Received: October 9, 2008
Accepted: February 13, 2009
Abstract published online: March 5, 2009
Full paper published online: August 31, 2009

J Venom Anim Toxins incl Trop Dis.
V.15, n.3, p.460-478, 2009.
Original paper.
ISSN 1678-9199.

IgG ANTIBODIES AGAINST PHOSPHOLIPASE A_2 FROM *Crotalus durissus terrificus*: CROSS-REACTION WITH VENOMS FROM *Bothrops* SPECIES FROM ARGENTINA

Rodríguez JP (1), De Marzi MC (2), Maruñak S (3), Teibler P (3), Acosta O (3), Malchiodi EL (2), Leiva LC (1)

(1) Department of Chemical Biology, School of Natural and Exact Sciences and Land Surveying, National University of the Northeast, UNNE, Corrientes, Argentina; (2) Department of Immunology, Center for the Study of Humoral Immunity, IDEHU, School of Pharmacy and Biochemistry, University of Buenos Aires, Buenos Aires, Argentina; (3) Department of Pharmacology, School of Veterinary Science, National University of the Northeast, UNNE, Corrientes, Argentina.

ABSTRACT: We examined the ability of IgG anti-crotalic PLA_2 to cross-react with *Bothrops* spp. venoms, from snakes found in the northeast of Argentina. Immunoblotting and ELISA tests showed that IgG anti-crotalic PLA_2 recognize antigens of bothropic venoms. Indirect hemolytic activity tests showed that the quantity of antibodies that neutralized 50% of *Crotalus durissus terrificus* venom (ED_{50}: 2.1 mg IgG anti-crotalic PLA_2/100 µg of venom) were also able to neutralize venom from other snakes in the following proportion: 34% of *B. alternatus*, 18% of B. diporus and 12% of B. jararacussu. Likewise, direct PLA2 activity neutralization tests showed a similar cross-neutralization pattern including 56% of *B. alternatus*, 29% *of B. diporus* and 30% of *B. jararacussu*. In addition, in a myotoxic activity neutralization test, measured by plasma activity of creatine kinase, 35% of *B. alternatus* venom and 26% of *B. diporus* venom were neutralized, while no neutralization was detected with *B. jararacussu* venom. This study presents original data concerning cross-reactions between bothropic venoms from Argentina and IgG anti-crotalic PLA_2. Our results suggest that anti-crotalic PLA_2 antibodies should not be used to neutralize PLA_2 activity of *B. alternatus*, *B. diporus* and especially *B. jararacussu* venoms; nor to enrich commercial antivenoms against these *Bothrops* species.

KEY WORDS: *Bothrops*, *Crotalus*, PLA_2, neutralization, cross-reaction.

CONFLICTS OF INTEREST: There is no conflict.

FINANCIAL SOURCE: SGCyT – UNNE, CONICET.

CORRESPONDENCE TO:
LAURA C. LEIVA, Departamento de Bioquímica, Facultad de Ciencias Exactas y Naturales y Agrimensura, Universidad Nacional del Nordeste, Corrientes, 3400, Argentina. Phone: +54 3783 457996 Fax: +54 3783 473930. Email: lcleiva@exa.unne.edu.ar.

INTRODUCTION

The most abundant snake species that inhabit northeastern Argentina are *Bothrops diporus* (*B.d.*), *Bothrops alternatus* (*B.a.*) and *Bothrops jararacussu* (*B.j.*) that belong to the Viperidae family. *Crotalus durissus terrificus* (*C.d.t.*) is the only subspecies of the genus *Crotalus* that is found in this subtropical region of South America (1). The venoms from these viperids contain phospholipases A_2 (PLA_2) that hydrolyze phospholipids at the *sn*-2 position in a calcium-dependent manner, releasing fatty acids and lysophospholipids (2). PLA_2 is the main enzyme responsible for signs and symptoms observed in crotalic envenomation, while it is only partially involved in manifestations of bothropic envenomation (3-5).

The toxicity of crotalic PLA_2 (*C.*PLA_2) enzyme is potentiated by a small non-enzymatic protein, crotapotin; therefore, both form a well known complex, crotoxin (6, 7). This lethal toxin exhibits *in vivo* a potent neuromuscular and myotoxic activity, and induces *in vitro* indirect hemolysis when red blood cells are in the presence of egg yolk (8, 9).

In contrast, PLA_2 from *Bothrops* spp. do not have an elevated lethality, but exhibit myotoxic, edematogenic, anticoagulant and hemolytic (indirect) activities, which in synergistic action with other components of the venom (thrombin-like enzymes, metalloproteases etc.) become a highly lethal secretion (10-12).

Snakebites from these species represent a clinical problem in northeastern Argentina and Brazil, and the treatment of choice for victims has been heterologous antiserum administration (13, 14). Most commercial antisera are produced in equines and whole venoms are employed for immunizations in order to obtain anti-bothropic (ABS) or anti-crotalic serum (ACS). A specific antiserum is indicated when the snake that bitten the patient is identified (13). However, there are also commercial antisera against venoms from several species of the same genus, or from different genera, in order to increase the neutralization spectrum, thus improving its versatility (15).

Over a century ago, Brazil (16) observed that ACS was apparently more efficient than ABS in treating *B. jararacussu* envenomation. More recently, a mixture of ACS and ABS has been reported to be more effective than ABS alone in neutralizing the myotoxic activity of *B. jararacussu* venom, suggesting that the addition of antibodies against *Crotalus* toxic components, such as *C.*PLA_2, could be valuable in the treatment of the intoxication (17, 18). Moreover, Beghini *et al.* (19) showed that it is possible to neutralize the neuromuscular blockade produced by *B.j.* and *C.d.t.* venom

with specific antibodies against crotoxin and PLA_2 from *Crotalus durissus cascavella.* On the other hand, in Argentina, De Roodt *et al*. (20) observed that ACS had a significant neutralizing capacity on hemorrhagic, necrotizing, procoagulant, proteolytic and lethal activities of bothropic venoms, all focused on proteases. Nevertheless, several toxic features of bothropic venoms related to PLA_2 – such as indirect hemolytic activity – appear not to be adequately neutralized by ACS. Thus, cross-neutralization of the PLA_2 activity of bothropic venoms in Argentina remains unclear up to now. On the other hand, neutralization of *B.a.* and *B.d.* venoms with antibodies against *C.*PLA_2 has not yet been reported.

