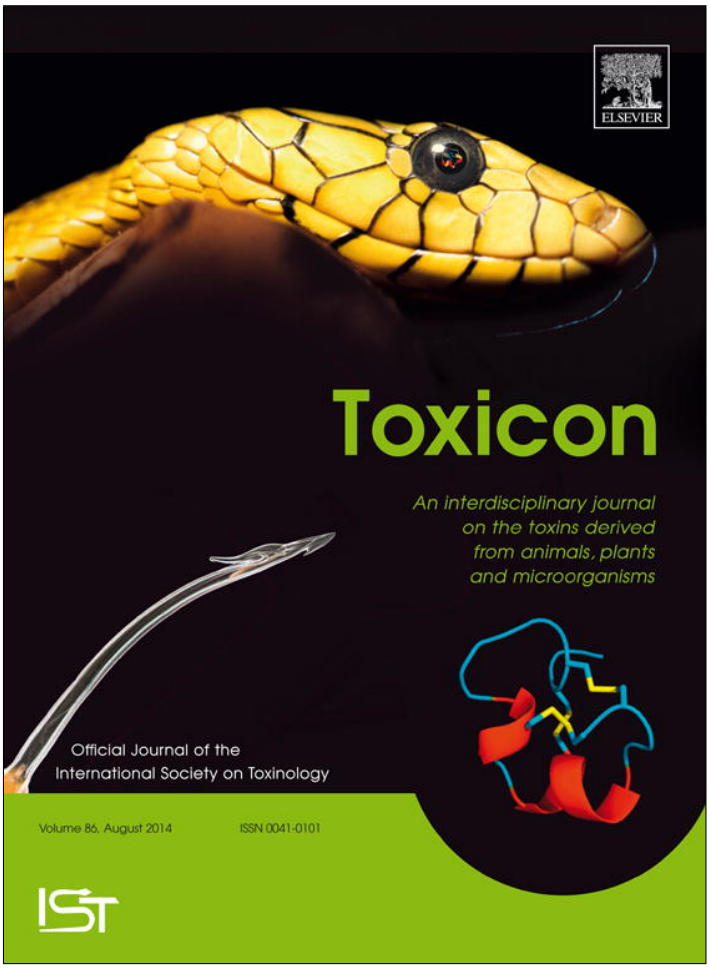

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Neutralisation of the pharmacological activities of *Bothrops alternatus* venom by anti-PLA_2 IgGs

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ABSTRACT

Basic phospholipases A_2 (PLA_2) are toxic and induce a wide spectrum of pharmacological effects, although the acidic enzyme types are not lethal or cause low lethality. Therefore, it is challenging to elucidate the mechanism of action of acidic phospholipases. This study used the acidic non-toxic Ba SpII RP4 PLA_2 from *Bothrops alternatus* as an antigen to develop anti-PLA_2 IgG antibodies in rabbits and used *in vivo* assays to examine the changes in crude venom when pre-incubated with these antibodies. Using Ouchterlony and western blot analyses on *B. alternatus* venom, we examined the specificity and sensitivity of phospholipase A_2 recognition by the specific antibodies (anti-PLA_2 IgG). Neutralisation assays using a non-toxic PLA_2 antigen revealed unexpected results. The (indirect) haemolytic activity of whole venom was completely inhibited, and all catalytically active phospholipases A_2 were blocked. Myotoxicity and lethality were reduced when the crude venom was pre-incubated with anti-PLA_2 immunoglobulins. CK levels in the skeletal muscle were significantly reduced at 6 h, and the muscular damage was more significant at this time-point compared to 3 and 12 h. When four times the LD_{50} was used (224 μg), half the animals treated with the venom-anti PLA_2 IgG mixture survived after 48 h. All assays performed with the specific antibodies revealed that Ba SpII RP4 PLA_2 had a synergistic effect on whole-venom toxicity. IgG antibodies against the venom of the Argentinean species *B. alternatus* represent a valuable tool for elucidation of the roles of acidic PLA_2 that appear to have purely digestive roles and for further studies on immunotherapy and snake envenoming in affected areas in Argentina and Brazil.

