, J.C., Batista-Silva, C., Santos, C.P., Groppo, F.C., Cogo-Müller, K., Oshima- Franco, Y., 2016. Anti-inflammatory and antibothropic properties of *Jatropha elliptica*, a Plant from Brazilian Cerrado Biome. Adv. Pharm. Bull. 6, 4, 573–79. https://doi.org/10.15171%2Fapb.2016.071.

Floriano, R.S., Nogueira, R.M., Sakate, M., Laposy, C.B., Motta, Y.P., Sangiorgio, F., David, H.C., Nabas, J.M., 2009. Effect of *Mikania glomerata* (Asteraceae) leaf extract combined with anti-venom serum on experimental *Crotalus durissus* (Squamata: Viperidae) envenomation in rats. Rev. Biol. Trop. 57, 4, 929–37.

Fuly, A.L., Calil-Elias, S., Martinez, A.M.B., Melo, P.A., Guimarães, J.A., 2003. Myotoxicity induced by an acidic Asp-49 phospholipase A_2 isolated from *Lachesis muta* snake venom: comparison with

lysophosphatidylcholine. Int. J. Biochem. Cell Biol. 35, 10, 1470-81. https://doi.org/10.1016/S1357-2725(03)00129-8.

Fuly, A.L., Calil-Elias, S., Zingali, R.B., Guimarães, A., Melo, P.A., 2000. Myotoxic activity of an acidic phospholipase A_2 isolated from *Lachesis muta* (bushmaster) snake venom. Toxicon 38, 7, 961-72. https://doi.org/10.1016/s0041-0101(99)00208-1.

Fuly, A.L., Machado, O.L., Alves, E.W., Carlini, C.R., 1997. Mechanism of inhibitory action on platelet activation of a phospholipase A_2 isolated from *Lachesis muta* (bushmaster) snake venom. Thromb. Haemost. 78, 5, 1372-80.

Gerez, J.R., Pinton, P., Callu, P., Grosjean, F., Oswald, I., Bracarense, A.P.L., 2015.Deoxynivalenol alone or in combination with nivalenol and zearalenone inducesystemic histological changes in pigs. Exp. Toxicol. Pathol. 67, 89-98. https://doi.org/10.1016/j.etp.2014.10.001 .

Gibson-Corley, K.N., Oliver, A.K., Meyerholz, D.K., 2013. Principles for valid histopathologic scoring in research. Vet. Pathol. 50, 6, 1007-15. https://doi.org/10.1177/0300985813485099.

Giovanni-De-Simone, S., Aguiar, A.S., Gimenez, A.R., Novellino, K,, Soares de Moura, R., 1997. Purification, properties, and N-terminal amino acid sequence of a kallikrein- like enzyme from the venom of *Lachesis muta rhombeata* (Bushmaster). J. Protein Chem. 16, 8, 809-18. https://doi.org/10.1023/a:1026372018547.

Graham, R.L., Graham, C., McClean, S., Chen, T., O'Rourke, M., Hirst, D., Theakston, D., Shaw, C., 2005. Identification and functional analysis of a novel bradykinin inhibitory peptide in the venoms of New World Crotalinae pit vipers. *Biochem. Biophys.* Res. Commun. 338, 3, 1587-92. https://doi.org/10.1016/j.bbrc.2005.10.130.

Grenier, B., Loureiro-Bracarense, A.P., Lucioli, J., Pacheco, G.D., Cossalter, A.M., Moll, W.D., Schatzmayr, G., Oswald, I.P., 2011. Individual and combined effects of subclinical doses of deoxynivalenol and fumonisins in piglets. Mol. Nutr. Food Res. 55,761–771. https://doi.org/10.1016/j.etp.2014.10.001

Gutierres, P.G., Pereira, D.R., Vieira, N.L., Arantes, L.F., Silva Júnior, N.J., Torres-Bonilha, K.A., Hyslop, S., Morais-Zani, K., Nogueira, R.M.B., Rowans, E.G., Floriano,
R.S. 2022. Action of Varespladib (LY-315920), a phospholipase a2 inhibitor, on the enzymatic, coagulant and haemorrhagic activities of *lachesis muta rhombeata* (South-American Bushmaster) Venom. Frontiers in Pharmacology 12, 812295. https://doi.org/10.3389/fphar.2021.812295

Gutiérrez, J.M., Lewin, M.R., Williams, D.J., Lomonte, B., 2020. Varespladib (LY315920) and methyl varespladib (LY333013) abrogate or delay lethality induced by presynaptically acting neurotoxic snake venoms. Toxins 12, 2, 131. https://doi.org/10.3390/toxins12020131.

Gutiérrez, J.M., Albulescu, L.O., Clare, R.H., Casewell, N.R., Abd El-Aziz, T.M., Escalante, T., Rucavado, A., 2021. The Search for Natural and Synthetic Inhibitors that Would Complement Antivenoms as Therapeutics for Snakebite Envenoming. Toxins (Basel) 13, 7, 451. https://doi.org/10.3390/toxins13070451.

Gutiérrez, J.M.; Lomonte, B. Phospholipase A2 myotoxins from Bothrops snake venoms. Toxicon, 1995, 33, 1405-1424. https://doi.org/10.1016/0041-0101(95)00085-Z.

Harder, C., Oliveira, A.L., Scriboni, A.B., Cintra, A.C.O., Schezaro-Ramos, R., Santos, M.G., Cogo-Müller, K., Miura, R.Y.H., Floriano, R.S., Rostelato-Ferreira, S., Oshima- Franco, Y., 2017. Pharmacological

properties of *Vochysia Haenkeana* (Vochysiaceae) extract to neutralize the neuromuscular blockade induced by Bothropstoxin-I (Lys49 Phospholipase A_2) myotoxin. Adv. Pharm. Bull. 7, 3, 433–9. https://doi.org/10.15171%2Fapb.2017.052.

Junqueira-de-Azevedo, I.L.M., Ching, A.T.C., Carvalho, E., Faria, F., Nishiyama Jr, M.Y., Ho, P.L., Diniz, M.R.V., 2006. *Lachesis muta* (Viperidae) cDNAs reveal diverging pit viper molecules and scaffolds typical of cobra (Elapidae) venoms: implications for snake toxin repertoire evolution. Genetics 173, 2, 877–89. https://doi.org/10.1534%2Fgenetics.106.056515.

Kazandjian, T.D., Arrahman, A., Still, K.B.M., Somsen, G.W., Vonk, F.J., Casewell, N.R., Wilkinson, M.C., Kool, J., 2021. Anticoagulant Activity of *Naja nigricollis* Venom Is Mediated by Phospholipase A_2 Toxins and Inhibi,ted by Varespladib. Toxins Basel 13, 5, 302. https://doi.org/10.3390%2Ftoxins13050302.

Leão-Torres AG, Pires CV, Ribelato AC, Zerbinatti MC, Santarém CL, et al. (2021) Protective action of N-acetyl-L-cysteine associated with a polyvalent 2 antivenom on the envenomation induced by Lachesis muta muta (South 3 American bushmaster) in rats. Toxicon, 198: 36-47. https://doi.org/10.1016/j.toxicon.2021.04.018.

Lewin, M.R., Gilliam, L.L., Gilliam, J., Samuel, S.P., Bulfone, T.C., Bickler, P.E., Gutiérrez, J.M., 2018. Delayed LY333013 (oral) and LY315920 (intravenous) reverse severe neurotoxicity and rescue juvenile pigs from lethal doses of *Micrurus fulvius* (eastern coral snake) venom. Toxins 10, 11, 479.
https://doi.org/10.3390/toxins10110479.

Lewin, M.R., Samuel, S., Merkel, J., Bickler, P., 2016. Varespladib (LY315920) appears to be a potent, broad-spectrum, inhibitor of snake

venom phospholipase A_2 and a possible pre-referral treatment for envenomation. Toxins 8, 9, 248.
https://doi.org/10.3390/toxins8090248.

Liu, C.C., Wu, C.J., Hsiao, Y.C., Yang, Y.H., Liu, K.L., Huang, G.J., Hsieh, C.H., Chen, C.K., Liaw, G.W., 2020. Snake venom proteome of protobothrops mucrosquamatus in Taiwan: delaying venom-induced lethality in a rodent model by inhibition of phospholipase A_2 activity with Varespladib. J. Proteomics 234, 104084.
https://doi.org/10.1016/j.jprot.2020.104084.

Lomonte, B.; Angulo, Y.; Calderón, L. An overview of lysine-49 phospholipase A2 myotoxins from crotalid snake venoms and their structural determinants of myotoxic action. Toxicon, 2003, 42, 885- 901. https://doi.org/10.1016/j.toxicon.2003.11.008.

Madrigal, M., Pla, D., Sanz, L., Barboza, E., Arroyo-Portilla, C., Corrêa-Netto, C., Gutiérrez, J.M., Alape-Girón, A., Flores-Díaz, M., Calvete, J.J., 2017. Cross-reactivity, antivenomics, and neutralization of toxic activities of *Lachesis* venoms by polyspecific and monospecific antivenoms. PLoS Neglected Tropical Diseases 11, 8, e0005793. https://doi.org/10.1371%2Fjournal.pntd.0005793.

