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Antivenom against *Crotalus durissus terrificus* venom: Immunochemical reactivity and experimental neutralizing capacity

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ABSTRACT

The venom of *Crotalus durissus terrificus* produces a neurotoxic and myotoxic syndrome that can lead to the death. Specific antivenom is the only treatment to neutralize the toxicity of the venom and the precocity in applying the antivenom is crucial for the efficiency of the treatment. We studied the variation of the immunochemical reactivity and neutralizing capacity of the specific antivenom on this venom in pre-incubation and rescue experiments, at different times. ELISA titers increased with longer venom-antivenom incubation times ($p < 0.05$) nevertheless incubation times had no effect on the neutralizing capacity of the antivenom. The antivenom dose necessary to rescue mice injected with 1.5 MMD (minimal mortal dose) 30 min after venom inoculation was over ten folds the dose of antivenom theoretically required to neutralize the same dose of venom according values obtained from pre-incubation experiments. Results showed that the *in vitro* immunochemical reactivity is not directly related to the neutralizing capacity. These observations underline the need for a rapid antivenom administration. Although preincubation experiments in mice are a powerful tool for the validation of the potency of the antivenoms in the productive process, it is clear that the nominal neutralization of the antivenoms must not be considered as a "stoichiometric" value regarding the venom to be neutralized in case of natural envenomation and emphasize the need of realization of clinical trials in order to evaluate the adequate doses of antivenom to be therapeutically used.

1. Introduction

The neutralizing capacity of an antivenom depends of the binding of the antibodies or its fragments to the different venom toxins and it is tested worldwide by preincubation assays (WHO, 1981, 2010, 2016; Theakston and Reid, 1983). Despite that pre-incubation assays does not represent the natural envenoming, since venom-antivenom are mixed previously the injection, these methods are easy to perform and their results are repetitive and extremely helpful for the study of the antivenom and as a tool for antivenom production (Gutiérrez et al., 1990, 2017; Theakston, 1990; Ownby, 1990; Araujo et al., 2008; Solano et al., 2010). The experiments that represent more natural conditions of the envenomation, the rescue experiments, are not routinely used for this purpose and are not used in the world to test antiophidic antivenoms (Gutiérrez et al., 1990; Theakston, 1990; Ownby, 1990). In addition, as an alternative to the neutralization experiments, assays based in the immunochemical reactivity of the antivenoms with the venoms have been proposed (Rungsiwongse and Ratanabanangkoon, 1991; Heneine et al., 1998; Maria et al., 1988; Barbosa et al., 1995).

However, in unpublished results, we did not observe relation

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between immunochemical reactivity and neutralizing capacity of several antivenoms, neither variation in the neutralizing capacity after different times of pre-incubation of the venom-antivenom. By these reasons, we studied these relationships in order to clarify the relation of the immunochemical reactivity with the neutralization at different times of incubation and the effectiveness of the antivenom in a situation near the natural envenoming, i.e. through rescue experiments applying the antivenom after venom injection. We studied these relationships using *Crotalus durissus terrificus* (henceforth *C. d. terrificus*) venom, a neurotoxic and myotoxic venom, and their specific antivenom.

2. Material and methods

2.1. Venom

A pool of *C. d. terrificus* venom was obtained from adult snakes (over 100 specimens) kept in the National Institute for Production of Biologicals of the National Administration of Laboratories and Institutes of Health "Dr. Carlos G. Malbrán" (henceforth INPB). The venom was milked by manual extraction, vacuum dried and stored at −20 °C until use.

2.2. Antivenom

The antivenom used was Anticrotálico antivenom (batch 335, expiration date 06/2009), produced by the INPB. The antivenom was composed by $F(ab)_2$ fragments of equine immunoglobulins, in liquid form, in a formulation of 10 ml vial.

The protein content of the antivenom determined by Biuret method using the kit Proti II (Wiener, Rosario, Argentina) was 68 mg/ml. The nominal neutralizing potency was 4 mg of venom neutralized by ml of antivenom.

2.3. Animals

CF-1 mice of 18–22 g were obtained from the Service of mice production of the INPB. Mice were maintained in plastic boxes with water and rodent food *at libitum*, with cycles of dark/light of 12 h. For the use and care of the animals the institutional guidance of care of animals, in agreement with those of the Public Health Service (2002) and the National Research Council (2011) were followed.