1. Introduction

The pit vipers inhabiting Central and South America belong to the genus Bothrops (family Viperidae, sub-family Crotalinae) and are responsible for most cases of deadly snakebites in America. *Bothrops alternatus* is a representative of this group of venomous snakes. Envenomation causes blood coagulation disorders, acute renal and

Abbreviations: PLA_2, phospholipase A_2; i.p., intra-peritoneal; s.c., subcutaneous; i.m., intramuscular; i.d., intra-dermal; MiHD, minimal indirect haemolytic dose; SVMPs, snake venom metallo-proteinases; ABS, anti-bothropic serum.

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respiratory failures and shock (Cardoso et al., 1993; Fan and Cardoso, 1995; Warrell, 1995; Ribeiro et al., 1998). The venom of Viperidae snakes is a rich source of PLA_2s.

PLA_2s are ubiquitous enzymes that catalyse hydrolysis of the C2 ester bond of 3-sn-phosphoglycerides, producing lysophospholipids and free fatty acids in a calcium-dependent reaction (Scott et al., 1990). These enzymes exert different toxic activities *in vivo*, notably neurotoxicity and myotoxicity (Kini, 2003; Montecucco et al., 2008). Furthermore, both acidic and basic PLA_2s are observed in venom, in variable proportions depending on the species. The basic isoforms appear to have the highest toxicity, especially among the neurotoxic and myotoxic enzymes (Rosenberg, 1986; Krizaj et al., 1993).

All acidic PLA_2s purified from viperid venoms contain a D residue at position 49. These acidic isoforms usually have higher catalytic activity than basic PLA_2s on conventional substrates *in vitro* (Rosenberg, 1986; Rosenberg, 1990; Santos-Filho et al., 2008). However, many acidic PLA_2s are not lethal or weakly lethal in mice (de Araújo et al., 1994; Andriao-Escarso et al., 2002). These enzymes are considered to have solely digestive roles (Fernandez et al., 2010).

We observed previously that isolated PLA_2 and a haemorrhagic metallo-proteinase, acting simultaneously, increases damage on muscle fibres *in vitro*. (Bustillo et al., 2012). Therefore, it is important to examine the effect of pre-incubating the venom with purified anti-PLA_2 IgGs obtained by immunising rabbits with the PLA_2 homologue to test whether all phospholipase A_2 enzymes are inhibited.

This study examined whether acidic PLA_2 in *B. alternatus* venom had pharmacological effects in co-operation with other enzymes in the venom. Antibodies obtained against a non-toxic acidic Ba SpII RP4 PLA_2 were used to study venom activity in *in vitro* and *in vivo* assays under partial blocking conditions.

2. Materials and methods

2.1. Reagents and venom

B. alternatus venom was obtained from several snakes kept in the serpentarium at Corrientes, in north-eastern Argentina. The venom was desiccated and stored at 20° C. When required, the venom was diluted with ammonium bicarbonate (1 M, pH 8.0). The small amount of insoluble material was centrifuged at 3000 rpm for 10 min, and the clear supernatant was used for assays. The purified phospholipase A_2, named Ba SpII RP4, was isolated previously (Garcia Denegri et al., 2010). The Sepharose affinity column (HiTrap Protein G HP 1 ml) and ÄKTAprime plus were purchased from GE Healthcare, and sodium phosphate 20 mM (pH 7.00), Glycine-HCl 0.1 M (pH 2.5), Tris–HCl 1 M (pH 9.00) and Tween 20 were purchased from Sigma Chemical Co.

2.2. Animals

The adult CF-1 male mice (20 g ± 5 g) and New Zealand white rabbits (3 kg) were obtained from the Animal House, University of Veterinary Sciences from University of North-eastern Argentina. Food (chow mice diet) was withdrawn 12–14 h before the experiment, but the animals had free access to water. The animals were maintained in a temperature-controlled room (23 ± 2° C), and the relative humidity was between 35% and 65%. The animal room was lit from 6 a.m. to 6 p.m. Rabbits were housed in single cages, and food and water were freely available. This study was approved by the Ethics and Biosafety Committee of the University of Veterinary Sciences at the University of North-eastern Argentina.

