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Biochemical comparison of venoms from young Colombian *Crotalus durissus cumanensis* and their parents

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ABSTRACT: *Crotalus durissus cumanensis*, a rattlesnake endemic to Colombia and Venezuela, is considered one of the most lethal snake species in Latin America. The aim of the present study was to compare the protein content and biological activity of the venom obtained from eight specimens of *C. durissus cumanensis,* namely two adults from different localities of Colombia and six offspring born in captivity. Protein profiles of crude venoms were analyzed by SDS-PAGE and RP-HPLC, and biological activities were evaluated for lethality, edema, defibrination, hemolytic and coagulant activities to assess individual venoms of adults and a pool of young snake venoms. Transient edema appeared rapidly after venom inoculation, whereas hemorrhagic effect was not observed. Differences in protein profiles, lethality, hemolytic, coagulant and defibrinating activities between both adult snake venoms were observed; those from the mother snake exhibited higher activities. Venoms from young snakes were similar to the one obtained from the mother, but the coagulant effect was stronger in offspring venoms. Notably, biological effects of the father snake venom were not comparable to those previously described for *C. durissus cumanensis* from Venezuela and *C. durissus terrificus* from Brazil, confirming the high variability of the venom from *Crotalus* species.

KEY WORDS: snakes, *Crotalus durissus cumanensis*, rattlesnake, venom.

CONFLICTS OF INTEREST: There is no conflict.

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INTRODUCTION

Snakebites represent a medical emergency in many regions of the world, particularly in tropical and subtropical areas where they are endemically distributed, affecting mainly agricultural workers and children. Although the precise global incidence of snakebites has proven difficult to estimate, it has been reported that there are at least 5 million snakebites per year worldwide, which results in 2.5 million clinical cases and an estimated 125,000 deaths (1). The incidence of snakebite mortality is especially high in Africa, Asia, New Guinea and Latin America. In Colombia, a total of about 3,000 snakebite accidents are reported yearly with an estimated mortality of 2 to 5% (2).

Poisoning by snakebite provokes medical emergencies that affect different tissues and organ systems depending on the species responsible for the bite. In the Americas, the most severe cases result from bites by members of the Viperidae and Elapidae families. *Crotalus* snakes (rattlesnakes), belonging to the Viperidae family, are geographically distributed from Canada to Argentina. *Crotalus durissus*, composed of 14 subspecies, has the widest geographical distribution (3). This species has been associated with intraspecific variability in the chemical composition of its venom and, consequently, in its clinical effects and related therapeutic strategies (4-7). Although symptoms are usually variable, snake venom is highly toxic and presents complex outcomes. In the case of *Crotalus,* local effects consist of moderate pain, edema and local hemorrhage without necrosis, whereas systemic manifestations are more severe and may cause considerable muscle damage (myotoxicity), bleeding (hemostatic abnormalities), renal damage (nephrotoxicity) and paralysis (neurotoxicity) (8).

Previous studies have shown great biochemical and pharmacological variation among venoms of *C. durissus* subspecies from different regions of South America, which appears more toxic than those of the same species from Central America (9-11). Diversity in venom composition and biological activities has also been associated with the age of snakes in *C. durissus durissus* (6, 12, 13). Moreover, in this species toxicity appears to be induced in part by crotoxin, a potent neurotoxin present in its venom in large quantities (14). *Crotalus durissus cumanensis* is a subspecies endemic to Colombia and Venezuela, particularly abundant in the Caribbean coast, Magdalena River valley and Llanos Orientales regions (15).

Recent studies on the biochemical functional characterization of *C. durissus cumanensis* venom from Venezuela have indicated high intraspecies variability whereas different isoforms of crotoxin complex from this venom have been isolated, purified and characterized by other researchers (16-21). In the current study, we present preliminary chemical and functional characterization of venom obtained from a snake family composed of adult *C. durissus cumanensis* (parents), captured in different regions of Colombia, and from six young offspring born in captivity.

MATERIALS AND METHODS

Snakes and Venoms

Eight specimens of *C. durissus cumanensis* were employed in the present study. Two of them were adults – the male was captured in Guajira (northern Colombia), while the female was from Tolima (central Colombia) – and the other six were their offspring (both genders) born in captivity. Snakes were kept at room temperature (23°C ± 5) in individual boxes (80 x 50 x 40 cm) at the snake holding facility in Caucaseco Scientific Research Center (Cali, Colombia), and were fed live mice every 15 days. Venom samples were extracted manually by glandular compression, collected in glass vessels, then centrifuged, lyophilized, and kept at –20°C until use. Venoms from all snakes were subjected to chemical analysis to determine their functional activities. Offspring were milked when they reached one year of age.

