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Use of EDTA in the treatment of local tissue damage caused by the *Bothrops alternatus* venom

[*Utilização do EDTA no tratamento da lesão tecidual local causado pelo veneno de Bothrops alternatus*]

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ABSTRACT

Twelve adult rabbits were distributed in three groups and received on the femoral biceps region, via intradermal injection (ID), 25µg of *Bothrops alternatus* venom dissolved in NaCl 0.9% and diluted in 0.25mL of phosphate buffered saline (PBS). Thirty minutes later, the group G1 received 0.25mL of phosphate buffered saline (PBS) ID while to G2 and G3 25mg of ethylenediamine tetraacetic acid (EDTA) dissolved in 0.25mL of PBS were administered via intramuscular (IM) and intravenous (IV) injection, respectively. Evaluations included local lesion and blood profile of all animals, before (time zero) and at 1, 2, 3, 4, 5, 6, 12, 18 and 24h after venom administration. All animal treated with PBS (G1) and EDTA IV (G3) presented increase of nociceptive stimuli at the site of inoculation of the venom, followed by moderate edema that persisted for 24h. Animals treated with IM EDTA (G2) only manifested increase of nociceptive stimuli at the site of injection 1h after treatment with discrete local edema between 12 and 24h. In relation to the local hemorrhagic halo no differences were found amongst the studied groups. Blood profile revealed significant decrease of segmented neutrophils in all groups. There was also increase in triglycerides and decrease in total protein and albumin in all groups. The local lesion was not altered by the treatments.

Keywords**:** *Rhinocerophis alternatus,* snake envenomation, myonecrosis, chelating agent

RESUMO

Doze coelhos adultos, distribuídos em três grupos, receberam, na região de bíceps femoral, por via intradérmica (ID), 25µg de veneno de Bothrops alternatus*, dissolvidos em NaCl 09%, diluído em 0,25mL de tampão salina fosfato (PBS). Trinta minutos após o desafio, o grupo G1 recebeu 0,25mL de (PBS) ID, e os grupos G2 e G3 receberam 25mg de ácido etilenodiamino tetra-acético (EDTA), dissolvidos em mL de PBS por via intramuscular (IM) e intravenosa (IV), respectivamente. Foram avaliados lesão local e perfil sanguíneo de todos os animais, antes – tempo zero, e à uma, às duas, três, quatro, cinco, seis, 12, 18 e 24 horas após a injeção do veneno. Tanto os animais tratados com PBS (G1) como os animais tratados com EDTA IV (G3) apresentaram aumento do estímulo nociceptivo no local da administração do veneno, seguido por moderado edema, que perdurou por 24h. Os animais tratados com EDTA IM (G2) somente manifestaram aumento do estímulo nociceptivo local uma hora após tratamento e discreto edema local entre 12 e 24 horas. Em relação ao halo hemorrágico, não houve diferença entre os três grupos estudados. No perfil hematológico, observou-se diminuição significativa dos neutrófilos segmentados nos três grupos estudados. Da mesma forma, houve aumento dos triglicerídeos e diminuição da proteína total e albumina em todos os grupos. Conclui-se que a lesão local não foi alterada pelos tratamentos.*

Palavras-chave: Rhinocerophis alternatus, *envenenamento ofídico, dermonecrose, quelante*

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INTRODUCTION

Snakes from *Bothrops* genus are responsible for the majority of snakebite envenomation in Brazil. *Bothrops alternatus* is one of the many species found in Brazilian territory and it is able to deliver a relative large volume of venom. The relevance of *B. alternatus* goes beyond human medicine, since it is also responsible for a great number of envenomations in domestic animals, particularly dogs, cattle, and horses (Santos *et al*., 2003; Oliveira *et al*., 2007; Soto-Blanco and Melo, 2014). Its venom causes edema, blistering, hemorrhage, dermonecrosis, and myonecrosis (Gutiérrez *et al*., 2009). These effects are mostly the consequence of the direct action of zinc dependent metalloproteinases (SVMPs) and myotoxic phospholipases A_2 (PLA_2). SVMPs induce hemorrhage, blistering, dermonecrosis, and general extracellular matrix degradation, whereas PLA_2 induce myonecrosis and cause cytotoxic changes to lymphatic vessels (Gutiérrez *et al*., 2009).