2.4. Immunochemical studies

2.4.1. Solid-phase enzyme immunoassay (ELISA)

The ELISA was carried out according to published procedures with some modifications (Giallongo et al., 1982). Solid-phase-adsorbed venom of *C.d. terrificus* was prepared by treating wells of MaxiSorp plates (NUNC-Apogent) with 100 μl solution of 5 μg/ml of venom in 100 mM sodium carbonate buffer (pH 9.5). After overnight incubation at 4 °C, the wells were aspirated and washed with three 250 μl aliquots of a washing buffer (PBS, pH 7.4, containing 0.5 mg/ml of Tween 20). The wells were then filled with 200 μl of blocking buffer (3% bovine defatted milk and 2 mg/ml of Tween 20 in PBS at pH 7.4). After incubation for 2 h at 37 °C, the wells were washed as described above and filled with 100 μl aliquots of the antivenom serially diluted in incubation buffer (PBS at pH 7.4 containing 0.3% defatted milk and 0.5 mg/ml of Tween 20). The incubation times at 37 °C were 5, 30 and 120 min. After washing, in each case, the bound horse $F(ab')_2$s were allowed to react with 100 μl per well of 1/5000 dilution of anti-horse IgG labeled with peroxidase (The Binding Site) in incubation buffer. After another hour, the wells were washed and filled with 100 μl of OPD solution (Sigma) and H_2O_2 as the substrate for peroxidase. The color development reaction was stopped by the addition of 100 μl of 3 N sulfuric acid and the plate was read at an absorbance of 490 nm in a Microplate Reader Model 550 (BIO-RAD). Data were analyzed by nonlinear regression using the sigmoidal dose-response equation of the Prism–StatMate program (GraphPad Software, San Diego, CA). Conventional titers (EC_{50}) were calculated from the midpoint of the curve and correspond to the reciprocal antivenom dilution for half of maximal binding. Each point was repeated by quintuplicate and all the assays were repeated by quadruplicate.

2.4.2. ELISA-urea assay to determine antibody fragments avidity

The avidity was measured as suggested by Bollen et al. (1996) with some modifications (de Andrade et al., 2013; da Rocha et al., 2017). Wells in plates coated with *C. d. terrificus* venom as described above were filled with 100 μl of 1/5000 dilution of the antivenom and incubated for 5, 30 or 120 min at 37 °C. After incubation, the wells were washed with PBS-Tween- defatted milk solution and filled with 100 μl of 8 M urea or 0.15 M NaCl solution and incubated for 2 h at 37 °C. Then, plates were washed 5 times and incubated with the conjugate (Anti-horse IgG peroxidase conjugate, Sigma at a dilution 1/5000). The reaction was developed as described above. Each point was repeated by quintuplicate and the assays were repeated by triplicate. The avidity was measured as the ratio of the absorbance of the sample treated with 8 M urea and the absorbance of the sample incubated with 0.15 M NaCl (Bollen et al., 1996; de Andrade et al., 2013).

2.5. Determination of lethal potency

The lethal potency of the venom was determined by intraperitoneal (i.p.) and by subcutaneous (s.c.) routes. Briefly, groups of 6 mice were injected with various doses of venom diluted in 0.15 M NaCl in a final volume of 0.5 ml (i.p.LD_{50}) or 0.3 ml (s.c.LD_{50}, in the back of mice). After 24 h the deaths were registered and the percentage of death versus the log of the venom dose were plotted. The median lethal dose (i.p.LD_{50} and s.c.LD_{50}) were estimated by non-linear regression of variable slope (Casasola et al., 2008) using the software Prism 5.0 and were defined as dose of venom that killed the 50% of the challenged mice during the period of observation. The minimal mortal dose (MMD) was estimated considering the minimal dose of venom that killed 100% of mice challenged.

2.6. Neutralization experiments

The protection provided by the antivenom was studied by pre-incubation and rescue assays.

2.6.1. Pre-incubation experiments

Five LD_{50} of the venom were mixed with various doses of the antivenom and incubated at 37 °C at different times after which the mixtures were injected by the i.p. route in groups of five C-F1 mice. The times of incubation of the antivenom were: 0 min, mixing venom plus antivenom in the syringe and immediately applying the mixture by the intra-peritoneal route (the mix was done individually for each animal), 1 min, 5 min, 10 min, 30 min, 60 min, 120 min and 180 min (in these cases mixing venom-antivenom and incubated at 37 °C during the correspondent times). After 24 h the number of surviving mice were registered and the percentage of survival versus the log of the dose of antivenom were plotted. The median effective dose was estimated by non-linear regression of variable slope (Casasola et al., 2008) using the software Prism 5.0. The median lethal dose (ED_{50}) was defined as the dose of antivenom that protected 50% of the challenged mice during the period

of observation.

