PARASPECIFIC NEUTRALIZATION OF THE VENOM FROM ADULTS AND YOUNG *CROTALUS ATROX* BY PARASPECIFIC SOUTH AMERICAN ANTIVENOMS.

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ABSTRACT

Crotalus atrox is one of the species of venomous snakes most commonly found in herpetological collections around the world and it is usually commercialized in the black market. Several collections have specimens but lack of the specific antivenom. We tested the toxicity of venom of adults and young snakes (2 to 3 years old) specimens of *C. atrox* in captivity and the para-specific neutralization provided by the antivenoms most used in Argentina. The i.p. lethal potency of the venoms were 100(95-105) µg and 43(42-45) µg/20g mouse and the indirect hemolytic activity was 7.9 (6.7-9.2) µg and 9.0(8.3-9.9) for adults and juvenile venoms. Despite the adult´s venoms lower lethal potency, these venoms were more difficult to neutralize, around 1.5 ml of antibothropic Antivenom (AB) were necessaries to neutralize 1 mg of venom in contrast to 0.54 ml required to neutralize young´s venoms. The neutralization by the Anticrotalic (AC) antivenom was despicable. The dose of AB required for the neutralization 5.0 LD_{50} of young snakes was in the range of those required for the neutralization of the specific venoms, nevertheless the dose required to neutralize venom from adults was 6 fold higher. The experiments using $2LD_{50}$ as challenge dose, showed similar results. The indirect hemolysis caused by both venoms was similarly neutralized by AB ($p<0.05$) while the AC did not show neutralizing activity. The myotoxicity determined by the increase of creatinquinase or by histopathology, was neutralized by both antivenoms, possibly due to the presence of myotoxins like K49 phospholipases present in this venom. Although the paraspecificity of AB has a potential use as treatment, especially in young snakes bites, the doses required in adult attacks are high. Despite AB seems to be useful for emergencies, these results suggest advantages in using specific antivenom for the treatment of these snakebites.

INTRODUCTION

Crotalus atrox ("Western diamond rattlesnake) (**Figure 1**), is one of the species of venomous snakes most commonly found in herpetological collections around the world and it is usually commercialized in the black market. Several collections have specimens but lack of the specific antivenom to treat their bites. For that reason, we tested the toxicity of venom from adults and young of *C. atrox* in captivity (**Figure 2**) and the para-specific neutralization provided by the antivenoms most used in Argentina

MATERIAL AND METHODS

Venoms: the venoms used were: **1)** *Crotalus atrox* venom: from bank of venoms from Área de Zootoxicología de la Cátedra de Toxicología of the Faculty of Medicine of the University of Buenos Aires: adults snakes (more than 10 years old) and young (1 to 3 years old), and a pool constituted from equals parts of each type of venoms. In addition as reference venom of *C. atrox* from Latoxan Laboratory (Portes-lès-Valence, Francia) was used. **2)** Venoms used as immunogens for the production of the antivenoms tested: were *B. neuwiedii*, *Bothrops alternatus* and *Crotalus durissus terrificus* from different regions of Argentina

Antivenoms: 1) Bivalente Antivenom (INPB-ANLIS "Dr. Carlos G. Malbrán", Buenos Aires, Argentina), specific immunogens: *B. alternatus* and *B. neuwiedii* complex venoms. Three different batches were used. **2)** Anticrotalico Antivenom (INPB), specific immunogens *Crotalus durissus terrificus.* **3)** For comparative purposes the mixing of Bivalente plus Anticrotalic as well as two commercial antibotrhopic − crotalic antivenoms were tested.

Determination of toxic and enzymatic activities and their neutralization: 1) Lethal potency and its neutralization (challenge 5 and 2 LD_{50}) were assessed by i.p. route in 18-22g CF-1 mice. **2)** Hemorrhagic activity and its neutralization (2 MHD) were studied in 250 Wistar rats. **3)** Indirect hemolytic activity and its neutralization (10 Indirect Hemolytic Doses) was studied in liquid medium with horse red blood cells and egg yolk. **4)** Myotoxic activity and its neutralization was determined by the injection in *Tibialis anterioris* muscle in Wistar rats of 100 µg of venom alone or preincubated with 90 µl of each antivenom, using Wistar rats (250-300 g) in a final volume of 100 µl. As negative 100 µl of 0.15 M NaCl or anti-*Latrodectus* antivenom (INPB) controls were inoculated as described. The myotoxic activity was determined by plasmatic measurement of creatinphosphokinase (CPK) levels and by the histopathological study of the muscles.

