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Effect of monospecific antibodies against baltergin in myotoxicity induced by *Bothrops alternatus* venom from northeast of Argentina. Role of metalloproteinases in muscle damage

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ABSTRACT

Myotoxicity, one of the most relevant local manifestations in envenomation by *Bothrops* genus, may result from a direct action of myotoxins or be due to an indirect vascular degeneration and ischemia. Baltergin, a snake venom metalloproteinase (SVMP), isolated from *Bothrops alternatus* venom has been used to obtain monospecific IgG, in order to determine the relative role of toxin in myotoxicity induced by whole venom. *Bothrops diporus* venom, another medical relevant genus of the northeastern region of Argentina, was also studied. Anti-baltergin IgG was able to neutralize completely the hemorrhagic activity of *B. alternatus* venom at an antibodies:venom ratio of 30:1 (w:w). However, mice injected with *B. diporus* venom showed a small spot remaining even at the highest ratio of IgG:venom assayed (50:1; w:w). Specific antibodies were efficient to neutralize the myotoxicity of *B. alternatus* venom at ratio 30:1 (w:w) but did not neutralize the same effects in *B. diporus* venom. Anti-baltergin polyclonal antibodies were useful tools for revealing the central role of SVMPs in the development of myotoxicity of *B. alternatus* venom, as well as, helping to suggest indirectly presence of potent myotoxic phospholipases A2 (PLA2s) in *B. diporus* venom.

1. Introduction

The most important events associated to the development of local tissue damage in viperid snakebites depend on a limited number of components, mainly phospholipases A2 (PLA2s) and metalloproteinases (SVMPs) (Gutierrez et al., 2010). PLA2s are responsible for inducing local myonecrosis and lymphatic vessel damage (Gutiérrez and Ownby, 2003) while SVMPs induce hemorrhage, extracellular matrix degradation, blistering and skin necrosis (Fox and Serrano, 2005; Gutiérrez et al., 2009).

Myotoxicity, a relevant local manifestation in envenomation by *Bothrops* genus, may result from a direct action of myotoxins upon the plasma membranes of muscle cells, or be due to an indirect vascular degeneration and ischemia (Gutiérrez and Lomonte, 1995; Ownby, 1998). The most abundant myotoxic components in viperid snake venoms are PLA2s, their toxicity is usually associated to basic proteins (Rosenberg, 1986; Gutiérrez and Lomonte, 1995). They are either Asp-49, catalytically-active variants or Lys-49, enzymatically inactive homologs (Ownby et al., 1999; Soares et al., 2004). PLA2s induce local myotoxicity, which

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affects predominantly the muscles located in the vicinity of the region where venom is injected (Milani et al., 1997).

On the other hand, intramuscular injection of many hemorrhagic metalloproteinases results in acute muscle cell injury by indirect myotoxic effect (Ownby et al., 1978; Queiroz et al., 1985; Ownby et al., 1990; Nikai et al., 1991; Gutiérrez et al., 1995; Rucavado et al., 1995b; Gay et al., 2005). The mechanism by which SVMPs induce myonecrosis is secondary to the ischemia caused as a consequence of bleeding and reduced perfusion (Gutiérrez and Rucavado, 2000).

Bothrops alternatus snake is one of the most important species causing envenomations in the northeastern area of Argentina. Baltergin is a member of P-III class of SVMPs isolated from this venom which is responsible to induce relevant local effects such as hemorrhage, edema and myotoxicity as well as, to cause systemic bleeding in mice (Gay et al., 2005, 2009). Moreover, two PLA2s BaTX (Ponce-Soto et al., 2007) and Ba SpII RP4 (Garcia Denegri et al., 2010), belonging to Lys-49 and Asp-49 PLA2s groups, respectively, were also isolated. The former is a basic protein that induces local myotoxicity in mice but its proportion in the whole venom is relatively low (Ponce-Soto et al., 2007). The latter is an acidic protein which did not exert either *in vivo* myotoxicity or *in vitro* cytotoxicity on myoblasts, despite being the most abundant PLA2 present in *B. alternatus* venom (Garcia Denegri et al., 2010).

