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IS THE SOUTH AMERICAN WATER SNAKE *HELICOPS ANGULATUS* (LINNAEUS, 1758) (DIPSADIDAE:XENODONTINAE) VENOMOUS?

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Abstract: The dipsadid genus *Helicops* comprises sixteen species ocurring in all South American countries, with the exception of Chile. Such aquatic snakes inhabit rivers and lagoons of Tropical areas; all species have enlarged rear teeth, although a sulcus is absent, reason why they are considered aglyphous snakes. Nonetheless, a well developed Duvernoy gland is present. The main objective of this study was to investigate the proteolytic and neurotoxic activities of the venom of *H. angulatus*. The crude venom lethal dose fifty (LD^{50}) was of 5.3 mg/kg. Venom proteins were tested by 15% SDS-PAGE, resulting in bands from 14 to 70 kDa. Six peaks were obtained by Superose 12 size exclusion chromatography with neurotoxic activity in peak 3. The neurotoxic clinical manifestations produced death in INH mice eight minutes postinjection with crude venom and its fractions. The proteolytic activities tested on gelatin and casein were positive with crude venom and size exclusion peak 1. Hemorrhagic activity was not present in the crude venom nor in its fractions. *Helicops angulatus* should be considered a middly venomous dipsadid snake.

Key Words: aglyphous, aquatic snake, Venezuela, *Helicops,* neurotoxic, proteolytic, venom.

Resumen: A. Estrella, A. Rodríguez-Torres, L. Serna, L.F. Navarrete, A. Rodriguez-Acosta. "¿Es venenosa la serpiente de agua suramericana *Helicops angulatus* (Linnaeus, 1758) (Colubridae:Xenodontinae)?". El género *Helicops* (Dipsadidae) contiene dieciséis especies de serpientes acuáticas distribuidas en todos los países suramericanos, con la excepción de Chile. Estas serpientes acuáticas viven en ríos y lagunas de áreas tropicales; todas las especies presentan colmillos en la parte trasera del maxilar, pero sin surcos, siendo por eso consideradas aglifas. Sin embargo, está presente una glándula Duvernoy bien desarrollada. El objetivo principal de este estudio fue investigar las actividades proteolíticas y neurotóxicas del veneno de la especie *H. angulatus*. La dosis letal cincuenta del veneno crudo fue de 5.3 miligramos/kg. Las proteínas del veneno se evaluaron por SDS-PAGE al 15%, dando como resultado bandas de 14 a 70 kDa. Se obtuvieron seis picos por cromatografia de exclusión molecular (Superose 12) con la actividad neurotóxica en el pico 3. Las manifestaciones clínicas neurotóxicas causaron la muerte de los ratones INH a los ocho minutos post-inyección, con el veneno crudo y sus fracciones. Las actividades proteolíticas evaluadas sobre gelatina y caseina, se observaron en el veneno crudo y el pico 1 de la cromatografía. No se detectó actividad hemorrágica en el veneno crudo o sus fracciones. *Helicops angulatus* debe ser considerada una serpiente dipsadida moderadamente venenosa.

Palabras Clave: aglifa, serpiente acuática, Venezuela, *Helicops*, neurotóxico, proteolítico, veneno.

INTRODUCTION

Until recently, the genus *Helicops* was included in the orthodoxal concept of the family Colubridae, which comprised the non-monophyletic assemblage of all colubroids, to the exception of the families Atractaspididae, Elapidae and Viperidae (e.g. Zug *et al*. 2003). However, recent phylogenetic studies provided considerable refinement of this system, recovering several monophyletic groups to be recognized at the familial status, such as the Colubridae *sensu stricto* and the Dipsadidae (Vidal *et al*. 2007, Zaher *et al.* 2009). According to these recent proposals, *Helicops* is presently allocated in the family Dipsadidae, subfamily Xenodontinae, forming the tribe Hydropsini along with the genera *Hydrops* and *Pseudoeryx* (Zaher *et al.* 2009).

In spite of the modern classifications mentioned above, studies focused on the venom activities of snakes species other than vipers, elapids and atractaspidids were based in the orthodoxal classification, considering species currently allocated in other families as belonging to the Colubridae *sensu lato* (e.g. Salomão 2003). Thus, although we accept that the genus *Helicops* is not to be considered

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a Colubridae *sensu stricto*, we will make use of the term "colubrids" (using quotation marks) throughout the text to designate snakes of "lower medical importance", i.e. Colubridae *sensu lato*. However, by lower medical importance, one must not understand harmless or not lethal, since several "colubrid" species are known for having caused fatal accidents to humans (Salomão *et al.* 2003).