Mice

Male or female BALB/c mice weighing 18 to 22 g were used throughout the study for functional assays of the venoms. Animals received food and water *ad libitum*. All the procedures involving mice were conducted following national and international recommendations and policies for animal use and were monitored by the Animal Ethics Committee of the University of Valle.

Electrophoretic Analysis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of venom samples was carried out according to the Laemmli method (22) using a Mini-Protean® II system (Bio-Rad Laboratories, USA) as previously described (17). Briefly, after dilution in run buffer, samples were loaded onto 12.5% acrylamide gels containing 0.1% SDS under non-reducing conditions, using Tris-glycine buffer (pH

8.3). Gels were stained with 0.1% Coomassie brilliant blue R250 and were analyzed using the GeneSnap® software (v 6.07 Synoptics Ltd., UK) and the GeneGenius/multigenius® equipment (Syngene, UK).

Chromatographic Analysis

Samples of *C. durissus cumanensis* venom were analyzed by reverse-phase high-performance liquid chromatography (RP-HPLC) on a Waters HPLC system (USA) consisting of 510 bombs, 717 PLUS autosampler and Waters 486 UV detector. Venom samples (1 mg/mL) were dissolved in 1% acetic acid and loaded onto the Zorbax 300SB-C8® (Agilent, USA) column (4.6 x 50 mm, 5 µm). The mobile phase was a mixture of phase A [20:80:0.083 (v/v) acetonitrile/water/TFA] and phase B [80:20:0.1 (v/v) acetonitrile/water/TFA], with a flow rate of 1.0 mL/minute and monitored at 280 nm.

Determination of Lethality

Lethality was determined in BALB/c mice after intraperitoneal injections of venom samples. Venoms were injected separately into groups of six mice each with doses ranging from 0.125 to 5 mg/kg, serially diluted in 0.2 mL of saline solution. Survival time of each animal was recorded for 48 hours and a LD_{50} value calculated according to the Spearman-Karber method (23).

Edema-Inducing Activity

Edema formation induced by the venoms was assayed according to the method of Yamakawa *et al*. (24). The time-course of edema was determined in mice injected into the subplantar surface of the left hind paw with 50 µL of saline solution containing 1.0, 2.5 or 5.0 µg of each venom. The other paw received the same volume of saline solution without venom as a control. Edema was measured at different intervals (30 minutes and 1, 2, 3, 4, 5, 6 and 24 hours). Results were calculated as the difference between values obtained before and after venom inoculation, compared with a control and plotted (expressed in millimeters) versus time of evaluation.

Hemorrhagic Activity

The minimal hemorrhagic dose (MHD) was determined by the Instituto Clodomiro Picado method (25), with crude venoms, and was defined as the amount of venom protein resulting in a 10-mm diameter hemorrhagic area. A series of four doses of 20, 40, 60 and 80 µg were used, and 0.1 mL of each was inoculated subcutaneously into the abdomen of each experimental BALB/c mouse. Mice were killed after one hour; the skin was removed and the hemorrhagic diameter in the subcutaneous tissue was measured to define the MHD.

Defibrinating Activity

Eight doses of venom (0.3, 0.6, 1.2, 2.5, 5.0, 10.0, 15.0 and 20.0 µg) were prepared in 200 µL of saline solution and three female mice per dose group were injected intravenously while three negative control mice were similarly injected with saline solution only. One hour after the injections, animals were bled and 200 µL of blood was collected in glass tubes and incubated for two hours at room temperature. The minimal defibrinating dose (MDD) was defined as the minimal dose of venom able to induce coagulation failure in blood samples of all injected mice.

Coagulant Activity

Coagulant activity of venom samples was determined using the method described by Instituto Clodomiro Picado (25). Briefly, 0.2 mL of human citrated plasma was incubated for five minutes at 37ºC and then doses ranging between 12 to 50 µg of venom in a total volume of 0.1 mL of saline solution were added. Coagulant activity was determined in triplicate preparations of these solutions and the mean clotting time was calculated. Normal human citrated plasma plus saline solution was used as coagulation control. The minimal coagulant dose (MCD) corresponds to the venom concentration that induced clotting development in 60 seconds.

