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Inhibition of proteases and phospholipases A$_2$ from *Bothrops atrox* and *Crotalus durissus terrificus* snake venoms by ascorbic acid, vitamin E, and B-complex vitamins

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ABSTRACT

The enzyme inhibition by natural and/ or low-cost compounds may represent a valuable adjunct to traditional serotherapy performed in cases of snakebite, mainly with a view to mitigate the local effects of envenoming. The objective of this study was to evaluate possible interactions between vitamins and enzymes that comprise *Bothrops atrox* and *Crotalus durissus terrificus* venoms, *in vitro*. Proteolysis inhibition assays (substrates: azocasein, collagen, gelatin and fibrinogen), hemolysis, coagulation, hemagglutination were carried out using different proportions of vitamins in face of to inhibit minimum effective dose of each venom. The vitamins were responsible for reducing 100% of breaking azocasein by *C.d.t.* venom, thrombolysis induced by *B. atrox* and fibrinogenolysis induced by both venoms. It is suggested the presence of interactions between vitamin and the active site of enzymes, for example the interactions between hydrophobic regions present in the enzymes and vitamin E, as well as the inhibitions exercised by antioxidant mechanism.

Key words: antiophidian properties, antioxidants, blood cells, hemostatic processes, natural inhibitors.

INTRODUCTION

The *Bothrops atrox* species can be found frequently in the Amazon region and is responsible for about 80% of snake bites in the region. Studies indicate that the envenoming by *B. atrox* venom is characterized by a strong inflammatory response triggering rupture of blood vessels, production of cytokines, leukocyte migration and cell death. Importantly, the lethality of the venom is associated with inflammatory factors induced by enzymes that

act as thrombin-like, which are primarily proteases (Barros et al. 1998).

Accidents with snakes of the genus *Crotalus* (Viperidae family) are less frequent, but because of the severity of the systemic response, they present high lethal potential, and the envenoming by these snakes is characterized by neurotoxic, coagulant and myotoxic action (Sgrignolli et al. 2011).

Crotalus durissus is a species that inhabits areas of the Central Brazilian Cerrado and arid and semi-arid regions of Northeast and fields and open areas in the south. The subspecies *Crotalus*

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durissus terrificus is prevalent in the southeastern and southern regions (Melgarejo 2003).

Among the components present in the venom of snakes, those showing the greatest potential as biotechnological agents and responsible for the most serious disorders within the envenoming are the phospholipases A_2 (PLA_2), proteases, lectins type C, hyaluronidases, disintegrins and L-amino acid oxidases.

The PLA_2s present in snake venoms are responsible for pharmacological effects involving blood incoagulability, myonecrosis, edema formation, inhibition of platelet aggregation and hypertension. These enzymes are also present in the pancreatic secretions, being widely distributed in animals and plants in nature (Burke and Dennis 2009).

Among the proteases present in snake venoms are the serineproteases that act selectively on factors of the coagulation cascade, with effect on platelet aggregation, fibrinolysis and coagulation (Braud and Wisner 2000) and metalloproteinases, dependent on the zinc ion, responsible for bleeding, tissue damage and local inflammation (Telesi and Machado 2008).

In literature, little has been discussed about the venom-vitamins interaction. Mukherjee et al. (1997) have shown that vitamin E inhibited the lipid peroxidation of the cell membrane of human erythrocytes induced by the *Vipera russeli* venom. Mohamed et al. (2013) obtained similar results with the synthesis of a lipophilic ascorbic acid derivative capable of inhibiting the activity of PLA_2 isolated from *Vipera russelli* venom. Soon the investigation of the interaction between vitamins and snake venoms seems to be necessary because vitamins are molecules that are closely linked to the performance of enzymatic functions present in organisms. In addition, various enzymes, such as phospholipases and proteases present in the venoms show functional and structural homology with enzymes present in the human body, enabling similarities between the inhibition of enzymes resulting from venoms and the likely effects on human endogenous enzymes.

Vitamins are the primary antioxidants obtained by feeding, being involved in processes such as cell migration, energy metabolism, vasomotor control, transportation of metal ions and glucose, amino acid formation, maturation of blood cells, cholesterol metabolism, decreased lipid membrane degradation - thereby reducing the formation of eicosanoids, repair of damage in DNA molecule, stimulation of the immune system and production of hormones (You et al. 2009, Du et al. 2011, Traber and Stevens 2011, Coquille et al. 2012).

The aim of this study was to evaluate the interaction between micronutrients (vitamins of B complex, vitamin E and ascorbic acid - together, and ascorbic acid alone) and enzymes present in the venom of *B. atrox* and *C.d.t.* snakes species, *in vitro*, focusing on the inhibition of proteases and PLA_2s acting on human cells and molecules.

