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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

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Abstract

In this study, we examined the potential use of *N*-acetyl-L-cysteine (NAC) in association with a polyvalent antivenom and as stand-alone therapy to reduce the acute local and systemic effects induced by *Lachesis muta muta* venom in rats. Male Wistar rats (300–350 g) were exposed to *L. m. muta* venom (1.5 mg/kg – i.m.) and subsequently treated with anti-*Bothrops*/*Lachesis* serum (antivenom:venom ratio 1:3 'v/w' – i.p.) and NAC (150 mg/kg – i.p.) separately or in association; the animals were monitored for 120 min to assess changes in temperature, locomotor activity, local oedema formation and the prevalence of haemorrhaging. After this time, animals were anesthetized in order to collect blood samples through intracardiac puncture and then euthanized for collecting tissue samples; the hematological-biochemical and histopathological analysis were performed through conventional methods. *L. m. muta* venom produced pronounced local oedema, subcutaneous haemorrhage and myonecrosis, with both antivenom and NAC successfully reducing the extent of the myonecrotic lesion when individually administered; their association also prevented the occurrence of subcutaneous haemorrhage. Venom-induced creatine kinase (CK) release was significantly prevented by NAC alone or in combination with antivenom; NAC alone failed to reduce the release of hepatotoxic (alanine aminotransferase) and nephrotoxic (creatinine) serum biomarkers induced by *L. m. muta* venom. Venom induced significant increase of leucocytes which was also associated with an increase of neutrophils, eosinophils and monocytes; antivenom and NAC partially reduced these

alterations, with NAC alone significantly preventing the increase of eosinophils whereas neither NAC or antivenom prevented the increase in monocytes. Venom did not induce changes in the erythrogram parameters. In the absence of a suitable antivenom, NAC has the potential to reduce a number of local and systemic effects caused by *L. m. muta* venom.

Keywords: Viperidae snake; *L. m. muta* venom; local and systemic toxicity; *N*-acetyl-L-cysteine, antivenom, neutralization.

1. Introduction

Envenomation by Viperidae snakes represent a critical public health problem in most countries of Latin America (Chippaux, 2017; Gutiérrez et al., 2020; Ochoa-Avilés et al., 2020). In Brazil, more than 24,000 cases of snakebites were officially notified in 2019 by Notifiable Diseases Information System of the Brazilian Ministry of Health (SINAN, 2020), being them mostly caused by *Bothrops* snakes (~85.5%), followed by *Crotalus* (~10.7%), *Lachesis* (~2.5%) 'Viperidae' and *Micrurus* (~1.3%) 'Elapidae' snakes. The low incidence of bites by *Lachesis* snakes probably reflects their low population density in the Amazon river basin (=*L. muta muta*) and Atlantic rainforest (=*L. muta rhombeata*), including their secretive behaviour and habitat preferences, although these envenomations are potentially severe (Campbell and Lamar, 2004; Costa and Bérnils, 2018; Diniz-Sousa et al., 2020; Nogueira et al., 2019).

Lachesis snakes – bushmaster or surucucu – (Viperidae: Crotalinae) represent the largest viper found in Americas and comprise four terrestrial oviparous species (*L. stenophrys*, *L. melanocephala*, *L. acrochorda* and *L. muta*) distributed in Central and

South America: (1) *L. stenophrys* is distributed along the Caribbean coast of Central America, (2) *L. melanocephala* occurs along the Pacific coast of southwestern Costa Rica and extreme western of Panama, (3) *L. acrochorda* is found in western Panama and northwestern Colombia and (4) *L. muta* is widely distributed in the Amazon river basin and east coast of Brazil (Campbell and Lamar, 2004; Costa and Bérnils, 2018; Diniz-Sousa et al., 2020; McDiarmid et a., 1999; Nogueira et al., 2019) (Figure 1). Together, these species comprising the second most frequent snakes involved in ophidian accidents in Americas, being exceeded only by those ones of the genus *Bothrops* (Magalhães et al., 2018; Diniz-Sousa et al., 2020).

Envenomation by *Lachesis* spp. is characterized by intense local pain accompanied by pronounced oedema and myonecrosis (Fuly et al., 2000, 2003; Damico et al., 2006, 2008, 2012; Ferreira et al., 2009), including severe systemic alterations such as haemorrhage (Rucavado et al., 1999; Sánchez et al., 1987, 1991, 1995), coagulopathy (Estevão-Costa et al., 2000; Fuly et al., 1997; Torres-Huaco et al., 2013), pulmonary thrombosis (Dias et al., 2016a) and cardiovascular disorders consisting of prolonged bradycardia and hypotension (Angel-Camilo et al., 2020; Dias et al., 2016a, 2016b; Diniz and Oliveira, 1992; Giovanni-De-Simone et al., 1997). These effects have been related to presence of non-enzymatic proteins, e.g., bradykinin-potentiating peptides and bradykinin receptor antagonists (Graham et al., 2005; Pinheiro-Júnior et al., 2018; Pla et al., 2013; Sanz et al., 2008; Soares et al., 2005), including a variety of enzymatically active proteins such as snake venom metalloproteases (SVMPs), serine proteases (SVSPs), phospholipases A_2 (PLA_2), C-type lectins and L-amino acid oxidase (Bregge-Silva et al., 2012; Cordeiro et al., 2018; Diniz-Sousa et al., 2018; Junqueira-de-Azevedo et al., 2006; Madrigal et al., 2012; Weinberg et al., 2004; Wiezel et al., 2019).