Hemolytic Activity

A gel containing a mixture of agar, blood and egg yolk was prepared in small Petri dishes. Three-millimeter-diameter wells were punched in the gel and 15 µL of different venom doses (1, 2, 4, 6 and 8 µg) prepared in saline solution were added in triplicate to wells. Plates were incubated at 37°C for 24 hours, hemolytic zones produced around each well were measured and the hemolytic dose was determined.

The indirect hemolytic activity was determined in terms of the minimal indirect hemolytic dose (MiHD), considered to be the minimum amount of venom that produced a 20-mm-diameter hemolytic zone (25).

RESULTS

Electrophoretic Analysis

Electrophoretic analysis demonstrated a high complexity in the venom composition as indicated by protein characterization (Figure 1). All venoms exhibited proteins of 15, 25, 36 and 60 kDa together with other more variable proteins. A greater complexity was observed in the venom of the snake captured in Guajira (Cd1, male) than in the specimen from Tolima (Cd2, female). Similarly, a series of high-molecular-weight proteins of 79, 100, 143 and 193 kDa were not detectable in Cd2. When the venoms of six offspring (Cd3-Cd8) were compared (Figure 1), they presented a predominance of low-molecular-weight proteins with similar ranges to those shown in the adult snakes. Analysis of these venoms using the GeneSnap® program showed a distinct overlap of bands with similar molecular weights in five of the venoms, whereas one presented some variations in the protein profile (Cd8), represented by an additional protein with 20 kDa and several bands with molecular weight greater than 25 kDa at high concentrations (Figure 2). A peak concentration revealed proteins with lower molecular weight (15 kDa) (Figure 2) identified as phospholipases A_2 (crotoxin basic chain-1) trough tandem mass spectrometry (data not shown). Additionally, another low-molecular-weight protein (approximately 12 kDa) was unique to the venom of male snakes (Figure 1).