In this work, we analyzed the ability of IgG anti-*C.*PLA_2 to cross-react with components of bothropic venoms, in order to determine the responsibility of these antibodies in the cross-neutralization between ACS and bothropic venoms. A better understanding of cross-reactions may contribute to the development of more versatile antivenoms.

MATERIALS AND METHODS

Venom and Toxin

Desiccated *C.d.t.* venom and bothropic venoms were obtained from the Zoo of Corrientes city. PLA_2 was purified from *C.d.t.* venom by gel filtration chromatography as described by Rodríguez *et al*. (21).

Animals

Male Swiss white mice weighing from 20 to 22 g were supplied by the animal facility of the School of Veterinary Science, UNNE. Mice were housed at 25°C on a 12-hour-light/dark cycle and had free access to food and water.

Male New Zealand white rabbits weighing 3 kg were individually housed with free access to food and water.

IgG anti-*C.*PLA_2

Rabbit polyclonal antibodies were obtained by immunization with 700 μg of PLA_2 per rabbit mixed with a volume of complete Freund's adjuvant. Boosts were administered on day 7, 14 and 28. Serum antibody levels were monitored by gel immunodiffusion

and ELISA. Blood was collected from a marginal ear vein, serum was separated by centrifugation and aliquots were stored at –70°C.

IgG anti-*C*.PLA_2 was obtained by ammonium sulfate precipitation, desionizated by dialysis against 50 mM Tris pH 8.5 and purified by anion exchanged chromatography. Briefly, 2 mL of redissolved immunoglobulin previously precipitated, was purified in a MonoQ® HR 5/5 column (Amersham Biosciences Biotech AB, Sweden) equilibrated with 50 mM Tris pH 8.5 and developed using a linear NaCl gradient. Purified IgG was dialyzed against phosphate buffered saline (PBS) solution and aliquots were stored at –70°C.

Enzyme-linked Immunosorbent Assay (ELISA)

Microtiter plates (96 wells) were coated overnight at 4°C with 100 μL of *C.d.t., B.a., B.j. or B.d.* venoms (5 $\mu g.mL^{-1}$) in PBS. The plates were washed and processed as described by Rodríguez *et al.* (21). Absorbance was read at 490 nm with a Multiskan® EX (Thermo Scientific, USA) multiwell plate reader.

Immunoblotting

C.d.t., B.a., B.d. or *B.j.* venoms (1 $mg.mL^{-1}$) were separated on 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) at 200 V for 45 minutes and the proteins were electrophoretically transferred to nitrocellulose membranes (0.45 mm) at 300 mA for one hour. Subsequently, membranes were blocked at room temperature for two hours in a solution of 5% non-fat milk, 0.05% tween-20. After washed three times in TBS (0.01 M Tris–HCl, 0.17 M NaCl, pH 7.6), membranes were incubated overnight with rabbit IgG anti-*C*.PLA_2 (0.1 $mg.mL^{-1}$ in TBS). After washing, bound antibodies were detected with goat anti-rabbit IgG peroxidase conjugate (Sigma, USA; 1:1000 in TBS) for one hour at room temperature with shaking. At the end of this incubation, blots were washed, developed with 4-chloro-1-naphthol (Sigma, USA; 0.03% in 0.05 M Tris–HCl, pH 7.6, containing 0.03% H_2O_2/OPD) and documented. Rabbit pre-immune serum was employed as negative control.

Indirect Hemolytic Activity Neutralization

Indirect hemolytic activity test was carried out in order to evaluate the IgG anti-*C*.PLA_2 ability to neutralize the phospholipase activity of *C.d.t.* venom as well as to cross-neutralize the PLA activity of bothropic venoms. To determine the amount of IgG required to neutralize 50% of *C*.PLA_2 activity (ED_{50}), 7.5 μg of *C.d.t.* venom was mixed with different dilutions of IgG anti-*C*.PLA_2 and then incubated for one hour at 37°C. Aliquots of 15 μL were added to wells in agarose-egg yolk-sheep erythrocyte gels (9). Plates were incubated at 37°C for 20 hours and the hemolytic halos were measured. Control samples included venom incubated without antibodies and antibodies incubated without venom. The degree of inhibition was calculated as percentage of activity, taking as 100% the diameter halo induced by venom alone (7.5 μg) , and ED_{50} calculated as milligram of antibodies/100 μg venom able to reduce by 50% the diameter of the hemolytic halo when compared to the effect produced by the venom alone.

In order to determine whether IgG anti-*C*.PLA_2 was able to cross-react with bothropic venoms, the amount of IgG corresponding to ED_{50} (158 μg) was pre-incubated with 7.5 μg of each bothropic venom. Then, the mixtures were added to wells in agarose-egg yolk-sheep erythrocyte gels. The hemolytic halos were measured after 20 hours of incubation at 37°C, and the percentage of cross-neutralization was calculated. Samples of bothropic venoms incubated with PBS were considered positive controls.

Direct PLA_2 Activity Neutralization

PLA_2 activity was assayed by a kinetic method according to Lôbo de Araújo and Radvanyi (22). The substrate was a solution of egg phosphatidylcholine (0.265%) in 10 mM $CaCl_2$, 100 mM NaCl, 100 mM phenol red and 7 mM Triton X-100. PLA_2 activity (as fatty acid liberated per min) was measured as changes of absorbance per minute, using a spectrophotometer UV/Visible CamSpec M330® (UK) (at 30°C, λ: 558 nm). The reaction was started by adding venom (7.5 μg) pre-incubated with IgG anti-*C*.PLA_2 (158 μg, corresponding to ED_{50}) in a final volume of 15 μL. The fatty acids released during the reaction were detected by recording changes in the absorbance per minute. Venom or antibodies pre-incubated with PBS were used as controls.

Myotoxic Activity Neutralization

Creatine kinase (CK) determination was performed in order to detect the maximum liberation in a 24-hour period after venom inoculation (23). Groups of five mice (CF1, 18 to 22 g) were intramuscularly injected in the right gastrocnemius with a myotoxic sublethal amount of desiccated crude venom, 1 μg of *C.d.t.* or 20 μg of bothropic venoms, dissolved in 100 μL of PBS. After 1, 3, 6, 12 and 24 hours of venom injection, mice were intraperitoneally anesthetized with chloral hydrate (300 mg/kg) for blood sample collection. Serum was obtained to analyze the activity of CK with the UV kinetic method (Randox Laboratories, UK) based on the measurement of creatinine formed after the reaction between ADP and phosphocreatine. CK activity was expressed in international units per litter (IU/L).