In Central and South America, envenomations caused by *Lachesis* spp. are treated with polyvalent antivenoms (Madrigal et al., 2017; Pla et al., 2013; Solano et al., 2018), whereas the efficacy of therapeutically useful adjuncts to treat local and systemic effects induced by these venoms needs further investigation (De Oliveira et al., 2014, 2016, 2020; Faioli et al., 2013; Marques et al., 2019). In Brazil, polyvalent antivenoms (=anti-*Bothrops*/*Lachesis* serum) used to treat envenomations by *Lachesis muta* are produced mainly by Instituto Butantan (IB, São Paulo, SP, Brazil), Fundação Ezequiel Dias (FUNED, Belo Horizonte, MG, Brazil) and Instituto Vital Brazil (IVB, Niterói, RJ, Brazil). These antivenoms consist in immunoglobulins raised in hyperimmunized horses using a pool of venoms that including five *Bothrops* snake's species (=*B. jararaca*, *B. alternatus*, *B. jararacussu*, *B. moojeni* and *B. neuwiedi*) and *Lachesis muta* (IB and IVB); there is also a second type of antivenom consisted in F(ab')2 fragments obtained by pepsin digestion of immunoglobulins raised in hyperimmunized horses using *Bothrops jararaca* and *Lachesis muta* venoms (FUNED).

In this study, we aimed to investigate the potential use of *N*-acetyl-L-cysteine (NAC), a drug known for scavenging a wide variety of reactive oxygen species (ROS) and increasing the intracellular level of glutathione (Briguori et al., 2011; Nicoletta et al., 2006; Paller and Patten, 1984; Thielemann and Rosenblut, 1990), to reduce the local and systemic effects induced by *L. m. muta* venom in rats. NAC is rapidly distributed and is free of systemic toxicity, being used therapeutically in the treatment of respiratory disorders (Fok, 2009; El-Hafiz et al., 2013; Moroz et al., 2019; Rogliani et al., 2019; Zhang et al., 2017). In recent studies, NAC has also demonstrated to be effective at inhibiting the haemorrhagic and nephrotoxic activities induced by Viperidae snakes from the New (Barone et al., 2014) and Old World (Sunitha et al., 2011, 2013).

According to Sunitha et al. (2011), NAC inhibited the haemorrhagic activity of *Echis carinatus* and *Vipera russelli* (=*Daboia russelii*) (Viperidae: Viperinae) venoms in mice; NAC also significantly decreased the hyaluronidase activity of *Echis carinatus* venom in vitro (Sunitha et al., 2013). In addition, Barone et al. (2014) reported that NAC improves the renal redox status and renal functions in mice exposed to *Bothrops jararaca* and *Crotalus durissus terrificus* (Viperidae: Crotalinae) venoms.

As the use of NAC has been shown to have a number of favourable physiological outcomes, we have systematically examined the potential use of this drug as an adjunct to the recommended polyvalent (anti-*Bothrops*/*Lachesis*) antivenom for envenomation by *Lachesis muta* and as a stand-alone therapy on the acute local and systemic effects of *L. m. muta* envenomation in rats using hematological, biochemical and histopathological approaches.

2. Material and methods

2.1. Reagents and venom

N-acetyl-L-cysteine (A7250) was obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA) and polyvalent antivenom (anti-*Bothrops*/*Lachesis* serum) was from Instituto Butantan (São Paulo, SP, Brazil). *Lachesis muta muta* venom was provided by Center for Biological Studies and Research of the Pontifical Catholic University of Goiás (Goiânia, GO, Brazil) through Dr. Nelson J. Silva Jr.; venom was obtained from one male adult snake from Altamira in the northern Brazilian state of Pará in the Brazilian Amazon (3°25′55″S and 51°56′6″W), lyophilized and stored at −20 °C until used.

147 *2.2. Animals*

148 Wistar rats (300–350 g; 2–3 months old) obtained from Central Bioterium of the
149 University of Western São Paulo (UNOESTE, Presidente Prudente, SP, Brazil) were
150 housed in plastic cages (2 animals/cage) with a wood-shaving substrate, at 23 ± 1 °C on
151 a 12-h light/dark cycle with lights on at 6 a.m. The animals had free access to food and
152 water. The experimental procedures were approved by an institutional Committee for
153 Ethics in Animal Use (CEUA/UNOESTE, Protocol No. 5808/2019) and were done
154 according to the general ethical guidelines for animal use established by the Brazilian
155 Society of Laboratory Animal Science (SBCAL) and Brazilian Federal Law No. 11.794
156 of October 8, 2008, in conjunction with the guidelines for animal experiments
157 established by the Brazilian National Council for Animal Experimentation (CONCEA).
158
159 *2.3. Experimental design*
160 The animals were transferred to the Experimental Bioterium of the University of
161 Western São Paulo (UNOESTE, Presidente Prudente, SP, Brazil) and allowed to adapt
162 for two weeks, under the same conditions as described above, before initiating the
163 experimental procedures. The animals were then randomly distributed in six
164 experimental groups (n = 6 per group) identified as: group **G1** (control) – intramuscular
165 (gastrocnemius muscle) injection of saline 0.9%; group **G2** (*N*-acetyl-L-cysteine
166 'NAC') – intraperitoneal injection of NAC at a dose of 150 mg/kg; group **G3** (*L. m.*
167 *muta* venom) – intramuscular (gastrocnemius muscle) injection of venom at a dose of
168 1.5 mg/kg; group **G4** (*L. m . muta* venom + antivenom) – intramuscular (gastrocnemius
169 muscle) injection of venom '1.5 mg/kg' followed by intraperitoneal injection of
170 antivenom at an antivenom:venom ratio of 1:3 (v/w); group **G5** (*L. m. muta* venom +