The immediate treatment with antivenom (antibothropic serum) is fundamental to avoid systemic complications and death. Antivenoms are only partially effective in the neutralization of *Bothrops*-induced local effects, and the search for novel toxin inhibitors represents a potential avenue for improving the treatment of this serious aspect of snakebite envenomation (Gutiérrez and Ownby 2003; Melo *et al*., 2005; Quesada *et al*., 2006). There are some hypotheses for this inefficiency, such as the difficulty of the antivenom to reach tissues that have been compromised by SVMP and PLA_2 and the limited potential of neutralization of the horse antibodies. Even in patients that received the antivenom, severe local alterations can cause permanent tissue damage (Herrera *et al*., 2016). Several studies approach new therapy options that do not replace the antivenom but promote a better tissue repair. In this way, extracts from several plant species were successfully tested, but extraction procedures and isolation of bioactive compounds are a limitation to its use (Melo *et al*., 2005, 2007; Sasidharan *et al*., 2011).

Ethylenediaminetetraacetic acid (EDTA) is an organic compound that acts as a chelating agent, forming very stable complexes with various metal ions. Its ability to chelate zinc would provide a potential inhibition of *Bothrops* venom metalloproteinases and phospholipases, responsible for the majority of hemorrhagic and tissue injuries. Therefore, injection of EDTA could reduce the damage of the envenomation *in situ* and prevent clinical complications. This therapeutic potential has been successfully evaluated for *Crotalus* spp. and *B. asper* venoms (Borkow *et al*., 1997; Rucavado *et al*., 2000) and it is suggested as an option for local treatment associated with the antivenom. These findings corroborate to the metal dependency of main enzymes in Bothropic venoms but further studies are required to elucidate EDTA's potential after envenomation is installed and to access different injection sites of the chelate. Considering the importance and severity of local lesions caused by bothropic envenomation, the present paper reports a study on the local and systemic effects of EDTA treatment in rabbits envenomed with *B. alternatus* venom.

MATERIAL AND METHODS

Crude venom was obtained from a collection of adult *B. alternatus* specimens (males and females) bred in captivity from Alfenas town in Minas Gerais State (Brazil).

The venom pool of *Bothrops alternatus* snakes was dried in a vacuum desiccator at room temperature immediately after milking and then stored at -20°C until the moment of utilization. Before experimental procedures, the venom was dissolved in NaCl 0.9% and then diluted in phosphate buffered saline/ bovine serum albumin (PBS/BSA) 0.1% for administration.

Protein concentration was determined by the Bradford method (Bradford, 1976). Polyacrylamide Gel Electrophoresis (PAGE) with sodium dodecyl sulfate (SDS) was performed according to Laemmil technique for 1h in the following conditions: 100V, 102Mm and 120w (Laemmil, 1970). Denaturant buffer, 10mL, was added to 20mg of the sample, incubated in water bath at 100ºC for 5 minutes for protein denaturation. Sample was applied in polyacrylamide gel at 12% (Mix Acrylamide 30%- Acrylamide 29% and 1% N-N-metil-bis-acylamide; Tris 1.5M, pH 8.8, sodium dodecyl sulfate at 10%, ammonium persulfate 10%, TEMED) (GE Healthcares, England) with stacking gel at 4% (Mix Acrylamide 30%-

Acrylamide 29% and 1% N-N-metil-bis-acylamide; Tris 1.0M, pH 6.8, sodium dodecyl sulfate at 10%, ammonium persulfate 10%, TEMED) (GE Healthcares, England) with standard molecular weight from BenchMark® Protein Lader (Invitrogen, USA). After electrophoresis, gels were stained with Coomassie Brillant Blue R-250 solution (0.1% Coomassie Brillant Blue R-250/Thermo Scientific-USA, 25% methanol/ Merck Germany, 5% acetic acid/Merk Germany) for 1h followed by acetic acid (5%) and methanol (10%) to visualize colored bands.