The genus *Helicops* comprises sixteen species of aquatic snakes occuring in moist habitats associated to brookes, rivers and lagoons of most South American countries, to the exception of Chile. Diet of *Helicops* species is mostly composed by frogs and fishes (Marques *et al.* 2005). Since biting is one of the most important defensive strategies of these snakes, accidents to humans have already been recorded for at least one species of the genus (Albolea 1998). Despite the presence of enlarged teeth that follow a diastema, such teeth in *Helicops* lack any traces of distinctive sulcus, characterizing an aglyphous dentition. Nonetheless, serous secretions are produced by a well differentiated Duvernoy gland.

The species approached in this study is *H. angulatus* (Linnaeus 1758), a vespertine species occurring in fishponds, watercourses, and rivers of South America (Peters and Orejas-Miranda 1970, Dixon and Soini 1986, Martins and Oliveira 1998, Ford and Ford 2002, Marques *et al*. 2005). This work comprises first experimental study of the neurotoxic and proteolitic action of the venom of *H. angulatus*. Other *Helicops* species were studied by Albolea (1998). Neurotoxic clinical symptoms were observed in mice injected with venom samples of *H. angulatus*, while proteolytic activity was tested on gelatine film and casein.

Even though some biological significances of "colubrid" snake venoms are known, information in the specialized literature is sparse and the subject represents an open field to be explored. Venoms of "colubrids" are a mixture of non-enzymatic and enzymatic toxins directed against the hemostatic and neurological systems of their prey (Lemoine and Rodríguez-Acosta 2003, Fry *et al*. 2003, Lemoine *et al*. 2004a). Most of these venom toxins have not been considered clinically important in envenoming because they are less than 30 kDa, and detect specific receptors often located on cell membranes (Chippaux and Goyffon 1998). Their response can be hemostatic, neurological, muscular, cardiovascular or undifferentiated. The toxicity is dose-dependant, relative to the amount of accessible receptors and/or the volume of venom inoculated (Chippaux 1999). Most toxins isolated from "colubrid" venoms fit in the three fingers toxins family, which is a family of nonenzymatic polypeptides containing 60-74 amino acid residues. Their mode of action and objectives are mainly neurotoxins, but cardiotoxins, myotoxins and hemostatic toxins are also present (Nkinin *et al*. 1997, Lemoine *et al*. 2004b, Rodríguez-Acosta *et al*. 2006). This work brings a contribution to the knowledge of "colubrid" venoms by partially isolating and characterising active components responsible for the *in vivo* neurotoxic action.

MATERIALS AND METHODS

Animals and venom

Male mice (INH strain) of 18 to 22 g were obtained from the animal facility of the National Institute of Hygiene "Rafael Rangel", Caracas, Venezuela. The mice were kept at temperature of 22-24°C , with a relative humidity of 45-70%, and a 12-h light/dark cycle. Animals were acclimated for about one week before beginning each experiment and received water and food *ad libitum*. The Animal House authorities' surveillance reports established that mice were free of known pathogenic bacteria, viruses, mycoplasmas, and parasites. The investigation complied with the bioethical standards taken from "Principles of Laboratory Animal Care" (Anonymous 1985).

Specimens of *H. angulatus* were collected in the area of the Morichal Largo River, Monagas state, Venezuela (Fig. 1), by sunset and then maintianed at the serpentarium of the Tropical Medicine Institute of the Universidad Central de Venezuela

The venom was gathered through a 50-mL plastic centrifuge tube transversely cut and covered on the top with Parafilm®. The snake was compeled to bite the Parafilm® with its rear teeth. The venom was extracted with a capillary tube. From each extraction, approximately 0.2 mL of venom was obtained (Rodriguez-Acosta *et al*. 2006). Venoms samples were centrifuged, pooled, lyophilized, and stored at -80°C before being used.

Protein determination

We followed the method of Lowry *et al*. (1951).