2.3. Production of specific antiserum

Anti-PLA_2 serum was obtained by successive immunisations of rabbits (3–4 kg weight) with an initial dose of 1 mg of the purified PLA_2, either intramuscularly or subcutaneously. The first injections were provided i.m. and included Freund's complete adjuvant in a 1:1 ratio. The subsequent boosters were provided s.c. at weekly intervals and contained 3-mg doses in incomplete adjuvant.

Rabbits were bled 10 days after the last PLA_2 antigen injection. Their sera were separated and stored in aliquots at 20° C. The antibody levels in the serum were monitored by gel immuno-diffusion (Ouchterlony, 1949) and ELISA (Chavez-Olortegui et al., 1997) analyses. The serum was then used for purification of specific antibodies by affinity chromatography.

2.4. Isolation of IgG by protein-G affinity chromatography

IgG antibodies were purified from the serum of rabbits immunised with Ba SpII RP4 PLA_2, using a Sepharose-protein G column (HiTrap Protein G HP 1 ml, GE Healthcare) in an ÄKTAprime plus system (GE Healthcare). The column was equilibrated with 20 mM sodium phosphate (pH 7.0), and 0.5 ml of serum (diluted 1:5 in PBS) was applied. Non-adsorbed proteins were removed by washing the column with the same buffer, and the IgGs were eluted with 0.1 M glycine-HCl (pH 2.5). The elution profile was monitored at 280 nm, and 1 ml fractions were collected and immediately neutralised with 70 μl of 2 M Tris–HCl (pH 9.0) prior to pooling and desalting by extensive dialysis against PBS (pH 7.4) for 24 h. After dialysis, the anti-PLA_2 IgG solution was concentrated by ultra-filtration using Amicon® membranes, and the rabbit IgG protein content was determined by the Biuret reaction. The Ouchterlony precipitation test was used to monitor the presence of IgG in the solution.

To verify the absence of other anti-toxins, specifically antibodies against metallo-proteinases, the ability of the anti-toxin to neutralise the major physiopathological properties of *B. alternatus* venom metallo-proteinases was examined, e.g., proteolytic activity was determined using the azocasein assay, and the haemorrhagic activity was monitored as described by Gonçalves and Mariano (Gonçalves and Mariano, 2000) with modifications (Peichoto et al., 2007). Then, 7.2 μg of venom and the corresponding dose of 25 mg/ml of IgG anti-PLA_2 with the MiHD selected were injected i.d. into each mouse. The animals were sacrificed two hours after injection, and the skin was removed. Haemorrhagic halos were immediately excised, fragmented, and added to tubes containing 4 ml of

Drabkin's reagent. The tissue was homogenised, and the reaction mixtures were incubated in the dark at room temperature for 24 h. Subsequently, the tubes were centrifuged at 1900 × g for 10 min. The absorbance of the supernatant at 540 nm was measured in a spectrophotometer (Boeco, Argentina), and the haemoglobin concentration was calculated. The isolated immunoglobulins were tested by SDS-PAGE (10% acrylamide resolution gels) under reducing/non-reducing conditions using 10% acrylamide (Laemmli, 1970), and their specificity was determined using by Ouchterlony and western blot analyses .

2.5. Immunoblotting analysis

Duplicate PLA_2 (1 mg/ml) and whole-venom (1 mg/ml) samples diluted in PBS (pH 7.2) were analysed by SDS-PAGE on a 12.5% gel under reducing conditions. The gel was run at 200 V for 45 min, followed by electro-transfer to a nitrocellulose membrane (0.45 mm) in a transfer tank at 300 mA for 1 h. Subsequently, the membrane was blocked at room temperature for 2 h in a solution containing 5% non-fat milk/0.05% Tween 20. After washing three times in Tris-buffered saline (TBS; 0.01 M Tris–HCl, 0.17 M NaCl, pH 7.6), the nitrocellulose membrane was cut down the middle, and the two halves were incubated separately overnight with anti-PLA_2 IgG (diluted to 0.1 mg ml^1 in TBS) or anti-venom serum (1:2.000 in TBS). After washing again with TBS, the bound antibodies were detected with a goat anti-rabbit IgG peroxidase conjugate (Jackson Immuno Research Laboratories) used as a secondary antibody (diluted 1:1.000 in TBS) for 1 h at room temperature with shaking. After this incubation, the blots were washed and revealed with 4-chloro-1-naphthol (Bio-Rad; 0.03% in 0.05 M Tris–HCl, pH 7.6, containing 0.03% H2O2/OPD).