In addition, the neutralizing potency of the antivenom was estimated as suggested by the Pharmacopeia of the State of Sao Paulo, Brazil (Ministerio de Saúde, 1996) with the following formula P (potency)= ([n-1]/[ED_{50} (μl)]) x LD_{50} (μg), where n-1 is the number of LD_{50} used as challenge dose (in this case 5-1 = 4), since the remaining venom is the responsible of the death of the half of mice, n-1 LD_{50} are neutralized, expressing the potency as mg of venom neutralized by ml of antivenom (Ministerio de Saúde, 1996). The challenge dose of 5 LD_{50} with different doses of antivenom and the incubation time by 30 min at 37 °C is used in Argentina and Brazil for the determination of the potencies of the therapeutic antivenoms (Ministerio de Saúde, 1996; Araujo et al., 2008). The assays for each incubation time were repeated 5 to 10 times. Only the assays that accomplished the requirement of two consecutive values of 0% protection and 100% protection and with at least two points in the log part of the dose-response curve were considered. Finally, 5 to 7 assays for each incubation time were selected (See Table 1 and Appendices 1 to 3).

2.6.2. Rescue experiments

The ED_{50}s in rescue experiment (rED_{50}) by the i.p. and intravenous (i.v.) routes were determined.

2.6.2.1. Selection of times and doses. The approximated ranges doses and the best times to be chosen for the neutralizing experiments were obtained by injecting antivenom in different doses at different times in groups of mice previously injected with 1.5 s.c. MMD (in this case, around 3.0s.c.LD_{50}). Two experiments were performed: 1) Twenty microliters of antivenom (around 12.5 i.p. ED_{50}) were administered at different times (0, 1, 5, 15, 20, 25, 30 min) to groups of mice (n = 5) previously injected with 1.5 MMD of venom by the s.c. route. The protection was expressed as the percentage of surviving mice in each time. 2) After 30 min of similar venom challenge, mice were treated with 30, 60, 120, 160 and 200 μl of antivenom by the i.p. route. The protection was expressed as the percentage of protection obtained with each dose.

2.6.2.2. Determination of the rescue neutralizing potency. After doses and times selection, six mice per dose level were injected with 1.5 MMD of venom by s.c. route as previously described. After 30 min, mice were injected by the i.v. or the i.p. route with several doses of the antivenom diluted in 0.15 M NaCl, in a final volume of 200 μl or in 500 μl/mouse respectively. Mice were controlled by 24 h and the neutralizing potency achieved by each route was expressed as ED_{50} (μl/mouse) and in potency (mg/ml), determined as explained. The rescue experiments were repeated 10 times, nevertheless for the analysis 8 (i.p.) and 7 (i.v.) experiments were selected (those that accomplished the requisites mentioned above).

2.7. Statistics

For lethality assays, seroneutralization and ELISA EC_{50}s, non-linear regression analysis of variable slope was used. To study the association between the incubation time and the EC_{50} and ED_{50}, it was used the linear regression analysis. For determination of the distribution of the neutralization results (ED_{50}s and potencies) data were analyzed by Kolmogorov-Smirnov test, and for comparison between the results the Man-Whitney test was used. For all the statistical analysis as well as for the determination of mean values, standard deviations, variation coefficients and 95% confidence intervals, the software Prism 5.0 (GraphPad, CA) was utilized.

Table 1
Values of the median effective doses (ED50) and potency (P) selected from the different experiments. The neutralization assays were repeated 5 to 10 times for each incubation time. Only the doses that accomplish the requirement of two consecutive doses of 0% protection and two consecutive doses of 100% protection, and two points in the log section of the dose-response curve. The ED50s are expressed as microliter (μl) of antivenom by mice and the P as milligram of venom (mgV) neutralized by ml of antivenom (mlAV). In both cases the confidence intervals are expressed into round brackets. The median value in each incubation is expressed and the minimal and maximal values are expressed into square brackets, separated by a dash. Only the experiments when the 100% and 0% of death (at least two consecutive doses of these values) and at least two points in the log part of the curve were accomplished, were considered.