Lethal dose (LD_{50})

The median lethal dose (LD_{50}) of the *H. angulatus* venom was estimated in 18 to 22-g female INH mice. A total of 0.1 mL of venom (at various concentrations) was injected intraperitoneally. The endpoint of lethality was calculated after 48 h. The LD_{50} were determined according to the Spearman-Kärber method (1978).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was run using 15% gels under reducing and non-reducing conditions. Molecular-weight markers (Bio-Rad, USA) were carried out in parallel, and gels were stained with Coomassie Blue R-250. *Helicops angulatus* venom and fractions (1 mg/mL) were dissolved

Fig. 1. Venezuelan specimen of *Helicops angulatus.* *Ejemplar venezolano de* Helicops angulatus.

in a proportion of 1:1 in the following solution: 0.5 M Tris-HCl, pH 6.8, with 10% (wt/vol) SDS, 10% (vol/vol) β-mercaptoethanol, 10% (vol/vol) glycerol, and 0.05% (wt/vol) bromophenol blue. The samples were then heated at 100°C for ten minutes. The relative masses were estimated by the Multi-Analyst PC version 1.1 (Bio-Rad) program.

Chromatographic analysis

The *H. angulatus* venom (2 mg) was diluted to 1.0 mL with 50 mM Tris-HCl buffer, pH 7.4, and separation was performed with a Superose 12 10/300 GL molecular exclusion chromatography column, pre-equilibrated with the 50 mM Tris-HCl buffer, pH 7.4 at 4 °C. The column was washed with 3-column volumes of the buffer at a flow rate of 1.0 mL/min. The venom proteins were eluted with equilibrating buffer, and proteins were detected at 280 nm. Each fraction size was 0.5 mL, and only the apexes of the peaks were analyzed for gelatinase activity.

Neurotoxic activity

To verify the neurological symptomatology produced by the crude venom and peaks 3 and 4 obtained from size exclusion chromatography (164 µg/20 g mouse weigth), six mice were subcutaneously injected with 100 µL of each sample. The mice were observed for neurotoxic effects (Lemoine and Rodríguez-Acosta 2003) for twenty minutes post injection or until death occurred.

Gelatinase assay

A modified method (Lemoine *et al*. 2004b) was used to test the gelatinase activity of *H. angulatus* crude venom and fractions. An x-ray film (Kodak X-OMAT) was soaked with distilled water and incubated at 37 °C for forty five minutes. After incubation, the film was completely dried and 10 µL of crude venom (10 mg protein/mL solution) and venom fractions diluted to 1/8 were located on the x-ray scientific imaging film containing a gelatine coating. The hydrolysis of gelatine on the x-ray film was observed after two hours incubation at 37 °C in a humid incubator by rinsing the x-ray film with distilled water. Serial dilutions were carried out to determine the minimum amount of venom necessary to produce a clear spot on the x-ray film. The titre was defined as the reciprocal of the highest dilution that produced a clear spot on the x-ray film. The specific gelatinase activity was calculated by dividing the titre by the amount of protein (µg) applied on the film. The assay was repeated three times.

Proteolytic activity on casein

Proteolytic activity, using casein as substrate, was tested by the Lomonte and Gutiérrez (1983) method. One millilitre of peak 1 (100 µg) was combined with 1.0 mL of 1% casein solution in 0.1 M phosphate buffered saline solution (pH 7.2). The solution mixture was reacted at 37 °C for thirty minutes and then the reaction was blocked by the addition of 4.0 mL of 5% trichloroacetic acid. After thirty minutes at room temperature, the mixture was centrifuged, and the supernatant was measured at an absorbance of 280 nm. The caseinolytic activity was calculated as U/mg.

Determination of hemorrhagic activity on mice skin

The venom hemorrhagic activity was assayed by the Gutierrez *et al*. (1998) method. One hundred microlitres of crude venom containing 82 µg protein/20 g of mouse body weight was injected intradermally into the abdominal skin of three male mice. The skins were isolated four hours later, and the diameters of the hemorrhagic spots on the inside surfaces were measured (Huang and Pérez 1980). *Bothrops colombiensis* venom (100 µL of 6.5 µg protein/20 g of mouse weight) and saline solution were used as positive and negative controls, respectively.

RESULTS

Lethality

The LD_{50} for *H. angulatus* venom was calculated to be 106 µg protein/20 g of mouse body weight (5.3 mg/kg). Peak 3 had an LD_{50} of 90 µg/20 g of mouse body weight (4.5 mg/kg).

Molecular exclusion chromatography

The fractionation of *H. angulatus* venom proteins was run on a Superose 12 10/300 GL chromatography column, resulting in six peaks (Fig. 2). All peaks were analyzed for neurotoxic and proteolytic activities.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

The 15% SDS PAGE revealed ~ 6 major protein bands with relative molecular masses (M_r) of approximately 13, 23.5, 28, 39, 47 and 70 kDa, under reducing conditions and M_r ~ 4 bands of approximately 22, 23.5, 47 and 70 kDa, under non-reducing conditions (Fig. 3). The gel was stained with Comassie blue stain.