In order to study the role of SVMPs in the muscle damage induced by *B. alternatus* venom, this work describes the ability of monospecific antibodies against baltergin to inhibit this effect in mice. *Bothrops diporus* venom was also assayed to compare the myotoxicity induced by both venoms.

2. Materials and methods

2.1. Venoms and reagents

B. alternatus and *B. diporus* venoms were purchased from Serpentarium of the local zoo, Corrientes, Argentina. The pools were desiccated and stored at −20 °C. When required, the venom was diluted with phosphate buffered saline solution, pH 7.2. The small amount of insoluble material was centrifuged and the clear supernatant was applied for studies. DEAE-Cellulose, Sephadex G-75, Bovine Serum albumin (BSA), Tween 20, Tris(hydroxymethyl) aminomethane (Tris) and o-phenylenediamine dihydrochloride (OPD) were purchased from Sigma–Aldrich, USA and HiTrap Protein G HP from Amersham Biosciences. Horseradish peroxidase-conjugated goat anti-rabbit IgG (HRP-anti-rabbit IgG) were purchased from Bio-Rad Laboratories, USA. NAC-activated creatine kinase (CK) was purchased from Randox. All others reagents were of analytical grade.

2.2. Animals

Animals were supplied by the Animal House, School of Veterinary Sciences, University of Northeastern Argentina. The assays were carried out with adult CF-1 male mice (18–22 g body weight). Food (chow mice diet) was withdrawn 12–14 h before the experiment, but the animals had free access to water. Temperature in the animal room was 23 ± 2 °C and the relative humidity was between 35% and 65%. Lights in the animal room were on from 6 a.m. to 6 p.m. Male New Zealand white rabbits weighing 3 kg were housed individually with free access to food and water. All experiments followed the ethical standards for animal experiments in toxinological research recommended by the International Society of Toxinology (Meier et al., 1993).

2.3. Baltergin isolation

Baltergin was isolated from *B. alternatus* crude venom (40 mg) as described in a previous work (Gay et al., 2005). In order to assay the isolated enzyme for purity, electrophoresis was performed on 12% polyacrylamide slab gels following the standard method (Laemmli, 1970).

2.4. Production and purification of IgG anti-baltergin

Baltergin (0.1–0.5 mg) was emulsified with complete Freund's adjuvant and i.m. and s.c. injected in male rabbits (2–2.5 kg). Boosts were administered on day 7, 14 and 28. Blood was collected from a marginal ear vein, the serum was separated by centrifugation and aliquots were stored at −70 °C. The antibody level in the serum of the rabbits was monitored by ELISA (Chavez-Olortegui et al., 1997) at the end of the experiment.

Anti-baltergin IgG was obtained by affinity chromatography with a liquid chromatography system ÄKTA Prime Plus (GE Healthcare). One mL of serum diluted in binding buffer (20 mM Na_2HPO_4, pH 7.0) and filtered on a 0.22-μm membrane was adsorbed on a 1 mL protein G column (HiTrap Protein G HP, Amersham Biosciences) equilibrated in binding buffer until all unbound material was washed out. IgG fractions were then eluted at a flow rate of 1.0 mL/min with 0.1 M glycine-HCl, pH 2.5 (elution buffer). The fractions were neutralized with 1 M Tris, pH 9.0, and concentrated with centrifugal filter devices (Amicon Ultra, Millipore).

2.5. Immunodiffusion test

Immunodiffusion assay was tested according to conventional method (Ouchterlony, 1949) with some modifications. Dissolved liquid agarose 7 mL was placed in 35 mm diameter petri dish. After the agarose hardened, four wells were punched, one in the center and three peripheral at 10 mm from the central well. The central well was filled with IgG ant-baltergin, and the surrounding wells with baltergin or venom having concentration 1 mg/mL (*B. alternatus*/*B. diporus*). After 48 h of incubation lines of precipitation were observed.