2.6. Neutralisation of the indirect haemolytic activity of B. alternatus snake venom with anti-PLA_2 IgGs raised in rabbits

Haemolytic activity was measured as described by Gutiérrez (Gutiérrez et al., 1986) to evaluate the amount of antibodies required to neutralise 100% of the enzymatic activity exhibited by 100 μg of venom (Effective Dose 100%, ED_{100}); this ED_{100} value was used for subsequent inhibitory assays. This method was based on the haemolytic activity of PLA_2 on red cells in the presence of egg yolk.

The ED_{100} was measured, and the venom dose was selected for each assay; therefore, the ratio of μg of anti-PLA_2 IgG/μg of venom was fixed. All mixtures were immediately used for different assays with prior incubation. A challenge dose of 6 μg of crude venom was used. A mixture of 25 ml of 1% (w/v) agar in PBS (pH 8.1) containing 0.3 ml of packed sheep erythrocytes, 0.3 ml of egg yolk in saline solution (1:3) and 0.25 ml of 0.01 M $CaCl_2$ was poured into plastic plates (135 × 80 mm) and allowed to solidify. Subsequently, 3 mm-diameter wells were filled with 15 μl of venom dilutions (400 μg/ml) or venom dilutions pre-incubated with different amounts of anti-PLA_2 IgG (18.7–0.58 mg/ml) in equal parts for 30 min at 37° C.

The plates were incubated in a moist chamber for 20 h at 37° C, and the diameter of the haemolytic halos was measured.

2.7. Neutralisation of myotoxicity

To determine the neutralising activity of the purified immunoglobulins, mice ($n = 4$) were intramuscularly injected in the right gastrocnemius muscle with 50 μg of venom (1 mg/ml, 50 μl) that was pre-incubated with 1250 μg of anti-PLA_2 IgG (25 mg/ml, 50 μl). The control groups received PBS (phosphate buffered saline) or venom (100 μl). After 3, 6 and 12 h, the mice were anaesthetised with chloral hydrate (300 mg/kg i.p.); blood samples were collected from the abdominal aorta in tubes without coagulant to obtain sera, and the CK levels were measured using a kinetic assay (Sigma–Aldrich, USA). Creatine kinase activity was expressed in International Units per litre, where one unit was defined as the amount of enzyme that transfers 1.0 mM of phosphate from phosphocreatine to ADP per min at pH 7.4 at 30° C.

2.8. Neutralisation of B. alternatus venom lethality

To examine venom neutralisation, animals were injected i.p. with 0.2 ml of venom solution containing a challenge dose of 2 LD_{50}, i.e., 112 μg (1.18 mg/ml, 95 μl) that was pre-incubated with 2.62 mg (25 mg/ml, 105 μl) of anti-PLA_2 antibodies. Other groups were injected similarly with increasing doses of venom: 4 DL_{50} i.e., 224 μg (4.5 mg/ml, 95 μl) with 5.25 mg of anti-PLA_2 IgG (35 mg/ml, 150 μl); these concentrations were used based on the Effective $Dose_{100}$ described above. The mixtures were incubated for 30 min at 37° C and then injected i.p. Control groups received the same volume of venom pre-incubated with PBS instead of anti-phospholipases A_2 antibodies. Dead animals were counted during the first 24 and 48 h.

2.9. Statistical analysis

Each experimental protocol was repeated at least three times. Student's *t*-test was used for statistical analysis of the data with a value of $P < 0.05$ indicating a significant difference.

3. Results

3.1. Purification of IgG antibodies from rabbits: specificity control

Ba SpII RP4 PLA_2 was purified previously from *B. alternatus* snake venom and used as an antigen for rabbit immunisation. After bleeding, the serum was applied to a Protein G antibody affinity column, and the IgG was eluted after a pH change in a second peak. Using electrophoretic analysis, two protein bands were observed with approximate molecular weights of 50 and 25 kDa under reduced conditions, consistent with the immunoglobulin G structure (data not shown).