MATERIALS AND METHODS

SNAKE VENOMS

The venoms of *B. atrox* and *C.d.t.* were commercially purchased from Bio-agents serpentarium (Batatais, SP, Brazil).

HUMAN BLOOD

The coagulant, thrombolytic, hemagglutinating and hemolytic activities were performed using human blood collected in tubes containing sodium citrate or without anticoagulant (thrombolytic activity).

The experiment was conducted in accordance with the standards of the Ethics Committee on Human Research (COEP) from Universidade Federal de Lavras, and has been approved by this committee (Nº CAAE/15258413.0.00000.5148).

VITAMINS

The vitamin complex containing vitamins C, E and B complex vitamins (beminal) was purchased from

a drugstore and the isolated vitamin C was acquired from a compounding pharmacy.

AZOCASEIN ACTIVITY

For purposes of this test, we made use of a methodology based on two methods previously described by Gutierrez et al. (1988) and Van der Walt and Joubert (1971) adapted by Wang et al. (2004). Thus, substrate (azocasein) concentration and dissolution conditions were maintained, obtaining proportions identical to those used by Van der Walt and Joubert (1971) to be incorporated into agarose gel (method of Gutierrez et al. (1988). The minimum dose azocaseinolytic of venoms, responsible for the formation of an activity halo of approximately 1.2 cm, has been previously determined being used in the inhibition assays. For gel preparation, azocasein, at 0.058%, was dissolved in 4 ml of Tris-HCl (pH= 9.6) and then added to the gel composition. The gels containing the samples remained in a cell culture chamber at 37°C for 12 hours. The formation of translucent halo around the opening in the gel was indicative of activity, where the halos were measured in millimeters for quantifying the proteolytic activity on azocasein. Mixtures of venoms (30μg) and vitamins previously incubated (30 minutes at 37°C) were evaluated in proportions of 1:1, 1:2.5, 1:5 and 1:10 (venom: vitamin; w/w), as well as controls containing venoms and vitamins in isolation.

PROTEOLYTIC ACTIVITY ON THE COLLAGEN AND GELATIN

The proteolytic analysis of collagen and gelatin (at 0.058%) was carried out through the preparation of a gel identical to the one described in the previous section with the change of the substrate for one of the proteins of interest. For displaying of results, the gel was subjected to coloring in starch black solution 0.25% (w/v) dissolved in water, followed by decoloration with 10% acetic acid. The analyzed samples were incubated in the same proportions described for azocaseinolytic activity.

CLOTTING ACTIVITY

The clotting time of citrated plasma (200 μL) was evaluated in assays with addition of vitamins to the plasma to previous incubation for a 5-minute period, and subsequent addition of venom and timing, as well as preincubations of vitamins with venom, for a 5-minute period, and subsequent plasma addition and timekeeping. Controls containing solely vitamins or venoms were also performed. The citrated plasma was placed in test tubes dipped in water bath at 37°C and kept until temperature stabilization with subsequent addition of the samples. The minimum coagulant dose was defined as the minimum amount of venom responsible for plasma clotting in a time interval between 1 and 1.5 minutes (Seliestre et al. 1990). The proportions of venom (10μg) and vitamin within the samples were 1: 0.5, 1: 1, 1: 2.5 and 1: 5 (w/w).

THROMBOLYTIC ACTIVITY

Thrombolytic activity was performed by distributing the collected blood into microtiter plates (100 μl per well) to obtain the thrombus. Then, several doses of venom were tested to determine the one responsible for a complete dissolution of the thrombus. The activity was quantified by the volume of liquid released from the thrombus, as described by Cintra et al. (2012). Controls containing only vitamins were also performed. The inhibitory potential of vitamins was measured after previous incubation (40 minutes at 37°C) of vitamins and venom (25μg), at the proportions 1:1, 1:5 and 1:10 (venom: vitamin; w/w) and subsequent addition to the thrombus.

HEMAGGLUTINATION ASSAY

The hemagglutination test was performed as described by Jaffé (1969) and Figueiros and Lajolo (2009). Ten mL of the used blood was immediately centrifuged and supernatant was discarded. The cells were suspended in saline solution (0.85%

Na$_2$HPO$_4$, KH$_2$PO$_4$, NaCl), followed by three wash steps with centrifugation at 1700g for 15 minutes. Then, the erythrocytes were suspended in the same solution (1: 20 v/v blood: saline solution) and distributed into microtiter plate (volume 100 µL/ well). The venom (40µg) and vitamin samples, previously incubated (30 minutes at 37°C), were then added, followed by incubation for another hour at room temperature. The *B. atrox* and *C.d.t.* venoms were tested in venom and vitamin proportions of 1: 0.5 and 1: 1 (w/w) for vitamins, isolated ascorbic acid and vitamin complex. The results were evaluated by a qualitative visual analysis, 2 hours after addition of samples, using an electronic magnifier.

