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Morphological alterations in salivary glands of *Rhipicephalus sanguineus* ticks (Acari: Ixodidae) exposed to neem seed oil with known azadirachtin concentration

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ABSTRACT

Neem (*Azadirachta indica*) has attracted the attention of researchers worldwide due to its repellent properties and recognized effects on the morphology and physiology of arthropods, including ticks. Therefore, this study aimed to demonstrate the effects of neem seed oil enriched with azadirachtin on salivary glands of *Rhipicephalus sanguineus* ticks, targets of great veterinary interest because of their ability to transmit pathogens to dogs. For this, *R. sanguineus* semi-engorged females were subjected to treatment with neem seed oil, with known azadirachtin concentrations (200, 400 and 600 ppm). After dissection, salivary glands were collected and evaluated through morphological techniques in light microscopy, confocal scanning laser microscopy and transmission electron microscopy, so that the possible relation between neem action and further impairment in these ectoparasites feed performance could be established. Neem oil demonstrated a clear dose-dependent effect in the analyzed samples. The agranular (type I) and granular acini (types II and III) showed, particularly in individuals treated with the highest concentrations of the product, cells with irregular shape, intense cytoplasmic disorganization and vacuolation, dilation of rough endoplasmic reticulum lumen, besides alterations in mitochondrial intermembrane space. These morphological damages may indicate modifications in salivary glands physiology, demonstrating the harmful effects of compounds present in neem oil on ticks. These results reinforce the potential of neem as an alternative method for controlling *R. sanguineus* ticks, instead of synthetic acaricides.

1. Introduction

Rhipicephalus sanguineus is undoubtedly the most important species involved in the transmission of infectious agents to dogs (Dantas-Torres, 2010). Those specimens belonging to tropical and subtropical lineages are capable of transmiting *Ehrlichia canis*, causative agent of the canine monocytic ehrlichiosis, and *Babesia canis*, responsible for transmitting canine babesiosis (Blagburn and Dryden, 2009). Besides, some research indicate that they may be potential vectors of *Leishmania chagasi*, which causes the canine visceral leishmaniosis (Dantas-Torres, 2010) and *Rickettsia rickettsii*, causative agent of Rocky Mountain spotted fever (Cunha et al., 2009). These ticks can be found in dogs living in both urban and rural areas, being highly adapted to live in human dwellings (Blagburn and Dryden, 2009; Dantas-Torres, 2010). Although dogs are their preferred hosts, *R. sanguineus* ticks may occasionally parasite a wide variety of domestic and wild animals (Dantas-Torres et al., 2006; Blagburn and Dryden, 2009), being found even in humans (Carpenter et al., 1990; Manfredi et al., 1999; Uspensky and Ioffe-Uspensky, 2002).

For this reason, there is a growing need for the development of new methods for tick control. In human and veterinary health field, tick control is performed mainly by the use of chemical compounds with acaricide action (Flamini, 2003; Jonjejan and Uilenberg, 2004), usually formulated based on synthetic products (Alves et al., 2012). However, such substances are toxic, causing environmental pollution and presenting high cost (Jonjejan and Uilenberg, 2004), besides promoting the selection of resistant strains of ticks (Blagburn and Dryden, 2009; Rosado-Aguilar et al., 2010).

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Substances obtained from plant extracts, however, have low cost, few residual effects and generate low incidence of resistance development (Rosado-Aguilar et al., 2010). In this sense, the use of leaves, fruits and seeds extracted from the neem tree (*Azadirachta indica*) has been intensified, due to the presence of biologically active ingredients with various modes of action (Vietmeyer, 1992; Atawodi and Atawodi, 2009). This Meliaceae is original from the South and Southeast of Asia, being also found in tropical and subtropical areas of America, Africa and Australia (Schmutterer, 1990). It is capable of controlling species that are considered plagues, apparently without harming the human being, animals or beneficial insects (Vietmeyer, 1992). Its most important and predominant active principle, azadirachtin, presents a broad action spectrum, including alterations in growth and reproduction of arthropods (Vietmeyer, 1992; Schmutterer, 1990; Brahmachari, 2004). Neem derivatives do not persist in the environment, once they are degraded by ultraviolet rays and rain (Brahmachari, 2004). For this reason, these compounds are considered excellent substitutes for many synthetic pesticides (Vietmeyer, 1992).

Under different formulations, neem extracts showed expressive effects on the control of several tick species, such as *Hyalomma anatolicum excavatum* (Abdel-Shafy and Zayed, 2002), *Rhipicephalus (Boophilus) decoloratus* (Choudhury, 2009), *and Rhipicephalus (Boophilus) microplus* (Srivastava et al., 2008; Broglio-Micheletti et al., 2010). Moreover, in *R. sanguineus* ticks, aqueous extracts of neem leaves caused significant morphological alterations in ovaries (Denardi et al., 2010, 2011, 2012; Remedio et al., 2015), synganglion and integument (Remedio et al., 2014).