Times	0 min		1 min		5 min		10 min		30 min		60 min		120 min		180 min	
Determinations	ED_{50} (μi/mouse)	P (mgV/mlAV)	ED_{50} (μi/mouse)	P (mgV/mlAV)	ED_{50} (μi/mouse)	P (mgV/mlAV)	ED_{50} (μi/mouse)	P (mgV/mlAV)	ED_{50} (μi/mouse)	P (mgV/mlAV)	ED_{50} (μi/mouse)	P (mgV/mlAV)	ED_{50} (μi/mouse)	P (mgV/mlAV)	ED_{50} (μi/mouse)	P (mgV/mlAV)
Experimental values	1.5 (1.4–1.6)	4.6 (4.2–5.0)	2.2 (1.8–2.8)	3.1 (2.5–3.9)	1.3 (0.7–2.1)	5.4 (3.3–9.4)	1.9 (1.7–2–0)	3.7 (3.4–4.0)	1.6 (1.5–1.6)	4.4 (4.2–4.5)	1.3 (1.1–1.5)	5.4 (4.5–6.4)	1.4 (1.3–1.4)	5.1 (4.8–5.5)	2.1 (1.5–2.8)	3.3 (2.4–4.6)
	1.6 (1.5–1.7)	4.4 (4.2–4.6)	2.2 (1.9–2.7)	3.1 (2.6–3.7)	2.1 (1.8–2.5)	3.3 (2.8–4.0)	2.5 (1.7–3.6)	2.8 (1.9–4.0)	1.6 (1.4–1.9)	4.3 (3.7–5.0)	1.8 (1.7–2.0)	3.8 (3.5–4.2)	2.7 (2.1–3.3)	2.6 (2.1–3.2)	2.2 (1.5 to 3-3)	3.1 (2.1–4.6)
	1.8 (1.6–2.1)	3.8 (3.4–4.3)	2.1 (1.7–2.5)	3.4 (2.8– 4.0)	1.4 (1.3–1.5)	4.9 (4.5–5.2)	1.3 (1.1–1.5)	5.5 (4.8 to 6.3)	1.6 (1.5–1.6)	4.4 (4.3–4.6)	1.9 (1.8–2.0)	3.7 (3.4–4.0)	1.9 (1.8–2.0)	3.6 (3.4–3.8)	1.8 (1.6–1.9)	4.0 (3.6–4.4)
	1.8 (1.7–2.0)	3.8 (3.6–4.2)	1.5 (1.1–2.0)	4.8 (3.5–6.4)	1.7 (1.5–2.0)	4.0 (3.4–4.8)	2.0 (1.8–2.2)	3.4 (3.1–3.8)	1.7 (1.7–1.8)	4.0 (4.0–4.1)	1.9 (1.8–2.1)	3.6 (3.3–3.9)	1.7 (1.6–1.8)	4.2 (4.0–4.4)	2.0 (1.8–2.2)	3.6 (3.2–4.0)
	2.0 (1.6–1.9)	3.5 (3.6–4.3)	1.5 (1.4–1.5)	4.8 (4.7–4.8)	1.5 (1.5–1.6)	4.5 (4.3–4.8)	1.5 (1.2–1.8)	4.6 (3.8–5.6)	1.5 (1.1–2.1)	4.7 (3.4–6.5)	1.8 (1.6–2.0)	3.9 (3.5–4.4)	1.9 (1.8–2.0)	3.7 (3.6 to 3.9)	1.8 (1.7–1.9)	3.8 (3.6–4.0)
			1.5 (1.4–1.7)	4.6 (4.2 to 5.1)	1.8 (1.8–2.1)	3.9 (3.3–4.5)									1.8 (1.4–2–3)	3.9 (3.0–5.1)
															2.1 (1.5–2.9)	3.3 (2.4–4.6)
Median with minimal and maximal values	1.8 [1.5–2.0]	3.8 [3.5–4.6]	1.8 [1.5–2.2]	4.0 [3.1–4.8]	1.6 [1.3–2.1]	4.3 [3.3–5.6]	1.9 [1.3–2.5]	3.7 [2.8–5.5]	1.6 [1.5–1.7]	4.4 [4.0–4.7]	1.8 [1.3–1.9]	3.8 [3.6–5.4]	1.9 [1.4–2.7]	3.7 [2.6–5.1]	2.0 [1.8–2.2]	3.6 [3.1–4.0]