Neurotoxic activity

The neurotoxic activity of *H. angulatus* venom was established by the neurological clinical symptoms observed in six mice inoculated intraperitoneally with peak 3 of size exclusion chromatography. In general, animals showed hyper-excitability, involuntary trembling and fasciculations, convulsions and finally died (Table 1).

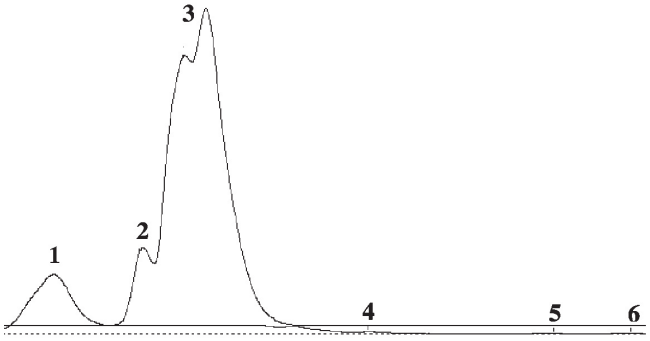

Fig. 2. Fractionation of *H. angulatus* venom by molecular exclusion chromatography on a Superose 12 10/300 GL column. Only peak 3 possessed neurotoxic activity. Peak 1 had proteolytic activity.

Fraccionamiento del veneno de H. angulatus *por cromatografia de exclusión molecular en una columna de Superose 12 10/300/GL. Solo el pico 3 tuvo actividad neurotóxica. El pico 1 tuvo actividad proteolítica.*

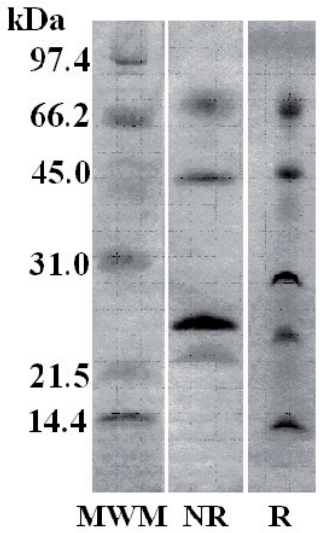

FIG. 3. Biochemical characterization of *H. angulatus* venom. Samples of 16 µg /10 µL of venoms were run in a 15% SDS-PAGE gradient gel under reducing (R) and non-reducing conditions (NR) and stained with Coomassie Blue. MWM: molecular weight markers.

Caracterización bioquímica del veneno de H. angulatus. *Muestras de 16 µg /10 µL de venenos fueron corridas en un gel de gradiente 15% SDS-PAGE bajo condiciones reductoras (R) y no-reductoras (NR) y coloreadas con Coomassie Blue. MWM: marcadores de peso molecular*

Gelatinase activity

Crude venom contained gelatinase activity up to dilutions of 1/2. Only peak 1 had proteolytic activity (up to 1/4 dilutions) (data not shown). The other five peaks did not demonstrate gelatinase activity.

Proteolytic activity on casein

The venom of *H. angulatus* showed a proteolytic activity of 98.6 ± 4.0 U/mg on casein.

Determination of haemorrhagic activity on mice

The tests of intradermal injections revealed no hemorrhagic activity for the venom of *H. angulatus* (data not shown).

DISCUSSION

According to clinical and epidemiological reports, the frequency of snakebites by "colubrid" snakes is increasing in Venezuela (Lemoine and Rodríguez-Acosta 2003, Diaz *et al*. 2004). Nowadays, "colubrids" are responsible for an important number of ophitoxemias and it may be a sign of the general abundance of these species (Rodriguez-Acosta *et al*. 2006) and the augmented intensity of patent access. The accidents produced by opisthoglyphous snakes are generally distinguished by local tissue injures, such as intense pain, edema and hemorrhages (Kamiguti *et al*. 2000; Lemoine and Rodríguez-Acosta. 2003). Nevertheless, there are studies (Fry *et al*. 2003) suggesting a complexity in "colubrid" venom that is comparable to the highly toxic venoms from Viperidae and Elapidae. Parallel work in our laboratory has decribed neurotoxic activity in a number of Venezuelan "colubrid" venoms (Lemoine and Rodríguez-Acosta 2003, Lemoine *et al*. 2004a, Lemoine *et al*. 2004b, Rodríguez-Acosta *et al*. 2006). Snake venom neurotoxins are mainly categorized into neurotoxins inhibiting synaptic transmission (postsynaptic and presynaptic neurotoxins) and neurotoxins which markedly facilitating it (dendrotoxin and fasciculin). Here, we have shown that *H. angulatus* venom displays *in vivo* neurotoxic activity, producing a flaccid paralysis and rapid death of experimental mice.