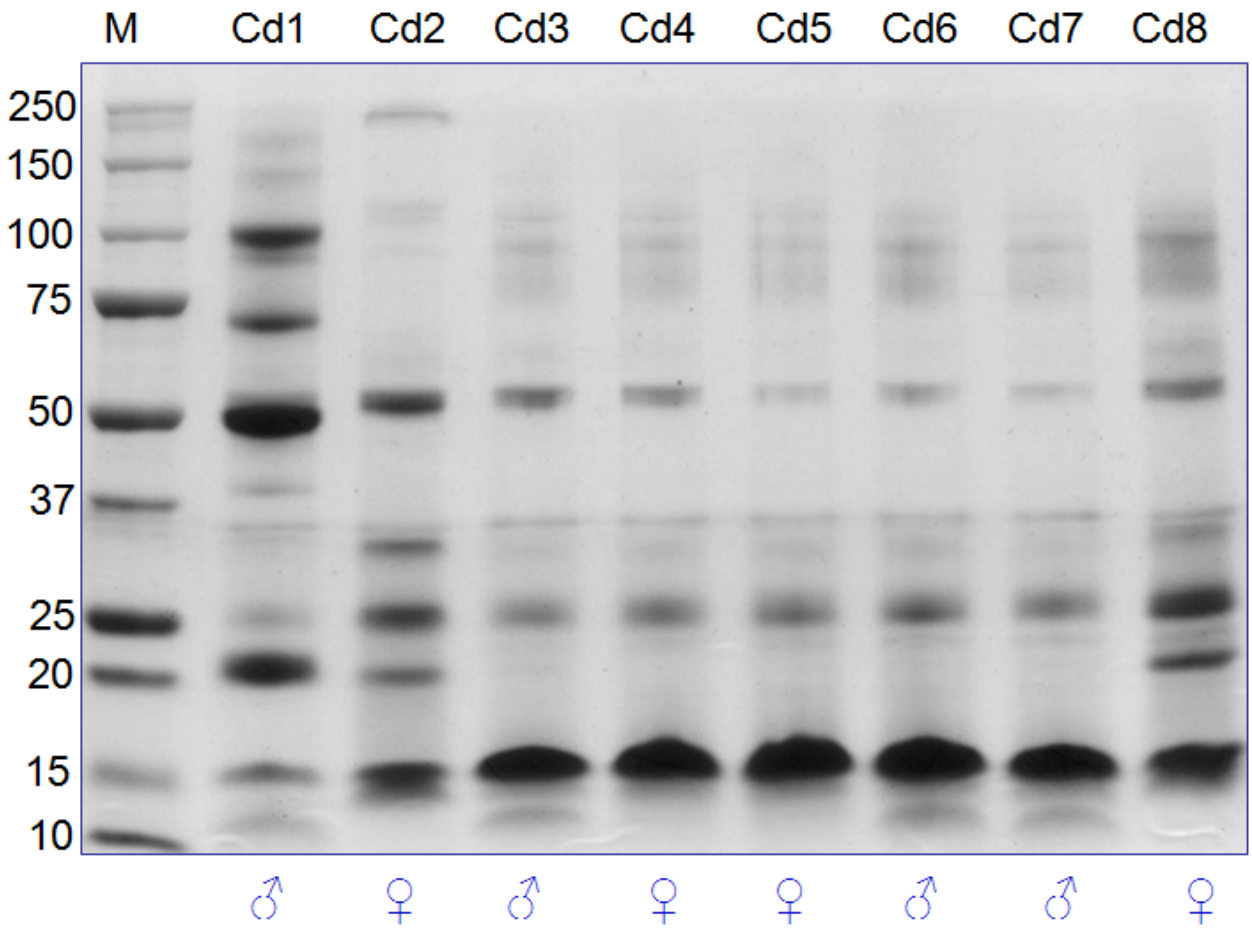


Figure 1. Electrophoretic analysis of *C. durissus cumanensis* venoms. Venoms from the Guajira male (Cd1), the Tolima female (Cd2), and male (Cd3, Cd6, Cd7) and female (Cd4, Cd5, Cd8) offspring were subjected to SDS-PAGE under non-reducing conditions.

