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# Morphological study of accessory gland of *Bothrops jararaca* and its secretory cycle

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ABSTRACT

The venom gland apparatus of *Bothrops jararaca* is composed of four distinct parts: main venom gland, primary duct, accessory gland and secondary duct. Despite the numerous studies concerning morphology and venom production and secretion in the main venom gland, there are few studies about the accessory gland and its secretion. We characterized the accessory gland of *B. jararaca* snake and determined the secretion cycle by morphological analysis using light and transmission electron microscopy. Our data showed that the accessory gland of *B. jararaca* has a simple secretory epithelium with at least six types of cells in the anterior region: two types of secretory cells, mitochondria-rich cells without secretory vesicles, horizontal cells, dark cells and basal cells, and in the posterior region a simple epithelium with two types of cells: seromucous cells and horizontal cells. Furthermore, the mucous secretory cells of the accessory gland show a delayed and massive exocytosis that occurs four days after the extraction of venom. Morphological analysis at different steps after venom extraction showed that the accessory gland has a long cycle of production and secretion, which is not synchronous with the main venom gland secretory cycle.



## 1. Introduction

*Bothrops jararaca* (Serpentes, Viperidae) is a Brazilian solenoglyphous venomous snake. The venom glandular apparatus of this snake consists of four distinct parts: the main venom gland, the primary duct, the accessory gland and the secondary duct that connects to the fang (Gomes and Puorto, 1993). The main venom gland contains at least four distinct cell types: secretory cells (79%), mitochondria-rich cells (2%), horizontal cells (10%) and dark cells (9%) (Mackessy, 1991). This gland has a basal-central lumen, where most of the venom is stored. After a bite or manual extraction of venom, the amount of venom inside the lumen decreases and stimulates a new cycle of venom synthesis and the secretory epithelium undergoes morphological and biochemical changes. The epithelial cells change their shape from cuboid to columnar, the cistern of the rough endoplasmic reticulum expands and venom is synthesized. The venom production cycle lasts around 30–50 days, peaking at 4 days after venom extraction. (Ben-Shaul et al., 1971; Carneiro et al., 1991; De Lucca et al., 1974; Kochva, 1960, 1987; Mackessy, 1991; Rotenberg et al., 1971).

The noradrenenergic innervation of the main venom gland has an essential role in the venom production cycle. Venom extraction activates noradrenergic innervation and the noradrenaline released acts on both α- and β-adrenoceptors and triggers the venom production cycle by activating the venom gland (Luna et al., 2009; Kerchove et al., 2004; Yamanouye et al., 1997, 2000). The stimulation of both α- and β-adrenoceptors triggers an intracellular signaling cascade that culminates with the regulation of

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the synthesis of proteins involved in venom gland activation (Luna et al., 2009; Kerchove et al., 2008; Yamanouye et al., 2000).

The accessory gland of the venom gland apparatus of viperid snakes is structurally more complex than the main venom gland. There are six types of cells in the accessory gland of *Crotalus viridis oreganus*: mucosecretory cells with large mucous granules, mitochondria-rich cells with apical vesicles, mitochondria-rich cells with electron-dense secretory granules, mitochondria-rich cells with numerous cilia, horizontal cells, and dark cells (Mackessy, 1991). Despite the numerous studies concerning the morphology of and venom production and secretion by the main venom gland, just a few studies have been conducted to understand the function of the accessory gland and its secretion (Gans and Kochva, 1965; Gennaro et al., 1963, 2007; Kochva and Gans, 1966; Mackessy, 1991; Mackessy and Baxter, 2006; Rhoades et al., 1967).

It has been suggested that the accessory gland secretion can keep the venom from draining continuously into the mouth of the snake or can facilitate the venom flow through the fang (Gans and Kochva, 1965). Another supposition is that this secretion can be a source of toxins, or even a possible site for activation of toxins of the venom, increasing their activities (Gennaro et al., 1963, 2007). However, no differences were found in the protein profile or in the composition of the secretion by reversed-phase high-performance liquid chromatography between the venom collected from the intact venom gland apparatus and that from the main gland only (Mackessy and Baxter, 2006).