NAC) – intramuscular (gastrocnemius muscle) injection of venom '1.5 mg/kg' followed by intraperitoneal injection of NAC '150 mg/kg'; group **G6** (*L. m. muta* venom + NAC + antivenom) – intramuscular (gastrocnemius muscle) injection of venom '1.5 mg/kg' followed by intraperitoneal injections of NAC '150 mg/kg' and antivenom '1:3 (v/w)'. The animals were monitored before (T_0) and at various intervals (T_{30}, T_{60}, T_{90} and T_{120} min) and then anesthetized with thiopental (1.8 mg/kg – i.p.) (Cristália®, São Paulo, SP, Brazil) to collect blood samples for hematological and biochemical analysis; the animals were afterwards euthanized by an overdose of thiopental and subjected to dissection of gastrocnemius muscle (right 'local of venom-injection' and left hind limbs), kidney, liver, lung and heart samples for histopathological analysis. To induce acute toxicity in rats, the dose of 1.5 mg of venom/kg was chosen based on previous investigations (Dias et al., 2016a, 2016b) and confirmed in pilot experiments in order to produce pronounced local and systemic alterations in at least 120 min post envenomation. The ability of anti-*Bothrops*/*Lachesis* serum produced by Instituto Butantan (IB) (São Paulo, SP, Brazil) to neutralize the *L. m. muta* venom-induced local and systemic effects in rats was assessed by administrating antivenom via intraperitoneal at an antivenom:venom ratio of 1:3 (v/w) immediately after exposing the animals to an intramuscular injection of venom (1.5 mg/kg); this antivenom:venom ratio was based on the manufacturer's stated neutralizing capacity for the antivenom (1 ml of antivenom neutralizes 3 mg of *L. muta* venom). The dose of 150 mg of NAC/kg was based on previous investigations (Oliveira Filho et al. 2015; Portella et al., 2004) and its lack of toxicity confirmed in pilot experiments. Fig. 2 summarizes the experimental design described in this section.

2.4. Clinical monitoring

The clinical monitoring (T_0 'basal', T_{30}, T_{60}, T_{90} and T_{120} min 'post exposure to venom') consisted in measuring the auricular temperature using a digital thermometer, locomotor activity using a semi-quantitative toxicity scale, local oedema formation using Image J software (National Institute of Health, Bethesda, Maryland, USA), including macroscopic monitoring for myonecrosis formation and occurrence of local or widespread bleeding. The animals were subsequently anesthetized by a non-lethal dose of thiopental (1.8 mg/kg – i.p.) and subjected to intracardiac puncture. Some blood samples were collected in vacuum EDTA tubes for hematological analysis and other ones in vacuum sodium citrate tubes for hemostatic analysis (BD Vacutainer®); blood samples for biochemical analysis were collected in vacuum tubes without anticoagulant (BD Vacutainer®). The animals were then placed in a dorsal position with the hind limbs positioned in the same orientation and images were taken of the ventral view 15 cm from both hind limbs, followed by analysis via Image J software to measure oedema; length calculations were determined by placing a centimeter scale close to the limbs (Supplementary material, Figure S1); values were expressed as a percentage of the magnitude of RHL and LHL observed in control animals 'G1', considered 100%. The animals were then exposed to an overdose of thiopental, followed by dissection of muscle (gastrocnemius), renal, hepatic, pulmonary and cardiac tissue samples for histopathological analysis. The action of antivenom and NAC to prevent the venom-induced lack of locomotor activity was determined based on a semi-quantitative toxicity scale (severity score) using the open field test. A cage with walls to prevent escape was divided by grid lines into nine squares (155 cm^2 each), with (0) indicating a complete absence of paralysis (more than 15 times crossing the grid lines), (1) indicating a slight

lack of locomotor activity (between 10 and 15 times crossing the grid lines), (2) indicating a moderate lack of locomotor activity (between 5 and 10 times crossing the grid lines) and (3) indicating a severe lack of locomotor activity (less than 5 times crossing the grid lines or no locomotor activity).

2.5. Haematological analysis

Erythrocytes, haemoglobin, haematocrit, mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), red blood cell distribution width (RDW-SD) and total leukocyte count were determined using the POCH-100 iV DIFF haematology analyser (Sysmex do Brasil Indústria e Comércio Ltda., São 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 (Panótico® – Laborclin Produtos para Laboratórios Ltda., Pinhais, PR, Brazil) and then analysed under an E-200 Nikon light microscope (Nikon Inc., Tokyo, Japan) at 100x magnification. The concentration of fibrinogen was determined by calorimetric technique using a refractometer ATC-ITREF-200 (Instrutemp Instrumentos de Medição Ltda., São Paulo, SP, Brazil) and the results were expressed as mg/dL. The total plasmatic 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.