Madrigal, M., Sanz, L., Flores-Díaz, M., Sasa, M., Núñez, V., Alape-Girón, A., Calvete, J.J., 2012. Snake venomics across genus *Lachesis*. Ontogenetic changes in the venom composition of *Lachesis stenophrys* and comparative proteomics of the venoms of adult *Lachesis melanocephala* and *Lachesis acrochorda*. J. Proteom. 77, 280-97. https://doi.org/10.1016/j.jprot.2012.09.003.

Magalhães, S.F.V., Peixoto, H.M., Moura, N., Monteiro, W.M., Oliveira, M.R.F., 2019. Snakebite Envenomation in the Brazilian Amazon: a Descriptive Study. Trans. R. Soc. Trop. Med. Hyg.113, 3,143-51. https://doi.org/10.1093/trstmh/try121.

Mann, P.C., Vahle, J., Keenan, C.M., Baker, J.F., Bradley, A.E., Goodman, D.G., Harada, T., Herbert Kaufmann, W., Kellner, R., Nolte, T., 2012. Rittinghausen S, Tanaka T, (2012). International harmonization of toxicologic pathology nomenclature: an overview and review of basic principles. Toxicol. Pathol. 40, sp. 4, 7S–13S. https://doi.org/10.1177/0192623312438738.

Melgarejo, A.R., 2009. Serpentes peçonhentas do Brasil. In: Cardoso, J.L.C., França, F.O.S., Wen, F.H., Málaque, C.M.S., Haddad Jr, V. (Eds.). Animais peçonhentos no Brasil: biologia, clínica e terapêutica dos acidentes. Sarvier/FAPESP, São Paulo, pp. 42–70.

Muniz, E.G., Noronha, M.D.D.N., Saraiva, M.D.G.G., Monteiro, W.M., Oliveira, S.S., 2021. Neutralization of Hemostatic Disorders Induced by Lachesis muta Venom using BrazilianAntivenoms. Toxicon 191,44–7. https://doi.org/10.1016/j.toxicon.2020.12.013.

Nogueira, C.C., Argôlo, A.J.S., Arzamendia, V., Azevedo, J.A., Barbo, F.E., Bérnils, R.S., Bolochio, B.E., Borges-Martins, M., Brasil-Godinho, M., Braz, H., Buononato, M.A., Cisneros-Heredia, D.F., Colli, G.R., Costa, H.C., Franco, F.L., Giraudo, A., Gonzalez, R.C., Guedes, T., Hoogmoed, M.S., Marques, O.A.V., Montingelli, G.G., Passos, P., Prudente, A.L.C., Rivas, G.A., Sanchez, P.M., Serrano, F.C., Silva Jr., N.J., Strüssmann, C., Vieira-Alencar, J.P.S., Zaher, H., Sawaya, R.J., Martins, M., 2019. Atlas of Brazilian snakes: verified point-locality maps to mitigate the Wallacean shortfall in a megadiverse snake fauna. South American Journal of Herpetology 14, sp. 1, 1–274.

Oliveira, I. C. F.; Gutiérrez, J. M.; Lewin, M. R.; Oshima-Franco, Y., 2020. Varespladib (LY315920) Inhibits Neuromuscular Blockade Induced by Oxyuranus Scutellatus Venom in a Nerve-Muscle Preparation. Toxicon 187, 101–04. https://doi.org/10.1016/j.toxicon.2020.08.023.

Pardal, P.P., Souza, S.M., Monteiro, M.R., Fan, H.W., Cardoso, J.L., Franca, F.O.,
Tomy, S.C., Sano-Martins, I.S., Sousa-e-Silva, M.C.C., Colombini, M., Kodera, N.F., Moura-da-Silva, A.M., Cardoso, D.F., Velarde, D.T., Kamiguti, A.S., Theakston, R.D.G., Warrell, D.A., 2004. Clinical trial of two antivenoms for the treatment of *Bothrops* and *Lachesis* bites in the north eastern Amazon region of Brazil. Trans R Soc Trop Med Hyg. 98, 1, 28–42. 10.1016/S0035-9203(03)00005-1.

Pinheiro-Júnior, E.L., Boldrini-França, J., Campos Araújo, L.M.P., Santos-Filho, N.A., Weinberg, M.L.D., Felicori, L.F., Bello, C.A., Magalhães, H.P.B., Almeida, A.P., Magalhães, A., Bendhack, L.M., Cilli, E.M., Arantes, E.C., 2018. LmrBPP9: A synthetic bradykinin-potentiating peptide from *Lachesis muta rhombeata* venom that inhibits the angiotensin-converting enzyme activity *in vitro* and reduces the blood pressure of hypertensive rats. Peptides 102, 1–7. https://doi.org/10.1016/j.peptides.2018.01.015.

Pla, D., Sanz, L., Molina-Sánchez, P., Zorita, V., Madrigal, M., Flores-Díaz, M., Alape- Girón, A., Núñez, V., Andrés, V., Gutiérrez, J.M., Calvete, J.J., 2013. Snake venomics of *Lachesis muta rhombeata* and genus-wide antivenomics assessment of the paraspecific immunoreactivity of two antivenoms evidence the high compositional and immunological conservation across *Lachesis.* J. Proteom. 89, 112–123. https://doi.org/10.1016/j.jprot.2013.05.028.

Rosa, J.G., Albuquerque, C.Z., Mattaraia, V.G.M., Santoro, M.L., 2019. Comparative study of platelet aggregation and secretion induced by bothrops jararaca snake venom and thrombin. Toxicon, 159, 50-60. https://doi.org/10.1016/j.toxicon.2019.01.003.

Rucavado, A., Sánchez, E.F., Franceschi, A., Magalhães, A., Gutiérrez, J.M., 1999. Characterization of the local tissue damage induced by LHFII,

a metalloproteinase with weak hemorrhagic activity isolated from *Lachesis muta muta* snake venom. Toxicon 37, 9, 1297-312. https://doi.org/10.1016/s0041-0101(98)00268-2.

Salvador, G.H.M., Gomes, A.A.S., Bryan-Quirós, W., Fernández, J., Lewin, M.R., Gutiérrez, J.M., Lomonte, B., Fontes, M.R.M., 2019. Structural basis for phospholipase A_2-like toxin inhibition by the synthetic compound Varespladib (LY315920). Sci. Rep. 9, 1, 17203. https://doi.org/10.1038/s41598-019-53755-5.

Salvador, G. H. M.; Borges, R. J.; Lomonte, B.; Lewin, M. R.; Fontes, M. R. M., 2021. The Synthetic Varespladib Molecule Is a Multi-Functional Inhibitor for PLA2 and PLA2- like Ophidic Toxins. Biochim. Biophys. Acta Gen. Subj. 1865, 7, 129913. https://doi.org/10.1016/j.bbagen.2021.129913.

Sánchez, E.F. (2004). Biochemical properties of a bushmaster snake venom serine proteinase (LV-Ka), and its kinin releasing activity evaluated in rat mesenteric arterial rings. J. Pharmacol. Sci. 96, 3, 333-42. https://doi.org/10.1254/jphs.fpj04005x.

Sánchez, E.F., Costa, M.I.E., Chávez-Olórtegui, C., Assakura, M.T., Mandelbaum, F.R., Diniz, C.R., 1995. Characterization of a hemorrhagic factor, LHF-I, isolated from the bushmaster snake (*Lachesis muta muta*) venom. Toxicon 33, 12, 1653-67. https://doi.org/10.1016/0041-0101(95)00097-6.

Sánchez, E.F.,, Magalhães, A., Diniz, C.R., 1987. Purification of haemorrhagic factor (LHF-I) from the venom of the bushmaster snake, *Lachesis muta muta*. Toxicon 25, 6, 611-19. https://doi.org/10.1016/0041-0101(87)90107-3.

Sánchez, E.F. Magalhães, A., Mandelbaum, F.R., Diniz, C.R., 1991.

Purification and characterization of the hemorrhagic factor II from the venom of the bushmaster snake (*Lachesis muta muta*). Biochim. Biophys. Acta 1074, 3, 347-56. https://doi.org/10.1016/0304-4165(91)90084-t.

Sanz, L., Escolano, J., Ferretti, M., Biscoglio, M.J., Rivera, E., Crescenti, E.J., Ângulo, Y., Lomonte, B., Gutiérrez, J.M., Calvete, J.J., 2008. Snake venomics of the South and Central American bushmasters. Comparison of the toxin composition of *Lachesis muta* gathered from proteomic versus transcriptomic analysis. J. Proteom. 71, 1, 46-60. https://doi.org/10.1016/j.jprot.2007.10.004.

Seo, T., Sakon, T., Nakazawa, S., Nishioka, A., Watanabe, K., Matsumoto, K., Akasaka, M., Shioi, N., Sawada, H., Araki, S.,2017. Haemorrhagic snake venom metalloproteases and human ADAMs Cleave LRP5/6, which disrupts cell-cell adhesions *in vitro* and induces haemorrhage *in vivo*. FEBS J. 284,11, 1657-71. https://doi.org/10.1111/febs.14066.