Thus, the aim of this study was to characterize the accessory gland of *B. jararaca* snake and determine the secretion cycle by morphological analysis using light and transmission electron microscopy. Our data showed that the accessory gland of the *B. jararaca* snake has seven types of cells. We also showed for the first time that the accessory gland also has a long production and secretion cycle as in the main venom gland. The time course of exocytosis in the mucous secretory cells observed suggests that the secretion from the accessory gland does not contribute significantly to the total venom bolus.

## 2. Material and methods

### 2.1. Animals and accessory glands

Adult female *B. jararaca* (Wied, 1824) snakes, weighing 200–400 g, were classified by the Laboratory of Herpetology, Instituto Butantan, and maintained as described by Breno et al. (1990). Animal care and procedures used were in accordance with the guidelines of the Animal Ethics Committee of Instituto Butantan (374/2007), Biomedical Science Institute of University of São Paulo (138/2009) and the Brazilian Institute for Environment and Renewable Natural Resources, an enforcement agency of the Brazilian Ministry of Environment (IBAMA, License 01/2009). Snakes were left without food for 40 days to prevent loss of venom and to make sure that most of the cells were in the quiescent stage. Fasting periods of 1–2 months are common in snakes living in the wild, but fasts can exceed one year (Secor and Nagy, 1994). The snakes were anesthetized with sodium pentobarbital (30 mg/kg, subcutaneous) and decapitated, and the accessory glands were removed and freed from connective tissue (Yamanouye et al., 2007). Accessory glands were obtained from snakes in which venom was not manually extracted and from snakes in which venom was extracted 1 h or 4, 7 or 15 days before they were killed by decapitation ($N = 3$ for each group), in order to observe changes that occur during the secretory cycle. To remove the venom, snakes were anesthetized with sodium pentobarbital (20 mg/kg, subcutaneous), and the venom was removed manually as described by Belluomini (1968).

### 2.2. Light and electron microscopy

The accessory glands were washed with 0.6% saline solution, and 1 $mm^3$ fragments were fixed in 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.2) containing 2% sucrose for at least 2 h at room temperature. Post-fixation was done in 1% osmium tetroxide in 0.1 M cacodylate buffer (pH 7.2) for 1 h at room temperature, and the fragments were then placed in 0.5% uranyl acetate containing 13.3% sucrose, overnight at 4 °C. Fragments were dehydrated with an ethanol series and propylene oxide, and then embedded in Epon resin. Semithin sections (1 μm thickness) were stained with toluidine blue (0.1% toluidine in 1% sodium borate). Ultrathin sections (50–70 nm thickness) were contrasted with uranyl acetate and lead citrate. The grids were examined with a LEO 906 E transmission electron microscope (Zeiss, Oberkochen, Germany) at 80 kv acceleration voltage. Images were acquired by a CCD camera MegaView III (Olympus) through the ITEM – Universal TEM Imaging Platform program (Olympus Soft Imaging Solutions GMBh, Germany). The height of cells of the anterior and posterior regions of the accessory glands from non-extracted snakes ($N = 3$ each) were measured using the measurement interface of the same software program.

For light microscopy, the accessory glands were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2), containing 3.5% sucrose, for at least 8 h at room temperature. The pieces were embedded in Historesin (Leica, Wetzlar, Germany). Sections of 5–6 μm thickness were obtained with a Microm HM 340 E microtome (Microm International GmbH, Walldorf, Germany) and were stained with toluidine blue. Images of the whole gland were taken with an Olympus SZX7 stereomicroscope (Olympus Corporation, Shindiuku, Tokyo, Japan). To determine the presence of acid mucopolysaccharides or neutral polysaccharides, sections were stained with alcian blue, pH 2.5, and Harry's hematoxylin or with periodic acid-Schiff (PAS), respectively. Semithin sections and histological or histochemical sections were analyzed with an Olympus BX51 light microscope coupled to a Q color 5 Olympus digital camera (Olympus Corporation, Shindiuku, Tokyo, Japan).

## 3. Results

### 3.1. Morphology of accessory gland of B. jararaca snake by light microscopy

The length of the accessory gland of non-extracted female snake was found to be 3.3 ± 0.1 mm ($N = 3$). It is