HEMOLYTIC ACTIVITY IN LIQUID MEDIUM

The blood collected (10 mL) in the presence of an anticlotting agent was immediately mixed with saline solution (2 mM NaH$_2$PO$_4$; 3 mM Na$_2$HPO$_4$; 154 mM NaCl; pH 7.4) and centrifuged at 700g (Fanem Baby®I Modelo 206 BL) for ten minutes. The plasma was removed, and the red cells were suspended in 5 mmol L^{-1} phosphate buffer, pH 7.4 and centrifuged under the same conditions. This washing procedure was repeated three times at 4°C. The 100% red blood cell concentrate was diluted to 2% hematocrit (137.33 µmol L^{-1} lipid, respectively), using the same buffer.

The hemolytic activity was evaluated by incubating 1 mL of erythrocyte suspension (2%) for 60 minutes at 37°C with the pre-incubated of venoms (20µg) with vitamins, at different ratios (1:1, 1:2.5, 1:5 and 1:10 w/w), followed by centrifugation at 1500g for seven minutes. The hemoglobin concentration was determined in the supernatant by measuring the absorbance at 540 nm (Shimadzu UV-160 1 PC) according to Rangel et al. (1997), with the modifications of Preté et al. (2010). The controls were performed using an erythrocyte suspension in PBS (c1 = mechanical hemolysis control) and distilled water (c2 = total hemolysis control) (Preté et al. 2010).

The hemoglobin concentration was determined using the equation:

$$\% \text{ hemolysis} = \frac{A_a - A_{c1}}{A_{c2} - A_{c1}} \times 100$$

in which Aa, Ac1 and Ac2 are, respectively, the absorbance of the sample and of the controls c1 and c2 at 412 nm.

FIBRINOGENOLYTIC ACTIVITY: POLYACRYLAMIDE GEL ELECTROPHORESIS WITH SODIUM SULFATE DODECYL TO VISUALIZATION

The fibrinogenolytic activity was carried out with modifications (Czaikoski et al. 2010). The vitamins were incubated with bovine fibrinogen (60 µg) for 90 minutes at 37°C in a final volume of 25 µl (PBS), as control. The samples were analyzed by polyacrylamide gel electrophoresis at 12% (acrylamide: bisacrylamide, 19:1) in the presence of SDS (SDS-PAGE) under denaturing conditions (Laemmli 1970). A sample control containing only fibrinogen was used for the visualization of the band pattern corresponding to α, β and γ chains of fibrinogen. The minimum fibrinogenolytic dose of venom, responsible for the degradation of α and β chains from fibrinogen has been previously determined (60µg to *B. atrox* and 80µg to *Crotalus durissus terrificus* venom) and used in inhibition assays.

The inhibitory potential of the vitamins on the proteolytic action of *B. atrox* and *C. d. t.* venoms was assessed by SDS-PAGE after preincubation of samples (1:1, 1:5 and 1:10, venom: vitamin; w/w) for 30 min. at 37°C, with subsequent addition of fibrinogen (60 µg) and incubation for 90 minutes at the same temperature. The reactions were stopped by addition of 10 µl of bromophenol blue solution [0.05 M Tris-HCl, pH 8.0, comprising 10% (v/v) glycerol, 10% (v/v) mercaptoethanol, 2% (w/v)

sodium dodecyl sulfate (SDS) and 0.05% (w/v) bromophenol blue] and boiling the samples for 5 minutes in a water bath (Laemmli 1970).

Electrophoretic run was held for a period of 2 hours at 90V. After run, gel was removed and colored for 40 minutes in 0.1% Coomassie Blue G-250 (w/v) dissolved in water: methanol: acetic acid (40: 50: 10, v/v) and destained in 10% acetic acid.

STATISTICAL ANALYSIS

Statistical analyses were performed with GraphPad Prism software, and the Student's t-test was chosen to compare the experimental mediums. Results with $p < 0.05$ were considered as significant. All assays were performed in triplicate.

RESULTS

PROTEOLYTIC ACTIVITY

The *B. atrox* venom presented proteolytic activity on substrates azocasein, collagen, and gelatin, while the *C.d.t.* showed activity only on azocasein.

