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ORIGINAL RESEARCH

Proteolytic, Hemorrhagic, and Neurotoxic Activities Caused by *Leptodeira annulata ashmeadii* (Serpentes: Colubridae) Duvernoy's Gland Secretion

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Objective.—The main goal was to explore the different toxin properties (proteolytic, hemorrhagic, and neurotoxic) of *Leptodeira annulata ashmeadii* Duvernoy's gland secretion (DGS).

Methods.—To separate and characterize the different proteins present in *L annulata ashmeadii* DGS, 20% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was run. To partially purify the proteolytic activity, Mono Q2 column ion-exchange chromatography was used. Hemorrhagic activity was analyzed in chicken embryos and mice (skin and peritoneum). Neurotoxic disorders were analyzed in mice inoculated with *L annulata ashmeadii* DGS.

Results.—The approximate relative mass of 21 protein bands was determined using the MultiAnalyst PC version 1.1 (Bio-Rad) program. *L annulata ashmeadii* DGS proved to have proteolytic, hemorrhagic, and neurotoxic activities.

Conclusions.—Given the properties of the secretions of its Duvernoy's gland, *L annulata ashmeadii* should be added to the list of venomous colubrids.

Key words: Colubridae, Duvernoy's secretion, hemorrhage, neurotoxic, proteolytic, *Leptodeira annulata ashmeadii*

Introduction

All snakes are carnivorous and must kill living animals for food. Many species rely on venom for this function. The Colubridae family of snakes is often thought of as a nonvenomous family relying on other mechanisms of obtaining prey such as constriction. Some colubrids, however, possess modified salivary glands (Duvernoy's glands) that produce secretions containing a mixture of enzymes[1] that break down cellular organization and hinder critical functions such as aeration, nerve conduction, and blood circulation. These species possess posterior grooved fangs (opisthoglyphous) through which toxic saliva from the Duvernoy's glands is drained.[2] Serious envenoming by these species has been reported, with symptoms similar to those seen with viper venom poisoning, which include edema, hemorrhage, paralysis, respiratory failure, and death.[3–7]

The snake genus *Leptodeira* is found in Mexico, Guatemala, Honduras, El Salvador, Nicaragua, Costa Rica, Panama, French Guiana, Trinidad, Tobago, Colombia, Venezuela, Brazil, Bolivia, Paraguay, Northern Peru, Argentina, and Ecuador.[5] *Leptodeira annulata ashmeadii* (false mapanare) is a nocturnal snake found in the subarboreal strata of forested formations and gallery forests.[5] Its food includes frog eggs, frogs, lizards, fish, tadpoles, toads, snakes, and its own eggs.[8]

The main goal of this study was to analyze the biochemical and biological properties of the Duvernoy's gland secretion (DGS) of *L annulata ashmeadii* and to assess its proteolytic, hemorrhagic, and neurotoxic activities.

Materials and methods

ANIMALS

Albino Swiss National Institute of Hygiene (NIH) strain male mice of 18 to 22 g obtained from the National

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Institute of Hygiene "Rafael Rangel," Caracas, Venezuela, were used. The investigation complied with the bioethical standards taken from *Principles of Laboratory Animal Care*.[9]

Wild snakes were captured from different geographic environments in Venezuela in vespertine and crepuscular tours (without transect delimitations), with strong emphasis on those areas of interest for the study (Aragua state, Venezuela), where museum opisthoglyphous snake (*Leptodeira* sp) references existed.

DUVERNOY'S GLAND SECRETION

The DGS was collected through a 50-mL plastic centrifuge tube transversely cut and covered on the top with Parafilm. The snake was forced to bite the Parafilm with its opisthoglyphous fangs. The DGS was milked with a micropipette. From each milking, approximately 0.3 mL of secretion was obtained.