The crude venom, purified PLA_2, IgG antibodies obtained in rabbits immunised with Ba SpII RP4 and ABS were used for Ouchterlony analysis (Fig. 1); the crude venom and purified PLA_2 were also examined by western blotting (Fig. 2 A, B). The anti-PLA_2 antibodies obtained from immunised rabbits showed a single precipitation band

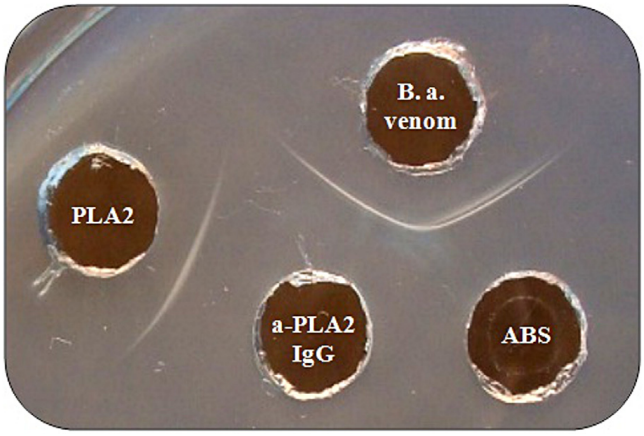

Fig. 1. Immuno-diffusion in 1% agarose gels in phosphate buffered saline (pH 7.2). The concentration of the PLA_2 enzyme and crude venom was 1 mg/ml. Note the reactivity of the PLA_2 enzyme and crude venom with anti-PLA_2 IgG.

when tested against purified PLA_2 enzyme and crude venom. As shown in Fig. 2A, ABS detected proteins in the *B. alternatus* venom and recognised purified PLA_2, yielding a specific band of 14 kDa. The same venom and enzyme solutions were incubated with antibodies raised against purified PLA_2 (Fig. 2B) to verify the specificity of these immunoglobulins. As expected, the anti-PLA_2 IgG recognised the single purified enzyme band and the corresponding band in the whole venom. Together, these results revealed the specificity of the antibodies to purified PLA_2; these antibodies were then used for further studies.

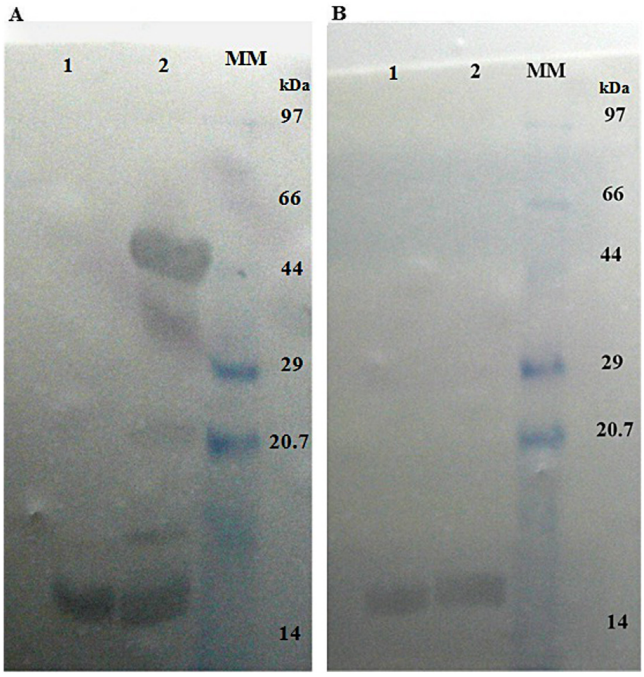

Fig. 2. Western blot analysis of purified PLA_2 (Lane 1) and *B. alternatus* venom (Lane 2). The samples were separated by SDS-PAGE under reducing conditions and transferred to a nitrocellulose membrane. The migration of molecular mass markers (MM) is shown on the right. A. The membrane was incubated with anti-venom antibodies. B. The membrane was incubated with anti-PLA_2 antibodies.

3.2. Quality control/quality assurance of anti-PLA_2 serum

To qualitatively examine the absence of anti-proteases in anti-PLA_2 IgG fractions, proteolytic activity and metallo-proteinase-derived haemorrhagic activity were measured.