3. Results

The immunochemical reactivity of the antivenom determined by ELISA varied in the different incubation times. The EC_{50}s showed a direct relation between the incubation time and the immunochemical reactivity in all the cases ($p < 0.05$). The reactivity was higher when the antivenom was incubated during 120 min, regarding 30 min incubation, and the last showed higher reactivity that than observed using the incubation time of 5 min. The EC_{50} values were for 5 min: 0.3765 (95% confidence intervals 0.3167 to 0.4476), 30 min: 0.1345 (c.i. 0.1219 to 0.1484) and 120 min: 0.097 (c.i. 0.0848 to 0.1111) (Fig. 1). The linear regression analysis of the EC_{50}s and the incubation times showed a direct correlation ($r^2 = 0.998$; n = 3; *p* 0.03).

The treatment with urea showed that antibody fragments strongly bind the venom at the different times. No differences in avidity were observed in the different incubation times, being in all the cases the index venom-antivenom treated with 8 M urea/venom-antivenom incubated with 0.15 M NaCl equal to1.0.

The lethal potency expressed as LD_{50} of the venom by the i.p. route was 1.74 μg/mouse (95% c.i. 1.5 to 2.0. μg) while considered by the s.c. route was around 5 fold higher, with a value of 8.55 μg/mouse (8.0–9.1 μg). The s.c. MMD (LD_{100}) was of 18 μg by mouse.

The incubation time did not affect the neutralizing potency. No relation was observed between the incubation times and the protection conferred by the antivenoms ($r^2 < 0.005$; $p > 0.8$; n = 8).

Pre-incubation experiments showed that the ED_{50} and P (potency) were very close in all the cases (Table 1 and Appendix 1). The variation expressed as coefficient of variation of the ED_{50}s obtained at the different times was close in all the cases: 0 min: 11.1%, 1 min: 21.3%, 5 min: 18.4%, 10 min: 25.0%, 30 min: 14.1%, 60 min: 14.2%, 120 min: 22.9% and 180min: 9.8%, surprisingly, the incubation times of 0 min (no incubation) and 180 min, were those with the lower coefficient of variation.

The values of ED_{50} ranged from 1.3 to 2.7 μl in the individual assays and in the different times, and from 1.6 to 2.0 μl considering the median value of the results in each different incubation time. The P ranged from 2.6 to 5.6 mg/ml (individually) and 3.6–4.4 mg/ml (different times of incubations). See Table 1. In almost all the cases the values of P were close to those declared in the vial of the antivenom by the producers. The ED_{50} at 30 min of incubation (the one required for the official antivenom control) was 1.6 μl (c.i. 1.5–1.7 μl; individually min.1.1, max. 2.1) with a P of 4.4 mg/ml. When values of the assays (ED_{50} and P) obtained at different times were compared, no statistical differences were observed between groups ($p > 0.05$) for the various times of incubation from 0 min to 180 min (See Appendices 2 and 3), being the only exception in which differences were observed the 30 min incubation time, regarding the time of 180 min of incubation ($p < 0.05$), although with very close values. See Table 1 and Appendices 2 and 3.

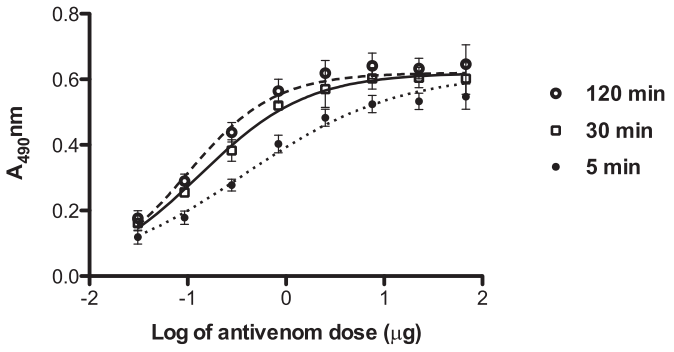

Fig. 1. ELISA studies of the reactivity *C. d. terrificus* venom – antivenom at different incubation times. The reactivity was determined by direct ELISA studies at different incubation times: 5 min (●), 30 min (□) and 120 min (○).

