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Research paper

Ultrastructural analysis of oocytes of *Rhipicephalus (Boophilus) annulatus* during postengorgement period as a tool to evaluate the cytotoxic effects of amitraz and deltamethrin on the germinative cells

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ABSTRACT

The present study utilizes the ultrastructural analysis of the fully engorged female *Rhipicephalus (Boophilus) annulatus* ticks, as a tool to evaluate the cytotoxic potential of deltamethrin and amitraz on the germinative cells. The ultrastructural analysis of the ovary of the normal (untreated) *R (B.) annulatus* revealed, oocytes in different stages of development, attached to the ovary wall by pedicel cells. The attachment site of oocyte to the pedicel cell was characterized by indentations of the plasma membrane. The oocyte was bound by three cell membranes *viz.*, plasma membrane, chorion and basal lamina. The stages of oocytes were differentiated ultrastructurally based on the features of their outer membrane and the number and size of lipid and yolk droplets. Detailed day wise analysis of ultrastructural changes in the ovary during the post-engorgement period revealed the occurrence of the degenerative changes from day five onwards. These appeared first in the oocytes followed by the germinal epithelium. The ovary of ticks treated with methanol (control), revealed similar topographies as that of a normal ovary except for the presence of very few oocytes with ring shaped nucleoli. Ultrastructurally, treatment with deltamethrin produced more prominent and extensive morphological alterations when compared to amitraz. In the case of ticks treated with amitraz, the oocytes of stage IV and V showed wavy and disrupted outer boundaries along with the loss of integrity of the yolk droplets. Uneven nuclear membranes of stage II oocytes and cristolysis of mitochondria of mature oocytes were the other changes noticed. Ticks treated with deltamethrin revealed prominent modifications such as, detachment of the basal lamina, wrinkled boundary, inconsistent nuclear membrane, ring shaped nucleoli and chromatin clumping in the case of the early stage oocytes (I and II), whereas swelling and cristolysis of mitochondria were seen in mature oocytes. The study further indicated that, in addition to the previous proven neurotoxic effects, these compounds act directly on the ovary of tick.

1. Introduction

The reproductive system of the ixodid female tick is mainly considered as a vital organ for the biological success of this group. A study of the reproductive characteristics of *Rhipicephalus (Boophilus) annulatus* in the laboratory through colony culture, showed that between 3140–5338 eggs ($P < 0.05$) were laid by each engorged female (Cen-Aguilar et al., 1998). The drugs which hinder the reproduction in ticks are considered superior to conventional acaricides for two reasons. First, they prohibit the evolution of subsequent generations. Second, since the reproductive system of the vertebrates and invertebrates are entirely different both physiologically and anatomically, the chances of the host toxicity are negligible. In addition to the cellular, molecular and pharmacological techniques (Abbott et al., 2012) for studying the modes of action of phytoacaricides on the reproductive system of ticks, several authors evaluated the histological and ultrastructural changes (Denardi et al., 2010; Arnosti et al., 2011; Denardi et al., 2011; Vendiramini et al., 2012; Sampieri et al., 2013; Remedio et al., 2015). Hence, the detailed analysis of normal histology and ultrastructure of the reproductive system of every tick species is very important. The

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Brazilian Center of Studies on Ticks Morphology (BCSTM) were performing several works principally aimed at the morpho-histology of the key systems of these ectoparasites (Denardi et al., 2004; Saito et al., 2005; Nunes et al., 2006; Oliveira et al., 2006, 2008; Furquim et al., 2008). The anatomical features of the ovary of *R. (B.) annulatus* and the changes during the development of the oocyte within the ovary during the postengorgement period were well analyzed by conventional microscopy (Sreelekha et al., 2015). The detailed ultrastructural analysis of the oocytes of *R. (B.) annulatus* was not extensively performed. Hence, there is an urgent need for a thorough ultrastructural exploration of the oocytes of *R. (B.) annulatus*. The present study tries to provide the basic information regarding the normal ultrastructure of the *R. (B.) annulatus* ovary.