The envenomation caused by this snake produced breathing paralysis, probably due to the dysfunctions at the neuromuscular junction level (Larreche *et al*. 2008). First, the mice presented immediate signs toward a paralysis, preceded by early non-specific signs and symptoms such as pruritus or itchy sensations ("face washing"), indicating the start of the envenomation. Then, they showed hyperexcitability, fasciculations, tremors, muscular contractions, convulsions and, eventually, coma. muscular tremors and contractions were observed. This clinical picture evolved quickly toward an upward breathing paralysis. The convulsions represented an attack of the central nervous system (Chippaux 2007). The sialorrhea, lacrimation, sweating and diarrhea absences seems to indicate that the neurotoxin(s) of *H. angulatus* do not have muscarinic effects. However, these latter symptoms could be present but may be missed due to an over saturation of the muscarinic acetylcholine postsynaptic receptors (Aubert *et al*. 1996, Larreche *et al*. 2008).

TABLE 1. Neurotoxic signs and symptoms in mice injected intraperitoneally with *H. angulatus* venom peak 3 (Superose 12 10/300 GL chromatography)*.
TABLA 1. *Signos y síntomas neurotóxicos en ratones inyectados intraperitonealmente con veneno de* H. angulatus *con actividad en pico 3 (Superose 2 10/300 chromatografía GL)*.*

Time (min)	Hyper-excitability	Face washing	Involuntary trembling and fasciculation	Tachypnea	Exophthalmos	Convulsion	Flaccid paralysis	Urinary sphincter relaxation	Death
1	1-6	1 ,2 ,4, 5	1, 2	-	-	-	-	-	-
3	-	-	3, 4, 5	1, 2, 3, 4, 5, 6	1,4,5,6	-	-	-	-
6	-	-	-	-	2,3	1,3,4	1-6	1,2,3, 6	1,2,3,6
8	-	-	-	-	-	4,5	-	4, 5	4, 5

Gel electrophoretic analyses showed few protein bands around 14 to 70 kDa. Several authors have reported that many neurotoxins are basic, low molecular weight proteins (Mackessy *et al*. 2006). From approximately 100 different molecules identified by Birrell *et al*. (2007) in Australian snake venoms, 62 components possessed molecular masses between 6 and 8 kDa and were cytotoxins and neurotoxins.

Proteolytic activity was an unimportant characteristic of *H. angulatus* venom, since crude venom and peak 1 were only positive on gelatin up 1/4 dilutions compared with other "colubrids" and *Bothrops* venoms (Furtado *et al*. 1991, Sanchez *et al*. 1992, Lemoine and Rodríguez-Acosta 2003, Rodriguez-Acosta *et al*. 2006). The proteolytic activity using casein as substrate was also not an important characteristic of *H. angulatus* venom, compared to other snake venoms. For instance, *Philodryas olfersii* (378±10.0 U/mg) and *P. patagoniensis* (291.0±7.0 U/mg) venoms described in the literature had higher proteolytic activities (Furtado *et al*. 1991, Sanchez *et al*. 1992, Teixeira Rocha *et al*. 2006).

Hemorrhage is one of the main pathophysiological effects provoked by Venezuelan snake venoms (Lemoine and Rodríguez-Acosta 2003, Lemoine *et al*. 2004a, Rodriguez-Acosta *et al*. 2006), in addition to the majority of Viperidae snake venoms (Ownby *et al*. 1984, França and Málaque 2003). However, in the *H. angulatus* venom we could not demonstrate cutaneous and/or intraperitoneal hemorrhages, despite the confirmed proteolytic activities. As it is known, hemorrhage produced by Viperidae (Bjarnason and Fox 1994, Sanchez *et al*. 1992) and Colubridae (Assakura *et al*. 1992, Mandelbaum *et al*. 1998) snake venoms is frequently attributed to metalloproteases. The weak proteolytic and strong neurotoxic activities shown by the venom of *H. angulatus* indicate it is mostly composed by neurotoxic molecules and a small fraction metalloproteases. These results corroborate the extensive distribution of neurotoxins among the highly developed Colubroidea superfamily of snakes.

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