2.6. Serum biochemical analysis

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 'CK-MB' (code 05401763-190), alanine aminotransferase 'ALT' (code 04718569-190) and creatinine 'Cr' (code 05401755-190), respectively. The assays were performed using a Cobas C111 analyser (Roche Holding AG, Basel, Switzerland).

2.7. Histopathological analysis

After collecting the blood samples, the animals were subsequently euthanized by an overdose of thiopental and subjected to dissection in order to collect tissue samples from the 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. Serial of 3–5 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 lesional score, as essentially described elsewhere (Gerez et al., 2015; Grenier et al., 2011).

266

267 *2.8. Statistical analysis*

268 The results were expressed as the mean ± SEM and statistical comparisons were
269 performed with Student's *t*-test or one-way ANOVA followed by the Tukey test, with
270 $p < 0.05$ indicated significance. In histopathological analysis, Dunn's test was applied to
271 evaluate the lesional score. All data were analyzed using Microcal Origin 8 SR4 v.
272 8.0951 (Microcal Software Inc., Northampton, MA, USA) and GraphPad Prism 4 v.
273 4.03 (GraphPad Software Inc., La Jolla, CA, USA) software.

274

275 **3. Results**

276 *3.1. Clinical manifestations produced by L. m. muta venom in rats and treatment*
277 *with antivenom and NAC*

278 *L. m. muta* venom (1.5 mg/kg – i.m.) administered into gastrocnemius muscle
279 (right hind limb 'RHL') produced pronounced oedema (Figure 3A) accompanied by
280 intense myonecrosis formation and subcutaneous haemorrhage (Figure $3B_3$).
281 Administration of antivenom (antivenom:venom ratio 1:3 'v/w' – i.p.) (G4) or NAC
282 (150 mg/kg – i.p.) (G5) reduced the venom-induced oedema by approximately 14% ($p <$
283 0.05 compared to G3 'venom' or G5 'venom + NAC', $n = 6$) and 5%, respectively, with
284 their combination promoting approximately 16% of reduction ($p < 0.05$ compared to G3
285 'venom' or G5 'venom + NAC', $n = 6$) (Figure 3A), whereas the myonecrosis caused
286 by venom was markedly reduced by both agents (Figures $3B_4$ and $3B_5$); antivenom and
287 NAC in combination also abolished venom-induced subcutaneous haemorrhage (Figure
288 $3B_6$). Fig. $3B_1$ and $3B_2$ show the unaffected hind limbs in animals exposed to an
289 injection of saline 0.9% (G1) or NAC alone (G2), respectively. No animal showed

290 evidence for oedema or necrosis formation in the contralateral gastrocnemius muscle
291 (left hind limb 'LHL'). There was no apparent systemic haemorrhage in the groups of
292 animals exposed to *L. m. muta* venom independently of the treatment. After 120 min,
293 following envenomation, locomotor activity was reduced by venom (severity score '3')
294 (G3) and the treatment with antivenom (G4) and NAC (G5), separately administered,
295 decreased the severity score to '2', whereas the combination of both agents (G6)
296 decreased the severity score to '1'. Table 1 summarizes the results of the clinical
297 assessment.
298
299 *3.2. Biochemical profile of rats exposed to L. m. muta venom and treated with*
300 *antivenom and NAC*
301 In those animals exposed to an intramuscular injection of *L. m. muta* venom (G3),
302 there was a significant increase in CK serum release after 120 min envenomation ($p <$
303 0.05 compared to G1 'control', $n = 6$); NAC administered alone (G2) did not affect the
304 basal CK serum level. Venom-induced CK release was significantly prevented by
305 antivenom (G4) and NAC (G5) when administered individually or combining both of
306 agents (G6) ($p < 0.05$ compared to G3 'venom' for all these treatments, $n = 6$) (Figure
307 4A). There was no alteration in CK-MB level in those animals exposed to *L. m. muta*
308 venom after 120 min envenomation (Figure 4B). Venom (G3) produced pronounced
309 increase of ALT serum ($p < 0.05$ compared to G1 'control', $n = 6$), which was
310 efficiently prevented by antivenom alone (G4) or associated with NAC (G6) ($p < 0.05$
311 compared to G3 'venom' for both treatments, $n = 6$); however, NAC alone (G5) failed
312 to prevent the venom-induced ALT release (Figure 4C). Venom (G3) also caused
313 significant increase in the creatinine serum level ($p < 0.05$ compared to G1 'control', *n*

= 6), with antivenom alone (G4) or associated with NAC (G6) ($p < 0.05$ compared to G5 'venom + NAC', $n = 6$) preventing partially this alteration; NAC alone (G5) did not prevent the increase of creatinine serum induced by venom (Figure 4D).