Silva, A.C., Ferreira, L.G., Duarte, M.E., Fujii, M.T., Sanchez, E.F., Noseda, M.D., Fuly, A.L., 2016. Protective effect of the sulfated Agaran isolated from the Red Seaweed *Laurencia aldingensis* against toxic effects of the venom of the snake, *Lachesis muta*. *Mar.* Biotechnol. 18, 6, 619-29. https://doi.org/10.1007/s10126-016-9722-8.

Silva-Carvalho, R., Gaspar, M.Z., Quadros, L.H.B., Lobo, L.G.G., Rogério, L.M.,
Santos, N.T.S., Zerbinatti, M.C., Santarém, C.L., Silva, E.O., Gerez, J.R., Silva Jr., N.J., Lamonte, B., Rowan, E.G., Floriano, R.S., 2021. *In Vivo* treatment with Varespladib, a phospholipase A_2 inhibitor, prevents the peripheral neurotoxicity and systemic disorders induced by *Micrurus corallinus* (Coral Snake) venom in rats. Toxicol. Lett. 356, 54-63. https://doi.org/10.1016/j.toxlet.2021.11.003.

SINAN, 2021. Sistema de Informação de Agravos de Notificação (SINAN), Ministério da Saúde, Brasil. http://www.saude.gov.br/saude-de-a-z/acidentes-por-animais-peconhentos#animais Acesso em 05.12.2022.

Soares, M.R., Oliveira-Carvalho, A.L., Wermelinger, L.S., Zingali, R.B., Ho, P.L., Junqueira-de-Azevedo, I.L., Diniz, M.R., 2005. Identification of novel bradykinin potentiating peptides and C-type natriuretic peptide from *Lachesis muta* venom. Toxicon 46, 1, 31–8. https://doi.org/10.1016/j.toxicon.2005.03.006.

Solano, G., Gómez, A., Corrales, G., Chacón, D., Estrada, R., León, G., 2018. Contributions of the snake venoms of *Bothrops asper*, *Crotalus simus* and *Lachesis stenophrys* to the paraspecificity of the Central American polyspecific antivenom (PoliVal-ICP).Toxicon144,1–6. https://doi.org/10.1016/j.toxicon.2018.01.016.

Stephano, M.A., Guidolin, R., Higashi, H.G., Tambourgi, D.V., Sant'Anna, O.A., 2005. The improvement of the therapeutic anti-*Lachesis muta* serum production in horses. Toxicon 45, 4, 467–73. https://doi.org/10.1016/j.toxicon.2004.12.006.

Stransky,. S, Costal-Oliveira, F., Lopes-de-Souza, L., Guerra-Duarte, C., Chávez- Olórtegui, C., Braga, V.M.M., 2018. *In vitro* assessment of cytotoxic activities of *Lachesis muta muta* snake venom. PLoS Negl. Trop. Dis. 12, 4, e0006427. https://doi.org/10.1371/journal.pntd.0006427.

Sunitha, K., Hemshekhar, M., Sebastian-Santhosh, M., Suresh-Kumar, M., Kemparaju, K., Girish, K.S., 2011. Inhibition of hemorrhagic activity of viper venoms by n-acetyl cysteine: involvement of n-acetyl and thiol groups. Curr. Top. Med. Chem. 11, 20, 2589–600. https://doi.org/10.2174/156802611797633401.

Sunitha, K., Suresh, P., Sebastian-Santhosh, M., Hemshekhar, M., Thushara, R.M., Marathe, G.P., Thirunavukkarasu, C., Kemparaju, K.,

Suresh-Kumar, M., Girish, K.S., 2013. Inhibition of hyaluronidase by N-acetyl cysteine and glutathione: role of thiol group in hyaluronan protection. Int. J. Biol. Macromol. 55, 39–46. https://doi.org/10.1016/j.ijbiomac.2012.12.047.

Torres-Huaco, F.D., Werneck, C.C., Vicente, C.P., Vassequi-Silva, T., Nery-Diez, A.C., Mendes, C.B., Antunes, E., Marangoni, S., Damico, D.C., 2013. Rapid purification and procoagulant and platelet aggregating activities of rhombeobin: a thrombin- like/gyroxin-like enzyme from *Lachesis muta rhombeata* snake venom. Biomed. Res. Int. 2013, 903292. https://doi.org/10.1155/2013/903292.

Tribuiani, N., Silva, A.M., Ferraz, M.C., Silva, M.G., Bentes, A.P., Graziano, T.S.,
Santos, M.G., Cogo, J.C., Varanda, E.A., Groppo, F.C., Cogo, K., Oshima-Franco, Y., 2014. *Vellozia flavicans* Mart. ex Schult. hydroalcoholic extract inhibits the neuromuscular blockade induced by *Bothrops jararacussu* venom. BMC Complement Altern. Med. 14, 48. https://doi.org/10.1186/1472-6882-14-48.

Tribuiani, N., Tavares, M.O., Santana, M.N., Fontana Oliveira, I.C., Amaral Filho, J.D., Silva, M.G., Santos, M.G., Cogo, J.C., Floriano, R.S., Cogo-Müller, K., Oshima-Franco, Y., 2017. Neutralising ability of *Terminalia fagifolia* extract (Combretaceae) against the *in vitro* neuromuscular effects of *Bothrops jararacussu* venom. Nat. Prod. Res. 31, 23, 2783–7. https://doi.org/10.1080/14786419.2017.1292506.

Varespladib, 2011. Am. J. Cardiovasc. Drugs 11, 2,137–43. https://doi.org/10.2165/11533650-000000000-00000.

Wang, Y., Zhang, J., Zhang, D., Xiao, H., Xiong, S., Huang, C., 2018. Exploration of the inhibitory potential of varespladib for snakebite envenomation. Molecules 23, 2,
391. https://doi.org/10.3390/molecules23020391.

Weinberg, M.L., Felicori, I.F., Bello, C.A., Magalhães, H.P.B., Almeida, A.P., Magalhães, A., Sanchez, E.F., 2004. Biochemical properties of a bushmaster snake venom serine proteinase (LV-Ka), and its kinin releasing activity evaluated in rat mesenteric arterial rings. J. Pharmacol. Sci. 96, 3, 333–42. https://doi.org/10.1254/jphs.fpj04005x

Wiezel, G.A., Bordon, K.C., Silva, R.R., Gomes, M.S., Cabral, H., Rodrigues, V.M., Ueberheide, B., Arantes, E.C., 2019. Subproteome of *Lachesis muta rhombeata* venom and preliminary studies on LmrSP-4, a novel snake venom serine proteinase. J. Venom Anim. Toxins Incl. Trop. Dis. 25, e147018. https://doi.org/10.1590/1678- 9199-jvatitd-1470-18.

Wiezel, G.A.,, Santos, P.K., Cordeiro, F.A., Bordon, K.C.F., Selistre-de-Araújo, H.S., Ueberheide, B., Arantes, E.C., 2015. Identification of hyaluronidase and phospholipase B in *Lachesis muta rhombeata* venom. Toxicon 107(Pt B), 359-68. https://doi.org/10.1016%2Fj.toxicon.2015.08.029.

Youngman, N.J., Walker, A., Naude, A., Coster, K., Sundman, E., Fry, B.G. 2020. Varespladib (LY315920) neutralises phospholipase A_2 mediated prothrombinase- inhibition induced by bitis snake venoms. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 236, 108818. https://doi.org/10.1016/j.cbpc.2020.108818.

Zamudio, K., Greene, H.W., 1997. Phylogeography of the bushmaster (*Lachesis muta*: Viperidae): implications for neotropical biogeography, systematics, and conservation. Biol. J. Linn. Soc. 62, 3, 421-42. https://doi.org/10.1006/bijl.1997.0162.

Zanotty, Y., Álvarez, M., Perdomo, L., Sánchez, E.E., Giron, M.E., Jimenez, J.C., Suntravat, M., Guerrero, B., Ibarra, C., Montero, Y.,

Medina, R., Navarrete, L.F., Rodríguez-Acosta, A., 2019. Mutacytin-1, a new C-type lectin-like protein from the Venezuelan cuaima (*Lachesis muta muta* linnaeus, 1766) (Serpentes: Viperidae) snake venom inducing cardiotoxicity in developing zebrafish (*Danio rerio*) embryos. Zebrafish 16, 4, 379–87. https://doi.org/10.1089/zeb.2019.1731.

Zinenko, O., Tovstukham, I., Korniyenko, Y., 2020. PLA_2 inhibitor varespladib as an alternative to the antivenom treatment for bites from Nikolsky's viper Vipera berus nikolskii. Toxins 12, 6, 356. https://doi.org/10.3390%2Ftoxins12060356.

ANEXO A - NORMAS DE PUBLICAÇÃO

TOXICON

An Interdisciplinary Journal on the Toxins Derived from Animals, Plants and Microorganisms

AUTHOR INFORMATION PACK

TABLE OF CONTENTS

ISSN: 0041-0101

DESCRIPTION

Toxicon has an open access companion *Toxicon: X* which has the same aims and scope, editorial board and peer-review process. To submit to *Toxicon: X* visit https://www.editorialmanager.com/TOXCX/default.aspx.

An introductory offer Toxicon: X - full waiver of the Open Access fee.