PROTEIN DETERMINATION

The protein determination method followed that of Lowry et al.[10]

LETHAL DOSE

The median lethal dose (LD_{50}) of the DGS was determined in 18- to 22-g female BALB/c mice. The DGS was obtained from snakes at the Serpentarium of the Tropical Medicine Institute of the Universidad Central de Venezuela. All of the collected DGS was pooled, lyophilized, and stored at −80°C until used. DGS was dissolved in distilled water and used for the different assays. Lethal toxicity was determined by injecting 0.1 mL of DGS (at various concentrations) intramuscularly in the mice. Saline controls were used. The endpoint of lethality was determined after 48 hours. The calculations for the LD_{50} were performed according to the Spearman-Karber method.[11]

SODIUM DODECYL SULFATE-POLYACRYLAMIDE GEL ELECTROPHORESIS

Electrophoresis using a Dual Mini Slam Kit AE-6450 (Atto Corp, Tokyo, Japan) chamber was performed.[6] Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to the Laemmli method,[12] using 20% gels under reducing conditions. Molecular-weight markers (Bio-Rad) were run in parallel, and gels were stained with Coomassie Blue R-250. *L annulata ashmeadii* DGS samples to be analyzed (1 mg/mL) were dissolved in a proportion of 1:1 in the following solubilizer 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 10 minutes. The relative masses were determined by the Multi-Analyst PC version 1.1 (Bio-Rad) program.

CHROMATOGRAPHIC ANALYSIS

The DGS (20 mg) was diluted to 1.0 mL with 50 mM Tris-HCl buffer, pH 7.0, and separation was performed with Mono Q2 column chromatography pre-equilibrated with the same buffer at 4°C. The column was washed with 3-column volumes of equilibrating buffer at a flow rate of 1.0 mL/min. The DGS proteins were eluted with a gradient of 0 to 1 M NaCl dissolved in 50 mM Tris-HCl, pH 7 to 9. The fraction size was 0.5 mL. Elution of protein was monitored at 280 nm.[13] To test proteolytic activity, the eluting peak tops were analyzed.

HEMORRHAGIC ACTIVITY EVALUATED IN CHICKEN EMBRYOS

Embryonic hen eggs incubated at 37°C for 5 days were cleaned with 70% alcohol, and the embryos were extracted by breaking the eggshells; they were then placed on petri dishes and incubated at 37°C for 3 hours. Circles of Whatman No. 2 filter paper of 3 mm in diameter were impregnated with 3 μL (0.75 μg) of DGS and applied to the chicken embryo vitelin vein.[14–16] Circles soaked with 3 μL (0.75 μg) of *Bothrops venezuelensis* venom were used as positive controls. Circles with saline solution were used for the negative controls.

DETERMINATION OF HEMORRHAGIC ACTIVITY ON SKIN

The DGS hemorrhagic activity was determined by a modification of the Kondo test.[17,18] One hundred microliters of DGS containing 5 to 50 μg protein/20 g of mouse weight was injected intradermally into the abdominal skin of 4 male NIH Swiss albino mice. The skins were removed 6 hours later, and the diameters of the hemorrhagic spots on the inside surfaces were measured.[19] Two diameters were obtained for the spot of hemorrhage by measuring the longest diameter of the spot and the diameter perpendicular to the first measurement. A minimal hemorrhagic dose was taken as the endpoint and defined as the concentration of DGS resulting in a 10-mm hemorrhagic spot. *B venezuelensis* venom (100 μL of 5.6 μg protein/20 g of mouse weight) and saline solution were used as positive and negative controls, respectively.

DETERMINATION OF HEMORRHAGIC ACTIVITY ON PERITONEUM

One hundred microliters of DGS containing 50 μg protein/20 g of mouse weight was injected intraperitoneally into 4 male NIH Swiss albino mice. *B venezuelensis* venom (100 μL of 5.6 μg protein/20 g of mouse weight) and saline solution were used as positive and negative controls, respectively.

NEUROTOXIC ACTIVITY

To determine the neurologic signs and symptoms possibly produced by DGS, 6 mice were subcutaneously injected with DGS 100 μL (5.6 μg protein/20 g of mouse weight), and the mice were observed for effects.

GELATINASE ASSAY

A modified method[20] was used to test the gelatinase activity of *L annulata ashmeadii* DGS. The x-ray film (Kodak X-OMAT) was washed down with distilled water and incubated at 37°C for 45 minutes. After incubation, the film was completely dried, and 25 μL of crude DGS, in addition to the fractions of dilutions from 1 to 128 (1.9 mg protein/mL solution) obtained from the ion chromatography, was placed on x-ray scientific imaging film with a gelatin coating. The hydrolysis of gelatin on the x-ray film was determined after a 4-hour incubation at 37°C in a humid incubator by washing the x-ray film with distilled water. Serial dilutions were performed to determine the minimum amount of DGS required to cause a clear spot on the x-ray film. The titer was defined as the reciprocal of the highest dilution that caused a clear spot on the x-ray film. The specific gelatinase activity was calculated by dividing the titer by the amount of protein (μg) applied on the film. The assay was repeated 3 times.