Aiming at determining IgG anti-*C*.PLA_2 ability to neutralize the myotoxic activity of bothropic venoms, groups of five mice (CF1, 18 to 22 g) received intramuscularly the same challenge amount of bothropic or crotalic venom pre-incubated with IgG anti-*C*.PLA_2 (420 μg or 21 μg corresponding to ED_{50}) at 37°C for one hour. Control mice were injected with PBS, venom or antibodies alone. According to the time in which each venom induced the maximum release of CK after inoculation, mice were intraperitoneally anesthetized with chloral hydrate (300 mg/kg) for blood sample collection. Then, serum CK level was determined.

In order to have a histological assessment of myotoxicity, tissue samples of injected muscle were taken and fixed with Bouin's solution for 24 to 48 hours. Thereafter, tissue was dehydrated in a graded alcohol series and embedded in paraffin. Sections of 5-mm thick were stained with hematoxylin and eosin. Control muscle tissue was processed similarly (24).

Survival Time

Groups of five animals were intramuscularly inoculated with a challenge dose of two LD_{50} venom (4 μg of *C.d.t.* or 70 μg of *B.a.* venom) pre-incubated with IgG anti-*C*.PLA_2 and continuously observed every hour. Control groups were inoculated with PBS or venom alone. Death was defined as lack of respiratory effort and movement during a 30-second period. An independent observer, blinded to drug injections, determined the time of death (25).

Statistical Analysis

All experiments were repeated at least three times and the results were expressed as the mean ± SD. The significance of differences between means was assessed by ANOVA followed by Tukey's test for multiple comparisons among groups. Values of $p < 0.05$ were considered significant.

RESULTS

PLA_2 Cross-reactivity

The reactivity determined by ELISA of IgG anti-*C.*PLA_2 against *C.d.t., B.a., B.d.* and *B.j.* venoms is shown in Figure 1. As expected, the greatest reactivity was observed with *C.d.t.* venom since its PLA_2 was used in the immunization process. All bothropic venoms revealed similar behavior with absorbance values around 50% of those obtained with *C.d.t* venom.

Immunobloting was performed in order to identify PLA_2 immunologically related between *Crotalus* and *Bothrops* genus. Figure 2A shows SDS-PAGE of crude *C.d.t.* and bothropic venoms. Bands ranging from 14 to 70 kDa can be observed under reducing conditions. Then, nitrocellulose membranes incubated with IgG anti-*C.*PLA_2 demonstrated that the reactivity of these specific antibodies was restricted to ~14 kDa components, corresponding to PLA_{2s} from crotalic and bothropic venoms (Figure 2B).

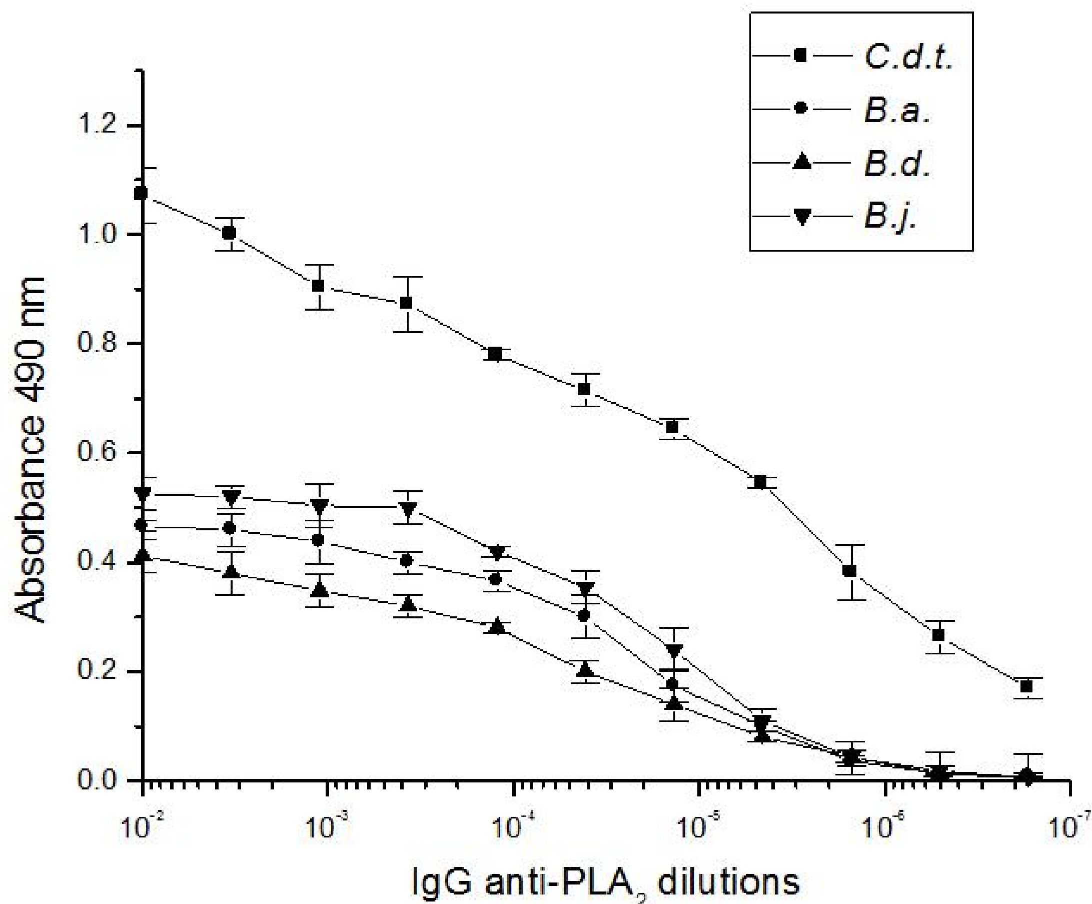

Figure 1. Cross-reactivity determined by ELISA. Plates were coated with antigen (5 μg/mL), incubated with rabbit IgG anti-*C*.PLA_2 antibodies at indicated dilutions and finally with an appropriate IgG-peroxidase conjugate and substrate (OPD). Absorbencies were read at 490 nm. As expected, the greatest reactivity was seen with *C.d.t.* since its PLA_2 was used in the immunization.