Toxicon's "aims and scope" are to publish: articles containing the results of original research on problems related to toxins derived from animals, plants and microorganisms papers on novel findings related to the **chemical**, **pharmacological**, **toxicological**, and **immunological** properties of natural **toxinsmolecular biological** studies of toxins and other **genes** from **poisonous** and **venomous** organisms that advance understanding of the role or function of toxins clinical observations on poisoning and envenoming where a new therapeutic principle has been proposed or a decidedly superior clinical result has been obtained. material on the use of toxins as tools in studying biological processes and material on subjects related to **venom** and **antivenom** problems. articles on the translational application of toxins, for example as drugs and insecticides epidemiological studies on envenoming or poisoning, so long as they highlight a previously unrecognised medical problem or provide insight into the prevention or medical treatment of envenoming or poisoning. Retrospective surveys of hospital records, especially those lacking species identification, will not be considered for publication. Properly designed prospective community-based surveys are strongly encouraged. articles describing well-known activities of venoms, such as antibacterial, anticancer, and analgesic activities of arachnid venoms, without any attempt to define the mechanism of action or purify the active component, will not be considered for publication in *Toxicon*. review articles on problems related to **toxinology**.

To encourage the exchange of ideas, sections of the journal may be devoted to Short Communications,Letters to the Editor and activities of the affiliated societies.

Toxicon strives to publish articles that are current and of broad interest and importance to the toxinology research community. Emphasis will be placed upon articles that further the understandingand knowledge of toxinology.

Types of paper

Full-Length Research Papers: Articles containing the results of original research on problems related to toxins derived from animals, plants and microorganisms.

Short Communications: Short communications differ from full manuscripts only in that the research study does not lend itself to an extended presentation. Even though brief, the Short communication should represent a complete, coherent and self contained study. The quality of Short Communications is expected to be as good as that of full articles, and both full articles and Short communications will be refereed in an identical manner. The form is identical to that for a full article except that the report should not be divided into Introduction, Materials and Methods, Results and Discussion. An abstract of not more than 75 words should be provided. The Short Communication may not be longer than five double-spaced typewritten pages (not including references, tables and figures) and should include not more than two tables of two figures or one of each.

Letters to the Editor: These may be published if judged by the Editor to be of interest to the broad field of toxinology or of special significance to a smaller group of workers in a specialized field of toxinology. They should be headed `Letter to the Editor' which should be followed by a title for the communication. Names of authors and affiliations should be at the end of the letter.

Announcements: *Toxicon* will only accept for publication announcements of great interest to toxinologists, such as notices of relevant meetings and symposia and activities of the International Society of Toxinology, The Brazilian Society of Toxinology, and the North American Society of Toxinology.

Reviews and mini-Reviews: *Toxicon* will publish reviews and mini-reviews on topics of interest to toxinologists. Suggestions for reviews or mini-reviews can be made at any time to the Editor-in-Chief or the relevant Associate Editor. In addition, articles of significant broad interest to toxinologists that are published in journals other than *Toxicon* may be abstracted in the Reviews section of *Toxicon*. Readers who feel that a particular article or book should be abstracted in this section are encouraged to bring their opinion to the attention of one of the Editor-in-Chief.

Clinical reports: *Toxicon* will publish clinical reports on poisoning or envenoming where a new therapeutic principle has been proposed or a decidedly superior clinical result has been established.Please consult the Clinical Reports Guidelines

AUDIENCE

Toxicologists, toxinologists, molecular biologists and chemists.

IMPACT FACTOR

2021: 3.035 © Clarivate Analytics Journal Citation Reports 2022

ABSTRACTING AND INDEXING

EDITORIAL BOARD

Venoms-based drug and insecticide discovery, Venom-derived ion channel modulators, Venomevolution, Toxin structure and function

Igor Križaj, Jožef Stefan Institute, Department of Molecular and Biomedical Sciences, Ljubljana, Slovenia

Toxinology,(animal venoms,envenomation,neurotoxins,anticoagulantprocoagulantsn tithrombotics,emorrhagins,myotoxins,molecular mechanisms of action, toxin receptors, venomics, venoms to drugs). Secreted phospholipases A2, their inhibitors and activators, physiological and pathological role. Proteomics and protein structure, structure-function relationships.

Gérard Lambeau, Institute of Molecular and Cellular Pharmacology, Valbonne, France Phospholipase A2, venom, mammals, phospholipase A2 receptors, phospholipids**Bruno Lomonte**, Costa Rica University, San José, Costa Rica

Snake venoms, myotoxic phospholipases A2, antibodies, venomics

Sulan Luo, Guangxi University, Medical School, Nanning, China

The structure and function of conotoxins (conopeptides) native to South China Sea and their molecular receptors (ion channels), molecular biology, electrophysiology, biotechnology and marine medicine,

and neuropeptides in marine organisms, among others.

Stephen P. Mackessy, University of Northern Colorado, Department of Biological Sciences, Greeley, Colorado, United States of America

Venom proteomics, evolution of venom systems, protein structure and function, herpetology **Frank Mari**, National Institute of Standards and Technology, Gaithersburg, Maryland, United States of America **Dietrich Mebs**, Goethe University Frankfurt Institute of General Medicine, Frankfurt am Main, Germany Toxinology, Natural Toxins

Wuelton Monteiro, Doctor Heitor Vieira Dourado Tropical Medicine Foundation, MANAUS, Brazil

Snakebites, Scorpion stings, Epidemiology, Public Health

Yehu Moran, Hebrew University of Jerusalem, Department of Ecology Evolution and Behavior, Jerusalem, Israel

Venom evolution, Cnidaria, sea anemones, non-coding RNA

Ashis K. Mukherjee, Institute of Advanced Study in Science and Technology, Guwahati, India

Venom toxins, commercial antivenom, antivenom production, peptidomimetic, drug discovery **Mario S. Palma**, IBRC-UNESP, Department Basic and Applied Biology, Laboratory of Structural Biology & Zoochemistry, Rio Claro, Brazil

Peptides, proteins, low molecular mass toxins, structural biology of toxins, Arthropod venoms

Gyorgy Panyi, University of Debrecen, Department of Biophysics and Cell Biology, Debrecen, Hungary

Kv1.3, scorpion toxin, T-cell activation, autoimmune diseases, molecular pharmacology, Kv channelgating, K channel inactivation

Mark A. Poli, US Army Medical Research Institute of Infectious Diseases, Fort Detrick, Maryland, United States of America

Toxicology; Immunodiagnostics

Lourival Possani, National Autonomous University of Mexico Biotechnology Institute, Cuernavaca, Mexico Scorpion venom components: isolation structure and function, Toxinology, Biochemistry, MolecularBiology, scopion venom components, isolation and function

Manuela Pucca, Federal University of Roraima, BOA VISTA, Brazil

Snakebite, scorpion toxins, immunotoxinology, autoimmune disease, immunomodulation

Franklin Riet-Correa, Federal University of Bahia, SALVADOR, Brazil

Plant poisoning, ruminants, horses, pathology, diagnostic laboratories

Adolfo Rafael de Roodt, University of Buenos Aires, Faculty of Medicine, Buenos Aires, Argentina

Venoms, Toxins, Antivenom, Antitoxins, Envenomation

Ashlee Rowe, The University of Oklahoma, Department of Biology, Norman, Oklahoma, United States of America

Voltage-gated sodium channels, scorpion venom, neurotoxins, sensory physiology

Helena Safavi-Hemami, University of Copenhagen, Department of Biomedical Sciences, København, Denmark

Venom, biomedicine, biochemistry, evolution, cone snails

Elda Sanchez, Texas A&M University Kingsville National Natural Toxins Research Center, Kingsville,

GUIDE FOR AUTHORS

Your Paper Your Way

We now differentiate between the requirements for new and revised submissions. You may choose to submit your manuscript as a single Word or PDF file to be used in the refereeing process. Only when your paper is at the revision stage, will you be requested to put your paper in to a 'correct format' for acceptance and provide the items required for the publication of your article.
To find out more, please visit the Preparation section below.

INTRODUCTION

[TOXICON] has an open access companion journal, [TOXICON: X]

Official Journal of The International Society on Toxinology (http://www.toxinology.org/), *Toxicon*'s "aims and scope" are laid down in the journal

as:

To publish:
• articles containing the results of original research on problems related to toxins derived from animals, plants and microorganisms
• papers on novel findings related to the chemical, pharmacological, toxicological, and immunologicalproperties of natural toxins
• molecular biological studies of toxin and other genes from poisonous and venomous organisms thatadvance understanding of the role or function of toxins
• clinical observations on poisoning and envenoming where a new therapeutic principle has been proposed or a decidedly superior clinical result has been obtained. Toxicon will not accept single-case reports unless they describe new, previously unreported, clinical features; envenomings or poisonings by rare animals, plants, fungi or microorganisms for which there is little or no clinical information in the literature; or treatment that employs a new therapeutic principle for which effectiveness is convincingly demonstrated. Such case reports must include: (1) expert species identification; (2) meticulous clinical documentation of symptoms, signs, laboratory data, treatment and clinical outcomes; (3) originality (adding to knowledge of the clinical phenotype); (4) where feasible, photographic documentation of clinical signs.
• material on the use of toxins as tools in studying biological processes and material on subjects related to venom-antivenom problems
• articles on the translational application of toxins, for example as drugs and insecticides
• epidemiological studies on envenoming or poisoning, so long as they highlight a previously unrecognised medical problem or provide insight into the prevention or medical treatment of envenoming or poisoning. Retrospective surveys of hospital records, especially those lacking species identification, will not be considered for publication. Properly designed prospective community-based surveys are strongly encouraged.
• articles describing well-known activities of venoms, such as antibacterial, anticancer, and analgesic activities of venoms, without any attempt to define the mechanism of action or purify the active component, will not be considered for publication in Toxicon
• review articles on problems related to toxinology.