Results

LETHALITY

The LD_{50} for *L annulata ashmeadii* DGS was determined to be 19.5 μg protein/20 g of mouse weight.

SODIUM DODECYL SULFATE-POLYACRYLAMIDE GEL ELECTROPHORESIS

To characterize the proteins present in *L annulata ashmeadii* DGS, we separated the proteins in SDS-PAGE. The gel showed approximately 21 protein bands (Figure 1).

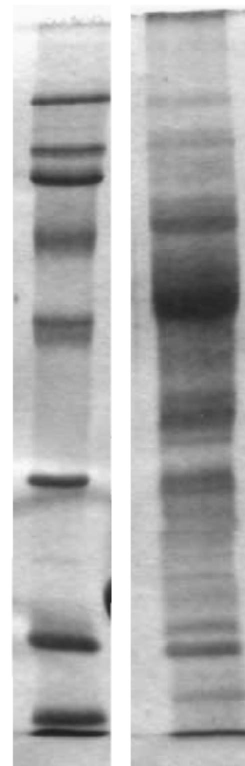

Figure 1. Separation of *Leptodeira annulata ashmeadii* Duvernoy's gland secretion (DGS) fractions by ion-exchange chromatography on a Mono Q2 column. Peaks 1, 3, 4, and 5 showed proteolytic activities.

ION-INTERCHANGE CHROMATOGRAPHY

The separation of *L annulata ashmeadii* DGS proteins was performed with Mono Q2 column chromatography, and it produced 9 peaks (Figure 2). All peaks were analyzed for proteolytic activity.

PROTEOLYTIC (GELATINASE) ACTIVITY

The material obtained from the 9 chromatographic peaks and the secretion from crude DGS were placed on x-ray film. The crude DGS showed proteolytic activity up to dilutions of 1:64. The substances from chromatography corresponding to peaks at P1, P3, P4, and P5 demon-

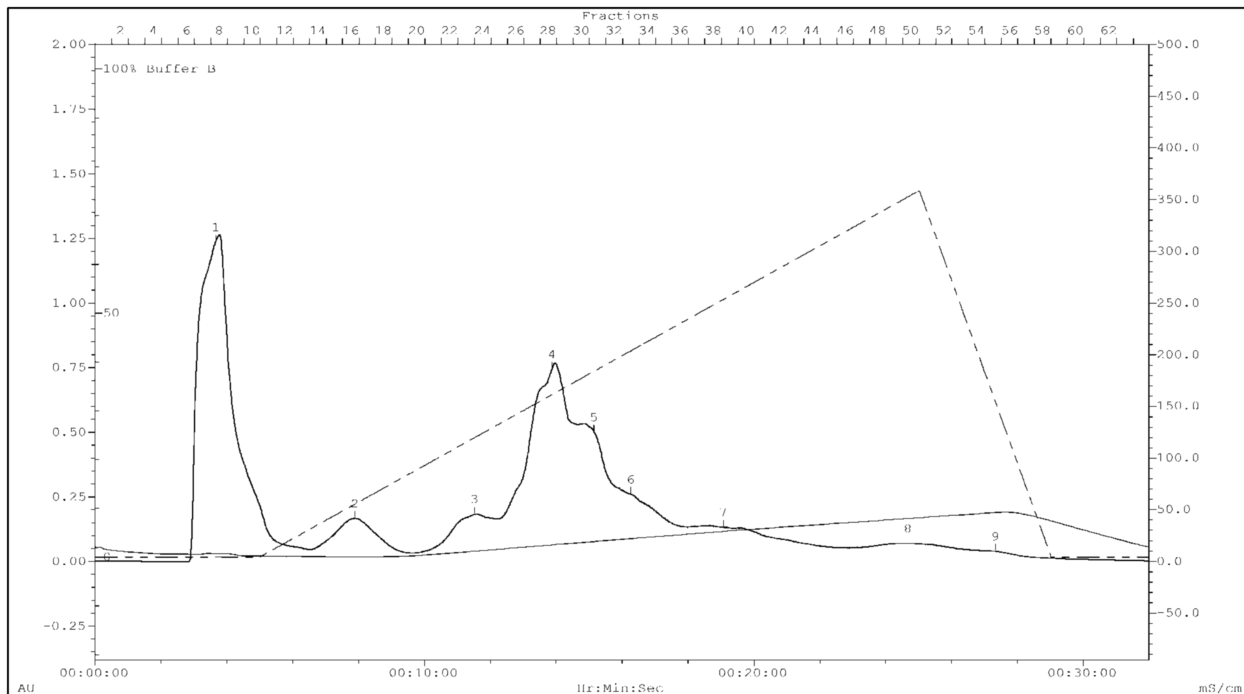

Figure 2. *Leptodeira annulata ashmeadii* Duvernoy's gland secretion (DGS) analysis performed in 20% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