2.6. Western blot analysis

Baltergin (0.25 mg/mL) or *Bothrops* venom (1 mg/mL) were separated on 12.5% SDS-PAGE at 200 V for 45 min and the proteins transferred electrophoretically to nitrocellulose membranes (0.45 mm) at 300 mA for 1 h. Subsequently, the membranes were blocked at room

temperature for 2 h in a solution of 5% non-fat milk 0.05% Tween 20. After washing three times in TBS (0.01 M Tris–HCl, 0.17 M NaCl, pH 7.6), the membranes were incubated overnight with IgG anti-baltergin. After washing, bound antibodies were detected with goat anti-rabbit IgG peroxidase conjugate (Sigma; 1:2000 in TBS) for 2 h at room temperature with shaking. At the end of this incubation, the blots were washed, developed with 4-chloro-1-naphthol (Sigma; 0.03% in 0.05 M Tris–HCl, pH 7.6, containing 0.03% H_2O_2/OPD) and documented.

2.7. Neutralization of hemorrhagic activity

One hundred μL of the mixtures, containing 20 μg of venom *B. alternatus*/*B. diporus* and different concentrations of IgG (ratios w:w; 1:10 to 1:50) were pre-incubated at 37 °C by 30 min and then, injected intradermally into the skin of groups of four mice as described by Kondo et al. (1960). The immunoprecipitates were removed previously by centrifugation of mixtures at 3000 × g for 5 min. This assay was also performed omitting the centrifugation step. Two hours later, mice were sacrificed by CO_2 inhalation, their skins were removed, and the areas of hemorrhagic lesions on the inner surface of the skin were observed. Controls containing venom alone and venom with non-immune IgG were also assayed.

2.8. Neutralization of myotoxic activity

2.8.1. Selection of doses and exposure time

Serum CK levels were evaluated at different times (0.5, 1, 3, 6, 12 y 24 h) and using different amounts of venoms (10, 20, 40 and 50 μg) in order to select adequate doses to perform the neutralization assay. Groups of 4 mice were injected i.m. in the right gastrocnemius (0.1 mL of final volume). After the indicated periods of time, mice were anesthetized with chloral hydrate i.p. 300 mg/kg to collect blood samples without using anticoagulant. Serum was obtained to analyze the activity of creatine kinase (CK) with the kinetic method U. V. based on the measurement of the creatinine formed in the reaction ADP/phosphocreatine (Randox; activity was expressed in units per liter).

2.8.2. Neutralization assay

In this case, 50 μg of *B. alternatus* venom or 10 μg of *B. diporus* venom, at a single ratio IgG:venom of 30:1 (w:w), were selected to perform the neutralization of the myotoxic activity. Groups of 4 mice were injected i.m. in the right gastrocnemius (0.1 mL of final volume). After 6 h of exposition, mice were anesthetized with chloral hydrate i.p. 300 mg/kg to collect blood samples without using anticoagulant. Serum was obtained to analyze the CK activity as was described previously. Controls containing venom alone and venom with non-immune IgG were also assayed.

2.8.3. Histological studies

In order to have a histological assessment of the ability of antibodies to neutralize myotoxicity, mice were sacrificed and samples of injected muscle were taken and fixed with Bouin solution for 24–48 h. Thereafter, the muscle was dehydrated in a graded alcohol series and embedded in paraffin. Sections 5 μm thick were stained with hematoxylin and eosin. Control muscles were processed in an identical manner. Necrosis was classified according to the method of Homma and Tu, based on the morphology of the necrotic fibers (Homma and Tu, 1971). The myolytic type was characterized by fibrillar material, alternating with clear areas. In the coagulative necrosis type, the fibers acquired a hyaline appearance and its distribution was homogeneous.

2.9. Statistical analysis

Data represent the mean ± standard deviation (SD) of at three or four replicates. Statistical significance was tested by one-way ANOVA and Tukey (HSD) and *p*-values inferior to 0.05 were considered significant.

3. Results

3.1. Immunoassays

Purified IgG obtained from rabbits immunized with baltergin showed the presence of specific antibodies when confronted to both venoms, producing a precipitation band by immunodiffusion test (Fig. 1). Immunoblotting was performed in order to identify SVMPs immunologically related to baltergin between *B. alternatus* and *B. diporus* venoms. Nitrocellulose membranes incubated with anti-baltergin IgG demonstrated that reactivity of these antibodies was restricted to 45–120 kDa components corresponding to region of P-III class of SVMPs in bothropic venoms (not shown).