Twelve adult male of New Zealand rabbits, mean weight of 3.5kg were used in this study. The animals were kept in metal cages (90x90x40 cm) and were maintained under a 12h light/dark cycle and controlled temperature (22±2ºC). Food and water were provided *ad libitum*. The experimental protocol was approved by the Ethics Committee in Animal Experimentation of the *Universidade Federal de Minas Gerais* (protocol number 274/2015).

The animals received 25µg of *B. alternatus* venom (Zeni *et al*., 2007) diluted in 0.25mL of PBS intra-dermal (ID) on the right femoral biceps, using a hypodermic syringe, and they were randomly distributed into three equal groups (n= 4): G1, G2 and G3. Thirty minutes after the challenge (venom inoculation), the animals were treated as follows: G1 received 0.25mL of PBS ID; G2 received 25mg of EDTA diluted in 0.25mL of PBS intramuscularly (IM) in the right femoral biceps; and G3 received the same volume and dose of EDTA intravenously (IV) by puncture of the right ear lateral vein, followed by a bolus of 1mL of 0.9% NaCl.

The local lesion evaluation was performed using a pachymeter before treatment (T0) and during the next 24 h as flows: 1, 2, 3, 4, 5, 6, 12, 18 and 24h. Clinical evaluation was also conducted at the same times. The blood was collected by puncture of the marginal vein of the left ear of each animal before envenomation (T0) and 24h after, to perform hematological and biochemical analyzes. Hematological parameters assessed were erythrocytes, hemoglobin, packet volume, mean corpuscular volume, mean corpuscular hemoglobin concentration, platelets, and leukocytes (Poch-100iV Diff® ROCHE).

Blood samples were stored in blood flasks without anticoagulant, centrifuged to obtain serum, to perform the biochemical profile (urea, creatinine, alanine aminotransferase, aspartate aminotransferase, glucose, triglycerides, protein and albumin measured by colorimetric assay using commercial kits (BIOCLIN®, Belo Horizonte, MG, Brazil) in a semi-automatic analyzer (TP Analyzer Basic, Thermoplate, São Paulo, Brazil).

After 24h, euthanasia was performed with 150mg/kg of intravenous Thiopental® at 5%, and necropsy exam was performed. At the time, the lesion size was visually measured with a ruler following muscle dissection. The urine samples from the bladder were immediately collected for evaluation.

The experiment followed a random design. Data was submitted to analysis of variance (ANOVA) and verification of normality distribution. Mean values of which variable study were compared using the Student-Newman-Keuls (SNK) test, paired T-test and Kruskal-Wallis (Sampaio, 2007). Statistical analyses were carried out with the aid of GraphPad Prism v5 (GraphPad Software, Inc.) and significant difference was established if $P<0.05$.

RESULTS

The SDS-PAGE of *B. alternatus* venom revealed bands ranging from 14 to 49kDa. Low intensity bands of 14kDa correspond to phospholipases (PLA_2); 28kDa are associated with type C lectins, and bands from 32 to 49kDa to serinoproteases and metalloproteases of classes P-I and P-III, respectively.

Animals from the control group (G1), which received *B. alternatus* venom and were treated with PBS showed increase sensitivity at the site of injection in the first hour of envenomation, followed by moderate edema during the first 24h. Animals treated with EDTA IM showed signs of increased sensitivity only in the first 2 hours following envenomation, corresponding to 1h after treatment. Mild signs of local edema were visualized at 12 and 24h. In addition, animals that received EDTA IV presented sensitivity and local edema throughout the 24h of experiment.