3.3. Haematological profile of rats exposed to L. m. muta venom and treatment with antivenom and NAC

In those animals exposed to *L. m. muta* venom alone (G3) or also in those ones treated with antivenom (G4 or G6) and NAC (G5 or G6), there was no significant alteration in total erythrocytes count, haemoglobin, haematocrit, mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), red blood cell distribution width (RDW) and total plasma protein after 120 min envenomation; NAC (G2) did not cause chances in these parameters when administered by itself. In addition, venom (G3) induced a slight increase in fibrinogen blood level and platelets count after 120 min envenomation; the treatments with antivenom (G4) and NAC (G5) avoided partially the increase of fibrinogen and platelets, with the association of these agents (G6) being more effective to avoid the increase of the former; NAC (G2) did not cause changes in these parameters when administered by itself (Supplementary material, Figure S2A and S2B). Venom (G3) also induced a mild increase in reticulocytes, with antivenom (G4) being effective at keeping the reticulocytes values close to normal whereas NAC (G5) slightly prevented the increase of reticulocytes by venom; the combined action of antivenom and NAC (G6) also kept the reticulocytes values close to normal. NAC alone (G2) did not cause alterations in reticulocytes values. (Supplementary material, Figure S2C).

338 *3.4. Leukocyte profile of rats exposed to L. m. muta venom and treatment with*
339 *antivenom and NAC*

340 No group showed alteration in lymphocytes count (Supplementary material,
341 Figure S3), whereas basophils and neutrophils precursor cells (myelocytes and
342 metamyelocytes) were not identified in any group. In addition, venom (G3) induced
343 significant increase in total leukocytes count after 120 min envenomation ($p < 0.05$
344 compared to G1 'control', $n = 6$), being moderately prevented by antivenom (G4) and
345 NAC (G5) or by both of agents associated (G6) ($p < 0.05$ compared to G3 'venom' for
346 all these treatments, $n = 6$); NAC alone (G2) did not interfere in the total leukocytes
347 count (Figure 5A). This increase in total leukocytes count was accompanied by a
348 pronounced increase of neutrophils ($p < 0.05$ compared to G1 'control', $n = 6$),
349 eosinophils ($p < 0.05$ compared to G1 'control', $n = 6$) and monocytes ($p < 0.05$
350 compared to G1 'control', $n = 6$) count. Antivenom (G4) and NAC (G5) administered
351 individually or in association (G6), moderately prevented the increase of neutrophils
352 caused by venom ($p < 0.05$ compared to G3 'venom' for all these treatments, $n = 6$),
353 with NAC alone (G2) unaffecting the neutrophils count (Figure 5B). Individual
354 administration of NAC alone (G5) ($p < 0.05$ compared to G3 'venom' or G4 'venom +
355 antivenom', $n = 6$) or in association with antivenom (G6) ($p < 0.05$ compared to G3
356 'venom', $n = 6$) produced greater protective action against the increase of eosinophils
357 caused by venom (G3) ($p < 0.05$ compared to G1 'control', n = 6) compared to
358 antivenom administered by itself (G4) ($p < 0.05$ compared to G3 'venom', $n = 6$),
359 whereas eosinophils were not identified in those animals treated with NAC alone (G2);
360 that is interesting to notice that the level of protection exhibited by NAC alone (G5) or
361 associated with antivenom (G6) on the venom-induced eosinophils increase were highly

similar, although only the protective action of NAC alone has been statistically greater when compared to antivenom therapy (G4) (Figure 5C). Venom-induced monocytes count increase (G3) was not prevented by antivenom (G4) and NAC (G5) administered individually or associating both of agents (G6) after 120 min envenomation (Figure 5D).

3.5. Morphological changes induced by L. m. muta venom in rats and treatment with antivenom and NAC

L. m. muta venom (G3) produced severe morphological changes in gastrocnemius muscle (RHL) characterized by haemorrhage, congestion, oedema, neutrophilic inflammatory infiltration, degeneration and myonecrosis ($p < 0.05$ compared to G1 'control', $n = 6$); the individual administration of antivenom (G3) and NAC (G4) prevented significantly the increase of the lesional score in gastrocnemius muscle ($p < 0.05$ compared to G3 'venom' for both treatments, $n = 6$), whereas their association (G6) was more effective to prevent the local myotoxicity ($p < 0.05$ compared to G3 'venom' or G4 'venom + antivenom' or G5 'venom + NAC', $n = 6$) (Figure 6A$_1$). There were no morphological changes in the contralateral gastrocnemius muscle (LHL) after 120 min envenomation (Figure 6A$_2$). *L. m. muta* venom did not induced relevant pulmonary (Figure 6B) and cardiac changes (Figure 6C). In liver, venom (G3) increased significantly the lesional score compared to control by causing mostly megalocytosis, congestion and cytoplasmic degeneration ($p < 0.05$ compared to G1 'control', $n = 6$); NAC administered by itself (G5) ($p < 0.05$ compared to G3 'venom', $n = 6$) or in association with antivenom (G6) ($p < 0.05$ compared to G3 'venom' or G4 'venom + antivenom', $n = 6$) produced greater protection than that seen with antivenom alone

(G4) on the venom-induced hepatotoxicity (Figure 6D). Venom (G3) also caused pronounced morphological changes in renal tissue characterized by acute tubular necrosis, cytoplasmic degeneration and congestion ($p < 0.05$ compared to G1 'control', $n = 6$); antivenom (G4) and NAC (G5) individually administered promoted significant reduction of these changes ($p < 0.05$ compared to G3 'venom' for both treatments, $n = 6$), while their association (G6) produced greater protection on the venom-induced nephrotoxicity ($p < 0.05$ compared to G3 'venom' or G4 'venom + antivenom' or G5 'venom + NAC', $n = 6$) (Figure 6E).