3.2. Neutralization of hemorrhagic activity

Anti-baltergin IgG were able to neutralize efficiently the hemorrhagic activity of *B. alternatus* and *B. diporus* venoms at an antibodies:venom ratio of 30:1 (w:w, Fig. 2A,B). However, mice injected with *B. diporus* venom showed a small spot remaining even at the highest ratio of IgG:venom assayed (50:1; w:w).

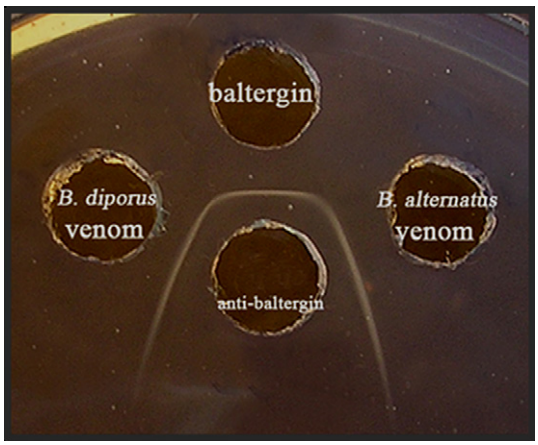

Fig. 1. Venom cross-reactivity determined by immunodiffusion. Note reactivity of baltergin and bothropic venoms with anti-baltergin IgG.

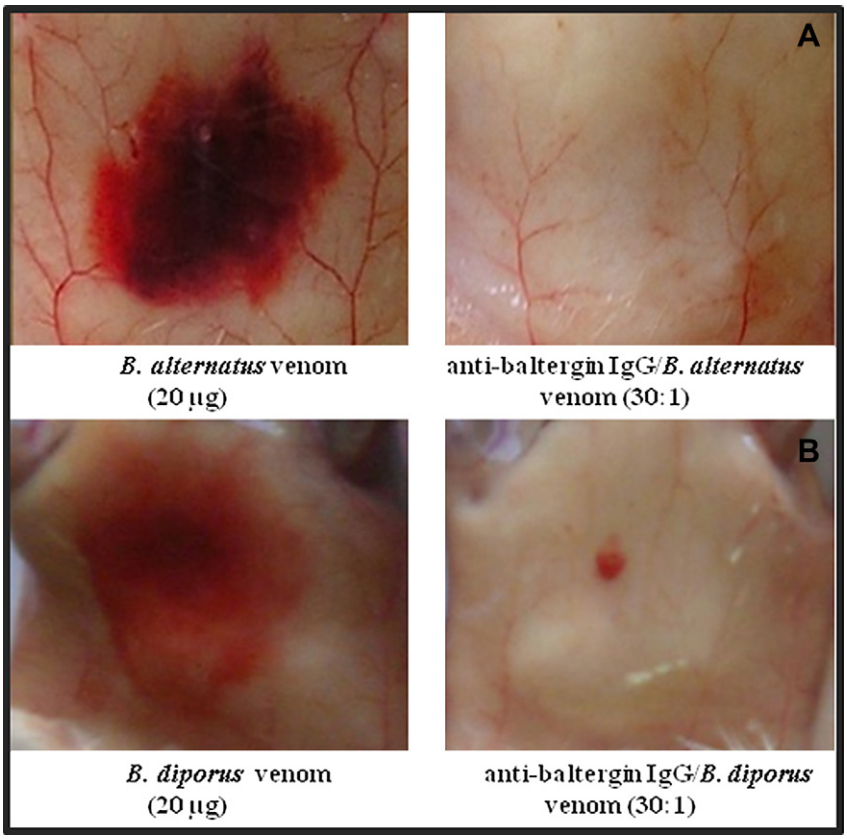

Fig. 2. Neutralization of hemorrhagic activity by skin method. **A**. Inside surface of skins from mice injected with *B. alternatus* venom alone (hemorrhagic spot) or venom pre-incubated with anti-baltergin IgG (ratio 1:30; w:w). **B**. Inside surface of skins from mice injected with *B. diporus* venom alone or venom pre-incubated with anti-baltergin IgG (ratio 1:30; w:w). Notice a small spot in the site of injection ($n = 4$).