3.2.1. Proteolytic activity

To examine whether the immune sera lacked antibodies against proteases, the whole venom (26 μg) was pre-incubated with 606 μg of IgG immunoglobulins. The absorbance at 450 nm (0.090 UA) was similar to the absorbance of the venom/PBS solution (0.086 UA).

3.2.2. Haemorrhagic activity

Both venom solutions produced similar halos on the internal surface of dissected skin in mice, as shown in Fig. 3. The haemoglobin concentrations were not significantly different. Therefore, we concluded that the sera lacked anti-haemorrhagin antibodies.

3.3. Neutralisation of B. alternatus snake venom toxicity using anti-PLA_2 immunoglobulins

3.3.1. Inhibition of (indirect) haemolytic activity from whole venom

The challenge dose used to neutralise venom-induced haemolysis by IgG was 6 μg, which was the amount of venom estimated from the minimal indirect haemolytic dose (MiHD) previously determined by Acosta (Acosta, 1999). Incubation of whole venom (6 μg) with increasing concentrations of anti-PLA_2 IgG ranging from 8.75 to 280 μg/well × 15 μl revealed that venom-mediated haemolytic activity was neutralised by 140 μg of the anti-PLA_2 IgG solution (Fig. 4). The ED_{100} was approximately 23.3 μg of IgG, which was defined as amount of immunoglobulins required to block the haemolytic activity induced by a microgram of venom. The immune sera obtained after immunisation reacted with Ba SpII RP4 PLA_2 and with all catalytically active PLA_2s in *B. alternatus* venom.

3.3.2. Neutralisation of myotoxicity

All PLA_2s in whole venom were blocked by pre-incubation with the anti-phospholipases A_2 IgG (25 mg/

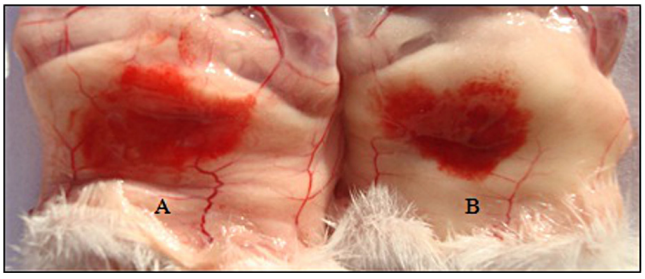

Fig. 3. Haemorrhagic activity of crude venom (A) and crude venom pre-incubated with anti-PLA_2 antibodies (B). Note the similarity between the haemorrhagic halos caused by metallo-proteinase action. The antibodies developed in rabbits had no specificity for haemorrhagic metallo-proteinases in *B. alternatus* venom. The photographs were captured 2 h post-injection.

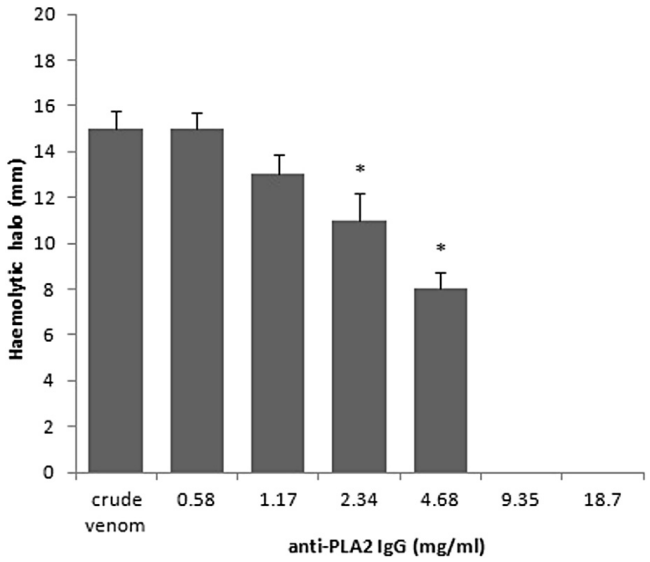

Fig. 4. Neutralisation of B.a. venom-induced haemolytic (indirect) effect by anti-PLA_2 sera. The MiHD of crude venom (6 μl) induced a haemolytic halo with a diameter of 15 mm. The anti-PLA_2 IgG serum preparation was tested at concentrations ranging from 0.58 to 18.7 mg/ml for its ability to neutralise (indirectly) the haemolytic activity of the phospholipases A_2 in whole venom. Rabbit anti-serum significantly reduced haemolytic activity at a concentration of 2.34 mg/ml. Bars represent the means ± SD ($n = 4$). Significant differences from the respective controls are indicated $^{*}P < 0.05$.