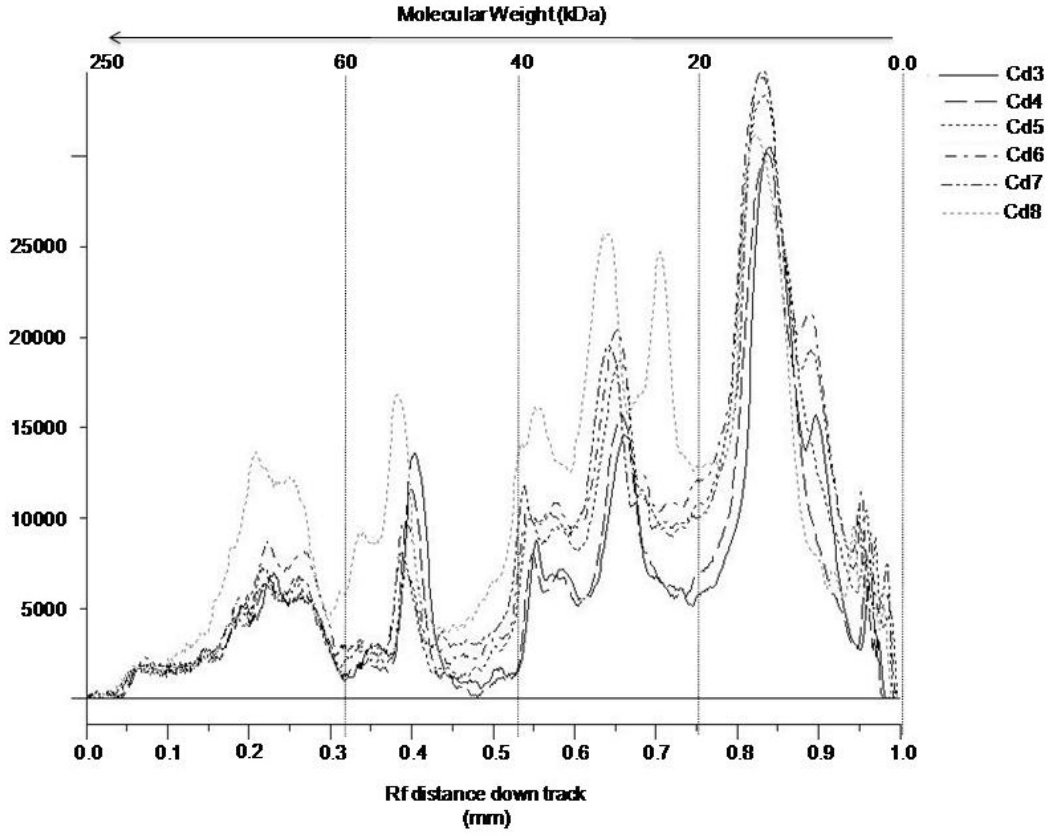
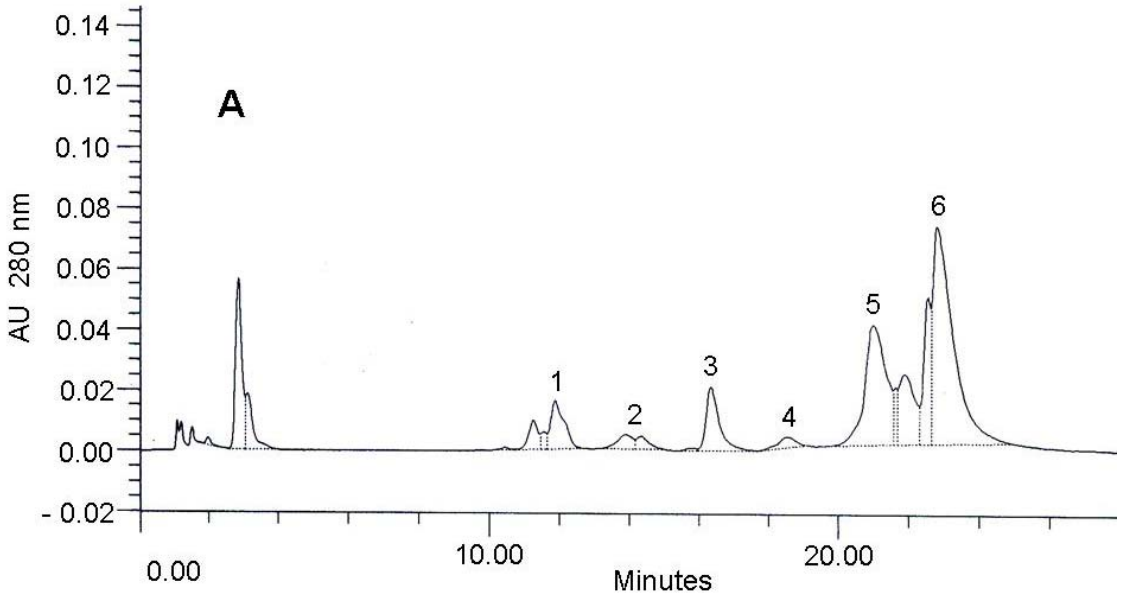


Figure 2. Comparative electrophoretic profiles of venoms from the offspring. The analysis was carried out using the GeneSnap® program after gel documentation. Cd3, Cd6, Cd7 were males and Cd4, Cd5, Cd8 were females.