3.3. Neutralization of myotoxic activity

3.3.1. Selection of doses and exposure time

Comparative assays demonstrated that *B. diporus* venom exhibited higher myotoxic activity than *B. alternatus* venom at all doses assayed. For instance, Fig. 3 displays serum CK levels after i.m. injection of 20 μg of *B. alternatus* or *B. diporus* venom at different exposure times. Both venoms showed the highest level after 6 h of venom injection but the CK value obtained for *B. diporus* venom was nearly seven times higher than for *B. alternatus* venom. The profiles were similar for the other doses tested showing in all cases a higher myotoxic effect for *B. diporus* venom (not shown). These results allowed to select the exposure time (6 h) and adequate doses of venoms to carry out the neutralization assays. Thus, 50 μg of *B. alternatus* and 10 μg of *B. diporus* venoms, which induce a peak of CK between 1000 and 2000 IU/L, were selected for testing with antibodies.

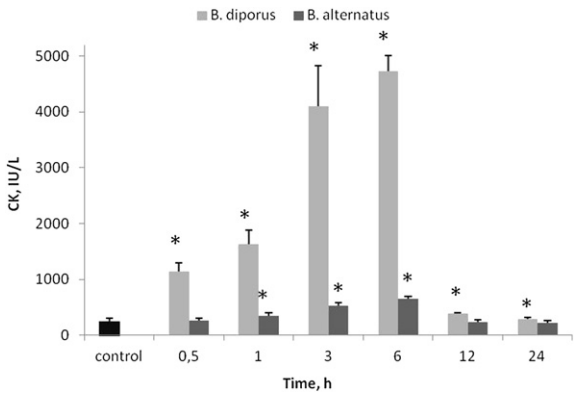

Fig. 3. Changes in serum CK levels of mice after i.m. injection of *Bothrops* venoms. Serum CK levels were determined at various time intervals after injection of 20 μg of venom (*B. alternatus* or *B. diporus*). Bars represent the mean ± SD ($n = 4$). $p < 0.05$ venom versus control group.

3.3.2. Neutralization assay

The ability of anti-baltergin IgG to neutralize the myotoxic activity of *B. alternatus* and *B. diporus* venoms was studied. *B. alternatus* and *B. diporus* crude venoms induced a mild increase in serum CK activity after 6 h of i.m. injection of 50 μg and 10 μg, respectively, compared to the control group (Fig. 4). After injection of venoms pre-incubated with anti-baltergin IgG (ratio 1:30; w:w), serum CK levels were not significantly different from control value for *B. alternatus*, whereas proteins from *B. diporus* venom, not recognized by antibodies, were able to

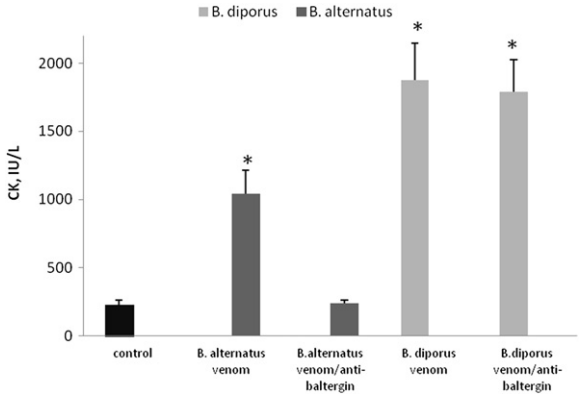

Fig. 4. Neutralization of myotoxic activity: serum CK levels after i.m. injection in mice. Serum CK levels were determined after injection of 50 μg of *B. alternatus* venom, 50 μg of *B. alternatus* venom + anti-baltergin IgG (ratio 1:30; w:w), 10 μg of *B. diporus* venom and 10 μg *B. diporus* venom + anti-baltergin IgG (ratio 1:30; w:w). Bars represent the mean ± SD ($n = 4$). $p < 0.05$ venom or venom/anti-baltergin IgG versus control group.

induce an increase in CK values similar to those induced by venom alone (Fig. 4).