And
To encourage the exchange of ideas, sections of the journal may be devoted to Short Communications, Letters to the Editor and activities of the International Society on Toxinology.

Toxicon strives to publish articles that are current and of broad interest and importance to the toxinology research community. Emphasis will be placed upon articles that further the understandingand knowledge of toxinology.

Types of paper

Full-Length Research Papers: Articles containing the results of original research on problems related to toxins derived from animals, plants and microorganisms.

Short Communications: Short communications differ from full manuscripts only in that the research study does not lend itself to an extended presentation. Even though brief, the Short communication should represent a complete, coherent and self contained study. The quality of Short Communications is expected to be as good as that of full articles, and both full articles and Short communications will

be refereed in an identical manner. The form is identical to that for a full article except that the report should not be divided into Introduction, Materials and Methods, Results and Discussion. An abstract of not more than 75 words should be provided. The Short Communication may not be longer than five double-spaced typewritten pages (not including references, tables and figures) and should include not more than two tables or two figures or one of each.

Correspondence: These may be published if judged by the Editor to be of interest to the broad field of toxinology or of special significance to a smaller group of workers in a specialized field of toxinology. They should be headed ` Correspondence ' which should be followed by a title for the communication. Names of authors and affiliations should be at the end of the letter.

Reviews and Short Reviews: Articles of interest to toxinologists which are published in journals other than *Toxicon* may be abstracted in the Reviews section of *Toxicon*. Readers who feel that a particular article or book should be abstracted in this section are encouraged to bring their opinions to the attention of one of the Review Editors. Mini-Reviews and proposals for mini-Reviews are welcome

Case reports: *Toxicon* will publish clinical reports on poisoning where a new therapeutic principle has been proposed or a decidedly superior clinical result has been established. Please observe the following: Case Reports Guidelines.

Submission checklist

You can use this list to carry out a final check of your submission before you send it to the journal for review. Please check the relevant section in this Guide for Authors for more details.

Ensure that the following items are present:

One author has been designated as the corresponding author with contact details:
• E-mail address
• Full postal address

All necessary files have been uploaded:
Manuscript:
• Include keywords
• All figures (include relevant captions)
• All tables (including titles, description, footnotes)
• Ensure all figure and table citations in the text match the files provided
• Indicate clearly if color should be used for any
figures in print*Graphical Abstracts / Highlights files*
(where applicable) *Supplemental files* (where
applicable)

Further considerations
• Manuscript has been 'spell checked' and 'grammar checked'
• All references mentioned in the Reference List are cited in the text, and vice versa
• Permission has been obtained for use of copyrighted material from other sources (including theInternet)
• A competing interests statement is provided, even if the authors have no competing interests todeclare
• Journal policies detailed in this guide have been reviewed
• Referee suggestions and contact details provided, based on journal requirements

For further information, visit our Support Center.

BEFORE YOU BEGIN

Ethics in publishing

Please see our information on Ethics in publishing.

Human and animal rights

If the work involves the use of human subjects, the author should ensure that the work described has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans; Uniform Requirements for manuscripts submitted to

Biomedical journals. Authors should include a statement in the manuscript that informed consent was obtained for experimentation with human subjects. The privacy rights of human subjects must always be observed.

Although prospective studies are generally preferable to retrospective studies, in some situations the latter may provide valuable information that is otherwise unobtainable. Retrospective reviews of existing medical records that are intended for publication require prior approval from an appropriate Institutional Review Board (IRB). These must protect the anonymity of patients involved. In single case reports, the patient's consent is required if they are identifiable. In all clinical prospective or retrospective studies, the approval number of the relevant IRB should be quoted in the manuscript.

All animal experiments should comply with the ARRIVE guidelines and should be carried out in accordance with the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines, EU Directive 2010/63/EU for animal experiments, or the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978) and the authors should clearly indicate in the manuscript that such guidelines have been followed. For all experiments involving humans or animals, details of the appropriate ethics committee approval(s) must be provided

Declaration of competing interest

Corresponding authors, on behalf of all the authors of a submission, must disclose any financial and personal relationships with other people or organizations that could inappropriately influence (bias) their work. Examples of potential conflicts of interest include employment, consultancies, stock ownership, honoraria, paid expert testimony, patent applications/registrations, and grants or other funding. All authors, including those *without* competing interests to declare, should provide the relevant information to the corresponding author (which, where relevant, may specify they have nothing to declare). Corresponding authors should then use this tool to create a shared statement and upload to the submission system at the Attach Files step. **Please do not convert the .docx template to another file type. Author signatures are not required.**

Submission declaration and verification

Submission of an article implies that the work described has not been published previously (except in the form of an abstract, a published lecture or academic thesis, see 'Multiple, redundant or concurrent publication' for more information), that it is not under consideration for publication elsewhere, that its publication is approved by all authors and tacitly or explicitly by the responsible authorities where the work was carried out, and that, if accepted, it will not be published elsewhere in the same form, in English or in any other language, including electronically without the written consent of the copyright- holder. To verify compliance, your article may be checked by Crossref Similarity Check and other originality or duplicate checking software.

Preprints

Please note that preprints can be shared anywhere at any time, in line with Elsevier's sharing policy. Sharing your preprints e.g. on a preprint server will not count as prior publication (see 'Multiple, redundant or concurrent publication' for more information).

Use of inclusive language

Inclusive language acknowledges diversity, conveys respect to all people, is sensitive to differences, and promotes equal opportunities. Content should make no assumptions about the beliefs or commitments of any reader; contain nothing which might imply that one individual is superior to another on the grounds of age, gender, race, ethnicity, culture, sexual orientation, disability or health condition; and use inclusive language throughout. Authors should ensure that writing is free from bias, stereotypes, slang, reference to dominant culture and/or cultural assumptions. We advise to seek gender neutrality by using plural nouns ("clinicians, patients/clients") as default/wherever possible to avoid using "he, she," or "he/she." We recommend avoiding the use of descriptors that refer to personal attributes such as age, gender, race, ethnicity,

culture, sexual orientation, disability or health condition unless they are relevant and valid. When coding terminology is used, we recommend to avoid offensive or exclusionary terms such as "master", "slave", "blacklist" and "whitelist". We suggest using alternatives that are more appropriate and (self-) explanatory such as "primary", "secondary", "blocklist" and "allowlist". These guidelines are meant as a point of reference to help identify appropriate language but are by no means exhaustive or definitive.

Reporting sex- and gender-based analyses

Reporting guidance

For research involving or pertaining to humans, animals or eukaryotic cells, investigators should integrate sex and gender-based analyses (SGBA) into their research design according to funder/ sponsor requirements and best practices within a field. Authors should address the sex and/or gender dimensions of their research in their article. In cases where they cannot, they should discuss this as a limitation to their research's generalizability. Importantly, authors should explicitly state what definitions of sex and/or gender they are applying to enhance the precision, rigor and reproducibility of their research and to avoid ambiguity or conflation of terms and the constructs to which they refer (see Definitions section below). Authors can refer to the Sex and Gender Equity in Research (SAGER) guidelines and the SAGER guidelines checklist. These offer systematic approaches to the use and editorial review of sex and gender information in study design, data analysis, outcome reporting and research interpretation - however, please note there is no single, universally agreed-upon set of guidelines for defining sex and gender.

Definitions

Sex generally refers to a set of biological attributes that are associated with physical and physiological features (e.g., chromosomal genotype, hormonal levels, internal and external anatomy). A binary sex categorization (male/female) is usually designated at birth ("sex assigned at birth"), most often based solely on the visible external anatomy of a newborn. Gender generally refers to socially constructed roles, behaviors, and identities of women, men and gender-diverse people that occur in a historical and cultural context and may vary across societies and over time. Gender influences how people view themselves and each other, how they behave and interact and how power is distributed in society. Sex and gender are often incorrectly portrayed as binary (female/male or woman/man) and unchanging whereas these constructs actually exist along a spectrum and include additional sex categorizations and gender identities such as people who are intersex/have differences of sex development (DSD) or identify as non-binary. Moreover, the terms "sex" and "gender" can be ambiguous—thus it is important for authors to define the manner in which they are used. In addition to this definition guidance and the SAGER guidelines, the resources on this page offer further insight around sex and gender in research studies.

Changes to authorship

Authors are expected to consider carefully the list and order of authors **before** submitting their manuscript and provide the definitive list of authors at the time of the original submission. Any addition, deletion or rearrangement of author names in the authorship list should be made only **before** the manuscript has been accepted and only if approved by the journal Editor. To request such a change, the Editor must receive the following from the **corresponding author**: (a) the reason for the change in author list and (b) written confirmation (e-mail, letter) from all authors that they agree with the addition, removal or rearrangement. In the case of addition or removal of authors, this includes confirmation from the author being added or removed.