Chromatographic Profile

Chromatographic profiles of both adult snakes (Figure 3) corroborated the variation observed by SDS-PAGE in protein profiles. Analysis of offspring venoms showed greater similarity with the Tolima snake (female) venom than with that of Guajira snake (male). In the venoms from the mother and offspring, there was a compound that eluted at 16 minutes (peak number 3) which corresponded to more than 50% of the total protein content, whereas in the male venom, the most concentrated compound (peak number 6) eluted at 23 minutes.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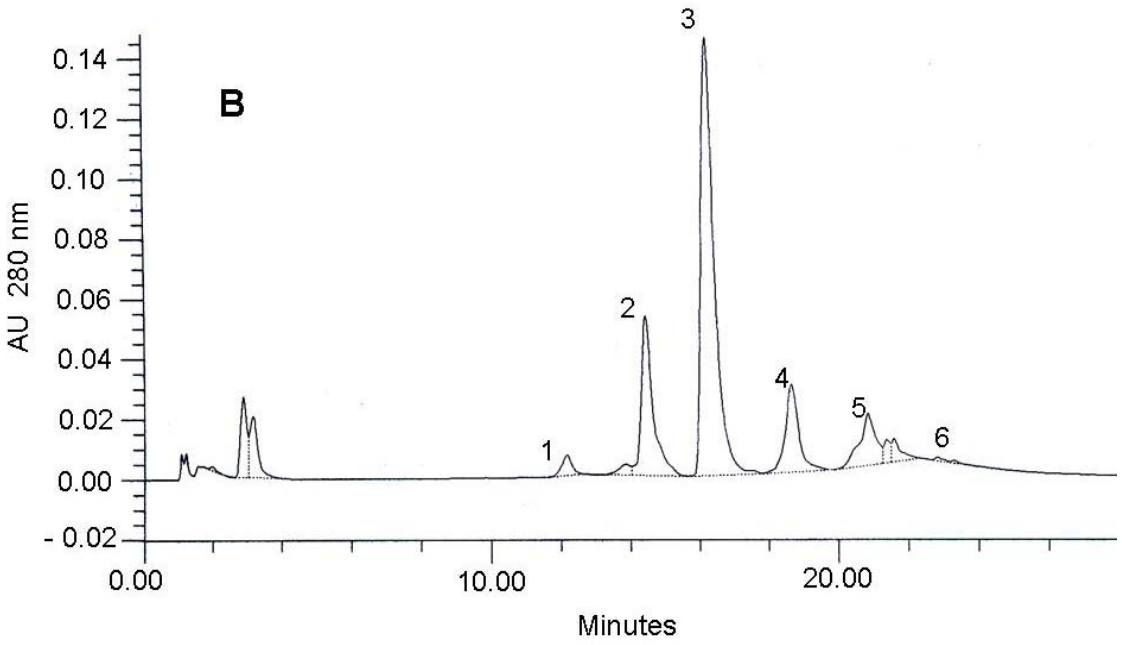
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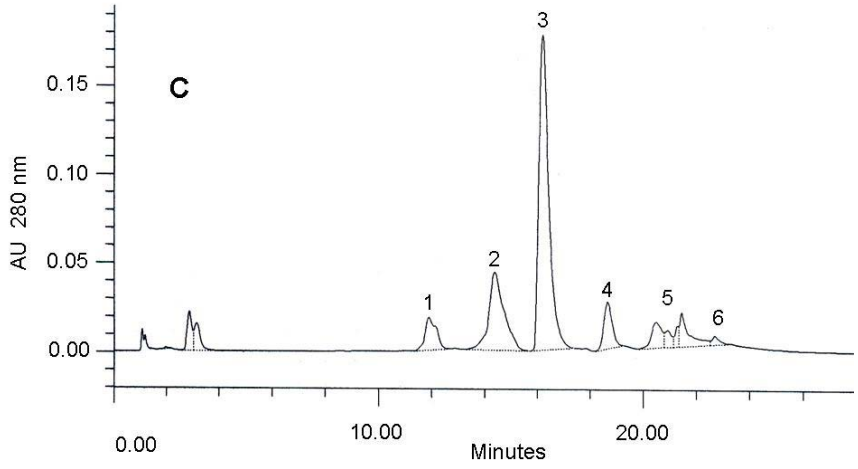

Figure 3. Reverse phase chromatographic protein profile of *Crotalus durissus* venom. Guajira male (**A**), Tolima female (**B**) and offspring pool (**C**). The retention times were 12 (1), 14 (2), 16 (3), 18 (4), 21 (5) and 23 (6) minutes.

Lethal Activity

Female snake venom was the most lethal and potent [LD_{50} = 0.47 mg/kg mouse (CI 95% 0.45-0.49)] compared to the male venom [LD_{50} = 2.69 mg/kg mouse (CI 95% 2.63-2.70)]. Young snake venoms showed similar lethal activity to female snake venom [LD_{50} = 0.41 mg/kg mouse (CI 95% 0.39-0.44)] (Table 1). In all cases, the effect was observed in the first eight hours after venom inoculation. Animals developed paraplegia, fasciculation and shortness of breath.