3.3.3. Histological studies

Histological observations of samples of gastrocnemius muscle injected with PBS retained a normal histological pattern (Fig. 5A). Muscle sections from mice injected with *B. alternatus* venom showed prominent damage characterized by intense hemorrhage, myonecrosis (associated to hemorrhagic areas), edema and polymorphonuclear inflammatory infiltrate (Fig. 5B). Sections of muscle from mice injected with *B. diporus* venom showed a similar profile of damage but the necrosis of muscle fibers was more extensive, not only associated to hemorrhagic areas (Fig. 5D).

Nevertheless, when venoms were previously neutralized with anti-baltergin IgG results obtained from each experiment were quite different. After neutralization of *B. alternatus* venom by the specific antibodies, histological samples showed intense infiltrate inflammatory in absence of hemorrhage and presence of normal muscle fibers in all fields observed, with exception of some necrotic cells near the site of injection (Fig. 5C). However, in the case of *B. diporus* venom pre-treated with anti-baltergin IgG, the most striking observation was the severe necrosis of muscle fibers, both myolytic and coagulative types. Intense edema and infiltrate inflammatory, in lack of hemorrhage, were also observed (Fig. 5E).

4. Discussion

B. alternatus and *B. diporus* are the main species involved in envenomations in Argentina (PNGCAM, 2007). Baltergin, a SVMP isolated from *B. alternatus* venom, has been used to obtain monospecific IgG in order to determine the relative role of metalloproteinases in myotoxicity induced by whole venom. SVMPs play a relevant role in the pathogenesis of local tissue damage characteristic of bothropic envenomations (Gutiérrez and Lomonte, 1989; Bjarnason and Fox, 1994). In the case of *B. alternatus* venom, baltergin was found to be responsible for causing local hemorrhage, myonecrosis and edema after inoculation in mice (Gay et al., 2005).

Purified polyclonal antibodies obtained through immunization of rabbits with baltergin were able to neutralize completely the local hemorrhagic activity of *B. alternatus* venom in mice. This finding demonstrates the importance of baltergin, and antigenically-related metalloproteinases, in the hemorrhagic action of the venom. In addition, hemorrhage induced by *B. diporus* venom was also neutralized by specific antibodies although with less effectiveness. It was noticed a small spot remaining in the site of the injection even at the highest ratio of IgG:venom assayed, likely due to insufficient amount of antibodies used or to the presence of metalloproteinases, weakly hemorrhagic, which were not recognized by the specific antibodies. Other authors have observed similar results using rabbit antiserum to the most potent hemorrhagic metalloproteinase isolated from *Bothrops asper* venom (BaH1) which was able to neutralize the hemorrhagic activity of several crude snake venoms with variable effectiveness (Rucavado et al., 1995a).

Local myotoxicity induced by *B. alternatus* venom in mice was efficiently neutralized by anti-baltergin IgG, suggesting that this venom is rich in hemorrhagic metalloproteinases and poor in highly myotoxic PLA2s. Besides, the delayed increase in CK release, 6 h after i.m. injection of crude venom, is characteristic of muscle damage induced by SVMPs (Andriao-Escarso et al., 2000). The muscular lesion caused by these proteins involves firstly the alteration of the microvasculature, minutes after injection, and a decrease of the irrigation in the affected area. However, the myonecrosis secondary to ischemia, is observed at relatively late time intervals after injection. Thereby, when damage is induced by SVMPs, areas of necrosis are always present in sections of muscle tissue with intense hemorrhage (Gutiérrez and Rucavado, 2000), such as was observed in the histological sections of mice treated with *B. alternatus* venom.

Two PLA2s, BaTX and Ba SpII RP4, were isolated from *B. alternatus* venom. BaTX, isolated from venom of Brazilian specimen, induced a conspicuous local myotoxic effect when it was injected by the i.m. route, showing a maximum increase in CK levels 2 h later (Ponce-Soto et al., 2007). Despite being a potent basic myotoxin, belonging to Lys-49 group of PLA2s, in the whole venom its proportion is relatively low since it has not induced significant changes in CK levels after neutralization of venom with anti-baltergin IgG. This result is also in agreement with observations of Andriao-Escarso et al. (2000), who demonstrated absence of basic PLA2s myotoxins after chromatographic fractionation of *B. alternatus* venom on CM-Sepharose column. Furthermore, it was reported that the proteome of *B. alternatus* venom is mainly a pool of acidic proteins, consisting of 43.1% of SVMPs and 7.8% of PLA2s, among other proteins families (Ohler et al., 2010).