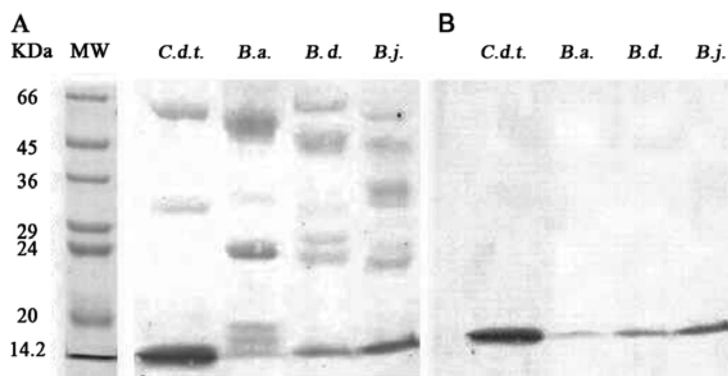

Figure 2. Venom cross-reactivity determined by immunoblotting. (**A**) SDS-PAGE of bothropic and crotalic venoms. (**B**) Immunoblotting showing the reactivity of *C.d.t.* venom and bothropic venoms to IgG anti-*C*.PLA_2.

Indirect Hemolytic Activity Neutralization

The amount of antibodies that neutralized 50% of 100 μg of *C.d.t.* venom was 2.1 ± 0.22 mg. Cross-neutralization of bothropic venoms was express as percentage of inhibition of indirect hemolytic activity (Table 1).

Table 1. Indirect hemolytic activity neutralization

	B.a.	***B.d.***	***B.j.***
Venom[a]	22.33 ± 0.76	25.83 ± 0.28	26.66 ± 0.57
Venom + IgG anti-*C.* PLA_2[b]	14.66 ± 0.50	21.16 ± 0.76	23.50 ± 0.50
Percentage of neutralization	34.32	18.06	11.87

[a]Indirect hemolytic activity exhibited by venoms (7.5 μg) expressed in millimeter of hemolytic halo; [b]hemolytic activity exhibited by venoms (7.5 μg) neutralized with IgG anti-*C.*PLA_2 (158 μg). Results are expressed as the mean ± SD. Data are representative of three experiments.

Direct PLA_2 Activity Neutralization

We analyzed whether the amount of IgG anti-*C.*PLA_2 that efficiently neutralize direct PLA_2 activity of *C.d.t.* venom (ED_{50}) was able to neutralize *Bothrops* venoms. Table 2 shows that *B.a*. venom was best cross-neutralized, while *B.d.* and *B.j.* showed similar low percentage of neutralization when IgG anti-*C.*PLA_2 was employed.

Table 2. Direct PLA_2 activity neutralization

	C.d.t.	***B.a.***	***B.d.***	***B.j.***
Venom[a]	0.332 ± 0.021	0.034 ± 0.007	0.062 ± 0.012	0.027 ± 0.007
Venom + IgG anti-*C.*PLA_2[b]	0.074 ± 0.015	0.015 ± 0.005	0.044 ± 0.009	0.019 ± 0.003
Percentage of neutralization	77.71	56	29.03	29.62

[a]Direct PLA_2 activity exhibited by venoms (7.5 μg) expressed as changes of absorbance/minute; [b]direct PLA_2 activity exhibited by venoms (7.5 μg) neutralized with IgG anti-*C.*PLA_2 (158 μg). Results are expressed as the mean ± SD. Data are representative of three experiments.

Myotoxic Activity Neutralization

To determine the time of the maximum release of CK for each venom, mice were inoculated with sublethal amount of desiccated venoms and CK activity was

determined in a 24-hour period. Venoms showed different CK releasing profile. *B.j.* venom showed the highest release of CK one hour after venom inoculation, *B.a*. venom presented the peak three hours after, whereas *C.d.t.* and *B.d.* venoms showed their peaks six hours after injection (Figure 3).

The ability of IgG anti-*C*.PLA_2 in neutralizing the myotoxic activity was evaluated 1, 3 and 6 hours after inoculation of *B.j., B.a.* and *B.d.* or *C.d.t.* venoms, respectively, corresponding to the maximum release of CK in plasma, as previously detected. Figure 4 shows that *B.a.* venom myotoxic activity is better cross-neutralized with 35% neutralization, whereas *B.d.* showed 26% of plasma CK level reduction. On the contrary, *B.j.* venom treated with IgG anti-*C*.PLA_2 showed no significant difference in animals injected with venom alone. Despite the myotoxic activity of *B.a.* and *B.d.* venoms was significantly cross-neutralized with IgG anti-*C*.PLA_2, the CK activity was high enough to show significant differences in the control group, indicating only a partial neutralization. On the other hand, *C.d.t.* venom neutralized with IgG anti-*C*.PLA_2 showed no differences in animals inoculated with PBS, demonstrating complete myotoxic activity neutralization (Figure 4).

Figure 5 illustrates the histological changes produced by *C.d.t.* venom with pronounced myonecrosis while animals treated with venom pre-incubated with IgG anti-*C*.PLA_2 maintained a normal appearance, and no tissue injury was identified. On the other hand, bothropic venoms produced extensive damage and no protective effect was detected after treatment with IgG anti-*C*.PLA_2.