In relation to the local hemorrhagic halo, there was no statistical difference between groups and interaction groups/times (Figure 2). Likewise, after necropsy, the area of the lesion also did not present a significant difference (Figure 2).

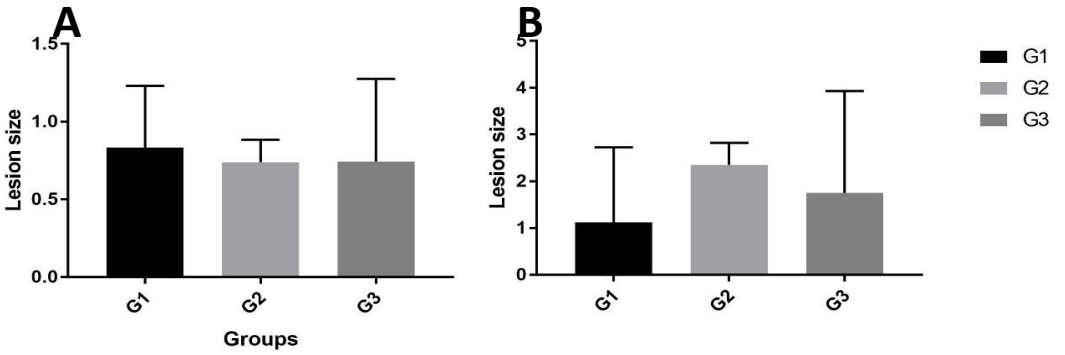

Figure 2. A- Visible lesion size in the leg of rabbits after 1h of envenomation with *Bothrops alternatus* venom and treated with PBS (G1), intramuscular EDTA (G2) and intravenous EDTA (G3). B- Lesions size evaluation after necropsy exam in the leg of rabbits envenomed with *Bothrops alternatus* venom and treated with PBS (G1), local EDTA (G2) and intravenous EDTA (G3).

The evaluation of hematological parameters showed no significant difference between groups and interaction groups/times considering hemoglobin and platelet mean values.

Animals from G2 (EDTA IM) presented a decrease in erythrocytes count in T24 in comparison to T0. Both G1 (PBS) and G2 (EDTA IM) showed decrease in packed cell in T24 in comparison to T0. MCV and MCHC were higher in T24 comparing with other groups and with T0.

The evaluation of the leukogram profiles showed no significant difference between groups and interaction groups/times considering total leucocytes, eosinophils, and basophils. However, there was a decrease in segmented leukocytes in 24h in both groups G1 and G2. It was observed an increase in lymphocytes count in G2 at 24h in comparison to T0. The monocytes were decreased in T24 in G3 (animals treated with EDTA IV) and increased in G1, when compared to T0 (Table 2).

Table 1. Hematological evaluation of rabbits envenomed with *Bothrops alternatus* venom and treated with PBS (G1), intramuscular EDTA (G2) and intravenous EDTA (G3)

		Groups			Reference values
Variable	Time	Control	IM EDTA	IV EDTA	5.2- 6.8
ERY (x 10^6 cel/µL)	T0	5.33±0.45	6.10±0.26 a	5.24±0.59	
	T24	4.81±0.55	5.59±0.41 b	5.44±0.28	
HB (g/dL)	T0	9.57±1.96	12.42±1.60	10.98±1.60	11.5- 15.1
	T24	10.10±1.42	11.80±1.02	11.25±0.44	
PV (%)	T0	37.00 Aa±2.58	43.00 Ba±2.44	36.25 A±4.11	36.6- 47.4
	T24	32.75 b±4.03	38.51 b±1.73	32.50±3.10	
MCV (fL)	T0	69.67±6.70	70.42±2.13	69.22±3.90	64.6- 76.2
	T24	67.95 A±1.97	69.02 A±2.86	69.80 B±5.38	
MCHC (%)	T0	25.87±4.91	29.05±4.65	30.20 a±1.57	29.5- 33.9
	T24	30.80 A±0.88	30.62 A±1.75	34.80 Bb±2.67	
PLA (x10^3 cel/µL)	T0	165.50±54.61	183.25±136.42	195.00±142.11	250- 610
	T24	216.25±62.08	228.00±70.61	299.75±122.28	