strated proteolytic activity. For substances from peak P3, dilutions of up to 1:2 showed proteolytic activity, and for substances from peaks P4 and P5, dilutions of up to 1:4 showed proteolytic activity (data not shown). Peaks from 6 to 9 did not show any activity.

HEMORRHAGIC ACTIVITY ANALYZED IN CHICKEN EMBRYOS

Figure 3 shows the hemorrhagic activity of the crude *L annulata ashmeadii* DGS in the chicken embryo vitelin vein. A conspicuous vascular blood extravasation was observed. Saline solution–negative and *B venezuelensis* venom–positive controls were also performed.

DETERMINATION OF HEMORRHAGIC ACTIVITY ON SKIN

L annulata ashmeadii DGS displayed hemorrhagic activity when analyzed by the use of intradermal injections in mice (Figure 4). The minimum hemorrhagic dose was 7.8 μg protein/20 g of mouse weight, indicating that this DGS was less active than the crude *B venezuelensis*–positive control venom (minimum hemorrhagic dose = 5.6 μg protein/20 g of mouse weight).

DETERMINATION OF HEMORRHAGIC ACTIVITY ON PERITONEUM

All mice injected intraperitoneally with *L annulata ashmeadii* DGS showed intense hemorrhagic activity. The saline solution–negative control and the *B venezuelensis* venom–positive control were also performed (Figure 5).

NEUROTOXIC ACTIVITY

L annulata ashmeadii DGS neurotoxic activity was demonstrated by the neurologic manifestations observed in 6 mice injected subcutaneously with this secretion (Table).

Discussion

Some snakes in the Colubridae family have been reported to have toxic secretions that are capable of causing severe symptoms.[4,7,21–24] The saliva of some "nonvenomous" colubrids can, on rare occasions, cause mild-to-moderate envenoming in humans. In the United States, people have had reactions to the bites of the cat-eyed snake (*Leptodeira septentrionalis*), the black-striped snake (*Coniophanes imperialis*), the western hognose snake (*Heterodon nasicus*), and others.[25] In Venezuela, only a few authors[6] have described the re-

Figure 3. *Leptodeira annulata ashmeadii* Duvernoy's gland secretion (DGS) hemorrhagic activity in peritoneum. **A,** Negative control (saline solution). **B,** Positive control (*Bothrops venezuelensis* venom). **C,** *L annulata ashmeadii* DGS.

sults obtained from electrophoretic analysis of DGS and the biological characteristics in the Colubridae *Philodryas viridissimus*. Eight bands of proteins have been described, and they were compared with proteins expressed by *Crotalus* (*Crotalus durissus cumanensis*, *Crotalus durissus ruruima*, *Crotalus vegrandis*, *Crotalus pifanorum*, and *Crotalus unicolor*) venoms. In the present work, the 106- and 38-kd bands observed in the *L annulata ashmeadii* DGS SDS-PAGE results seemed to share the same molecular weight as some *Crotalus* venom fractions, and they corresponded to proteolytic venom enzymes already described for these species[26]; work is in progress to verify this. The DGS of *Leptodeira* has not been as thoroughly investigated as those of Viperidae snakes.

A comparison of the *L annulata ashmeadii* DGS LD_{50} value (19.5 μg protein/20 g of mouse weight) with the LD_{50} values of venoms from other snake species such as the Viperidae *C vegrandis* (3.12 μg protein/20 g of mouse weight) shows the significant toxicity of this DGS, which could produce various symptoms in humans bitten by this snake.