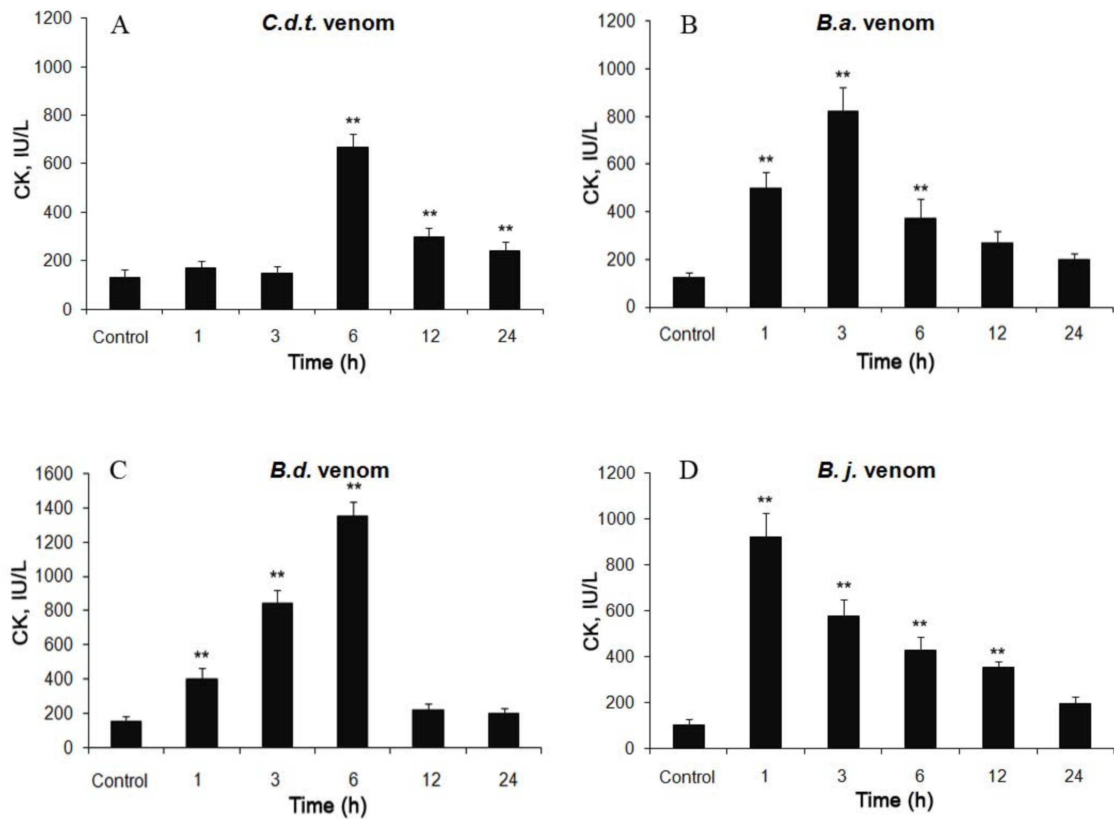

Figure 3. Myotoxic activity of *C.d.t.* venom and bothropic venoms. (**A**) Effects of *C.d.t.* venom and (**B**, **C**, **D**) bothropic venoms on mouse skeletal muscle were measured by plasma CK levels (IU/L). Each point represent the mean $\pm$ SD of five samples. **$p < 0.001$; compared with the corresponding control group.

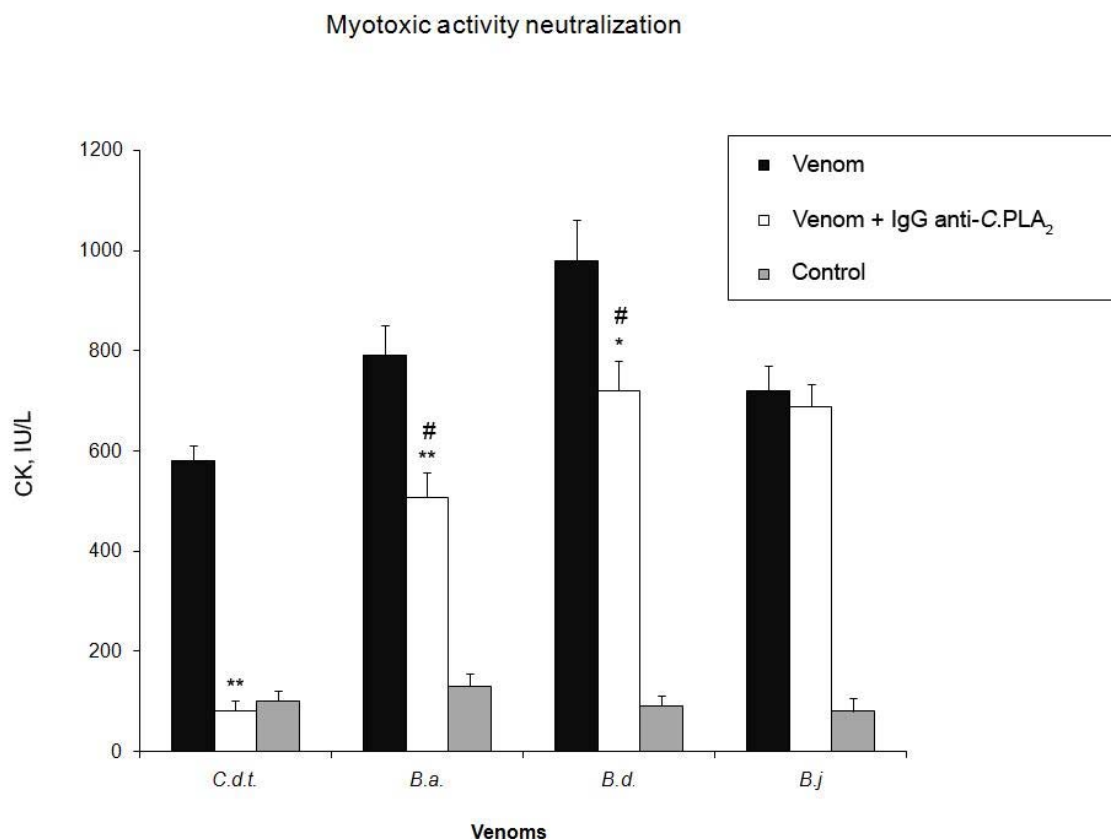

Figure 4. Neutralization of myotoxic activity. Effects of *C.d.t.* venom and bothropic venoms on mouse skeletal muscle measured by plasma CK levels (IU/L) and their neutralization by IgG anti-*C.*PLA_2. Neutralization was carried out at the time of the maximum liberation of serum CK: one hour (*B.j.*), three hours (*B.a.*) or six hours (*C.d.t.*; *B.d.*). Each point represent the mean $\pm$ SD of five samples. # $p < 0.001$, compared with the control group; * $p < 0.01$ or ** $p < 0.001$ compared with the group inoculated with venom alone.