Table 1. Functional activities of *C. durissus cumanensis* venom

Activity	*C. durissus cumanensis* venom		
	Female	**Young**	**Male**
Coagulant activity (µg)[a]	27.7 ± 2.3	11.7 ± 0.6	29.3 ± 1.2
Hemolytic (µg)[b]	11.3 ± 0.7	14.7 ± 0.4	192 ± 0.7
Lethality (µg/g)[c]	0.47 (0.45 – 0.49)	0.41 (0.39-0.44)	2.69 (2.63 – 2.70)
Hemorrhagic[d]	Absent	Absent	Absent
Defibrinating MDD (µg)[e]	0.6	2.5	Absent

[a]Minimum coagulant dose (MCD).
[b]Minimum hemolytic dose (MiHD); each response was the mean of three observations.
[c]Median lethal dose (LD50); data within parenthesis represent 95% confidence intervals.
[d]Minimum hemorrhagic dose (MHD); each response was the mean of three observations; control: *Bothrops asper* venom.
[e]Minimum defibrinating dose (MDD): each response was the mean of three observations

Edema-Inducing Activity

Edema developed rapidly after injection of both male and female venoms. The maximum reaction was observed at 30 minutes, subsequently disappearing within 24 hours (Figure 4). Similar behavior was observed for venoms of young snakes and no differences were noticed when different doses were employed.

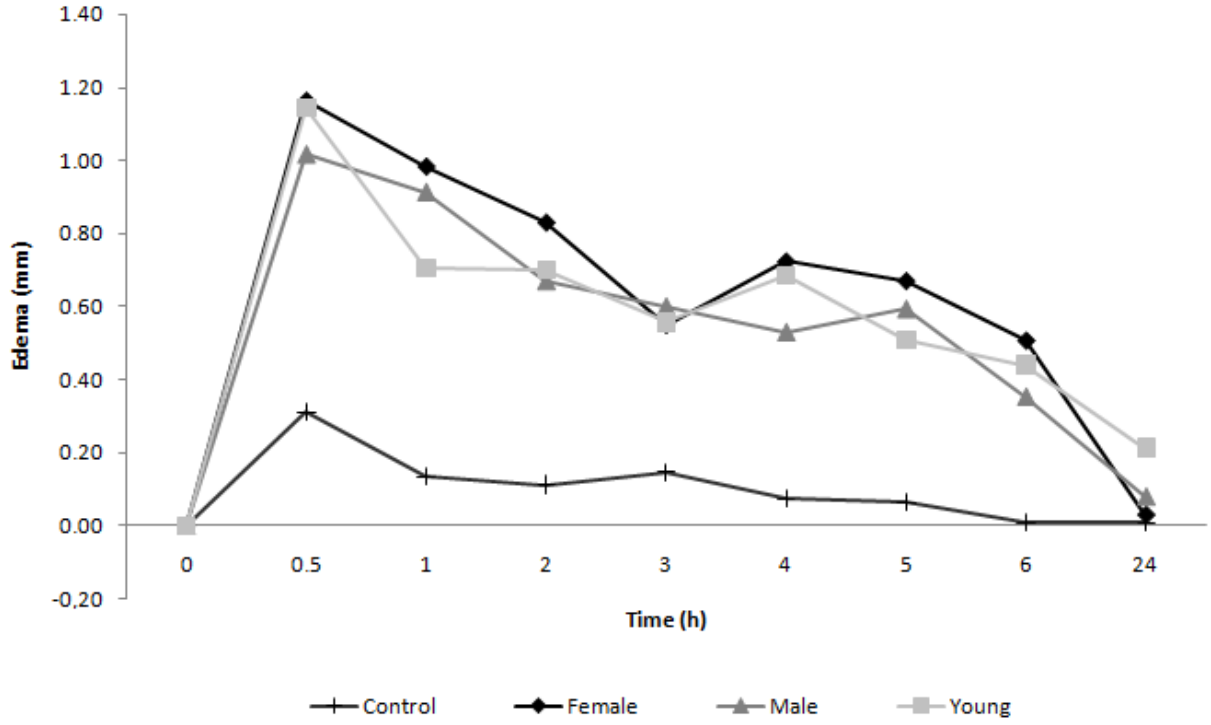

Figure 4. Edematous activity of *C. d. cumanensis* venom. BALB/c mice were inoculated with 2.5 µg of each venom into the subplantar region of the hind paw.

Hemorrhagic and Hemolytic Activity

Minimal hemorrhagic dose (MHD) could not be estimated for any of the venoms as none of them showed any hemorrhagic effect at the different tested doses. In terms of hemolysis, female venom exhibited hemolytic activity with the venom dose of 11 ± 0.7 µg while young snake venom did the same with a 14.7 ± 0,4 µg dose, the male venom, in turn, required significantly higher concentrations (192 ± 0.9 µg of venom) to induce hemolysis (Table 1).