The greatest inhibitions, obtained to the proteolytic activity induced by *B. atrox* venom, were observed in a proportion of 1: 10 with reduced activity 63% and 83%, after incubation with ascorbic acid and vitamin complex, respectively (Fig. 1a).

Ascorbic acid and vitamin complex inhibited the azocaseinolytic activity with greater efficiency at the proportion 1: 10, where the *C.d.t.* venom had

its activity reduced to 5% in preincubation with ascorbic acid and vitamin complex (Fig. 1b). At the ratio of 1: 5, ascorbic acid also reduced the *C.d.t.* activity to 5%, while in the presence of vitamin complex activity was reduced to approximately 65%. At ratios of 1: 2.5 and 1: 1, inhibition was observed only in the presence of vitamin complex reducing the proteolytic activity to 70 and 50%, respectively (Fig. 1b).

None of the vitamin treatments proved to be effective in inhibiting proteolysis of collagen (data not shown).

The vitamin complex and ascorbic acid were effective in inhibiting gelatinolytic activity induced by *B. atrox*, and inhibitions were observed in all evaluated proportions (Fig. 2). The most effective ratio of 1:10 induced activity reduction to approximately 65% and 52% for vitamin complex and ascorbic acid, respectively (Fig. 2).

CLOTTING ACTIVITY

Either the preincubation of vitamin with plasma (Table I) and with venoms (Table II), both proposed for coagulant testing, showed significant results and extended clotting time for ascorbic acid and vitamin complex.

Preincubation with venom and vitamins stood out between the two treatments (Table II). This suggests, therefore, possible interactions between toxins and vitamin molecules. In addition,
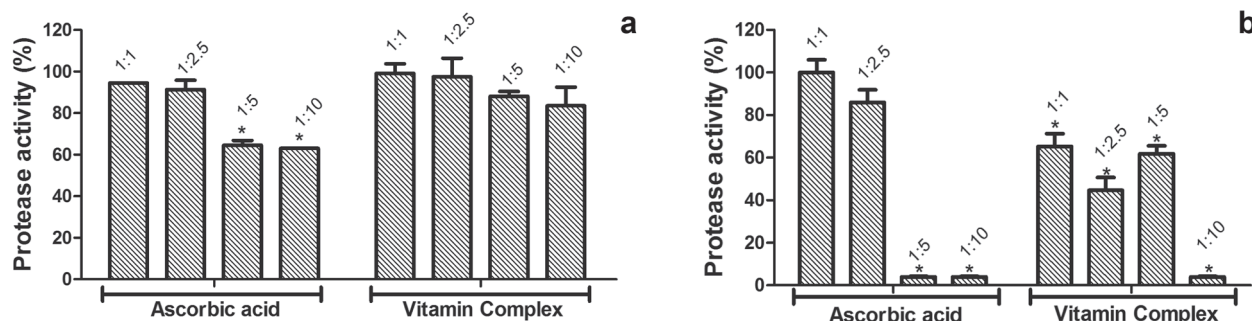

Figure 1 - Vitamins effect on azocaseinolytic activity. (**a**) *Bothrops atrox* venom previously incubated with ascorbic acid or vitamin complex in different ratios. (**b**) *Crotalus durissus terrificus* venom previously incubated with ascorbic acid or vitamin complex in different ratios.

the largest proportions, such as 1: 2.5 and 1: 5, promoted the highest inhibition for both venoms (Table II).

Hence, lower inhibitions were observed for preincubated vitamins with plasma, demonstrating non-significant changes in coagulation time induced by *C.d.t.* venom (Table I).

On the part of vitamins, higher proportions could not be assessed due to the saturation limit amounts of the reaction environment; however, variations in incubation time and concentration of ions can be further evaluated.
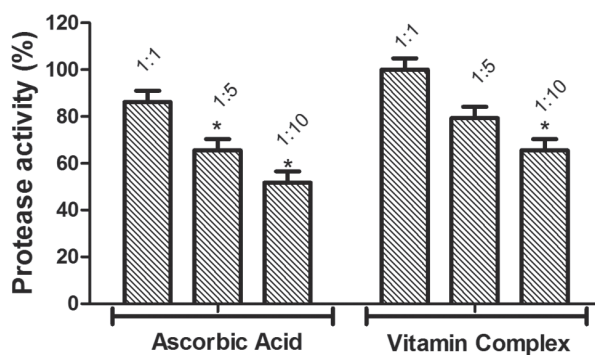

Figure 2 - Vitamins effect on gelatinolytic activity induced by *Bothrops atrox* venom. The controls average containing only venom was considered as 100% activity.

TABLE I
Coagulant activity induced by *Crotalus durissus terrificus* and *Bothrops atrox* venoms on human citrated plasma preincubated with vitamins in different ratios (for 5 min. at 37°C).