Only in exceptional circumstances will the Editor consider the addition, deletion or rearrangement of authors **after** the manuscript has been accepted. While the Editor considers the request, publication of the manuscript will be suspended. If the manuscript has already been published in an online issue, any requests approved by the Editor will result in a corrigendum.

Article transfer service
This journal uses the Elsevier Article Transfer Service to find the best home for your manuscript. This means that if an editor feels your manuscript is more suitable for an alternative journal, you might be asked to consider transferring the manuscript to such a journal. The recommendation might be provided by a Journal Editor, a dedicated Scientific Managing Editor, a tool assisted recommendation, or a combination. If you agree, your manuscript will be transferred, though you will have the opportunity to make changes to the manuscript before the submission is complete. Please note that your manuscript will be independently reviewed by the new journal. More information.

Copyright

Upon acceptance of an article, authors will be asked to complete a 'Journal Publishing Agreement' (see more information on this). An e-mail will be sent to the corresponding author confirming receipt of the manuscript together with a 'Journal Publishing Agreement' form or a link to the online version of this agreement.

Subscribers may reproduce tables of contents or prepare lists of articles including abstracts for internal circulation within their institutions. Permission of the Publisher is required for resale or distribution outside the institution and for all other derivative works, including compilations and translations. If excerpts from other copyrighted works are included, the author(s) must obtain written permission from the copyright owners and credit the source(s) in the article. Elsevier has preprinted forms for use by authors in these cases.

Elsevier supports responsible sharing
Find out how you can share your research published in Elsevier journals.

Role of the funding source

You are requested to identify who provided financial support for the conduct of the research and/or preparation of the article and to briefly describe the role of the sponsor(s), if any, in study design; in the collection, analysis and interpretation of data; in the writing of the report; and in the decision to submit the article for publication. If the funding source(s) had no such involvement, it is recommended to state this.

Open access

Please visit our Open Access page for more information.

Language (usage and editing services)
Please write your text in good English (American or British usage is accepted, but not a mixture of these). Authors who feel their English language manuscript may require editing to eliminate possible grammatical or spelling errors and to conform to correct scientific English may wish to use the English Language Editing service available from Elsevier's Author Services.

Submission

Our online submission system guides you stepwise through the process of entering your article details and uploading your files. The system converts your article files to a single PDF file used in the peer-review process. Editable files (e.g., Word, LaTeX) are required to typeset your article for final publication. All correspondence, including notification of the Editor's decision and requests for revision, is sent by e-mail.

Submit your article
Please submit your article via https://www.editorialmanager.com/TOXCON/default.aspx.

Referees

Authors are required to suggest the names and addresses of a minimum of three individuals who could expertly review their manuscript. These suggested reviewers should not be from the same institutions as the authors, or have published or collaborated with the authors in the past three years. **At least one of the suggested reviewers must be a member of the Editorial Board.** The Editors reservethe right to use these or other reviewers.

PREPARATION

Queries

For questions about the editorial process (including the status of manuscripts under review) or fortechnical support on submissions, please visit our Support Center.

NEW SUBMISSIONS

Submission to this journal proceeds totally online and you will be guided stepwise through the creation and uploading of your files. The system automatically converts your files to a single PDF file, which is used in the peer-review process.

As part of the Your Paper Your Way service, you may choose to submit your manuscript as a single file to be used in the refereeing process. This can be a PDF file or a Word document, in any format or lay- out that can be used by referees to evaluate your manuscript. It should contain high enough quality figures for refereeing. If you prefer to do so, you may still provide all or some of the source files at the initial submission. Please note that individual figure files larger than 10 MB must be uploaded separately.

References

There are no strict requirements on reference formatting at submission. References can be in any style or format as long as the style is consistent. Where applicable, author(s) name(s), journal title/ book title, chapter title/article title, year of publication, volume number/book chapter and the article

number or pagination must be present. Use of DOI is highly encouraged. The reference style used by the journal will be applied to the accepted article by Elsevier at the proof stage. Note that missing data will be highlighted at proof stage for the author to correct.

Formatting requirements

All manuscripts must have double or 1.5-line spacing, and must contain **both page numbers and consecutive line numbering.** Articles must be divided into clearly numbered sections as outlined below. Aside from this, there are no strict formatting requirements, but all manuscripts must contain the essential elements needed to convey the details of the study, including Abstract, Keywords, Highlights, Introduction, Materials and Methods, Results, Discussion, Conclusions, and Figures/Tables with Captions.

If your article includes Videos and/or other Supplementary material, this should be included in yourinitial submission for peer review purposes.

Figures and tables embedded in text

Please ensure the figures and the tables included in the single file are placed next to the relevant text in the manuscript, rather than at the bottom or the top of the file. The corresponding caption shouldbe placed directly below the figure or table.

Peer review

This journal operates a single anonymized review process. All contributions will be initially assessed by the editor for suitability for the journal. Papers deemed suitable are then typically sent to a minimum of two independent expert reviewers to assess the scientific quality of the paper. The Editor is responsible for the final decision regarding acceptance or rejection of articles. The Editor's decision is final. Editors are not involved in decisions about papers which they have written themselves or have been written by family members or colleagues or which relate to products or services in which the editor has an interest. Any such submission is subject to all of the journal's usual procedures, with peer review handled independently of the relevant editor and their research groups. More information on types of peer review.

REVISED SUBMISSIONS

Use of word processing software

Regardless of the file format of the original submission, at revision you must provide us with an editable file of the entire article. Keep the layout of the text as simple as possible. Most formatting codes will be removed and replaced on processing the article. The electronic text should be prepared in a way very similar to that of conventional

manuscripts (see also the Guide to Publishing with Elsevier). See also the section on Electronic artwork.
To avoid unnecessary errors you are strongly advised to use the 'spell-check' and 'grammar-check' functions of your word processor.

LaTeX
You are recommended to use the latest Elsevier article class to prepare your manuscript and BibTeXto generate your bibliography.
Our Guidelines has full details.

Article structure

Subdivision - numbered sections
Divide your article into clearly defined and numbered sections. Subsections should be numbered
1.1 (then 1.1.1, 1.1.2, ...), 1.2, etc. (the abstract is not included in section numbering). Use this numbering also for internal cross-referencing: do not just refer to 'the text'. Any subsection may be given a brief heading. Each heading should appear on its own separate line.

Introduction
State the objectives of the work and provide an adequate background, avoiding a detailed literaturesurvey or a summary of the results.

Material and methods
Provide sufficient details to allow the work to be reproduced by an independent researcher. Methods that are already published should be summarized, and indicated by a reference. If quoting directly from a previously published method, use quotation marks and also cite the source. Any modifications to existing methods should also be described.

Experimental
Provide sufficient details to allow the work to be reproduced by an independent researcher. Methods that are already published should be summarized, and indicated by a reference. If quoting directly from a previously published method, use quotation marks and also cite the source. Any modifications to existing methods should also be described.

Experimental procedures

All animal experiments should be carried out in accordance with the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines, the European Communities Council Directive of 24 November 1986 (86/609/EEC) or the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978) and the authors should clearly indicate in the manuscript that such guidelines have been followed. ***All animal studies need to ensure they comply with the ARRIVE guidelines. More information can be found at http://www.nc3rs.org.uk/page.asp?id=1357.***

Theory/calculation
A Theory section should extend, not repeat, the background to the article already dealt with in the Introduction and lay the foundation for further work. In contrast, a Calculation section represents apractical development from a theoretical basis.

Results
Results should be clear and concise.

Discussion
This should explore the significance of the results of the work, not repeat them. A combined Results and Discussion section is often appropriate. Avoid extensive citations and discussion of published literature.

Conclusions
The main conclusions of the study may be presented in a short Conclusions section, which

may standalone or form a subsection of a Discussion or Results and Discussion section.

Appendices
If there is more than one appendix, they should be identified as A, B, etc. Formulae and equations in appendices should be given separate numbering: Eq. (A.1), Eq. (A.2), etc.; in a subsequent appendix, Eq. (B.1) and so on. Similarly for tables and figures: Table A.1; Fig. A.1, etc.

Essential title page information

• ***Title.*** Concise and informative. Titles are often used in information-retrieval systems. Avoid abbreviations and formulae where possible.
• ***Author names and affiliations.*** Please clearly indicate the given name(s) and family name(s) of each author and check that all names are accurately spelled. You can add your name between parentheses in your own script behind the English transliteration. Present the authors' affiliation addresses (where the actual work was done) below the names. Indicate all affiliations with a lower- case superscript letter immediately after the author's name and in front of the appropriate address. Provide the full postal address of each affiliation, including the country name and, if available, the e-mail address of each author.
• ***Corresponding author.*** Clearly indicate who will handle correspondence at all stages of refereeing and publication, also post-publication. This responsibility includes answering any future queries about Methodology and Materials. **Ensure that the e-mail address is given and that contact details are kept up to date by the corresponding author.**
• ***Present/permanent address.*** If an author has moved since the work described in the article was done, or was visiting at the time, a 'Present address' (or 'Permanent address') may be indicated as a footnote to that author's name. The address at which the author actually did the work must be retained as the main, affiliation address. Superscript Arabic numerals are used for such footnotes.