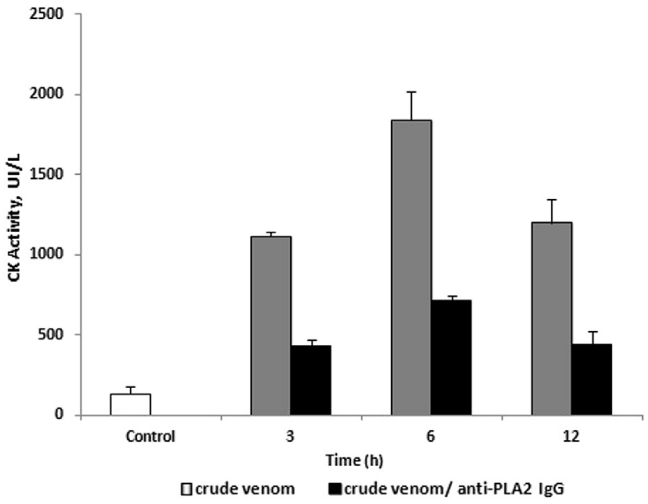

Fig. 5. Neutralisation of myotoxic activity. Variation of CK levels by venom with or without pre-incubation with anti-PLA_2 immunoglobulins at different time intervals (3, 6 and 12 h). A significant variation in CK plasma levels (*, $P < 0.05$) occurred when crude venom (50 μg) was tested (maximum peak at 6 h) with respect to the control (*). Significant changes were observed (#, $P < 0.05$) upon pre-incubation of the *B. alternatus* venom with anti-phospholipase A_2 IgG (1:25); pre-incubation reduced the myotoxicity compared to crude venom. Bars represent the means ± SD ($n = 4$). Significant differences from respective controls are indicated: $^{*}P < 0.05$ for venom versus control group and $^{\#}P < 0.05$ for incubation of venom and anti-PLA_2 antibodies (1:25) with respect to crude venom.

ml, 50 μl) at the effective dose (ED_{100}) measured in the previous assay (*2.6*). The ability of anti-PLA_2 IgG to neutralise myotoxic activity was monitored at 3, 6 and 12 h after injection of *B. alternatus* venom that was pre-incubated with the antibodies. The selected time intervals after injection correspond to maximal CK release into circulation. The anti-PLA_2 IgG inhibited the myotoxic activity of *B. alternatus* venom (1 mg/ml, 50 μl) by ~37–39% at 3 and 12 h (Fig. 5). The CK level in the skeletal muscle was significantly lower at 6 h, and the muscular damage was worse at 6 h compared to 3 and 12 h (Fig. 5).

3.3.3. Neutralisation of lethal activity

Four mice were injected intraperitoneally with a challenge dose of $2LD_{50}$, equivalent to 112 μg of *B. alternatus* venom (1.18 mg/ml; 95 μl) that was pre-incubated with 2.61 mg of anti-PLA_2 IgG (25 mg/ml; 105 μl) for 30 min at 37° C. The ratio *IgG anti-PLA_2:crude venom* was calculated in a previous experiment (*2.6*). After 48 h, this treated group survived. Conversely, the control group (venom pre-incubated with PBS) die within approximately four hours after injection. To increase the relative amount of proteins required for venom lethality, the challenge dose was doubled (4 LD_{50}: 224 μg) while maintaining the antigen/antibodies ratio. Half of the animals treated with the venom-IgG mixture survived after 48 h, whereas the other half of the animals did not survive longer than 10 h. The control group (venom/PBS) died at 90 min post-injection.