On the other hand, Ba SpII RP4, the most abundant Asp-49 PLA2 present in *B. alternatus* venom from Argentinean specimen, is an acidic protein which did not exert either *in vivo* or *in vitro* myotoxicity (Garcia Denegri et al., 2010). Only when Ba SpII RP4 was added to myoblasts culture

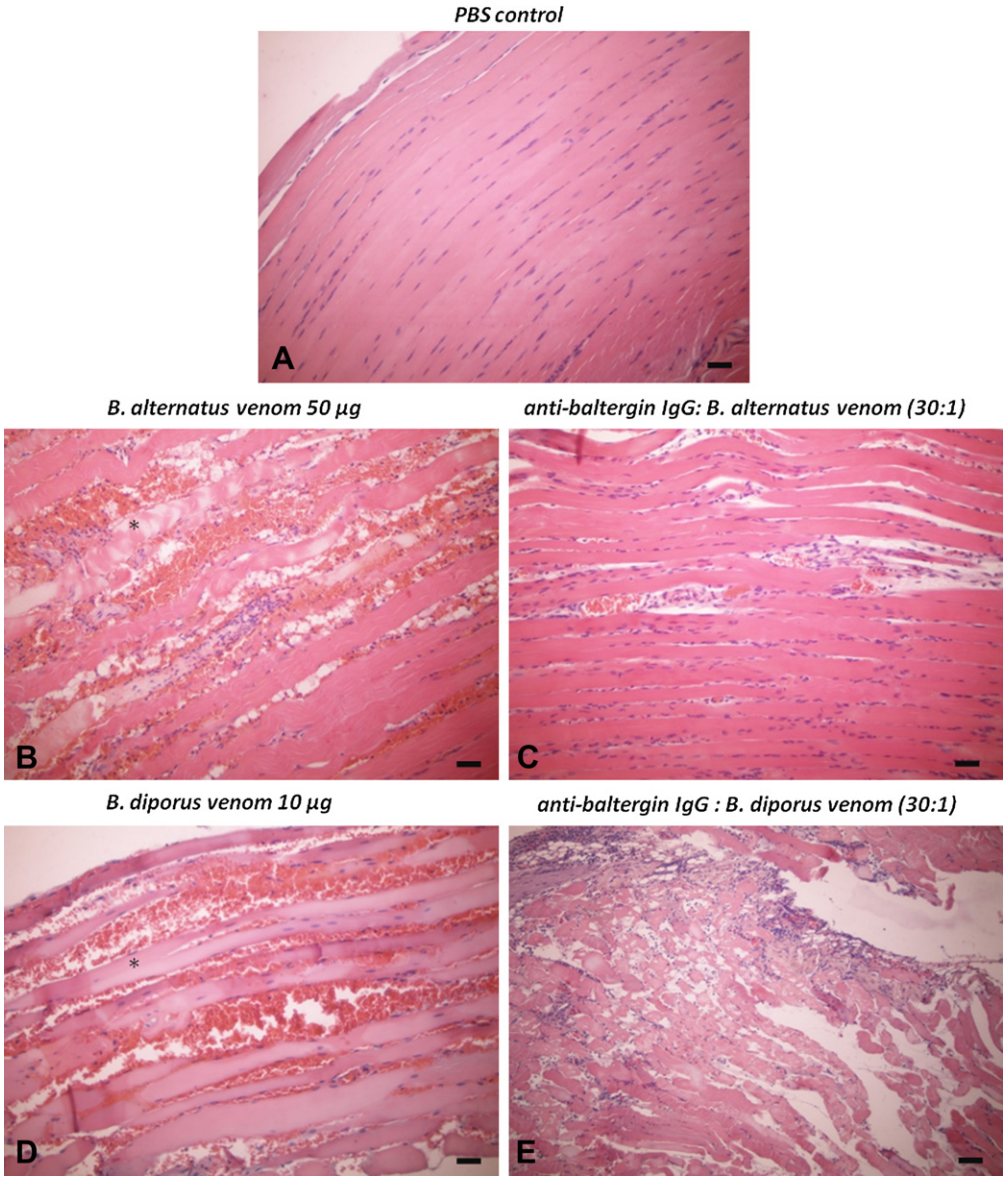

Fig. 5. Neutralization of myotoxic activity: histological sections of gastrocnemius muscle of mice. Stained with H-E. **A**. Control: normal muscle tissue. Bar = 30 µm. **B**. Six hours after injection of 50 µg of *B. alternatus* venom. Note presence of necrotic fibers restricted to areas of prominent hemorrhage (asterisks) and intense infiltrate inflammatory. Bar = 30 µm. **C**. Six hours after injection of 50 µg of *B. alternatus* venom pre-incubated with anti-baltergin IgG (ratio 1:30; w:w). Normal integer fibers are preserved. Absence of hemorrhage and moderate infiltrate inflammatory are also observed. Bar = 30 µm. **D**. Six hours after injection of 10 µg of *B. diporus* venom. Intense hemorrhage and myonecrosis (asterisks) are observed. Edema indicated by the increased spacing between muscle cells is also noted. Bar = 30 µm. **E**. Six hours after injection of 10 µg of *B. diporus* venom pre-incubated with anti-baltergin IgG (ratio 1:30; w:w). Notice presence of severe myonecrosis and intense edema in absence of hemorrhage. Infiltrate inflammatory is also observed. Bar = 60 µm.

simultaneously with baltergin, a cytotoxic action was observed, proving the existence of synergism *in vitro* between these enzymes (Bustillo et al., 2012). Therefore, the neutralization of hemorrhagic SVMPs in the *B. alternatus* venom may be enough to significantly reduce the local muscle damage since they are responsible to induce vascular lesion with ensuing ischemia and, probably, acting synergistically with other toxins.

