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Fipronil-induced cell death in salivary glands of *Rhipicephalus sanguineus* (Latreille, 1806) (Acari: Ixodidae) semi-engorged females

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ABSTRACT

The tick *Rhipicephalus sanguineus* is currently considered an urban plague. For this reason many studies are intended to find methods to control these ectoparasites. Thus, the present study analyzed the ultrastructural modifications of the salivary glands cells of semi-engorged females of *R. sanguineus* resulting from their exposition to Fipronil (active ingredient of Frontline®). The studied individuals were divided into four groups. Group 1 was exposed to distilled water (control) and groups 2, 3 and 4 were exposed to 1, 5 and 10 ppm of Fipronil, respectively. The salivary gland of ticks subjected to the acaricide showed accelerated process of cell death by atypical apoptosis, as well as augmented cell damages as the concentration of the chemical compound was increased. The acaricide toxicity at cellular level was demonstrated by remarkable changes of elements of the cytoskeleton and spherocrystals (extremely hard inorganic structures). However, tick defense mechanisms, such as the observed autofagic vacuoles proved the cells attempt to preserve their integrity and minimize the devastating action of this chemical compound on the salivary glands.

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1. Introduction

The tick *Rhipicephalus sanguineus* belongs to the Ixodidae family and has the dog as its natural host (Walker, 1994). The introduction of dogs in houses as companion animals and consequently its ectoparasites facilitates the propagation of biopathogens that can cause diseases both to the dog and the human being; therefore ticks are nowadays considered "urban plagues".

The feeding success of these ectoparasites is the result of the action of their salivary glands, organs that are responsible for the fixation of the tick in the host (Moorhouse and Tatchell, 1966), by osmoregulation (Sonenshine, 1991), the inhibition of the host defense mechanisms such as coagulation and inflammation (Nuttall and Strickland, 1908; Künsberg, 1911; Pawlowsky and Chodukin, 1929; Walker et al., 1985; Bowman et al., 1997; Ribeiro and Mather, 1998; Paesen et al., 1999) and by the digestion of the tissues (Lavoipierre and Riek, 1955; Walker et al., 1985). Therefore, salivary glands are organs indispensable for the feeding process and therefore make ticks very biological successful organisms.

The tick salivary glands are paired organs found in its celomatic cavity and contain approximately 1400 acini each (Walker et al., 1985). Many histological descriptions of salivary glands have been made in different species of ixodides (Walker et al., 1985; Fawcett et al., 1986; Sonenshine, 1991; Coons and Alberti, 1999; Furquim et al., 2007, 2008). In summary, there are three different types of acini in females (I, II and III) and four in males (I, II, III and IV). Type I acini cells are agranular and have the osmoregulatory process as the main function, while types II, III and IV acini cells are granular and responsible for the other previously mentioned functions of the gland (Walker et al., 1985; Fawcett et al., 1986; Coons and Alberti, 1999).

Due to the fact that *R. sanguineus* are considered plagues, the studies about this biological model have been focused on finding new and more efficacious methods of tick control. The chemical control through the use of acaricide compounds is still considered the most efficient one despite all inconveniences related to it, such as development of resistance by the ticks and environmental and animal products contaminations. Consequently, new chemical acaricides are often being developed and tested in animals; among them is Fipronil, the active ingredient of Frontline®. It acts inhibiting the development of ticks in pets (Taylor, 2001) and studies about its toxicity state that the compound presents neurotoxic action in arthropods in general (Rauh et al., 1990; Sattelle, 1990; Cole et al., 1993). Also, it acts in the reproductive process by altering both the structure and function of germinative cells of *R. sanguineus* females (Oliveira et al., 2008, 2009). However, little is known about the consequences of its utilization in non-target organs.

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The present study aimed to evaluate, at ultrastructural level, possible effects of different concentrations of Fipronil (1, 5 and 10 ppm) in salivary glands of semi-engorged females of *R. sanguineus* ticks looking for morphophysiological changes induced by the acaricides. Furthermore, to understanding mechanisms used by the salivary gland cells in an attempt to preserve their integrity, even in the presence of the toxic compound.

2. Material and methods

2.1. R. sanguineus female ticks

Semi-engorged *R. sanguineus* females were obtained from 50 fasting couples fed on New Zealand White rabbits during two infestations (25 couples/infestation). All the infestation procedure was made in hosts that had never been infested before, according to methodology described elsewhere (Bechara et al., 1995).

For the present study 60 specimens were used, distributed as follows: Group 1 (control) – 15 females exposed to distilled water; Groups 2, 3 and 4 (tests) – females exposed to 1, 5 and 10 ppm of Fipronil, respectively (15 females per group).

The experiments were carried out in the Histology and Electronic Microscopy Laboratories of the Biology Department at the Institute of Biosciences, São Paulo State University, Rio Claro-SP, Brazil.