Thus, the present work aimed to evaluate the effects of the oil extracted from neem seeds (*A. indica*) on the morphology of the salivary glands of *R. sanguineus* female ticks. These organs are remarkably versatile, vital for the biological success of these animals (Sauer et al., 1995). To this end, such structures were analyzed using techniques in conventional light microscopy, confocal laser scanning microscopy and transmission electron microscopy, in order to generate information that can complement the results previously obtained for *R. sanguineus* treated with neem and reinforce the use of neem extracts in the control of this tick species.

2. Material and methods

2.1. R. sanguineus *ticks*

Unfed male and female ticks were obtained directly from the colony maintained in a Biological Oxygen Demand (BOD) incubator under controlled conditions (25 ± 1 °C, 80% humidity, in a 12 h photoperiod), in the Biosciences Institute vivarium of São Paulo State University (UNESP), Rio Claro/SP, Brazil.

2.2. Azadirachta indica *(neem)*

Neem oil was provided by Professor Moacir Rossi Forim, from Universidade Federal de São Carlos (UFScar). Neem seeds (2,5 kg) were ground in a Tecnal TE 631/2 mill and, then, subjected to an extraction process with hexane (5 extractions with duration of 3 days each), in order to obtain the neem oil. After this, the seeds went through a methanol extraction by 3 sequent extractions performed using an Ultra-Turrax T-20. After filtration, the solvent was evaporated under reduced pressure to provide an extract rich in azadirachtin, which was subsequently mixed with the previous extracted oil in order to enrich it with azadirachtin. The measurement of azadirachtin concentration in the crude extract of neem oil was made through the same procedures described by Forim et al. (2010). A concentration of 1000 parts per million (ppm) of azadiractin in the crude extract was determined, and from this oil the dilutions used in this experiment were made.

2.3. Experimental procedures

2.3.1. Preparation of neem dilutions

The crude oil extracted from neem seeds was diluted at concentrations of 20, 40 and 60% in a 10% aqueous solution of ethanol, according to an adaptation of the methodology described by Ndumu et al. (1999). Thus, it was possible to obtain dilutions with specific concentrations of azadirachtin: 200 ppm (20%), 400 ppm (40%) and 600 ppm (60%). The solutions were mixed on a magnetic stirrer and applied topically on ticks. The dilutions were made daily during the experiment, and kept in a light-protected recipient, in order to avoid alterations of the acaricidal properties of neem oil.

2.3.2. Bioassay

A total of sixty couples of *R. sanguineus* were released inside special feeding chambers set on the back of naive New Zealand White female rabbits (without prior exposure to tick infestation), following the methodology described by Bechara et al. (1995). Five rabbits were used in this experiment, one for each group (CI, CII, TI, TII and TIII). The procedures for application of neem dilutions were performed inside these feeding chambers, during engorgement of females.

Five groups were established in this experiment: Control Groups (CI and CII) and Treatment Groups (TI, TII and TIII). In the Treatment Groups, the solutions of neem oil were applied topically on ticks attached on the back of rabbits with a soft brush for three days, twice a day. The applications begun 24 h after attachment of tick to the host, at the concentrations of 20% (TI), 40% (TII) and 60% (TIII). The ticks from Control Groups (CI and CII) were subjected to the same procedure, with applications of distilled water and 10% aqueous ethanol, respectively. Approximately 5 mL of solution was used in each application. On the fourth day of infestation, semi-engorged females were collected and maintained under controlled conditions in a BOD incubator before dissection for removal of samples. The collected ticks were dissected under a Leica EZ4 stereomicroscope in Petri dishes containing phosphate-buffered saline (PBS) solution (NaCl 0.13 M, Na_2HPO_4 0.017 M, KH_2PO_4 0.02 M, pH 7.2) for withdrawal of salivary glands samples.

All experimental procedures performed in this study were approved by the Ethics Committee in Animal Use, CEUA, UNESP, Rio Claro/SP, Brazil, protocol number 2206, decision number 021/2012.

2.4. Morphological analysis

2.4.1. Light microscopy (LM)

For histological analysis, the collected material was immediately fixed in formaldehyde (4% paraformaldehyde solution) for 72 h and, then, transferred to sodium phosphate buffer solution (NaCl 0.13 M, Na_2HPO_4 0.017 M, KH_2PO_4 0.02 M, pH 7.2), for 24 h. The samples were subsequently subjected to dehydration in an ascending series of ethanol (70, 80, 90 and 95%, for 20 min in each solution), overnight infiltration in Leica historesin, followed by polymerization with Leica historesin plus a hardener agent.

The resin blocks containing the material were sectioned on a Leica RM2255 microtome (thickness: 3 μm), and the sections subjected to staining with hematoxylin and eosin technique (HE), for the observation and description of the general morphology of the tissue (Junqueira and Junqueira, 1983). The microscopic slides obtained were mounted in Canada synthetic balsam and the material was photographed in a Leica DM150 light photomicroscope, equipped with a Leica ICC50HD camera, by means of the Leica LAS v.3.8 software.