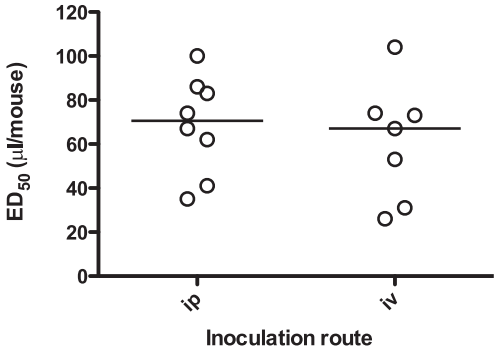

Fig. 2. Neutralizing capacity of the antivenom in rescue experiments. The neutralizing capacity of the antivenom in rescue experiments 30 min after the injection of 1.5 MMD (27 μg) of venom. The different ED_{50} values of the rescue experiments by the intraperitoneal (i.p.) or the intravenous route (i.v.) are show. Bars indicate the medians. Despite that the i.v. route seems to provided higher protection, the differences between the two inoculation routes did not show statistical differences ($p > 0.05$). Only the experiments when the 100% and 0% of death (at least two consecutive doses of these values) and at least two points in the log part of the curve were accomplished, were considered.

When the adjustments for rescue experiments were performed, inverse relation between protection provided by 20 μl (12.5 ED_{50} determined by the preincubation assay) and the time elapsed after the challenge with venom was observed. The protection observed were 100% (1 and 5 min after venom injection), 60% (10 min), 40% (15 min) and 20% (20 and 25 min) and 0% in longer times. When antivenom was applied in higher doses 30 min after venom injection, only using 60 μl or higher doses, protection was observed. See Appendix 4.

The rescue experiments were performed challenging the mice with 1.5 MMD of venom by s.c. route and applying the antivenom doses 30 min after the challenge, either by the i.p. or i.v. routes. The rED_{50} (rescue ED_{50}) were 71 μl (median; minimal 35 μl and maximal 100 μl) using the i.p. route (n = 8) and 67 μl (median; minimal 26 μl and maximal 104 μl) using the i.v. route (n = 7). Despite that i.v. route could seems more potent than the i.p., no statistical differences were observed ($p > 0.05$). See Fig. 2.

Potencies expressed in milligrams of venom neutralized by 1 ml of antivenom or in milliliters of antivenom required to neutralize 1 mg of venom, for preincubation experiment (incubation 30 min) and rescue experiments are shown in Fig. 3.

4. Discussion

The venoms are formed by a large number of proteins which both toxicologically and immunologically are highly variable between species, inclusive between individuals of the same snake species (Chippaux et al., 1991; de Roodt et al., 2011). The only tool to treat snakebites envenoming is the use of antivenoms and their therapeutic effect is due by the direct contact of the antivenom molecules with their targets. This happens immediately during the incubation experiments but it is more difficult to achieve when the venom and the antivenom are injected separately (both route and time) in the same organism. The antivenom-venom encounter is random, nevertheless the efficiency of an antivenom meets an additional challenge: to bring the appropriate antibodies in contact with the specific toxic proteins of the venom in the victim's body. This is what this work tried to study.