Vitamin effect on plasma clotting time			
Sample	Clotting time (min.)	Sample	Clotting time (min.)
C.d.t. (10 µg)	1.05 ± 0.04	*B. atrox* (10 µg)	1.15 ± 0.02
C.d.t. + AA (1:0.5)	1.21 ± 0.03	*B. atrox* + AA (1:0.5)	1.31 ± 0.05
C.d.t. + AA (1:1)	1.12 ± 0.01	*B. atrox* + AA (1:1)	1.34 ± 0.05
C.d.t. + AA (1:2.5)	1.31 ± 0.03*	*B. atrox* + AA (1:2.5)	1.49 ± 0.09*
C.d.t. + AA (1:5)	1.39 ± 0.04*	*B. atrox* + AA (1:5)	2.07 ± 0.06*
C.d.t. + VC (1:0.5)	1.18 ± 0.03	*B. atrox* + VC (1:0.5)	1.39 ± 0.04*
C.d.t. + VC (1:1)	1.10 ± 0.02	*B. atrox* + VC (1:1)	1.56 ± 0.04*
C.d.t. + VC (1:2.5)	1.16 ± 0.01	*B. atrox* + VC (1:2.5)	2.23 ± 0.05*
C.d.t. + VC (1:5)	1.16 ± 0.04	*B. atrox* + VC (1:5)	2.24 ± 0.05*

Results expressed in clotting time represent averages of triplicates and the standard deviation (SD). The significance asterisks result from comparative analysis of treatments with control containing only venom. AA = ascorbic acid. VC = vitamin complex.

TABLE II
Clotting activity induced by *Crotalus durissus terrificus* and *Bothrops atrox* venom on human citrated plasma proceeding with preincubation of vitamins with the venoms in different ratios (for 5 min. at 37°C).

Vitamin effect on plasma clotting time			
Sample	Clotting time (min.)	Sample	Clotting time (min.)
C.d.t. (10 µg)	1.02 ± 0.02	*B. atrox* (10 µg)	1.29 ± 0.01
C.d.t. + AA (1:0.5)	1.17 ± 0.03	*B. atrox* + AA (1:0.5)	1.29 ± 0.01
C.d.t. + AA (1:1)	1.41 ± 0.07*	*B. atrox* + AA (1:1)	1.39 ± 0.01*
C.d.t. + AA (1:2.5)	1.42 ± 0.03*	*B. atrox* + AA (1:2.5)	1.36 ± 0.04*
C.d.t. + AA (1:5)	2.40 ± 0.05*	*B. atrox* + AA (1:5)	2.01 ± 0.01*
C.d.t. + VC (1:0.5)	1.41 ± 0.05*	*B. atrox* + VC (1:0.5)	2.03 ± 0.03*
C.d.t. + VC (1:1)	1.14 ± 0.02	*B. atrox* + VC (1:1)	1.56 ± 0.03*
C.d.t. + VC (1:2.5)	1.26 ± 0.07	*B. atrox* + VC (1:2.5)	2.04 ± 0.04*
C.d.t. + VC (1:5)	2.54 ± 0.04*	*B. atrox* + VC (1:5)	2.18 ± 0.04*

Results expressed in clotting time represent averages of triplicates and the standard deviation (SD). The significance asterisks result from comparative analysis of treatments with control containing only venom. AA = ascorbic acid. VC = vitamin complex.

THROMBOLYTIC ACTIVITY

Only the *B. atrox* venom induced lysis of the thrombus. The vitamin complex preincubated with venom at 1: 5 and 1: 10 ratios was able to reduce the thrombolytic activity to 15 and 3.3%, respectively (Fig. 3). However, ascorbic acid showed higher inhibitory potential, being responsible for reducing the venom activity to 47, 8 and 3.8% at ratios of 1: 1, 1: 5 and 1: 10, respectively (Fig. 3).

HEMAGGLUTINATION ASSAY

Only the *B. atrox* venom induced hemagglutination activity. This activity was completely inhibited after preincubation of the venom with ascorbic acid at the ratio of 1: 0.5, whereas in higher proportions (1: 1, 1: 2.5 and 1: 5), partial agglutination
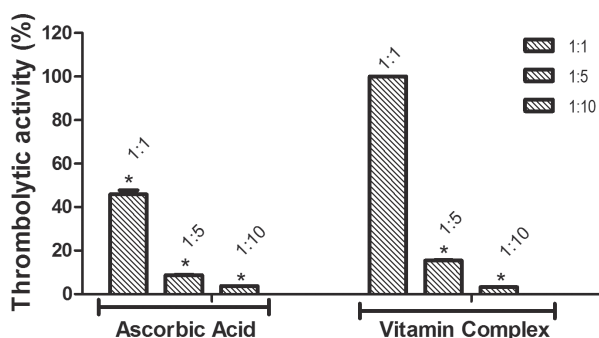
of the erythrocytes was observed. Absence of hemagglutination was also observed after preincubating the venom with vitamin complex at a ratio of 1: 1 (data not shown).