Peaks 1, 3, 4, and 5 obtained from *L annulata ashmeadii* electrophoresis were partially purified, with peak 1 presenting the highest proteolytic activity (1:64 dilutions). Improved purifications of closely eluting fractions (eg, 3–7 and 8 and 9) are in accordance with chromatographic results from SDS-PAGE analysis, showing that the crude purification proteins in peak 4 may also be present in 3 and 5 and that, thus, all toxic effects may be due to a few proteins—or even just a single protein. Therefore, an advanced analysis of toxicity or more systematically purified fractions is necessary as well as an additional analysis of constituent proteins in toxic fractions using Western blots and/or mass spectrophotometry to identify individual toxins.

Chicken embryos were used to measure hemorrhagic activity and antivenom efficacy in Viperidae and Elapidae venom,[15] only because this represented an alternative to the traditional method of using mice or rats. To our knowledge, this is the first time that this method in Colubridae DGS studies has been performed. By using chicken embryos in early developmental stages, we avoided the infliction of pain, because the reflex arcs in embryos have not yet developed. In addition, this proved to be very economical, given the cost of chicken embryos and the quantity of DGS used: only 3 μL of DGS was needed to clearly demonstrate high hemorrhagic activity. On the other hand, the use of mice clearly revealed skin and peritoneum hemorrhagic activity produced by *L annulata ashmeadii* DGS, demonstrating that

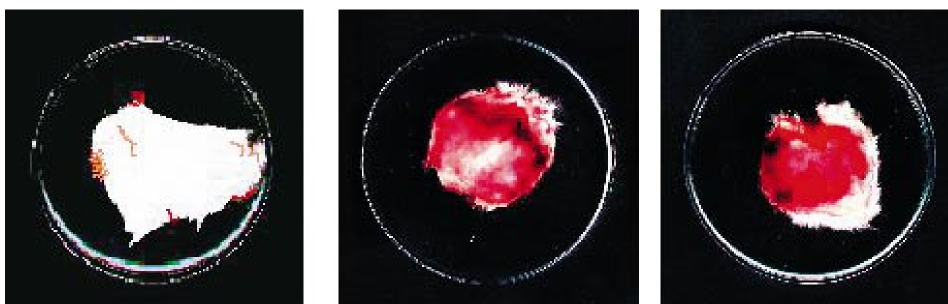

Figure 4. *Leptodeira annulata ashmeadii* Duvernoy's gland secretion (DGS) hemorrhagic activity in mice skin. **A,** Negative control (saline solution). **B,** Positive control (*Bothrops venezuelensis* venom). **C,** *L annulata ashmeadii* DGS.

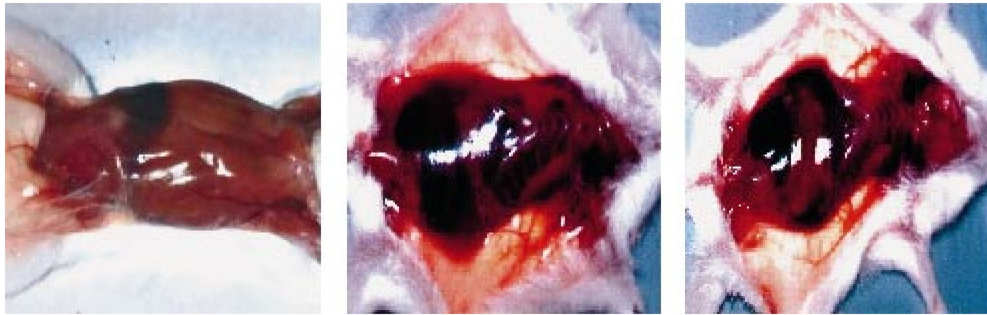

Figure 5. *Leptodeira annulata ashmeadii* Duvernoy's gland secretion (DGS) hemorrhagic activity on chicken embryos. **A,** Negative control (saline solution). **B,** Positive control (*Bothrops venezuelensis* venom). **C,** *L annulata ashmeadii* DGS.

this snake produces very strong hemorrhagic action in mammal tissues.

One author (Mebs[27]) has described large hemorrhagic areas within mice internal organs produced by Nordic *L annulata* DGS. He commented that 1 mg of DGS was sufficient to kill a mouse of 20-g weight 2 or 3 days after being injected subcutaneously. In our experiments, 56 μg of *L annulata ashmeadii* DGS was sufficient to kill mice of 20-g weight 21 minutes after subcutaneous injection. This quantity is approximately equivalent to what the snake can inject in a bite, since it corresponds to the mean amount produced during a milking from a medium-sized specimen (45–55 cm). The ecological characteristics of each subspecies could influence DGS lethality, with the tropical subspecies being more lethal, as observed in this work.