In the ELISA the immunochemical reactivity of the antivenoms

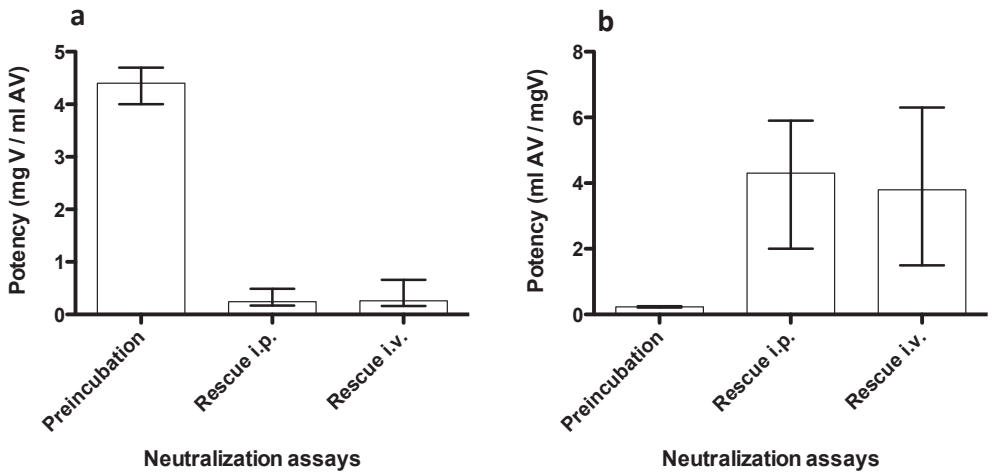

Fig. 3. Neutralizing capacity of the antivenom expressed as potency. The neutralizing capacity of the antivenom in experiments is expressed in milligrams of venom (V) neutralized by 1 mL of antivenom (AV) (mgV/mlAV, Fig. 3.a) or as the volume of antivenom expressed in milliliters required to neutralize 1 mg of venom (ml/mg, Fig. 3.b) in the preincubation or in rescue experiments. The challenge doses were: 8.7 μg/mouse in preincubation experiment (5.0 i.p. LD_{50}) and 27 μg/mouse in rescue experiments (1.5 s.c. MMD). It is clear that the potency obtained in preincubation experiments is different ($p < 0.05$) regarding those obtained from rescue experiments. The different rescue ED_{50} values observed in the rescue experiments by intraperitoneal (i.p.) or intravenous routes (i.v.) did not show statistical differences ($p > 0.05$). Error bars indicate the minimal and maximal values.

increased with the incubation time (Fig. 1), in agreement with results described by Solano et al. (2010), which observed higher turbidity of the mix antivenom – *B. asper* venom in concordance with incubation times. Despite the immunochemical reactivity increased with longer incubation times, these had no effect on the neutralizing activity of the venom.

Avidity experiments showed strong binding of the fragments since there were no differences in reactivity after treatment with 8 M urea since 5–120 min.

The evaluation of the antivenoms in the world is performed by pre-incubation assays (WHO, 1981, 2010, 2016). These assays are easy to carry on, provided repetitive results and valuable information on the neutralizing potency of the antivenoms, and are crucial for the study and production of antivenoms (Gutiérrez et al., 1990; Ownby, 1990; Theakston, 1990; Solano et al., 2010). Although data from experimental animals cannot be directly extrapolated to humans (Theakston and Reid, 1983), pre-incubation assays have shown to be reliable tools for working in preparation and evaluation of antivenoms (Schlottler, 1952; Christensen, 1966; WHO, 1981, 2010, 2016; Theakston and Reid, 1983; Gutiérrez et al., 1990; Araujo et al., 2008; Solano et al., 2010). However, the finding of methods for a better evaluation of the effectiveness of the antivenoms, were discussed. Among these, the rescue assays (or sequential procedures) were proposed as a more realistic assay to determine the usefulness of the antivenoms (Schlottler, 1952; Christensen, 1966; Gutiérrez et al., 1990; Chippaux and Goyffon, 1998; Dzikouk et al., 2002). In addition, the information provided by this type of assessment is valuable to understand neutralization in the context of the dynamics of envenoming (Gutiérrez et al., 2017). Nevertheless, despite the more realistic conditions regarding the neutralizing potency of the antivenoms, these assays are affected by several factors among that can be cited the type of venom, pharmacokinetics of venom compounds and antivenoms and physiological characteristics of the animal models (Gutiérrez et al., 1990, 2017; Ownby, 1990; Theakston, 1990).

Under natural conditions, in a snakebite the venom is injected into the tissues of the victim while the antivenom is administered after the bite by the i.v. route (if correctly applied). On the contrary, in preclinical tests, the venom is mixed with antivenom for 30 min incubation - according to WHO standards - and the mixture is administered intravenously or intraperitoneal (WHO, 1981, 2010, 2016). It follows that the crucial stage of the venom/antivenom challenge is optimized in the second case, which does not reflect at least two facts: a) the delay between the introduction of the venom and the administration of the antivenom and b) the place of introduction and separate distribution of venom and antivenom in the victim's organism. The difference between these two factors represents constraints limiting the chance of the encounter of the venom and antivenom.

By these reasons we tested the antivenom in rescue assays on order to study the neutralizing capacity of an antivenom of known neutralizing potency in mice in an assay closer to a natural envenomation in mice.

For rescue assays, we preferred to use 3 s.c. LD_{50} (around 1.5 MMD) instead of 5 LD_{50} (for which the gap would have been probably much higher) that should require more volume of antivenom and/or shorter times for their administration. We used the i.p. and i.v. routes of inoculation because despite the i.p. route has around the half of distribution rate regarding the i.v., this route allows to use higher volumes (Shimizu, 2004; Turner et al., 2011). Despite the different rate of distribution, the neutralizing doses obtained from the two methods in this study, were quite similar.