On the contrary, the myotoxic activity of the *B. diporus* venom was not neutralized by the anti-baltergin IgG. Histological sections of treated muscle exhibited intense necrosis of fibers even in absence of hemorrhage. The myotoxic effect, in this case, is likely due to the presence of highly myotoxic PLA2s rather than SVMPs. PLA2s that exert local myotoxicity, have a strongly cationic face which is to interact with negatively charged phospholipids in membranes (Falconi et al., 2000; Murakami and Arni, 2003; Murakami et al., 2005). This event probably occurs earlier than indirect muscle damage induced by SVMPs and would explain the quick release of CK and, the lower doses of *B.*

diporus venom used, to induce similar CK levels than *B. alternatus* venom. Hence, even if the hemorrhagic activity of *B. diporus* venom is neutralized, muscle damage is not significantly reduced, indicating that the role of SVMPs in myonecrosis is only marginal in venoms which contain direct myotoxic factors such as basic PLA2s.

In other bothropic venoms with high content of PLA2s, the myotoxic activity is mainly due to the action of potent basic PLA2 myotoxins. This is the case of *B. asper* venom which contains 29–45% of PLA2s (Alape-Giron et al., 2008) and several well characterized myotoxins (Gutiérrez and Lomonte, 1995, 1997). Monospecific antiserum against a myotoxic PLA2 (myotoxin I) was able to neutralize more than 75% of the myotoxic activity of crude *B. asper* venom, demonstrating that the group of basic PLA2s myotoxins is the most important factor in the induction of myonecrosis by this venom (Lomonte et al., 2009).

In conclusion, neutralization assays using monospecific polyclonal antibodies, were useful for revealing the central role of SVMPs in the development of myotoxicity by *B. alternatus* venom, as well as, they allowed us to suggest presence of potent myotoxic PLA2s in *B. diporus* venom. In order to know the composition and the different proportions of proteins present in both venoms, additional proteomic studies would be necessary to carry out.

Ethical statement

All experiments followed the ethical standards for animal experiments in toxinological research recommended by the International Society of Toxinology. 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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Conflicts of interest

The authors declare that there are no conflicts of interest.

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