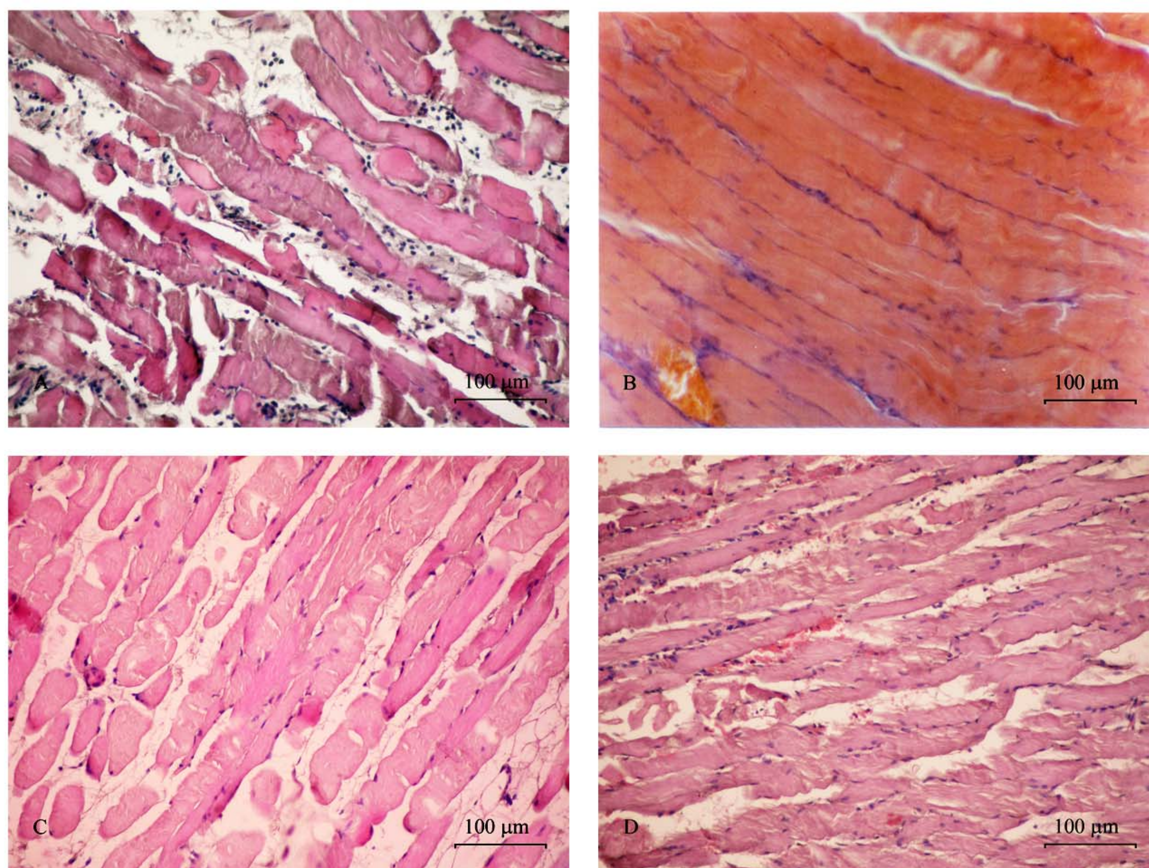

Figure 5. Histological studies on neutralization of myotoxic activity. Longitudinal section of mouse gastrocnemius muscle. (**A**) *C.d.t.* venom; (**B**) *C.d.t.* venom pre-incubated with IgG anti-*C*.PLA_2; (**C**) *B.j.* venom; (**D**) *B.j.* venom pre-incubated with IgG anti-*C*.PLA_2. Magnification 20x.

Survival

To analyze the resistance of mice inoculated with venom neutralized by anti-*C*.PLA, we measured survival time after inoculation of two LD_{50} of venom, or the same amount of venom neutralized with IgG anti-*C*.PLA_2 (Figure 6). At this challenge level, all animals inoculated with *C.d.t.* venom succumbed in five hours, whereas all animals inoculated with *C.d.t.* venom pre-incubated with IgG anti-*C*.PLA_2 survived. On the other hand, all mice inoculated with *B.a.* venom died in four hours, while mice inoculated with venom pre-incubated with IgG anti-*C*.PLA_2 exhibited a delay in the time of death of 2 to 3 hours. These results reveal partial cross-neutralization of PLA_2 activity of *B.a.* venom when anti-*C*.PLA_2 is used.