Highlights

Highlights are mandatory for this journal, as they increase the discoverability of your article via search engines. They consist of a short collection of bullet points that capture the major findings of your research as well as new methods that were used during the study (if any). Please take a look at the examples here: example Highlights.Highlights should not be be used to provide a summary of background information or methods.

Highlights should be submitted in a separate editable file in the online submission system. Please use 'Highlights' in the file name and include 3 to 5 bullet points (maximum 125 characters, including spaces, per bullet point). Highlights should NOT contain abbreviations/acronyms

Abstract

A concise and factual abstract is required. The abstract should state briefly the purpose of the research, the principal results and major conclusions. An abstract is often presented separately from the article, so it must be able to stand alone. For this reason, References should be avoided, but if essential, then cite the author(s) and year(s). Also, non-standard or uncommon abbreviations should be avoided, but if essential they must be defined at their first mention in the abstract itself.

Graphical abstract
A graphical abstract is mandatory for this journal. It should summarize the contents of the article in a concise, pictorial form designed to capture the attention of a wide readership online. Authors must provide images that clearly represent the work described in the article. Graphical abstracts should be submitted as a separate file in the online submission system. Image size: please provide an image with a minimum of 531 × 1328 pixels (h × w) or proportionally more. The image should be readable at a size of 5 × 13 cm using a regular screen resolution of 96 dpi. Preferred file types: TIFF, EPS, PDF or MS Office files. You can view Example Graphical Abstracts on our information site.

Keywords

Immediately after the abstract, provide a maximum of 6 keywords, using American spelling and avoiding general and plural terms and multiple concepts (avoid, for example, 'and', 'of'). Be sparing with abbreviations: only abbreviations firmly established in the field may be eligible. These keywordswill be used for indexing purposes.

Abbreviations

Define abbreviations that are not standard in this field in a footnote to be placed on the first page of the article. Such abbreviations that are unavoidable in the abstract must be defined at their first mention there, as well as in the footnote. Ensure consistency of abbreviations throughout the article.

Acknowledgements

Collate acknowledgements in a separate section at the end of the article before the references and do not, therefore, include them on the title page, as a footnote to the title or otherwise. List here those individuals who provided help during the research (e.g., providing language help, writing assistanceor proof reading the article, etc.).

Formatting of funding sources

List funding sources in this standard way to facilitate compliance to funder's requirements:

Funding: This work was supported by the National Institutes of Health [grant numbers xxxx, yyyy]; the Bill & Melinda Gates Foundation, Seattle, WA [grant number zzzz]; and the United States Institutesof Peace [grant number aaaa].

It is not necessary to include detailed descriptions on the program or type of grants and awards. When funding is from a block grant or other resources available to a university, college, or other research institution, submit the name of the institute or organization that provided the funding.

If no funding has been provided for the research, it is recommended to include the following sentence:

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Math formulae

Please submit math equations as editable text and not as images. Present simple formulae in line with normal text where possible and use the solidus (/) instead of a horizontal line for small fractional terms, e.g., X/Y. In principle, variables are to be presented in italics. Powers of e are often more conveniently denoted by exp. Number consecutively any equations that have to be displayed separately from the text (if referred to explicitly in the text).

Footnotes

Footnotes should be used sparingly. Number them consecutively throughout the article. Many word processors build footnotes into the text, and this feature may be used. Should this not be the case, indicate the position of footnotes in the text and present the footnotes themselves separately at theend of the article.

- Make sure you use uniform lettering and sizing of your original artwork.
- Preferred fonts: Arial (or Helvetica), Times New Roman (or Times), Symbol, Courier.
- Number the illustrations according to their sequence in the text.
- Use a logical naming convention for your artwork files.
- Indicate per figure if it is a single, 1.5 or 2-column fitting image.
- For Word submissions only, you may still provide figures and their captions, and tables within asingle file at the revision stage.
- Please note that individual figure files larger than 10 MB must be provided in separate source files.

A detailed guide on electronic artwork is available.

You are urged to visit this site; some excerpts from the detailed information are given here.

Formats

Regardless of the application used, when your electronic artwork is finalized, please 'save as' or convert the images to one of the following formats (note the resolution requirements for line drawings,halftones, and line/halftone combinations given below):
EPS (or PDF): Vector drawings. Embed the font or save the text as 'graphics'.
TIFF (or JPG): Color or grayscale photographs (halftones): always use a minimum of 300 dpi.TIFF (or JPG): Bitmapped line drawings: use a minimum of 1000 dpi.
TIFF (or JPG): Combinations bitmapped line/half-tone (color or grayscale): a minimum of 500 dpiis required.

Please do not:

- Supply files that are optimized for screen use (e.g., GIF, BMP, PICT, WPG); the resolution is too low.
- Supply files that are too low in resolution.
- Submit graphics that are disproportionately large for the content.

Color artwork

Please make sure that artwork files are in an acceptable format (TIFF (or JPEG), EPS (or PDF), or MS Office files) and with the correct resolution. If, together with your accepted article, you submit usable color figures then Elsevier will ensure, at no additional charge, that these figures will appear in color online (e.g., ScienceDirect and other sites) regardless of whether or not these illustrations are reproduced in color in the printed version. **For color reproduction in print, you will receive information regarding the costs from Elsevier after receipt of your accepted article**. Please indicate your preference for color: in print or online only. Further information on the preparation of electronic artwork.

Figure captions

Ensure that each illustration has a caption. A caption should comprise a brief title (**not** on the figure itself) and a description of the illustration. Keep text in the illustrations themselves to a minimum butexplain all symbols and abbreviations used.

Tables

Please submit tables as editable text and not as images. Tables can be placed either next to the relevant text in the article, or on separate page(s) at the end. Number tables consecutively in accordance with their appearance in the text and place any table notes below the table body. Be sparing in the use of tables and ensure that the data presented in them do not duplicate results described elsewhere in the article. Please avoid using vertical rules and shading in table cells.

References

Citation in text

Please ensure that every reference cited in the text is also present in the reference list (and vice versa). Any references cited in the abstract must be given in full. Unpublished results and personal communications are not recommended in the reference list, but may be mentioned in the text. If these references are included in the reference list they should follow the standard reference style of the

journal and should include a substitution of the publication date with either 'Unpublished results' or 'Personal communication'. Citation of a reference as 'in press' implies that the item has been accepted for publication.

Reference links

Increased discoverability of research and high quality peer review are ensured by online links to the sources cited. In order to allow us to create links to abstracting and indexing services, such as Scopus, Crossref and PubMed, please ensure that data provided in the references are correct. Please note that incorrect surnames, journal/book titles, publication year and pagination may prevent link creation. When copying references, please be careful as they may already contain errors. Use of the DOI is highly encouraged.

A DOI is guaranteed never to change, so you can use it as a permanent link to any electronic article. An example of a citation using DOI for an article not yet in an issue is: VanDecar J.C., Russo R.M., James D.E., Ambeh W.B., Franke M. (2003). Aseismic continuation of the Lesser Antilles slab beneath northeastern Venezuela. Journal of Geophysical Research, https://doi.org/10.1029/2001JB000884. Please note the format of such citations should be in the same style as all other references in the paper.

Web references
As a minimum, the full URL should be given and the date when the reference was last accessed. Any further information, if known (DOI, author names, dates, reference to a source publication, etc.), should also be given. Web references can be listed separately (e.g., after the reference list) under a different heading if desired, or can be included in the reference list.

Data references
This journal encourages you to cite underlying or relevant datasets in your manuscript by citing them in your text and including a data reference in your Reference List. Data references should include the following elements: author name(s), dataset title, data repository, version (where available), year, and global persistent identifier. Add [dataset] immediately before the reference so we can properly identify it as a data reference. The [dataset] identifier will not appear in your published article.

Preprint references
Where a preprint has subsequently become available as a peer-reviewed publication, the formal publication should be used as the reference. If there are preprints that are central to your work or that cover crucial developments in the topic, but are not yet formally published, these may be referenced. Preprints should be clearly marked as such, for example by including the word preprint, or the name of the preprint server, as part of the reference. The preprint DOI should also be provided.

References in a special issue
Please ensure that the words 'this issue' are added to any references in the list (and any citations inthe text) to other articles in the same Special Issue.

Reference management software
Most Elsevier journals have their reference template available in many of the most popular reference management software products. These include all products that support Citation Style Language styles, such as Mendeley. Using citation plug-ins from these products, authors only need to select the appropriate journal template when preparing their article, after which citations and bibliographies will be automatically formatted in the journal's style. If no template is yet available for this journal, please follow the format of the sample references and citations as shown in this Guide. If you use reference management software, please ensure that you remove all field codes before submitting the electronic manuscript. More information on how to remove field codes from different reference management software.