ERY: erythrocytes (x 10^6 cel/µL); HB: hemoglobin (g/dL); PV: packed volume (%); MCV: mean corpuscular volume (fL); MCHC: mean corpuscular hemoglobin concentration (g/dL); PLA: platelets (x10^3 cel/ µL). Reference values for the species according to Campbell, 2007. *Means followed by the different upper case letter in the lines and by the different lower case letters in the columns differ according to the analysis of variance and SNK and paired T test ($P \leq 0.05$).

Table 2. Leucocytes profile of rabbits envenomed with *Bothrops alternatus* venom and treated with PBS (G1), local EDTA (G2) and intravenous EDTA (G3)

		Groups			Reference values
Variable	Time	Control	IM EDTA	IV EDTA	
LEU	T0	8.08±1.98	8.15±4.14	5.78±2.37	6.30-10.06
	T24	9.82±0.81	8.03±1.92	8.65±1.86	
SEG	T0	4.00 Aa±3.36	5.98 Aa±3.36	2.24 Ba±2.03	1.49-3.21
	T24	3.31 Ab±2.07	3.79 Ab±0.99	2.95 Aa±2.50	
LIN	T0	1.24±1.10	1.76 a±0.58	1.36±1.39	3.36-7.00
	T24	2.50±2.57	3.74 b±1.58	2.53±1.97	
EOS	T0	0±0	0.07±0.13	0±0	0.001-0.15
	T24	0±0	0±0	0±0	
BAS	T0	0±0	0.02±0.05	0.02±0.04	0
	T24	0.03±0.07	0.03±0.04	0±0	
MON	T0	0.138 a±0.16	0.37±0.12	0.23±0.22	0.05-0.45
	T24	0.70 Ab±0.61	0.47 AB±0.14	0.27 B±0.38	

LEU: leukocytes 10^3cel/µL; SEG: segmented leukocytes 10^3cel/µL; LIN: lymphocytes 10^3cel/µL; EOS: eosinophils 10^3cel/µL; BAS: basophils 10^3cel/µL; MON: monocytes 10^3cel/µL. Reference values for the species according to Campbell, 2007. Means followed by the different upper case letter in the lines and by the different lower case letters in the columns differ according to the analysis of variance and SNK and paired T test ($P \leq 0.05$). EOS and BAS were analyzes by Kruskal-Wallis ($P \leq 0.05$).

The evaluation of the biochemical profile showed no significant difference between groups and interaction groups/times considering creatinine, ALT and glucose levels and relation between albumin/globulin (Table 3). Urea levels were increased in G2 (EDTA IM) at T24 when compared to T0. Although there was no change in the enzymatic values of AST, it can be observed that 24h after treatments, the group G3, that received intravenous EDTA, had the lowest values of AST. Triglycerides were increased in G2 and G3 at T24. Protein and albumin levels were lower at T24 in all groups.