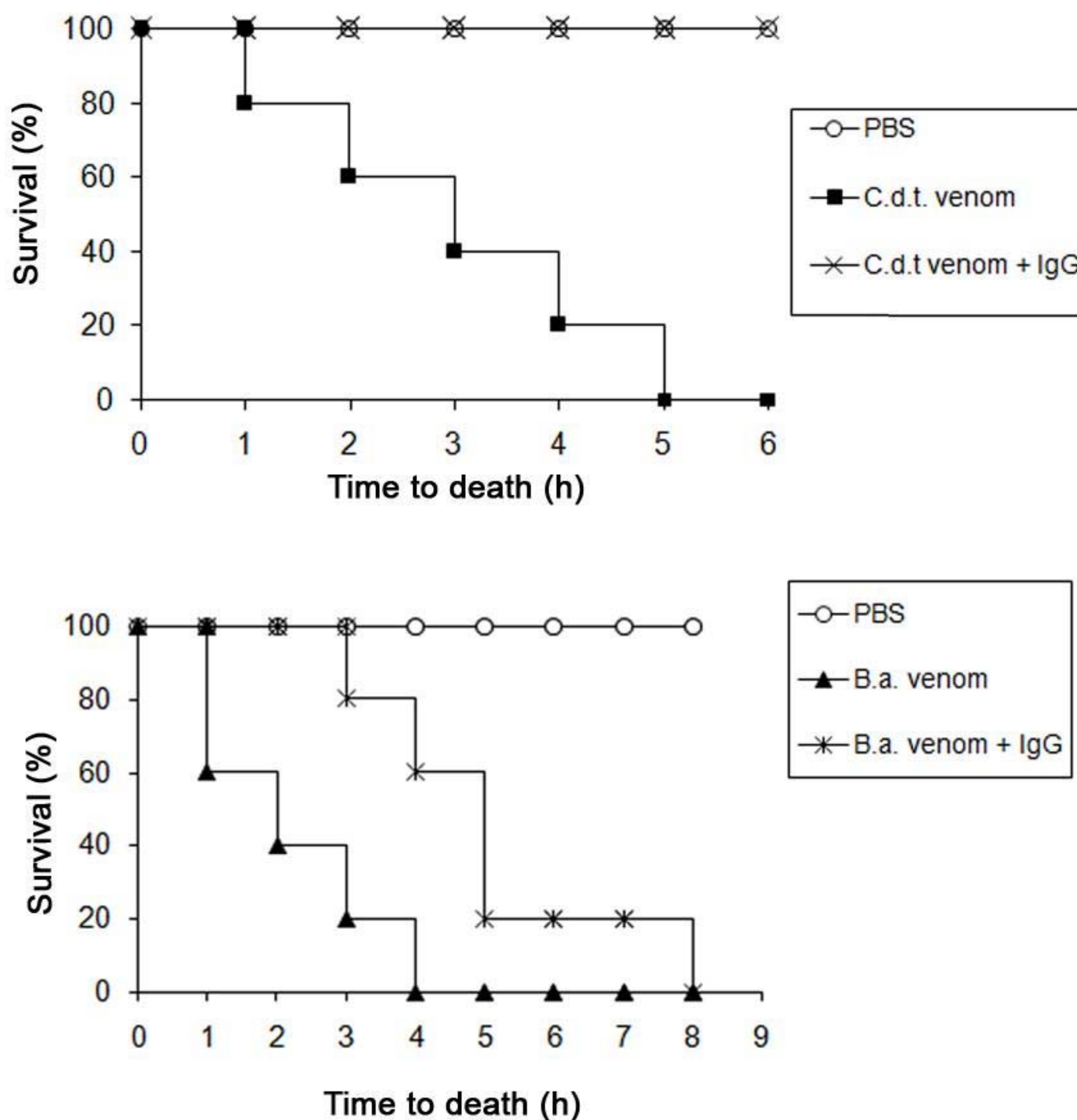

Figure 6. Survival of mice treated with *C.d.t.* venom*, B.a.* venom or these venoms pre-incubated with IgG anti-*C*.PLA. Note that animals inoculated with *B.a.* venom pre-incubated with IgG anti-*C*.PLA_2 resisted at least for three hours compared with animals inoculated with venom only.

DISCUSSION

The present study was undertaken to assess the cross-reaction of IgG anti-*C*.PLA_2 against specific bothropic venoms from Argentina, in order to employ it as antivenom or to enrich commercial antisera with these antibodies.

Immunoblot assays showed that IgG anti-*C.*PLA_2 recognized PLA_2 from all Argentinean bothropic venoms. Additionally, ELISA results confirmed the reactivity of IgG anti-*C.*PLA_2 to bothropic venoms. It is not rare to find considerable cross-reactivity among subclasses of venom PLA_2, since they have an extensive structural homology (8, 19, 26). Although there is a high level of immunochemical cross-reactivity among them, bothropic venoms showed lower reactivity than *C.d.t.* venom, as expected, since *C.*PLA_2 was used as antigen in rabbit inoculations.

Overall, these results are consistent with *in vitro* and *in vivo* biological neutralization tests. We demonstrated, by indirect hemolytic activity and direct PLA_2 neutralization tests, that IgG anti-*C.*PLA_2 partially cross-neutralizes bothropic venoms. The amount of antibodies that efficiently neutralized 50% of *C.d.t.* venom indirect hemolytic activity, or 77% of direct PLA_2 activity, was not enough for neutralizing bothropic PLA_2 biological activities; *B.a.* venom was better cross-neutralized than the others whereas a lower level of cross-reaction was obtained with the other bothropic venoms, among them, *B.j.* venom resulted the worst neutralized. These tests were carried out employing specific antibodies anti-*C.*PLA_2 and agreed with those developed with antivenom against whole *C.d.t.* venom (20).

Myotoxic activity neutralization test demonstrated that IgG anti-*C.*PLA_2 had a certain degree of protective effect on *B.a.* venom, in opposition to *B.d.* and *B.j.* Mice inoculated with antivenom plus *B.a.*-antigen complex exhibited a lower releasing of CK compared with mice inoculated with venom only. These tests show that IgG anti-*C.*PLA do not neutralize neither PLA_2 (Asp49) – responsible for indirect hemolytic activity, edematogenesis and myotoxicity – nor PLA_2 (Lys49) – present in bothropic venoms and particularly powerful in *B.j.* venom.

Thus, these results reveal that cross-neutralization of hemorrhagic and lethal activities obtained by other authors with ACS or ACS/ABS mixtures cannot be attributed to a reaction between antibodies against crotoxin and bothropic myotoxins (Lys49).

C.d.t. venom presents enzymes with thrombin-like activity; consequently, cross-neutralization of hemorrhagic and lethal activities may be explained by the cross-reaction between antibodies against crotalic and bothropic thrombin-like enzymes (27).

Survival test results were in accordance with those obtained from biological activity neutralization tests. The antivenom did not cross-neutralize the lethal activity of *B.a.*

venom, as expected, since it presents other lethal components. However, mice injected with a antivenom-antigen complex exhibited a delay of the survival time compared with animals inoculated with venom alone. Mice treated with *B.j.* or *B.d.* venom neutralized with IgG anti-*C.*PLA_2 did not present significant differences compared with animals inoculated with venom only. This test, like other studies stated before, showed that antibodies against thrombin-like enzymes could play a key role neutralizing the reduction of blood fibrinogen provoked by the enzymes in cross-neutralizations developed with ACS or ABS/ACS mixtures.

In conclusion, our results further reveal that the cross-reactivity detected in PLA_2 of bothropic venoms from Argentina to specific IgG antibodies anti-*C.*PLA_2 does not present the appropriated pharmacological effect required for a complete neutralization of the PLA_2 activity of whole bothropic venoms.

Thus, other components of bothropic and crotalic venoms must be studied as molecular targets to generate antibodies, either employed alone or enriching commercial antivenoms with IgG anti-*C.*PLA antibodies.

ACKNOWLEDGEMENTS

This work was financially supported by the Science and Technique General Office – UNNE (SGCyT UNNE, PI 051/06) and National Council on Scientific and Technological Investigations (CONICET, PIP 6298). J. P. Rodríguez has received a fellowship from SGCyT – UNNE Res. 697/06 C.S.

REFERENCES

1. Acosta de Pérez OC, Koscinczuk P, Teibler P, Sánchez Negrette M, Ruiz R, Maruñak S, Bogarin G. Hemorrhagic and edema-forming activity and histological changes in the mouse footpad induced by venoms from Argentinean *Bothrops* and *Crotalus* genera. Toxicon. 1998;36(8):1165-72.

2. Dennis EA. Diversity of group types, regulation, and function of phospholipase A_2. J Biol Chem. 1994;269(18):13057-60.