2.2. Fipronil testing (CAS # 120068-37-3)

Fipronil or (RS)-5-amino-1-(2,6-dichloro-α,α,α-trifluoro-*p*-tolyl)-4-trifluoro methylsulfinylpyrazole-3-carbonitrile was obtained from the so-called Regent® 800 WG (BASF) with 80% degree of purity.

The concentrations of Fipronil used were based on DL_{50} of 10 ppm determined by Oliveira et al. (2008). The doses correspond to 10% of the DL_{50} (1 ppm), 50% of the DL_{50} (5 ppm) and 100% of the DL_{50} (10 ppm). The control group was exposed only to placebo (distilled water).

After being washed in running water and sieved, the 60 females were dried in soft absorbent paper. Afterwards, 45 specimens (groups 2, 3 and 4) remained immerse for 2 min in Petri dishes containing the different concentrations of Fipronil, while the 15 control females (Group 1) were immerse in distilled water for the same period. Soon after, they were dried with absorbent paper and put in a BOD incubator for 7 days.

The research protocol was approved by the Ethics Committee in Animal Research of the University of Araras, state of São Paulo, Brazil, under the protocol #010/2009.

2.3. Scanning Electron Microscopy (SEM)

The salivary glands of fasting semi-engorged females of *R. sanguineus* were fixed in Karnovsky solution for 2 h, and then subjected to a crescent series of 10-min acetone baths – 70%, 80%, 90%, 95% and 100% (the last one twice).

The material was taken to the critical point and fixed to a metal support with double sided adhesive tape where it received alternate layers of gold and carbon.

Only the salivary glands of fasting individuals were analyzed and photographed by Scanning Electron Microscope PHILIPS 505, SEM.

2.4. Transmission Electron Microscopy (TEM)

All the ticks maintained in the refrigerator for thermal shock anesthesia were dissected in a phosphate buffered saline (PBS) solution (NaCl 7.5 g/L, Na_2HPO_4 2.38 g/L e KH_2PO_4 2.72 g/L).

The salivary glands were fixed in 2.5% glutaraldehyde for 2 h, postfixed in 1% OsO_4 for 2 h, contrasted in 2% uranyl acetate and dehydrated in a graded acetone series (50%, 70%, 90%, and twice in 100%). Afterwards the material was immersed in a mixture of acetone and resin (1:1) for 12 h, embedded in Epon Araldite for 12 h and then in pure Epon resin and polymerized at 60 °C for 72 h. The material was sectioned and ultrathin sections were contrasted with uranyl acetate and lead citrate during 25 and 10 min, respectively.

Afterwards, screens containing ultrathin sections of the material were examined and photographed in a PHILLIPS 100 Transmission Electron Microscope at the Institute of Biosciences, São Paulo State University, Rio Claro-SP, Brazil.

3. Results

3.1. Scanning Electron Microscope (SEM)

The ultra morphology of the salivary glands of *R. sanguineus* semi-engorged females from the control groups showed that these organs are constituted by round acini connected to different kinds of glandular ducts (intermediate, acinar and common excretory) which conduct the salivary secretions (Fig. 1A and B).

The females subjected to the treatment groups with Fipronil in different concentrations presented significant loss of acinar turgidity as well as a decrease in the number of integral acini as the concentration of Fipronil increased. The acini of the individuals subjected to the concentration of 1 ppm were less turgid (Fig. 1C and D).

The salivary glands of the individuals from Group 3 (subjected to concentration of 5 ppm) presented gland tissue completely disorganized with few integral acini (Fig. 1D) and the loss of turgidity of the acini from the individuals of this group was bigger than the ones from that group (Fig. 1E).

The alterations observed in the individuals from group 4 (subjected to concentration of 10 ppm) were huge, being the acini completely shrunken and totally irregular (Fig. 1G and H).

No apparent changes were observed in the ultramorphology of the salivary duct system (Fig. 1A–G)

3.2. Transmission Electron Microscopy (TEM)

3.2.1. Group 1 females – control

Results of the *R. sanguineus* semi-engorged females at the scanning ultrastructural level confirm the ones described by Pereira et al. (2009) using histological and histochemical techniques. In addition, they bring new information also.

The semi-engorged females (6 days of feeding) of this group presented the glandular tissue in the beginning of natural degeneration process (ectoparasite natural biology), which is characterized by the presence of apoptotic bodies. As the distinction of the different types of acini was so difficult they were named as undetermined acini. However, integral cells can still be observed and ultrastructural details were seen among these cells, such as the presence of highly electrondense cell junctions (Fig. 2A and B).

Moreover, numerous and deep membrane invaginations can be seen in the basal domain of some cells, forming an extense membranous labyrinth where several mitochondria can be seen (Fig. 2C). Due to the cell degeneration phase, evident membrane retraction separated from the basal lamina was observed (Fig. 2C); in this region secretion vesicles containing material with varied electrondensity can be found.

The cytoplasm of these cells was full of rough lamellar endoplasmatic reticulum with evident widening of its cisterns due to the degeneration phase, as well as lipid droplets and mitochondria

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