The need of higher doses of antivenom required to rescue mice can partially be explained by the characteristics of this venom. The main component of *C. d. terrificus* venom is the myotoxic and neurotoxic presynaptic phospholipase A_2 called crotoxin, that can represent over the half of the venom mass (Breithhaupt et al., 1974; Calvete et al., 2010). Amaral et al. (1997) showed a highly significant decrease in the concentration of crotoxin in patients' plasma arriving at the hospital related with the time elapsed post-bite. These results suggested that the clearance of crotoxin is fast and it is quickly distributed in deep tissues, probably linked to neurological receptors or muscle. This could partially explain the need of fast treatment with antivenom.

Aside from the limitations described above, another difference must be taken into account. The animal model used - the mouse - cannot be considered as an human suffering a snake bite and treated with antivenom. Toxic substances for one as for the other may be different due its different anatomy and physiology (Gutiérrez et al., 2017).

The ED_{50} and P of the antivenom, very stable after various times of incubation, was not useful to predict the dose of antivenom

needed to neutralize a determinate dose of venom *in vivo*. The P of the antivenom obtained at 30 min of incubation at 37 °C following the technique used for the control of the antivenom indicate a neutralizing capacity of 4.4 mg/ml (theoretically 0.23 ml of antivenom to neutralize 1 mg of venom), nevertheless when the P of the same antivenom was estimated in the rescue experiments, it was lower: 0.25 mg/ml (4.3 ml/mg) and 0.26 mg/ml (3.8 ml/mg) using the i.p. or the i.v. routes, respectively (Fig. 3).

The dose determined by preincubation assay, was not useful for the determination of the potency in rescue assays since this did not follow the "law of multiple proportions" and, consequently, did not give twice as high a potency if the antibody concentration is doubled (Schlottler, 1952; Christensen, 1966; Ownby, 1990). The lack of proportionality that we showed in the rescue experiment confirms that the law of multiple proportions cannot be applied for the dosage of antivenoms. Antivenoms must only be applied after well-designed clinical assays to evaluate their dosage (Chippaux et al., 2010). The estimation for calculating the dosage of a therapeutic antivenom by simply multiplying the amount of antivenom necessary to neutralize 1 mg of venom by the number of milligrams of venom suspected to have been injected (Schlottler, 1952; Christensen, 1966; Belluomini, 1968; Ownby, 1990; Min. de Salud, 2000; Min. de Salud, 2014) should not be used as a tool for dosage.

These results in neutralizing experiments were very consistent and reliable since we used the same venom, same antivenom and same strain of mice for all the experiments. Results clearly showed that the neutralizing capacity of the antivenom obtained by the assays used in most of the countries and recommended by the Pharmacopeias (Ministerio de Saúde, 1996; Farmacopea de los Estados Unidos Mexicanos, 2000; WHO, 1981, 2010, 2016), cannot be used as a tool to predict the exact or approximate neutralization capacity of the antivenom in rescue assays, even in experimental and controlled conditions.

Preincubation and rescue experiments are methodologically quite different with distinct concentrations and times of contact of the reactants. In the pre-incubation experiments, venom-antivenom are mixed in a tube with the absence of external factors able to cause interactions, and thus exist higher reaction rate and extent. In the rescue experiment the volumes of distribution of venom and antivenom in the mice body are larger and the venom-antivenom binding is influenced by pharmacokinetics and pharmacodynamics parameters, with a lower rate and extent of reaction. Nevertheless, *in vivo* techniques are closer to reality.

Differences between the nominal neutralizing dose of the antivenom and the dose required for neutralization *in vivo* could also explain many therapeutic failures in envenomations treated with highly potent antivenoms, or at least why very high doses of antivenom fail to cure patients (Baldé et al., 2013) and reinforce the need of well-designed clinical assays in order to establish the correct dosage of an antivenom.

The neutralizing capacity determined by pre-incubation experiments is an important and valuable tool for the production and control of antivenoms, nevertheless should not be considered a stoichiometric measure to the dosage of antivenoms, since its nominal potency can only be applied to the experimental conditions in a same system (i.e. using the same venom, antivenom and animals) and not to other experimental conditions. If the change in the methodology of preclinical testing is not an urgent necessity -except for the replacement of mice for ethical reasons-, this reinforces the need for clinical trials to assess the real efficacy and dosage, and also safety, of antivenoms in humans.

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.toxicon.2017.10.009.

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