3. Rangel-Santos A, Dos-Santos EC, Lopes-Ferreira M, Lima C, Cardoso DF, Mota I. A comparative study of biological activities of crotoxin and CB fraction of venoms from *Crotalus durissus terrificus*, *Crotalus durissus cascavella* and *Crotalus durissus collilineatus.* Toxicon. 2004;43(7):801-10.

4. Andrião-Escarso SH, Soares AM, Rodrigues VM, Angulo Y, Díaz C, Lomonte B, et al. Myotoxic phospholipases A_2 in *Bothrops* snake venoms: effect of chemical modifications on the enzymatic and pharmacological properties of bothropstoxins from *Bothrops jararacussu*. Biochimie. 2000;82(8):755-63.

5. Zuliani JP, Fernandes CM, Zamuner SR, Gutiérrez JM, Teixeira CFP. Inflammatory events induced by Lys-49 and Asp-49 phospholipase A_2 isolated from *Bothrops asper* snake venom: role of catalytic activity. Toxicon. 2005;45(3):335-46.

6. Hendon RA, Fraenkel-Conrat H. Biological roles of the two components of crotoxin. Proc Natl Acad Sci USA. 1971;68(7):1560-3.

7. Breithaupt H, Habermann E. Byochemistry and pharmacology of phospholipase A_2 and its complex from *Crotaluss durissus terrificus* venom as influenced by crotapotin. Toxicon. 1972;10:525-6.

8. Kini RM. Excitement ahead: structure, function and mechanism of snake venom phospholipase A_2 enzymes. Toxicon. 2003;42(8):827-40.

9. Gutiérrez JM, Avila C, Rojas E, Cerdas L. An alternative *in vitro* method for testing the potency of the polyvalent antivenom produced in Costa Rica. Toxicon. 1988;26(4):411-3.

10. Gutiérrez JM, Lomonte B. Phospholipase A_2 myotoxins from *Bothrops* snake venoms. Toxicon. 1995;33(11):1405-24.

11. Gutiérrez JM, Lomonte B. Local tissue damage induced by *Bothrops* snake venoms. A review. Mem Inst Butantan. 1989;51:211-23.

12. Bjarnason JB, Fox JW. Hemorrhagic toxins from snake venoms. J Toxicol Toxins Rev. 1988;7:121-209.

13. Chipaux JP, Goyffon M. Venoms, antivenoms and immunotherapy. Toxicon. 1998;36(6):823-46.

14. Otero Patiño R, Núñez Rangel V, Barona J, Díaz Cadavid A, Saldarriaga Córdoba MM. Características bioquímicas y capacidad neutralizante de cuatro antivenenos polivalentes frente a los efectos farmacológicos y enzimáticos del veneno de *Bothrops asper* y *Porthidium nasutum* de Antioquia y Chocó. Iatreia. 2002;15(1):5-15.

15. Russell FE. Snake venoms immunology: historical and practical considerations. J Toxicol Toxin Rev. 1988;7(1):1-82.

16. Brazil V. Contribuição ao estudo do veneno ophidico. III. Tratamento das mordeduras de cobras. Rev Med São Paulo. 1903;6:265-78.

17. Dos-Santos, MC, Gonçalves LR, Fortes-Dias CL, Cury Y, Gutierrez JM, Furtado MF. Estudo comparativo do efeito neutralizante dos soros antibotrópico (Sab) e anti-crotálico (Sac) sobre a atividade miotóxica do veneno de *Bothrops jararacussu* (Bjssu) em camundongos. In: 1º Simpósio da Sociedade Brasileira de Toxinologia: Peçonhas e Envenenamentos no Brasil, 1990, São Paulo, SP. Anais do Simpósio; 1990.

18. Dos-Santos MC, Gonçalvez LR, Fortes-Dias CL, Cury Y, Gutiérrez JM, Furtado MF. A eficácia do antiveneno botrópico-crotálico na neutralização das principais atividades do veneno de *Bothrops jararacussu*. Rev Inst Med Trop S Paulo. 1992;34(2):77-83.

19. Beghini DG, Cruz-Höfling MA, Randazzo-Moura P, Rodrigues-Simioni L, Novello JC, Hyslop S, Marangoni S. Cross-neutralization of the neurotoxicity of *Crotalus durissus terrificus* and *Bothrops jararacussu* venoms by antisera against crotoxin and phospholipase A_2 from *Crotalus durissus cascavella* venom. Toxicon. 2005;46(6):604-11.

20. De Roodt AR, Dolab JA, Fernandez T, Segre L, Hajos SE. Cross-reactivity and heterologous neutralization of crotaline antivenoms used in Argentina. Toxicon. 1998;36(7):1025-38.

21. Rodríguez JP, De Marzi M, Maruñak S, Malchiodi EL, Leiva LC, Acosta O. Rabbit IgG antibodies against phospholipase A_2 from *Crotalus durissus terrificus* neutralize the lethal activity of the venom. Medicina (B Aires). 2006;66(6):512-6.

22. Lôbo de Araújo A, Radvanyi F. Determination of phospholipase A_2 activity by a colorimetric assay using a pH indicator. Toxicon. 1987;25(11):1181-8.

23. Gay CC, Leiva LC, Maruñac S, Teibler P, Acosta de Perez O. Proteolytic, edematogenic and myotoxic activities of a hemorrhagic metalloproteinase isolated from *Bothrops alternatus* venom. Toxicon. 2005;46(5):546-54.

24. Acosta O, Leiva LC, Peichoto ME, Maruñak S, Teibler P, Rey L. Hemorrhagic activity of the Duvernoy's gland secretion of the xenodontine colubrid *Philodryas patagoniensis* from the north-east region of Argentina. Toxicon. 2003;41(8):1007-12.

25. Richardson WH, Tanen DA, Tong TC, Betten DP, Carstairs SD, Wiliams SR, Cantrell FL, Clark RF. Crotalidae polyvalent immune Fab (Ovine) antivenom is effective in the neutralization of South American viperidae venoms in a murine model. Ann Emerg Med. 2005;45(6):595-602.

26. Harrison RA, Wüster W, Theakston RD. The conserved structure of snake venom toxins confers extensive immunological cross-reactivity to toxin-specific antibody. Toxicon. 2003;41(4):441-9.

27. Maruñak SL, Acosta OC, Leiva LC, Ruiz RM, Aguirre MV, Teibler P. Mice plasma fibrinogen consumption by thrombin-like enzyme present in rattlesnake venom from the north-east region of Argentina. Medicina (B Aires). 2004;64(6):509-17.