4. Discussion

The systemic effects produced by *Lachesis* envenomation such as consumption coagulopathy (Fuly et al., 1997; Estevão-Costa et al., 2000; Torres-Huaco et al., 2013), haemorrhagic disturbances (Sánchez et al., 1987, 1991, 1995; Rucavado et al., 1999), hypotension (Diniz e Oliveira, 1992; Giovanni-De-Simone et al., 1997; Dias et al., 2016a), bradycardia (Soares et al., 2005; Sanz et al., 2008; Pla et al., 2013) and renal disturbances (Damico et al., 2007; Alves, 2010) may be satisfactorily attenuated or even prevented with early administration of a suitable antivenom. However, the limited availability of antivenom in addition to the difficulties in accessing health services in certain regions of Brazil are the likely factors resulting in death by accidents involving *Lachesis* snakes. In this study, rats exposed to *L. m. muta* venom (1.5 mg/kg – i.m.) exhibited 1) pronounced local oedema accompanied by intense myonecrosis and haemorrhage, 2) important haematological changes such as leukocytosis, neutrophilia, eosinophilia and monocytosis, 3) significant increase in serum markers indicating myotoxicity (CK), hepatotoxicity (ALT) and nephrotoxicity (creatinine), 4) skeletal

410 muscle, hepatic and renal morphological alterations after 120 min envenomation.
411 Treatment with a single peritoneal administration of anti-*Bothrops*/*Lachesis* serum (IB)
412 (venom:antivenom ratio 1:3 'v/w' – i.p.) did ameliorate both the local and systemic
413 myotoxicity, e.g., oedema and myonecrotic lesions were reduced alongside reduced
414 levels of serum CK, however, the antivenom did not prevent the development of a local
415 subcutaneous haemorrhage. In addition, the antivenom also prevent the hepatotoxic
416 action of *L. m. muta* venom as assessed through serum ALT release. On the other hand,
417 the antivenom only partially prevented the nephrotoxicity induced by *L. m. muta* venom
418 and offered limited protection against the increase in leukocytes involved in mediating
419 the inflammatory response, e.g., neutrophils and monocytes.

420 Studies with antivenom supporting therapeutic tools have been limited in regard
421 to envenomation by *Lachesis* spp., however, there have been a few reports describing
422 the neutralizing properties of plant extracts (De Oliveira et al., 2014, 2016; Marques et
423 al., 2019) and their isolated metabolites (De Oliveira et al., 2020), including marine
424 sponge extracts (Faioli et al., 2013). This study is the first to demonstrate the protective
425 action of an antioxidant drug (*N*-acetyl-L-cysteine) with well-known molecular and
426 pharmacology properties (Paller & Patten, 1984; Thielemann & Rosenblut, 1990;
427 Nicoletta et al., 2006; Fok, 2009; Briguori et al., 2011; Zhang et al., 2017; Moroz et al.,
428 2019; Rogliani et al., 2019) on the experimental envenomation by *Lachesis* (=*L. m.*
429 *muta*) in rats.

430 *N*-acetyl-L-cysteine (NAC) has potent antioxidant activity and has the capacity to
431 scavenge a variety of ROS or indirectly acting as a precursor of L-cysteine which is a
432 limiting factor in the biosynthesis of intracellular glutathione, a natural antioxidant
433 agent and substrate for several antioxidant enzymes (Paller & Patten, 1984; Thielemann

& Rosenblut, 1990; Gillissen & Nowak, 1998; Nicoletta et al., 2006; Briguori et al., 2011; Aldini et al., 2018). NAC also shows efficient mucolytic action due its ability to break the disulphide bridges of high molecular weight glycoproteins present in bronchial mucus, resulting in the reduction of its viscosity, important for treating of respiratory disorders (Hurst et al., 1967; Fok, 2009; Aldini et al., 2018; Zhang et al., 2017; Rogliani et al., 2019). In addition, NAC has potential to reverse the toxic effects induced by acetaminophen (Prescott, 1983; Whitehouse et al., 1985; Holdiness, 1991), to prevent haemorrhagic cystitis induced by cyclophosphamide and doxorubicin hydrochloride (Palma et al., 1986), including renal disorders caused by contrasts (Sun et al., 2013). These well-known favourable therapeutic properties of NAC, aided by its rapid distribution and relatively low toxicity (Holdiness, 1991; Atkuri et al., 2007; Mokhtari et al., 2017), suggests there is a therapeutic application of this drug in treating the local and systemic toxicity caused by Viperidae snake venoms (Sunitha et al., 2011, 2013; Barone et al., 2014).