Table 3. Biochemical profile of rabbits envenomed with *Bothrops alternatus* venom and treated with PBS (G1), local EDTA (G2) and intravenous EDTA (G3)

		Groups			Reference values
Variable	Time	Control	IM EDTA	IV EDTA	
URE	T0	61.50±9.05	55.30 a±8.51	68.78±21.73	47.3-57.7a
	T24	69.30±20.55	65.51 b±5.05	66.25±18.19	
CRE	T0	2.92±0.41	2.69±0.24	2.59±0.39	0.8-2.9b
	T24	2.86±0.38	2.92±0.10	2.57±0.41	
ALT	T0	66.01±21.62	66.32±5.59	61.53±44.02	<100b
	T24	69.10±24.25	84.01±15.16	69.54±32.88	
AST	T0	97.44±49.74	95.20±22.38	65.65±30.17	<100b
	T24	72.35 AB±20.30	110.48 A±36.14	46.81 B±22.10	
GLU	T0	95.58±17.09	113.30±13.26	99.70±42.27	89-144b
	T24	125.33±13.22	136.88±18.18	117.51±22.34	
TRI	T0	85.32 a±48.32	76.85 a±29.77	74.52 a±40.63	72-90a
	T24	93.36 a±54.38	123.32 b±54.09	121.80 b±66.11	
TP	T0	6.54 Aa±0.27	8.44 Ba±1.15	6.32 Aa±0.56	5-8.5 b
	T24	5.29 b±0.50	6.05 b±0.45	5.56 b±0.55	
ALB	T0	5.53 ABa±0.86	6.43 Aa±1.06	4.70 Ba±0.26	3-3.34b
	T24	4.29 Ab±0.43	4.65 Ab±0.22	4.12 Ab±0.16	
ALB/GLO	T0	3.46±0.16	3.19±0.44	2.83±0.50	
	T24	5.07±2.91	3.44±0.8	3.03±0.74	

URE: urea (mg/dL); CRE: creatinina (mg/dL); ALT: alanine aminotransferase (UI/L); AST: aspartate aminotransferase (UI/dL); GLU: glucose (mg/dL); TRI: triglycerides (mg/dL); TP: total protein (mg/dL); ALB: albumin (mg/dL); ALB/GLO: albumin/globulin. (%). References values according to a-Spinelli *et al*., 2012, b-Campbell, 2007 *Means followed by the different upper case letter in the lines and by the different lower case letters in the columns differ according to the analysis of variance and SNK and paired T test ($P \leq 0.05$).

Urinalysis was evaluated, but no significant difference was found in the parameters evaluated (Table 4). Variables were transformed into numbered categorizes in order to provide statistical analysis (Kruskal-Wallis). Blood found in urine was categorized according to the number of crosses in the urinalysis tape as follows: 0 to 5 crosses corresponded to 0, 5 to 10, 10 to 100, 100 to 200 and 200 to 300 corresponded to 1, 2, 3 and 4, respectively. Glucose was determined as low corresponded to 0, normal to 1, and high to 2. Bilirubin with 1 cross corresponded to 1 and 2 crosses corresponded to 2. Leukocyte count of 25 corresponded to 1. Protein levels of 0, 30 and 100 corresponded to classes 0, 1 and 2, respectively.

Table 4. Urinalysis profile of rabbits envenomed with *Bothrops alternatus* venom and treated with PBS (G1), local EDTA (G2) and intravenous EDTA (G3)

	Groups		
Variable	G1	G2	G3
BLO	0.75±0.95	2.25±2.06	0.25±0.5
URO	1±0	1±0	1±0
BIL	0±0	0.75±0.95	0±0
PRO	30±0	53.33±0.95	30±0
NIT	0±0	0±0	0±0
KET	0±0	0±0	0±0
GLU	0±0	1±0	1±0
DEN	0.75±0.5	1.00±0.01	1.02±0
LEU	0.5±0.57	6±0.57	0±0
pH	6.5±1	6±0.81	5±0

BLO: blood; URO: urobilinogen; BIL: bilirrubin; PRO: protein; NIT: nitrite; KET: ketone; GLU: glucose; DEN: density; LEU: leukocytes. Means do not differ according to Kruskal-Wallis post test (P≤0.05).

DISCUSSION

The use of synthetic venom inhibitors with high diffusibility, long shelf life, and easy acquisition that could be applied shortly after the envenomation represents an alternative to overcome limitations in antivenom therapy in regards of local tissue damage (Rucavado *et al*., 2000).