Reference formatting
There are no strict requirements on reference formatting at submission. References can be in any style or format as long as the style is consistent. Where applicable, author(s) name(s), journal title/ book title, chapter title/article title, year of publication, volume number/book chapter and the article number or pagination must be present. Use of DOI is highly encouraged. The reference style used by the journal will be applied to the accepted article by Elsevier at the proof stage. Note that missing data will be highlighted at proof stage for the author to correct. If you do wish to format the references yourself they should be arranged according to the following examples:

Reference style
Text: All citations in the text should refer to:

1. *Single author:* the author's name (without initials, unless there is ambiguity) and the year ofpublication;

2. *Two authors:* both authors' names and the year of publication;
3. *Three or more authors:* first author's name followed by 'et al.' and the year of publication. Citations may be made directly (or parenthetically). Groups of references can be listed either first alphabetically, then chronologically, or vice versa.
Examples: 'as demonstrated (Allan, 2000a, 2000b, 1999; Allan and Jones, 1999)…. Or, as demonstrated (Jones, 1999; Allan, 2000)… Kramer et al. (2010) have recently shown …'
List: References should be arranged first alphabetically and then further sorted chronologically if necessary. More than one reference from the same author(s) in the same year must be identified by the letters 'a', 'b', 'c', etc., placed after the year of publication.
Examples:
Reference to a journal publication:
Van der Geer, J., Hanraads, J.A.J., Lupton, R.A., 2010. The art of writing a scientific article. J. Sci.Commun. 163, 51–59. https://doi.org/10.1016/j.Sc.2010.00372.
Reference to a journal publication with an article number:
Van der Geer, J., Hanraads, J.A.J., Lupton, R.A., 2018. The art of writing a scientific article. Heliyon.19, e00205. https://doi.org/10.1016/j.heliyon.2018.e00205.
Reference to a book:
Strunk Jr., W., White, E.B., 2000. The Elements of Style, fourth ed.
Longman, New York.Reference to a chapter in an edited book:
Mettam, G.R., Adams, L.B., 2009. How to prepare an electronic version of your article, in: Jones, B.S.,Smith , R.Z. (Eds.), Introduction to the Electronic Age. E-Publishing Inc., New York, pp. 281–304.
Reference to a website:
Cancer Research UK, 1975. Cancer statistics reports for the UK.
http://www.cancerresearchuk.org/aboutcancer/statistics/cancerstatsreport/ (accessed 13 March 2003).
Reference to a dataset:
[dataset] Oguro, M., Imahiro, S., Saito, S., Nakashizuka, T., 2015. Mortality data for Japanese oak wilt disease and surrounding forest compositions. Mendeley Data, v1. https://doi.org/10.17632/ xwj98nb39r.1.
Reference to software:
Coon, E., Berndt, M., Jan, A., Svyatsky, D., Atchley, A., Kikinzon, E., Harp, D., Manzini, G., Shelef, E., Lipnikov, K., Garimella, R., Xu, C., Moulton, D., Karra, S., Painter, S., Jafarov, E., & Molins, S., 2020. Advanced Terrestrial Simulator (ATS) v0.88 (Version 0.88). Zenodo. https://doi.org/10.5281/zenodo.3727209.

Journal abbreviations source
Journal names should be abbreviated according to the List of Title Word Abbreviations.

Taxonomy and toxin nomenclature

Authors should ensure that they are using the most up-to-date taxonomic description of any animal or plant described in their manuscript.For some taxa, up-to-date taxonomic information is readily available online (e.g., for spiders at http://www.wsc.nmbe.ch). For new, unidentified,or potentially cryptic species, a voucher specimen should be deposited in a natural history museum, or other facility, as appropriate.

Authors are encouraged to use rational nomenclature for all toxins described in their manuscript. *Ad hoc* naming schemes only serve to introduce confusion and make it difficult to compare toxins and establish evolutionary relationships. Suggested nomenclature has been published for spider toxins (King et al., Toxicon 52, 264-276, 2008; PMID 18619481), centipede toxins (Undheim et al., Mol. Biol. Evol.31, 2124-2148, 2014; PMID 24847043),sea anemone toxins (Oliveira et al., Toxicon 60, 539-550, 2012; PMID 22683676), and some classes of scorpion toxins (Tytgat et al., Trends Pharmacol. Sci. 20, 444-447, 1999;PMID 10542442).

Video

Elsevier accepts video material and animation sequences to support and enhance your scientific research. Authors who have video or animation files that they wish to submit with their article are strongly encouraged to include links to these within the body of the article. This can be done in the same way as a figure or table by referring to the video or animation content and noting in the body text where it should be placed. All submitted files should be properly labeled so that they directly relate to the video file's content. In order to ensure that your video or animation material is directly usable, please provide the file in one of our recommended file formats with a preferred maximum

size of 150 MB per file, 1 GB in total. Video and animation files supplied will be published online in the electronic version of your article in Elsevier Web products, including ScienceDirect. Please supply 'stills' with your files: you can choose any frame from the video or animation or make a separate image. These will be used instead of standard icons and will personalize the link to your video data. For more detailed instructions please visit our video instruction pages. Note: since video and animation cannot be embedded in the print version of the journal, please provide text for both the electronic and the print version for the portions of the article that refer to this content.

Data visualization

Include interactive data visualizations in your publication and let your readers interact and engage more closely with your research. Follow the instructions here to find out about available data visualization options and how to include them with your article.

Supplementary material

Supplementary material such as applications, images and sound clips, can be published with your article to enhance it. Submitted supplementary items are published exactly as they are received (Excel or PowerPoint files will appear as such online). Please submit your material together with the article and supply a concise, descriptive caption for each supplementary file. If you wish to make changes to supplementary material during any stage of the process, please make sure to provide an updated file. Do not annotate any corrections on a previous version. Please switch off the 'Track Changes' option in Microsoft Office files as these will appear in the published version.

Research data

This journal requires and enables you to share data that supports your research publication where appropriate, and enables you to interlink the data with your published articles. Research data refers to the results of observations or experimentation that validate research findings. To facilitate reproducibility and data reuse, this journal also encourages you to share your software, code, models, algorithms, protocols, methods and other useful materials related to the project.

Below are a number of ways in which you can associate data with your article or make a statement about the availability of your data when submitting your manuscript. When sharing data in one of these ways, you are expected to cite the data in your manuscript and reference list. Please refer to the "References" section for more information about data citation. For more information on depositing, sharing and using research data and other relevant research materials, visit the research data page.

Data linking

If you have made your research data available in a data repository, you can link your article directly to the dataset. Elsevier collaborates with a number of repositories to link articles on ScienceDirect with relevant repositories, giving readers access to underlying data that gives them a better understanding of the research described.

There are different ways to link your datasets to your article. When available, you can directly link your dataset to your article by providing the relevant information in the submission system. For moreinformation, visit the database linking page.

For supported data repositories a repository banner will automatically appear next to your publishedarticle on ScienceDirect.

In addition, you can link to relevant data or entities through identifiers within the text of your manuscript, using the following format: Database: xxxx (e.g., TAIR: AT1G01020; CCDC: 734053; PDB: 1XFN).

Research Elements

This journal enables you to publish research objects related to your original research – such as data, methods, protocols, software and hardware – as an additional paper in Research Elements.

Research Elements is a suite of peer-reviewed, open access journals which make your research objects findable, accessible and reusable. Articles place research objects into context by providing detailed descriptions of objects and their application, and linking to the associated original research articles. Research Elements articles can be prepared by you, or by one of your collaborators.

During submission, you will be alerted to the opportunity to prepare and submit a Research Elementsarticle.

More information can be found on the Research Elements page.

Data statement

To foster transparency, we require you to state the availability of your data in your submission if your data is unavailable to access or unsuitable to post. This may also be a requirement of your funding body or institution. You will have the opportunity to provide a data statement during the submission process. The statement will appear with your published article on ScienceDirect. For more information, visit the Data Statement page..

AFTER ACCEPTANCE

Online proof correction

To ensure a fast publication process of the article, we kindly ask authors to provide us with their proof corrections within two days. Corresponding authors will receive an e-mail with a link to our online proofing system, allowing annotation and correction of proofs online. The environment is similar to MS Word: in addition to editing text, you can also comment on figures/tables and answer questions from the Copy Editor. Web-based proofing provides a faster and less error-prone process by allowing you to directly type your corrections, eliminating the potential introduction of errors.

If preferred, you can still choose to annotate and upload your edits on the PDF version. All instructions for proofing will be given in the e-mail we send to authors, including alternative methods to the onlineversion and PDF.

We will do everything possible to get your article published quickly and accurately. Please use this proof only for checking the typesetting, editing, completeness and correctness of the text, tables and figures. Significant changes to the article as accepted for publication will only be considered at this stage with permission from the Editor. It is important to ensure that all corrections are sent back to us in one communication. Please check carefully before replying, as inclusion of any subsequent corrections cannot be guaranteed. Proofreading is solely your responsibility.

Offprints

The corresponding author, at no cost, will be provided with a PDF file of the article via e-mail (the PDF file is a watermarked version of the published article and includes a cover sheet with the journal cover image and a disclaimer outlining the terms and conditions of use). For an extra charge, paper offprints can be ordered via the offprint order form which is sent once the article is accepted for publication.

AUTHOR INQUIRIES

Visit the Elsevier Support Center to find the answers you need. Here you will find

everything from Frequently Asked Questions to ways to get in touch.
You can also check the status of your submitted article or find out when your accepted article willbe published.