Figure 1

Natural distribution of *Crotalus atrox.*

Figure 2

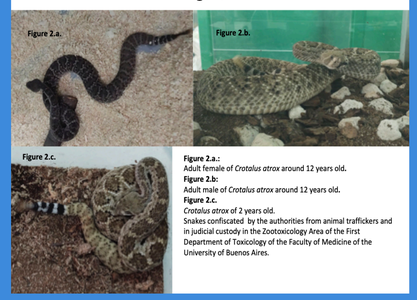


Figure 2.a.: Adult female of *Crotalus atrox* around 12 years old.
Figure 2.b: Adult male of *Crotalus atrox* around 12 years old.
Figure 2.c. *Crotalus atrox* of 2 years old.
Snakes confiscated by the authorities from animal traffickers and in judicial custody in the Zootoxicology Area of the First Department of Toxicology of the Faculty of Medicine of the University of Buenos Aires.

RESULTS

The toxic potencies are expressed in **Table 1** and **Figure 1.** Neutralization of Lethality is shown in **Figures 4, 5, 6** and **Table 2.** Comparative hemorrhagic activity is shown in **Figure 7.** Neutralization of Hemorrhagic activity is shown in **Figure 8.** Indirect Hemolitic activity neutralization is showed in **Table 3.** Miotoxic activity and its neutralization is showed in **Figure 9.**

Table 1

Venom	LD_{50} µg/mouse	LD_{50} µg/g	LD_{50}/mg venom	MHD (µg)	MCD-P (µg)	MCD-F (µg)	Indirect Hemolysis (µg)
C. atrox Adults	10,,1 (95.2 - 105.2)	5,01 (4.76 - 5.26)	10,0	59 (±10)	No Detectable	No Detectable	7.9 (6.7-9.2)
C. atrox Youngs	42.3 (40,7 – 44,5)	2.12 (2.04 - 2.23)	23,6	NR	No Detectable	NR	9 (8.3-9.9)
Pool	58.6 (46.9 – 73.2)	2.93 (2.35 - 3.66)	17,1	NR	No Detectable	NR	10.2 (8.7-12.1)
Latoxan	95 (81 - 110)	4.75 (4.05 - 5.50)	10.5	67 ((±10)]	No Detectable	No Detectable	NR
B. alternatus	82 (80-85)	4.1 4.00 - 4.25	12.2	78 (±46)	5.1 (±1.4)	151 (±9)	9.5 (8.3 - 10.5)
B. diporus	77 (51 - 116)	3.35 (2.55 - 5.80	13.0	368 (±43)	35 (±5)	591 (±13)	NR
C. d. terrificus	2 (1,6 -1,9 (1.6 - 1.9)	0.1 (0.080 -0.095)	500.0	No Detectable	NR	NR	NR
Arce et al.,	112 (95 - 132)	5,6 (4.75 - 6.60)	8.9	NC	No Detectable	NR	NR
Minton y Weinstein *C. atrox* Adults	100 ± 16,8* 340 ± 88**	2.5 ± 0.84* 17.0 ± 4.4**	100.018.5	NC	NR	NR	NR
Minton y Weinstein *C. atrox* Youngs	53.6 and 56.8 ** 257.2**	2.68 and 2.84** 12.86 **	18,5	NC	NR	NR	NR

***= intravenous; **= subcutaneous route.**

TABLE 2
Neutralization by Bivalent Antivenom

C. atrox venoms	Challenge Doses 5 mg/mg	2 mg/mg
Young specimens	13,5 (11,5-15,75)	6,25 (5,75-6,75)
Adults	36 (16,75-77)	16,5 (12,5-22)
Pool	11,75 (8,5-14,5)	5,25 (4,75-5,5)

TABLE 3
Neutralization of Indirect Hemolysis (ED_{50})

Antivenoms	Venom from young snakes	Venom from adult snakes	Pool of both venoms
Bival. (Antibothropic)	40,3 µl (20,0 - 81,0)	40,8 µl (37,2-44,7)	65,1 µl (58,8 - 72,1)
Anticrotálico	388,4 µl (94,5-1596)	No neutralization	221,1 µl (84,3 - 584,7)

Figure 4

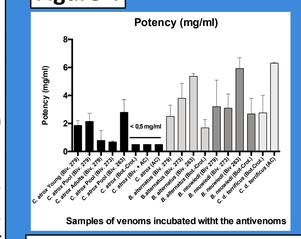


Figure 5

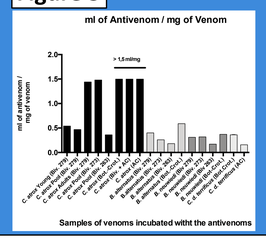
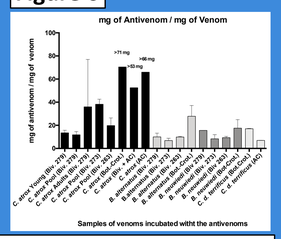


Figure 6



Figures 4 to 6. Neutralization of the lethality by antivenoms. The specific (*Bothrops* and *C. d. terrificus* venoms) and paraspecific (*C. atrox*) neutralization by the Antibothropic (Biv), Anticrotalic (Ac), their mix and two Polivalent Bothropic-Crotalic (B-C) antivenoms. Note the high doses required for the neutralization of the venom from Adults regarding the required for the venom of young *C. atrox*. Deviation bars indicate 95% c.i.