In this study, we have investigated the action of NAC on the acute envenomation by *L. m. muta* induced in rats using two different approaches that consisted of the administration of NAC alone or in association with a polyvalent antivenom, immediately post exposing the animals to venom. In this context, NAC showed great therapeutic potential on some local and systemic aspects of the envenomation by *L. m. muta* in rats such as: 1) NAC prevented the local oedema only when used in association with antivenom, 2) NAC alone reduced significantly the local myonecrosis formation comparable to antivenom, whereas the association of these agents produced greater protection, 3) NAC prevented the occurrence of subcutaneous haemorrhage when used in association with antivenom, 4) NAC alone prevented the systemic myotoxicity and in

458 association with antivenom as measured through serum CK release, 5) NAC also
459 reduced hepatotoxicity in association with antivenom as measured through serum ALT
460 release and 6) partially reduced the inflammatory responses similarly to the action of
461 antivenom alone. The suppressive action of NAC on the numbers of eosinophils during
462 the envenomation process by *L. m. muta* venom may be related to the ability of this
463 drug to inhibit specifically the functional responses of eosinophils (Martinez-Losa et al.,
464 2007) and through its action as an anti-inflammatory as reported by El Hafiz et al.
465 (2013), when used in patients with chronic obstructive pulmonary disease.

466 *L. m. muta* venom produced pronounced morphological changes in gastrocnemius
467 muscle, without affecting the contralateral muscle. These local alterations, e.g.,
468 haemorrhage, congestion, oedema, neutrophilic inflammatory infiltration, degeneration
469 and myonecrosis, are commonly caused by *Lachesis* spp. venoms (Fuly et al., 2000,
470 2003; Damico et al., 2006, 2008, 2012; Ferreira et al., 2009; De Oliveira et al., 2016).
471 NAC alone and when used in association with antivenom prevented the local
472 morphological alteration as measured through lesional score, corroborating with that
473 delay of the local myonecrosis formation previously mentioned. *L. m. muta* venom also
474 altered markedly the renal and hepatic morphological aspects which were associated
475 with the increase of serum creatinine and ALT release, respectively, with NAC alone
476 and in association with antivenom avoiding efficiently the increase of the lesional score
477 for both tissues. We have not observed important cardiac and pulmonary damage in any
478 group of animals exposed to *L. m. muta* venom, as measured through serum CK-MB
479 release and histopathological analysis of the heart and lungs tissue samples; however,
480 cardiovascular disorders and occurrence of pulmonary thrombosis have been previously
481 seen in animal experimentation models (Diniz e Oliveira, 1992; Dias et al., 2016a,

2016b; Zanotty et al., 2019; Angel-Camilo et al., 2020) and in human cases reports (Jorge et al., 1997) involving *Lachesis* spp. venoms and their toxins. Here, we have also shown the renal and hepatic toxicity of *L. m. muta* venom characterized by increase of serum creatinine and ALT release, respectively, accompanied by increase in the lesional score. Although the hepatotoxicity by *Lachesis* venoms has not been previously reported, the renal injuries comprises one of the most important consequences of the envenomation by these snakes (Damico et al., 2007; Alves, 2010). NAC alone failed to prevent the increase in the serum biomarkers but managed to prevent the renal and hepatic tissue damage.

Hence, NAC administered by itself effectively prevents the *L. m. muta* venom-induced local (i.e. delaying the myonecrosis formation) and systemic myotoxicity (i.e. preventing serum CK release), haematological alterations (i.e. leukocytosis, neutrophilia and eosinophilia) and helps to decrease skeletal muscle, hepatic and renal morphological damage. These effects were comparable to those ones seen in animals treated with antivenom alone. In association with a polyvalent antivenom (=anti-*Bothrops*/*Lachesis* serum), NAC has additional therapeutic benefit to produce greater protection than that seen with antivenom alone such as: preventing local haemorrhage, eosinophilia and skeletal muscle, hepatic and renal morphological damage. However, it is worth noting that NAC was not able to reverse the changes in ALT, either alone or in the presence of antivenom which may suggest that, in this regard, the mechanism of action of the venom does not result in the production of ROS and is refractory to the effects of NAC.

In accordance with these findings, Sunitha et al. (2011) demonstrated that NAC successfully prevented the occurrence of subcutaneous haemorrhage in mice exposed to

the Asian vipers *Echis carinatus* and *Vipera russelli* (=*Daboia russelii*) (Viperidae: Viperinae) venoms. The same group also reported that NAC inhibited efficiency the hyaluronidase activity of *Echis carinatus* venom (Sunitha et al., 2013). This biological property of NAC may be associated to its protective action on the local damage produced by Viperidae venoms, since the degradation of hyaluronic acid in the extracellular matrix by venoms hyaluronidase represent a primordial factor to promote the diffusion of the venom and, consequently, potentializing their toxins and increasing the local damage (Girish et al., 2002; Kemparaju and Girish, 2006; Bala et al., 2018). In addition, Barone et al. (2014) showed that NAC prevented the increase of serum creatinine in mice exposed to *Crotalus durissus terrificus* (Viperidae: Crotalinae) venom, although it failed to prevent the increase of serum creatinine in those animals exposed to *Bothrops jararaca* (Viperidae: Crotalinae) venom.