HEMOLYTIC ACTIVITY IN LIQUID MEDIUM

The two treatments evaluated, ascorbic acid and vitamin complex, reduced hemolytic activity, highlighting the vitamin complex which reduced the hemolytic activity induced by the *B. atrox* venom to 27% at the proportions of 1: 2.5 and 1: 5 (Fig. 4a). Although ascorbic acid in all tested proportions has shown significant inhibition against the *C.d.t.* venom, the greater inhibitions of hemolysis induced by this venom were obtained after incubation with vitamin complex at ratios of 1: 5 and 1: 10, with activity reduced to about 50% (Fig. 4b).

FIBRINOGENOLYTIC ACTIVITY

The two treatments evaluated were effective in inhibiting the degradation of fibrinogen molecules, induced by the *B. atrox* venom (Fig. 5a) and *C.d.t.* venom (Fig. 5b). Some fibrinopeptides can be seen on line 4 of Figure 5a, which corresponds to the treatment with ascorbic acid at a ratio of 1: 5, showing partial inhibition of proteolytic activity. Figure 5b shows 100% inhibition of the activity induced by the *C.d.t.* venom, for both treatments.
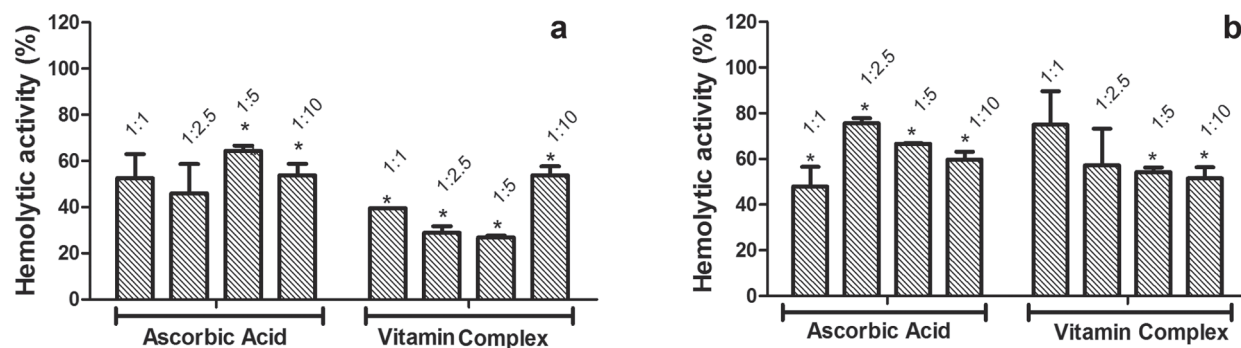

Figure 3 - Vitamins effect on thrombolytic activity induced by *Bothrops atrox* venom. The controls average containing only venom was considered as 100% activity.

Figure 4 - Vitamins effect on hemolytic activity. (**a**) *Bothrops atrox* venom previously incubated with ascorbic acid or vitamin complex in different ratios. (**b**) *Crotalus durissus terrificus* venom previously incubated with ascorbic acid or vitamin complex in different ratios. Data presents average of triplicate and the values obtained to the venom alone were converted to 100% activity.

However, smaller and larger doses were also evaluated (data not shown) and shown to be without effect, only being presented the ratio of 1: 5 and this one is considered as corresponding to the equivalent molar concentrations between fibrinogenolytic protease and vitamins.
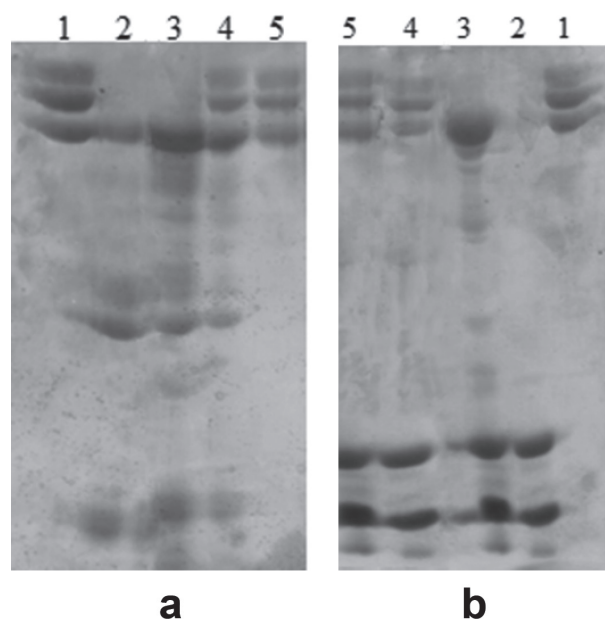