Snake venom SVMPs are responsible for the hemorrhagic activity. They are classified in various groups (P-I–IV), according to their domain composition. P-III SVMPs, comprising metalloproteinase, disintegrin-like and cysteine-rich domains, exert more potent hemorrhagic activity than P-I SVMPs, which present only the metalloproteinase domain. SVMPs degrade various components of the basement membrane and are able to hydrolyze endothelial cell membrane proteins, such as integrins and cadherins, involved in cell–matrix and cell–cell adhesion. SVMPs induce a hemorrhage extremely rapidly (within few minutes), with capillary endothelial cells showing drastic structural alterations (Gutierrez *et al*., 2005).

Taking into consideration that SVMPs are a major component of *Bothrops* venom (Gutiérrez and Lomonte, 1989) and are responsible for the pathophysiology of local hemorrhage, edema, myonecrosis (Gutierrez *et al*., 1995), blister formations (Rucavado *et al*., 1998), and dermonecrosis (Moura-da-Silva *et al*., 1996; Gutiérrez *et al*., 2009), they represent a viable target for therapeutic agents. Different chelating substances were previously used with success in treatment of local tissue damage for both *Crotalus* (Borkow *et al*., 1997) and *Bothrops* genus, including EDTA (Borkow *et al*., 1997; Rucavado *et al*., 2000).

EDTA treatment can chelate metallic ions, including zinc, through the conversion of the metallic ion to its anionic form, creating a complex of metal-EDTA (Nowack and Sigg, 1996). Considering that the SVMPs present in the venom are zinc-dependent, EDTA treatment could be beneficial to neutralize its effects (Bjarnason and Fox, 1994).

The present study showed that local and intravenously treatment with EDTA 30 minutes after ID inoculation of *B. alternatus* venom in rabbits were not able to reduce local tissue damage during the first 24h of observation. Lesion size was not reduced by the treatments. Animals that received intramuscular EDTA showed larger lesions (G2) and the highs values of AST, suggesting higher muscular damage,

with reduced edema and sensitivity. The intravenous venom injection showed tendency to decrease lesion size in comparison to other treatment group, but edema and sensitivity were considered moderate. Thus suggesting that IM injection of EDTA can reduce local edema formation and therefore sensitivity at the site of inoculation, while intravenous injection of EDTA seems to increase local sensitivity.

Edema and sensitivity are multifactorial signs not only affected by inflammatory response. Direct damage by hemorrhagic toxins can cause liquid extravasation followed by pain, independently of inflammatory mediators (Gutiérrez and Lomonte, 1989; Teixeira *et al*., 2009; Gutiérrez *et al*., 2009). This result provides insights that local EDTA can possibly partially neutralize SVMP activity.

Previous work that evaluated EDTA as a successful treatment option used culture cell analysis and rat animal model with previous or immediate injection of the drug (Borkow *et al*., 1997; Rucavado *et al*., 2000). This report uses rabbits, considered a model for skin dermonecrosis in envenomation cases (Elston *et al*., 2005), and treatment following thirty minutes of envenomation, that represents a typical time response in snake bites treatment (Ribeiro and Jorge, 1997). Delayed treatment with EDTA showed only partial or absent inhibition of the proteolytic, hemorrhagic and dermonecrotic activity of *B. asper* venom (Rucavado *et al*., 2000), in accordance to our findings.