We determined that *L annulata ashmeadii* DGS produces various neurotoxic symptoms in envenomed mice. Several bibliographic references were found describing Colubridae neurotoxic alterations.[28] The fractions that cause neurotoxic symptoms would involve presynaptic and postsynaptic neurotoxins. The presynaptic toxins operate by inhibiting the release of acetylcholine at the neuromuscular junction. Neurotoxic phospholipase A is found in some colubrid venoms.[29] The involvement of this substance in nervous system depolarization and nerve block phenomena has been demonstrated in Viperidae venom (crotoxin component).[30] The band with a molecular mass of 14 kd found in *L annulata ashmeadii* DGS (Figure 1) may be related to the neurotoxic phospholipase A2 (from Viperidae—rattlesnakes and pit vipers—belonging to class II pancreatic phospholipase A2) with a molecular mass of 14 kd, which could explain the neurotoxic activity (flaccid paralysis) described in the current work.

There have been reports of a central neurotoxic reaction from phospholipase A2, whose mechanism remains unknown. However, the neurotoxicity is probably

Neurotoxic signs and symptoms in 6 mice intraperitoneally injected with *Leptodeira annulata ashmeadii* Duvernoy's gland secretion*

Time (min)	*Dyspnea*	*Equilibrium alterations*	*Posterior limb paralysis*	*Flaccid paralysis*	*Abdominal contraction*	*Bradyspnea*	*Aggressive-ness*	*Pain*	*Pares-thesia*	*Urinary sphincter relaxation*	*Death*
1	1s	1s	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .
3	3s	3s	. . .	. . .	. . .	. . .	2s	2s	. . .	. . .	. . .
5	2s	5s	3s	. . .	. . .	. . .	1s	3s	. . .	. . .	. . .
7	5s	. . .	. . .	. . .	3s	. . .	. . .	. . .	. . .	. . .	. . .
13	6s	. . .	4s	1s	4s	. . .	. . .	. . .	. . .	. . .	. . .
15	4s	2s	6s	3s	6s	. . .	. . .	. . .	. . .	. . .	. . .
21	. . .	. . .	2s	5s	. . .	1s	. . .	. . .	. . .	1s	1s–3s
23	. . .	. . .	1s	4s	. . .	3s	. . .	. . .	4s	. . .	2s
56	. . .	. . .	5s	6s	. . .	. . .	. . .	. . .	5s	. . .	. . .
75	. . .	. . .	. . .	. . .	. . .	5s	. . .	. . .	6s	6s	6s
81	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	5s	4s–5s

*Only 6 mice were used to test neurotoxic symptoms. Each mouse is represented by a number from 1s to 6s.

associated with the union of this toxin with cerebral receptors, specifically those that have previously been identified as type N (neuronal) receptors, characterized by the clinical production of convulsions and epilepsy.[31]

To our knowledge, this is the first time that the neurotoxic signs and symptoms produced by *L annulata* DGS have been described. The most remarkable activities were dyspnea, equilibrium alterations, and involuntary movements, which appeared 1 minute after the DGS injection. After 5 minutes, mice developed posterior limb paralysis and demonstrated increased aggressiveness. Flaccid paralysis appeared 13 minutes after injection, and all animals died, probably by respiratory paralysis, before 120 minutes.

Opisthoglyphous colubrids have for a long time been ignored by most toxin researchers, since it was thought that their venom was of little importance because of its minor effect on humans. But in the case of many opisthoglyphous snakes, the venom may cause significant reactions in humans. In Venezuela, Gorzula[32] described swelling and hypersensitivity of a finger and wrist after a bite from this snake, and there are reports of human death caused by the venom of these ''harmless'' species.[5,7,33] Other aspects that have contributed to this lack of attention include the small quantity of venom and the low lethal potential of most opisthoglyphous snakes. On the other hand, Hayes et al,[34] who determined the quantity and proportional distribution of DGS delivered to dermal tissues compared to the viscera during a bite by the colubrid snake *Boiga irregularis*, found that only 54% of the secretion reached the viscera and that the rest stayed in the integument.

The results obtained in our study (with respect to hemorrhagic, neurotoxic, and proteolytic activities) were much more striking than we had anticipated. This work represents an analysis of the toxic potential that this species possesses and that has hitherto been neglected by researchers in the area.

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