In conclusion, the antioxidant drug *N*-acetyl-L-cysteine (NAC) shows potential as a supporting therapeutic agent to attenuate the local effects of the acute envenomation by *Lachesis m. muta*. We have shown that this approach offers some additional protection preventing the occurrence of local haemorrhage and extension of myonecrosis. NAC also contributes to minimize some systemic aspects of envenomation, e.g., hepatotoxicity and inflammation, caused by this venom. This study is the first to show the value of NAC when treating *Lachesis* envenomation. Together, these findings may further support the notion of the use of a drug intervention following envenomation that would ameliorate the effects of envenomation in the absence of antivenom therapy.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

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840

841 **Legends**

842 Figure 1. *Lachesis muta muta* and its distribution throughout Amazon river basin in
843 South America (green area); the blue area along the east coast shows the distribution of
844 the subspecies *L. m. rhombeata* fount in the Atlantic rainforest, as essentially described
845 elsewhere (Nogueira et al., 2019). Photos: Marcus Buononato.
846

847 Figure 2. Representation of the experimental design for this investigation. In groups G4,
848 G5 and G6, "+" indicates the combined action of venom vs. AV, venom vs. NAC or
849 venom vs. NAC and AV, respectively. AV: antivenom, NAC: *N*-acetyl-L-cysteine.
850

851 Figure 3. Local haemorrhage, oedema and myonecrosis formation induced by *L. m.*
852 *muta* venom in rats and treatment with antivenom and NAC. (A) Venom (1.5 mg/kg)
853 produced significant oedema formation (expressed as a percentage of the RHL
854 dimension observed in control animals 'G1', considered 100%), being reduced by
855 antivenom alone or associated with NAC. (B) Antivenom and NAC administered
856 individually prevented the venom-induced myonecrosis formation, with their
857 association also reducing remarkably the points of subcutaneous haemorrhage [B_1

(control), B_2 (NAC), B_3 (venom), B_4 (venom + antivenom), B_5 (venom + NAC) and B_6 (venom + NAC + antivenom)]. In A, the columns are the mean ± SEM ($n = 6$); *$p <$ 0.05 compared to control 'saline' group 'G1', $^{\#}p < 0.05$ compared to venom group 'G3' and $^{\diamond}p < 0.05$ compared to venom + NAC group 'G5'. RHL: right hind limb (ventral view), LHL: left hind limb (ventral view), filled white arrow: indicates myonecrosis formation, opened white arrow: indicates points of haemorrhage (in B_3, the points of haemorrhage are unclear on the extensive myonecrosis formation).

Figure 4. Examination of blood-derived plasma protein biomarkers for systemic toxicity induced by *L. m. muta* venom in rats and treatment with antivenom and NAC. (A) Venom produced pronounced myotoxicity characterized by the increase of creatine kinase (CK) release, which was prevented by antivenom and NAC or by combination of both agents. (B) There was no evidence for cardiotoxicity as assessed through CK-MB 'myocardial band' release into the blood. (C) Venom caused hepatotoxicity characterized by increase of alanine aminotransferase (ALT) release, with only the antivenom being able to prevent the release of this biomarker. (D) Venom induced nephrotoxicity characterized by increase of serum creatinine (Cr), with both of agents failing to produce significant protection. The columns are the mean ± SEM ($n = 6$). *$p <$ 0.05 compared to control 'saline' group 'G1', $^{\#}p < 0.05$ compared to venom group 'G3' and $^{\diamond}p < 0.05$ compared to venom + NAC group 'G5'.

Figure 5. Leukocyte changes induced by *L. m. muta* venom in rats and treatment with antivenom and NAC. (A) Venom significantly increased the total leukocyte which was partially prevented by antivenom and NAC or combining both of agents. (B) Venom

882 also promoted an increase of neutrophils, with the treatments producing partial
883 protection. (C) The increase of eosinophils induced by venom was significantly
884 prevented by NAC itself or associated with antivenom; note that NAC has a property to
885 avoid the expression of eosinophil cells when administered alone. (D) Antivenom and
886 NAC or their association failed to prevent the increase of monocytes induced by venom.
887 The columns are the mean ± SEM ($n = 6$). *$p < 0.05$ compared to control 'saline' group
888 'G1', $^{\#}p < 0.05$ compared to venom 'alone' group 'G3' and $^{\blacklozenge}p < 0.05$ compared to
889 venom + antivenom group 'G4'.
890
891 Figure 6. Morphological aspects of tissues isolated from rats exposed to *L. m. muta*
892 venom and treated with antivenom and NAC. (A) Lesional score of right 'local of
893 venom injection' (1) and left 'contralateral' (2) gastrocnemius muscles; venom
894 produced pronounced myotoxicity which was prevented mainly associating antivenom
895 and NAC. (B) Lack of morphological changes in lung. (C) Lack of morphological
896 changes in heart. (D) Venom caused moderate changes in lesional score of liver, being
897 mostly prevented by NAC and its association with antivenom. (E) Venom caused severe
898 nephrotoxicity, with antivenom and NAC producing partial protection whereas their
899 association was effective at preventing the renal changes induced by venom. The
900 columns are the mean ± SEM ($n = 6$). *$p < 0.05$ compared to control 'saline' group
901 'G1', $^{\#}p < 0.05$ compared to venom 'alone' group 'G3', $^{\blacklozenge}p < 0.05$ compared to venom +
902 antivenom group 'G4' and $^{\diamond}p < 0.05$ compared to venom + NAC group 'G5'.