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Functional assessment of toad parotoid macroglands: A study based on poison replacement after mechanical compression

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ABSTRACT

Toads have a pair of parotoid macroglands behind the eyes that secrete poison used in passive defence against predators. These macroglands are composed of juxtaposed alveoli, each one bearing a syncytial gland, all connected to the exterior by ducts. When the parotoids are bitten, the poison is expelled on the predator oral mucosa in the form of jets, causing several pharmacological actions. After poison release, the empty secretory syncytia immediately collapse in the interior of their respective alveoli and gradually start refilling. After parotoid manual compression, simulating a predator's bite, we studied, by means of morphological methods, the replacement of the poison inside the alveoli. The results showed that after compression, a considerable number of alveoli remained intact. In the alveoli that were effectively affected the recovery occurs in different levels, from total to punctual and often restrict to some areas of the syncytia. The severely affected alveoli seem not recover their original functional state. The fact that only a part of the parotoid alveoli is compressed during an attack seems to be crucial for toad survival, since the amphibian, after being bitten by a predator, do not lose all its poison stock, remaining protected in case of new attacks.

1. Introduction

The chemical defence among amphibians, especially in toads, although based on the effect of potent toxins, is generally passive, opposing the active defence of venomous animals, such as snakes, capable of injecting venom through bites (Jared and Antoniazzi, 2009). As a number of salamanders and other anurans, when toads (Bufonidae) are threatened, they exhibit a sequence of stereotyped postures consisting of inflating the lungs, assuming a stiffened and voluminous form, tilting the body and exposing one of the parotoids towards the attacker (Brodie, 1983; Toledo and Jared, 1995; Williams et al., 2000; Jared et al., 2009; Toledo et al., 2011; Mailho-Fontana et al., 2014). In this mechanism of passive defence, jets of poison are released only in the case an external mechanical pressure (for example, a bite of a predator) is exerted onto the parotoids. Spontaneous release of poison jets among anurans is very rare, and has been described so far only in the Amazonian toad *Rhaebo guttatus* (Jared et al., 2011; Mailho-Fontana et al., 2014). In urodelans, spontaneous poison release was reported in *Salamandra salamandra*, species able of ejecting poison from the paratoids (Brodie, 1983;

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Brodie and Smatresk, 1990). "Paratoid" macroglands differ from the anuran "parotoids" for being located on the middle dorsum, running in line along the salamander backbone (Tyler et al., 2001).

Studies on the structure and function of skin glandular accumulations in amphibians are limited. Among them, toad parotoids seem to be the most studied structures, with many descriptions of their anatomy and general histology (Tronchet, 1952; Hostetler and Cannon, 1974; Cannon and Hostetler, 1976; Toledo et al., 1992; Barthalmus, 1994; Almeida et al., 2007; Jared et al., 2009; Antoniazzi et al., 2013; Mailho-Fontana et al., 2014). In these studies the parotoids are described as thickened portions of dorsal skin in which, besides the presence of the mucous and granular glands that are similar to those present in the rest of the skin, other much larger bottle-shaped syncytial glands are present, arranged side by side in individual alveoli moulded within the *stratum compactum*. These larger glands are connected to the outside by a very narrow opening delimited by a thick epithelium that lines the duct. The structural changes involving the mechanism of parotoid poison release in toads have been well described, featuring a peculiar system of poison expelling dependent on an external force, such as a bite from an aggressor, to trigger the process (Jared et al., 2009; Mailho-Fontana et al., 2014). Furthermore, exclusive morphological features have been described in toad parotoids such as a blood-vessel network (Hutchinson and Savitzky, 2004) and differentiated glands surrounding each one of the main ducts (Jared et al., 2009; Mailho-Fontana et al., 2014). Based on the exclusive structural complexity of these macroglands, we suggested that they should be considered as true cutaneous organs in toads, especially evolved to act as efficient means of passive defence (Jared et al., 2009).

Aside from the morphological data, the biochemical and biological effects of toad poison secretions, especially regarding the parotoids, are relatively well studied (Pasquarelli et al., 1987; Rossi et al., 1997; Maciel et al., 2003; Shimada et al., 2006; Tempone et al., 2007, 2008; Sciani et al., 2013), since they represent an important veterinary issue, mainly affecting dogs (Pineau and Romanoff, 1995; Sakate and Lucas de Oliveira, 2000; Jared and Antoniazzi, 2009).

Although the morphology of toad parotoids immediately after mechanical compression have already been described (Jared et al., 2009), their subsequent recovery process for poison replacement has never been studied. Since parotoids are constituted by syncytial units devoid of lumen, which collapse and lose most of their content when expelling the secretion, the poison replacement process is expected to be distinct from that normally seen in other glands, such as in snake venom glands (Carneiro et al., 1991).

This study aimed at the morphophysiological description of the recovery process of the parotoid secretory alveoli after manual compression, simulating the bite of a predator, using the common Brazilian southeastern toad *Rhinella icterica* as a model. From the analysis of the data obtained, we present some considerations about the significance of the parotoid morphological structure for toad passive defence.

2. Material and methods

2.1. Animals

Fifty-two adult males of *Rhinella icterica* Spix 1824 (Fig. 1) (mean SVL = 98.7 ± 6.9 mm) were collected in Cunha (Brazilian Atlantic Forest, State of São Paulo) and maintained in the vivarium of the Laboratory of Cell Biology, Instituto Butantan. The toads were kept in tanks containing clay roof tiles as shelters and a pot with water. The animals were feed weekly with crickets, cockroaches and new-born mice. Temperature was kept between 24 and 28 °C and high air humidity was maintained by washing the tank every day.

The toads had both parotoid glands manually compressed for poison extraction. At intervals varying from 2 h to 105 days, groups of three animals were anesthetized with a lethal dose of Thiopental (50 mg/kg) and had their parotoids removed for histological examination. Three toads used as negative controls did not have the parotoids compressed and were prepared in the same conditions. The toads were handled according to the procedures indicated in the Guidelines for Animal Experimentation established by the Brazilian College for Animal Experimentation.

Besides the three specimens serving as negative controls, two other specimens from the same population were used as positive controls: 1) one toad, belonging to the same group submitted to the initial parotoid compression, was kept in the vivarium for 330 days and had the parotoids removed using the same procedures described above; 2) the other toad was noticed at the time of collection to have a deformed parotoid, presumably caused by a predator's bite, and was, thus, prepared for parotoid examination.

Samples of the dorsal and ventral skin from all specimens were removed for morphological comparison with the parotoids.

2.2. Histology

All samples were fixed in Bouin fixative. After fixation, the parotoids were cut transversely to the gland larger axis into three pieces and embedded in paraffin. The samples

Fig. 1. Male of *Rhinella icterica*.