Administration of subcutaneous EDTA 5 to 10 minutes after experimental envenomation with *B. atrox* significantly reduced hemorrhage, edema and necrosis in rabbits (Flowers and Goucher, 1965). The chelate preincubation with *B. asper* (Borkow *et al*., 1993), *B. jararaca* (Tanizaki *et al*., 1989) and *B. jararacussu* (Mazzi *et al*., 2004) SVMP also reduced hemorrhagic (Borkow *et al*., 1993; Mazzi *et al*., 2004) and dermonecrotic potential of the toxins (Leon *et al*., 1998), without reducing myonecrosis and edema formation. And intraperitoneal injection of *B. asper* venom SVMP preincubated with EDTA also significantly reduced leukocyte infiltration (Fernandes *et al*., 2006), while *in vitro* EDTA assays showed reduction in platelet aggregation and tromboxane formation by inhibiting PLA_2 from *Naja naja atra* venom (Ouyang and Huang, 1984).

Bothrops envenomation causes different alterations in blood and biochemical parameters according to the species evaluated and methodological approaches. Previous work using rabbits and similar methodology with *B. alternatus* venom showed that animals that were only treated with venom had leukocytosis with eosinopenia seven days after injection (Melo *et al*., 2005). In the present work, evaluations after 24h showed no alterations in total leukocyte count, with reduced number of segmented cells and a higher number of monocytes and lymphocytes, indicating a possible leukocyte migration to the site of inoculation (Arruda *et al*., 2003), thus reducing blood levels of segmented cells and increasing relative percentage of monocytes and lymphocytes.

Animals treated with intramuscular EDTA presented the same pattern of leukocyte migration, with minor changes in erythrocytes, without signs of anemia and discrete decrease in erythrocyte count and globular volume that could be attributed to local discrete hemorrhage at the site of bite due to capillary lesions and degradation of basal membrane (Farsky *et al*., 1999). Previous research did not evaluate leukocyte profile and EDTA treatment, and so far, no scientific data supports or denies these changes.

Our results showed that platelets were not affected by the venom or treatments in the first 24h of evaluation, probably because of the low dose administrated or the short evaluation time. Although various venom components, such as proteins of the C-type lectin-like family, serine proteinases, SVMPs, and disintegrins exert effects that impair platelet function, by thrombocytopenia (platelet sequestration associated with severe hemorrhage and endothelial lesion) or inhibition of platelet aggregation (Gutiérrez *et al*., 2016). This result also suggests that EDTA treatment does not interfere with platelets count, regardless of the application site.

Biochemical evaluations showed minor significant alterations in control group, showing that the dose administrated is considered low and more suitable for local tissue damage evaluation. Intravenous and intramuscular treatment with

EDTA caused increase in triglycerides values, a marker for cell membrane rupture in snakebites caused by PLA_2 and SVMP.

Protein and albumin levels decreased in all groups after 24h, despite the fact that albumin/globulin relation did not change. G2 presented higher levels of protein at T0, but still in the normal range value for the species (4.5-12.2md/dL) (Ozkan *et al*., 2012). Albumin is an acute negative phase protein common to inflammatory process (Silva *et al*., 2005). Albumin synthesis is altered by a variety of stress factors, including bothropic envenomation, causing total protein reduction (Telles *et al*., 2014).

Urea levels were discreetly higher in animals treated intramuscularly with EDTA, but not accompanied by creatinine increase. Urinalysis also did not show alterations, and both the venom and EDTA, probably did not affect kidney function at the doses and time frames used. In higher doses using the same venom, acute renal insufficiency can be established considering direct and indirect effects of by PLA_2 and SVMP in kidney cells (Morais *et al*., 2013).

In conclusion, our observations indicate that *in situ* and intravenous injection of chelating agent EDTA 30 minutes after ID *B. alternatus* venom injection in rabbits does not alter lesion size. Previous studies showed beneficial effects of EDTA, but the chelating agent was used almost immediately after envenomation or in the same solution containing the venom (Borkow *et al*., 1997; León *et al*., 1998; Rucavado *et al*., 2000). The treatment-initiating half hour after envenomation is according to the fact that most snakebites occur in the rural region and medical care provided in the cities requires dislocation (Caiaffa *et al*., 1997). EDTA is possible beneficial effects should be further assed and administration in a shorter amount of time should be evaluated.

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