Defibrinating and Coagulant Activities

As observed in Table 1, all venoms presented coagulant activity. The female venom needed 27.7 µg to produce the effect whereas the male venom required 29.3 µg and the offspring venoms, 11.7 µg. While the female venom showed defibrinating activity in all tested doses down to 0.6 µg, venoms from young snakes required doses of at least 2.5 µg to show coagulant activity and the male venom did not exhibit this activity at any of the tested doses (Table 1).

DISCUSSION

In this study we confirmed the great variability previously observed in the protein content and biological activities of adult snake venom from *C. durissus cumanensis* (16, 17). Variability in venom composition has been attributed to environmental and individual factors such as habitat, origin, seasonality, diet, age and gender (1). In the current work, the environmental factors including habitat, seasonality and diet were not considered because adult animals had been kept in captivity for more than ten years under similar feeding and environmental conditions, and young snakes for at least one year. Therefore, the observed differences between adult snakes may be due to potential genetic diversity, possibly associated with their different geographic origins. Additionally, the great similarity among protein profiles of male and female offspring might also discard the gender factor. We cannot explain the similarities among the venoms from the mother and from the offspring; therefore, further studies are required to clarify how genes connected with venom proteins are transmitted from parents to offspring.

The venoms from the female and from the young snakes were particularly similar in their hemolytic and defibrinating activities and lethality, but they all differed from the male adult venom as well as from those previously reported for *C. durissus cumanensis* from Venezuela and for other *Crotalus* subspecies from Brazil, Costa Rica and Guatemala, which suggests a geographic association in these effects (6, 10, 16, 17, 26). Besides the great similarities among mother and young snake venoms, there were surprising differences since the adult female venom presented greater coagulant activity and lower defibrinating property.

Although lethality is likely of multifactorial origin, both neurotoxicity and hemolytic activities appear to play a preponderant role as lethality has been associated with the presence and concentrations of the PLA enzyme (27). It has been proposed that the

lethality of *C. durissus terrificus* venom is mainly due to the action of a crotoxin complex, which constitutes a major component (60%) of the venom (28, 29). Crotoxin is a very potent neurotoxin that is composed of a PLA_2 and a chaperone molecule (crotapotine) and is capable of blocking neuromuscular transmission. In this study the young and female venoms presented a higher concentration of this protein than the male venom, as observed in the electrophoretic and chromatographic profiles, therefore suggesting a higher lethal potential than the male venom. Indeed, in this study, the female and offspring venoms were more fatal than male venom. The latter was also less lethal than several other subspecies (6).

In general, it appears to exist a gradient in toxicity as female and young snake venoms showed a lethality rate comparable to that reported for *C. durissus cumanensis* from Venezuela, but were less toxic than venom from Brazilian snakes and more lethal than those of Central America species (6, 7, 11, 16, 17).

Regarding the defibrinating activity it was significantly lower in the Colombian snakes than in the *C. durissus cumanensis* from Venezuela (16, 17). In terms of coagulation activity adult snakes appear to behave similarly in Colombian and Venezuela (2002) Furtado et al (13), including the fact that newborn and young *C. durissus durissus* and *C. durissus terrificus* have been significantly more potent than the adult snake venoms (6, 14). It has been demonstrated that coagulant activity is due to the action of thrombin-like enzymes (11) in others *C. durissus* species. It is likely that similar components are present in *C. durissus cumanensis* venoms.

An interesting feature of this study is the fact that venoms tested herein induced a pronounced edematous activity, but poor hemorrhagic activity. Edema activity has been associated to metalloproteinases that also cause hemorrhage. Therefore, the absence of hemorrhagic activity in these venoms may suggest that the edema is induced by proteins different from these proteinases with edematous activity like myotoxins and phospholipases A_2 (19, 20, 30). In previous studies with *C. durissus* venoms from other South American regions, no edematous or hemorrhagic activity was observed (9-11, 31). This is in contrast to the positive results reported for *C. durissus* snakes from Central and North America (11). Nevertheless, hemorrhagic activity has been reported for *C. durissus cumanensis* venoms of Venezuela (16-17).

Finally, we have confirmed the presence of great variability in *C. durissus cumanensis* venom that did not appear to be associated with age, gender, diet or habitat, but rather by genetic factors not identified yet. Complementary studies are

ongoing in an attempt to better understand the difference in protein profiles and functional differences of venoms and their potential importance for envenoming therapy in humans.

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