Figure 5 - Vitamins effect on fibrinogenolytic activity. (**a**) Samples: 1- Fibrinogen (60 µg); 2- *Bothrops atrox* venom (60 µg); 3- Fibrinogen + *B. atrox* venom; 4- Fibrinogen + *B. atrox* venom + ascorbic acid – AA (300 µg) and 5- Fibrinogen + *B. atrox* venom + vitamin complex – VC (300µg). (**b**) Samples: 1- Fibrinogen (60 µg); 2- *Crotalus durissus terrificus* venom (80 µg); 3- Fibrinogen + *C. d. terrificus* venom; 4- Fibrinogen + *C. d. terrificus* venom + ascorbic acid – AA (300 µg) and 5- Fibrinogen + *C. d. terrificus* venom + vitamin complex – VC (300µg).

DISCUSSION

As observed during caseinolytic activity tests, the inhibition differential can be related to the greater expression of proteases from snake venoms of the species *B. atrox* (69.3% serineproteases plus metalloproteases) compared to the species *C.d.t.* (29.2% serineproteases plus metalloproteases) (Guércio et al. 2006, Georgieva et al. 2010, Kohlhoff et al. 2012). Therefore, the vitamin doses evaluated were not appropriate to inhibit

substantially all classes of proteases expressed in the *B. atrox* venom, although they have been shown to be effective in inhibiting the *C.d.t.* venom.

The lack of inhibition observed for both vitamin samples, evaluated on the proteolysis of collagen molecules, is probably due to a quick action of several proteases from the venom, which act on coagulation factors, platelets and bleeding induction, requiring additional tests to exploit the concentration-time relation in order to observe the inhibitory effect (Escalante et al. 2011).

The different structures of the evaluated substrates, azocasein, gelatin and collagen, suggest the action of different proteases on each of them, enabling the interaction of these with inhibitors also in a variable form, which explains the variations in inhibition percentages observed at each test.

The ascorbic acid and the vitamin complex have drastically inhibited (inhibition of ≈ 100%) the fibrinogenolytic activity induced by both venoms evaluated. The result is in agreement with the data obtained in coagulation assay, since the fibrinogenolysis is directly related to blood clot development (Braud and Wisner 2000). Thus, the same way as vitamins proved effective in prolonging plasma-clotting time, by partially inhibiting coagulant toxins, they were also able to inhibit degradation of fibrinogen.

In the coagulant assay, the lower inhibition response by vitamin incubation with plasma can be related to the less interaction between vitamins and constituents of the plasma that participate in clot cascade, in comparison to the interactions between vitamins and molecules as PLA_2 and proteases, present in crotalic and bothropic venoms, act on coagulation. In addition, different amounts of these molecules in the composition of venoms can interfere in the process, as well as variations in the ionic composition of the same (enzyme cofactor) that are able to interact with vitamins.

Antioxidant compounds have been suggested as being able to interact with binding sites of

cofactors within protease structure, and modify their structure; thus, interfering with the binding of cofactors and consequently resulting in reduced enzymatic activity (Patiño et al. 2013, Oliveira et al. 2014). For instance, thiol groups have been described with the ability of both chelating enzyme cofactors and interfering with enzymatic redox reactions in pharmacological tests induced by viperid venoms (Sunitha et al. 2011). Another result that confirms this hypothesis is the inhibition of proteolytic activity by rosmarinic acid, cinnamic acid and their analogues (Aung et al. 2011). Thus, the aforementioned interaction seems to be one of the inhibition mechanisms performed by vitamins with antioxidant activity on the various activities induced by different proteases, such as coagulant, proteolytic on various substrates and thrombolytic activities.

The inhibition of proteases, in addition to increasing vitamin survival time and decrease severity of toxic signs also contributes in preserving the cardiovascular system changes by inhibiting blood pressure lowering and depressed atrial contractility (Shabbir et al. 2014).

The hemagglutinating activity exerted by C-type lectins may vary in composition of the different batches of venoms used, since this protein class has increased expression in juvenile snake specimens and semi-adults and lower expression in adults (Cruz et al. 2005). Thus, the absence of activity observed for the *C.d.t.* venom may be related to maturity of the snakes used to obtain the samples of venom used in evaluations.