4. Discussion

B. alternatus venom contains primarily metallo-proteinases (43.1%) and several types of serine proteinases (24.1%), whereas PLA_2 comprise only 7.8% (Öhler et al., 2010).

Our previous studies demonstrated that the most abundant phospholipase A_2 from *B. alternatus* venom is Ba SpII RP4, a catalytically active enzyme that has low to no toxicity, i.e., it is not lethal and does not have myotoxic effects even at doses as high as 200 μg. Consistent with these observations, PLA_2 is an immunogen with low or no toxicity. Therefore, it was possible to produce rabbit-specific antibodies using a large amount of enzyme (3 mg) without affecting animal health.

Antibodies against Ba SpII RP4 recognised the catalytically active PLA_2s in *B. alternatus* venom. This observation suggests that these enzymes have similar epitopes. Therefore, the role of PLA_2 in venom pathophysiology was examined indirectly by pre-incubation of *B. alternatus* venom with anti-PLA_2 antibodies.

Injection of mice with venom pre-incubated with anti-PLA_2 IgG caused less severe myotoxicity compared to injection with crude venom. Acidic PLA_2s with weak to no myotoxic activity are specific to *B. alternatus* venom (Öhler et al., 2010). Notably, when the activity of these enzymes is blocked, the CK levels decreased significantly compared to treatment with whole venom. The residual activity observed is most likely due to haemorrhagins; this is consistent with the observations of Gay et al. (Gay et al., 2013), who demonstrated that myotoxic injury is almost entirely prevented when snake venom metallo-proteinases (SVMPs) in *B. alternatus* venom are neutralised. These observations might be explained by the synergistic activity of PLA_2s and SVMPs in which the PLA_2s are not toxic to muscular fibres but cause significant muscular lesions in the presence of metallo-proteinases. This synergism was

observed in murine myoblasts (C2C12) *in vitro* by Bustillo and col. (Bustillo et al., 2012). *B. alternatus* venom is considered to have no basic PLA_2 (Öhler et al., 2010); however, Ponce-Soto et al. (Ponce-Soto et al., 2007) described a Lys49 myotoxic phospholipase A_2. Based on transcriptomic and proteomic studies, Cardoso et al. (Cardoso et al., 2010) observed that *B. alternatus* venom lacks basic PLA_2. Therefore, considering the trace level (<1%) of this basic PLA_2 in venom, its overall contribution to venom activity is likely to be minimal.

Furthermore, mice treated with *B. alternatus* venom pre-incubated with anti-PLA_2 antibodies showed increased survival. Considering that (i) metallo-proteinases (SVMPs) are the most abundant toxins in *B. alternatus* venom and represent 50% of the known venom components (Öhler et al., 2010), (ii) the baltergin LD_{50} is 206.4 μg/mouse (i.p.), and (iii) 2 LD_{50} (112 μg) of crude venom represents only 40 μg of the metallo-proteinase content based on proteomic studies from Öhler et al. (2010), the level of these haemorrhagic and proteolytic enzymes used was insufficient to induce death in half of the treated animals. Therefore, it was necessary to increase the dose ($4LD_{50}$) of *B. alternatus* venom pre-incubated with the corresponding amount of anti-toxin to observe SVMP activity. Because PLA_2 activity was blocked, 50% of the injected mice died. The control mice died within 2 h after i.m. injection.

Our observations provide insight into the functions of this enzyme family in animal/human envenoming; PLA_2 from *B. alternatus* venom might enhance the individual toxicity of other toxins such as proteinases and have a significant effect on the targeted organs in the snakebite victim. This is not consistent with previous studies (Fernandez et al., 2010) in which the acidic PLA_2 in snake venoms only had a minor role in digestion.

In conclusion, *B. alternatus* venom toxicity is caused by synergy among several components and not by individual highly toxic components. In addition, this study reveals that specific antibodies can be used to elucidate the activity of individual enzymes in complex mixtures.

Ethical statement

The present study was approved by the Ethics at Biosafety Committee of Facultad de Ciencias Veterinarias, Universidad Nacional del Nordeste, Argentina.

This work not includes plagiarism, forgery, use or presentation of other researcher's works as one's own, fabrication of data.

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Conflict of interest

The authors declare that there are no conflicts of interest.

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