Figure 9

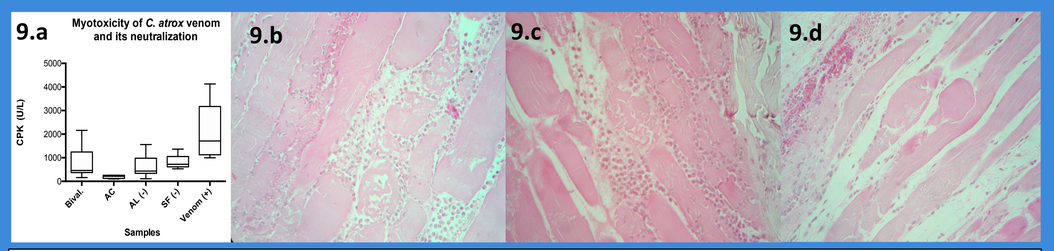


Figure 9.a. Levels of CPK in rats injected with C. atrox venom alone or princubated with antivenoms (Bival.= antibothropic, AC: Anti crotalic. AL= Anti-Latrodectus and SF= 0.15 M NaCl, both negative controls. **Figure 9.b. Venom of *C. atrox*.** Extense coagulative necrosis. Myocitolisis. Hemorrhagic areas. Acute severe inflamation. Interfibrilar edema. **Figure 9.c. Muscle injected with venom preincubated with Bival Antivenom**. Focal necrosis, intense acute inflamatory infiltration, dissecting the fibers. Mild interfibrilar edema. **Figure 9.d. Venom preincubated with antiCrotalic antivenom**. Hemorrhagic areas, perivascular inflammation, interfibrilar edema. Focal necrosis. In all the cases Hematoxiline and eosine, and 250x of augmentation, were used.

Figure 3

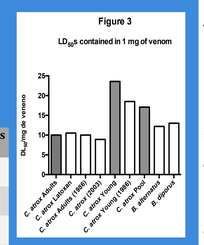


Figure 3. Comparative theoretical median lethal doses contained in 1 mg of *Crotalus atrox* venoms or in 1 mg of the bothopic venoms used as immunogens.1986: data from Sherman and Minton 1986; 2003= data from Arce et. al 2003.

DISCUSSION

The venom showed toxic ontogenic variation according to previous results, nevertheless these differences were found in addition in the capacity of the antivenoms for venoms neutralization. This is important when a treatment with paraspecific antivenom must be applied, since the amount of antivenom required for the neutralization of venom from adults specimens is higher regarding the required for young specimens venom.

In addition must be seriously considered the mixing of venoms that constitute the pools of venom for antivenom production and evaluation, since data on toxicity and neutralization drastically vary regarding the type of venom used and/or mixed.

Anti-*C. d. terrificus* antivenom or its mixture with antibothropic antivenoms did not show good protection.

In these preclinical tests the venom of the young animals could be neutralized by the antibothropic antivenom but the neutralization of the lethality of venom from adults is very low and would require very high doses of antivenom. By this reason in case of posession of this specie of snake and in front of the lack of antivenom the use could be used only as an heroic measure for treatment.

Figure 7

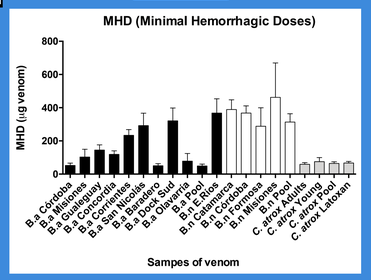


Figure 7. Comparative hemorragic activity of *C. atrox* and the Bothropic venoms used as immunogens for the Anti-Botrhopic antivenom. Note the high hemorrhagic potency of *C. atrox* venom. *C. d. terrificus* venoms).

Figure 8

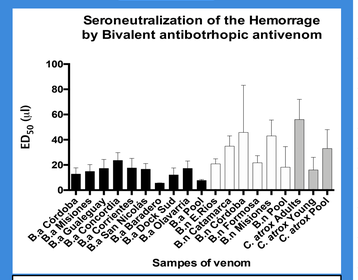


Figure 8 shows the specific and paraspecific neutralization of Bothropic antivenoms, note the high doses required for the neutralization of *C. atrox* Adult venom and the low dose for the young *C. atrox* venom neutralization. Deviation bars indicate 95% c.i.

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