The C-type lectins are related to platelet and erythrocytes aggregation (Zelensky and Gready 2005), acting by binding to carbohydrates present in cell membranes (Kassab 2001). Thus, vitamins could be interacting with the glycidic ligands present on the surface of erythrocytes and preventing the lectin-binding present in the *B. atrox* venom, resulting in complete inhibition of hemagglutinating activity observed in this study.

Interactions of lectin present in the venom with possible inhibitory molecules are poorly explored, with no indications of possible action mechanisms of vitamins, such as lectin inhibitors (Oliveira et al. 2016).

The hemolytic activity partial inhibition, whose induction is primarily attributable to PLA$_2$s, may represent the absence of interactions between vitamin molecules and phospholipases in venoms, as well as the difficulties of interaction of vitamins with membrane components, such as phospholipids, resulting in low protection against the catalytic action exerted by phospholipases.

However, there are reports in literature about a lipophilic ascorbic acid derivative, synthesized in laboratory, which due to its changes in polarity, can be effective in inhibiting PLA$_2$ activity isolated from *Vipera russelli* (Mohamed et al. 2013). Moreover, venom proteases may also contribute to hemolysis induction, being, thus, necessary to inhibit several phospholipases and proteases by vitamins, hindering their action.

Vitamin E, present in the vitamin complex, can also have inhibitory effect against lipid peroxidation of the cell membrane of human erythrocytes, this being one of the possible mechanisms for hemolytic activity reduction (Mukherjee et al. 1997).

Vitamins of the B complex may contribute to repair mechanisms, acting on the damage caused to cellular membrane by free radicals generated by toxin activity (Huang et al. 2010), as well as ascorbic acid (antioxidant) which can neutralize radicals reducing damage to the membranes. This way, the vitamins E, B complex and ascorbic acid, present in the vitamin complex, probably act in synergy through several action mechanisms, reducing the hemolytic activity induced by the toxins.

The inhibitory effects provided by ascorbic acid and the B-complex vitamins, analyzed in this paper, on the biological activities induced by bothropic and crotalic venoms, have mechanisms still not clarified; however, part of these effects

come from the antioxidant activity of some vitamins (e.g. ascorbic acid), as well as interactions between hydrophobic regions of others (e.g. vitamin E). Nevertheless, further studies are required to expand the mechanistic discussions about interactions between vitamins and toxins and of these with animal cells and molecules, aiming to formulate therapies complementary to serotherapy, increasing treatment efficiency.

CONCLUSIONS

The vitamin complex evaluated, as well as the isolated ascorbic acid, show inhibitory potential on activities, mainly the ones induced by proteases and PLA$_2$, the major classes of toxins present in the snake venoms from the Viperidae family.

Once vitamins can be easily obtained in pharmaceutical formulations certified by ANVISA (Brazilian National Health Surveillance Agency), they represent a safer alternative and can bring benefits to snakebite victims.

There is a lack of knowledge on the interaction of vitamins with the different classes of enzymes present in snake venoms, since existing studies describe only interactions between lipophilic vitamins and phospholipases.

Therefore, several additional studies should be conducted to extend the information on inhibitory mechanisms, administration forms and dosages which may be effective as adjuvants of traditional serotherapy.

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RESUMO

A inibição enzimática por compostos naturais e/ou de baixo custo pode representar um valioso adjuvante ao tratamento soroterápico tradicional realizado em casos de ofidismo, com vistas a amenizar principalmente os efeitos locais do envenenamento. Objetivou-se avaliar *in vitro* possíveis interações entre vitaminas e enzimas presentes nas peçonhas de *Bothrops atrox* e *Crotalus durissus terrificus*. Ensaios de inibição de proteólise (substratos: azocaseína, colágeno, gelatina e fibrinogênio), hemólise, coagulação, hemaglutinação, foram realizados utilizando diferentes proporções de vitaminas em face de inibir à dose mínima efetiva de cada peçonha. As vitaminas foram responsáveis por reduzir 100% a quebra da azocaseína por peçonha de *C.d.t.*, a trombólise induzida por *B. atrox* e a fibrinogenólise induzida por ambas as peçonhas. Sugere-se a presença de interações de vitaminas com o sítio ativo das enzimas, como por exemplo, interações entre regiões hidrofóbicas presentes nas enzimas e vitamina E, assim como, inibição de algumas atividades por mecanismo de ação antioxidante.

Palavras-chave: propriedades antiofídicas, antioxidantes, células sanguíneas, processos hemostáticos, inibidores naturais.

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