The Brazilian group also analyzed the functional changes in the ovaries and salivary glands of ticks exposed to stressful conditions, such as the effects of chemical, synthetic, and natural compounds (Oliveira et al., 2008, 2009; Pereira et al., 2009; Roma et al., 2009, 2010; Denardi et al., 2010; Denardi et al., 2012; Remedio et al., 2015). They confirmed that these compounds produced a direct harmful effect on the germ cells, contradicting the earlier concept that acaricides act specifically in the tick nervous system, and any changes in other organs result from their indirect effect. In India, amitraz and deltamethrin are the commonly used acaricides for the control of ticks (Pradeep et al., 2012; Haque et al., 2014; Ghosh and Nagar, 2014). The recommended concentration for the on host application in cattle is 25 ppm for deltamethrin and 250 ppm for amitraz respectively (Mustafa et al., 1993; Nicolas et al., 2008). However, the effect of these acaricides on the cytomorphology of the tick reproductive system was not previously compared. Hence, the present study evaluates the cytotoxic effects of amitraz and deltamethrin on the germinative cells of *R (B.) annulatus.*

2. Materials and methods

2.1. Ticks and acaricides

The fully-engorged adult female *R. (B.) annulatus* that dropped to the ground from the naturally infested animals with no prior exposure to formulations containing deltamethrin or amitraz were collected manually. They were used within one hour after the dropping for the immersion experiments. Pure compounds of deltamethrin and amitraz (purity of 99 per cent) (AccuStandard, New Haven, CT, USA) were used in the present study. The dose for amitraz and deltamethrin were fixed as 300 and 30 ppm respectively in the present study, based on the results of adult immersion test (AIT) performed to find out the minimum effective concentration of these chemicals against *R. B. annulatus* (unpublished). The acaricides were diluted in methanol (100%) to achieve these concentrations.

2.2. Transmission electron microscopy

Eighty fully engorged female ticks were used for studying the ultrastructure of the ovary. They were divided into three groups of 20 ticks each and immersed for 2 min in separate beakers containing 10 mL each of methanol (100%), amitraz (300 ppm) and deltamethrin (30 ppm) respectively. Methanol was previously identified as safe for dissolving acaricides for assessing acaricidal properties, because of its low adult mortality and inhibition of fecundity (Ravindran et al., 2011). Hence, methanol was used as a vehicle control for the preparation of the acaricide standard solutions used in the study. Twenty normal ticks without any treatment (untreated controls) were also maintained throughout the study. The ticks were then recovered from the respective solutions, dried on an absorbent tissue paper towel, and placed in a separate plastic specimen tube (25 × 50 mm). They were kept in the biological oxygen demand incubator (BOD) at 28 °C and 80% relative humidity. After 24 h, they were taken out from the incubator, dissected in 0.9 per cent saline solution, and the ovaries were removed under a stereozoom microscope (M/s Carl Zeiss, Germany).

For the ultrastructural studies, the ovaries were fixed in Karnovsky's fixative for 36 h. The specimens were then prepared for electron microscopy as per standard protocols (Luft, 1961). Semi-thin sections were cut at 500 nm using an ultra-microtome (Leica, Germany) and stained with toluidine blue. The areas were selected, and subsequently ultra-thin sections (60 nm) were further cut and lifted on copper grids, stained by uranyl acetate and lead citrate. Later, they were examined and photographed in an electron microscope (FEI 200volts) at the All India Institute of Medical Sciences (AIIMS), New Delhi, INDIA.

3. Results

3.1. Ovary of untreated ticks

Ultrastructural analysis of the ovary of *R (B.) annulatus* revealed oocytes in different stages of development. The oocytes were attached to the ovary wall by the pedicel cells. The indentations of the plasma membrane were seen at the site of attachment of the oocyte to the pedicel cell. The oocytes were bound by three membranes *viz.,* plasma membrane, chorion and basal lamina. The stages of the oocytes were differentiated ultrastructurally based on the characters of their outer membrane and the number and size of lipid and yolk droplets (Table 1).

Oocyte I: The external membrane was disorganized with partially formed basal lamina. The cytoplasm was filled with a large number of rough endoplasmic reticulum. Mitochondria studded with a sizable number of cristae, were seen throughout the cytoplasm. Free ribosomes were also present within the cytoplasm. Enlarged nucleus was observed in most of the oocytes. The karyoplasm of this nucleus was occupied by euchromatin. The enlarged nucleolus with more granular content was also appreciated in this stage (Fig. 1A–C).

Oocyte II: The peripheral membrane was better organized in stage II oocytes with fully developed basal lamina and plasma membrane, compared to the stage I oocyte. The two layers of the basal lamina, the outer thinner and inner thicker layers were well appreciated, in this stage of oocyte. Abundant mitochondria were seen inside the oocytes. Prominent rough endoplasmic reticulum and small lipid droplets were seen during this stage of oocytes also. Clusters of lipid droplets were seen throughout the cytoplasm (Fig. 1D–F).

Oocyte III: In the case of stage III oocytes, the cytoplasm was filled with numerous small lipid and few yolk droplets. The size and number of the lipid droplets were increased at this stage of the oocyte compared to stage II oocyte. Chorion was deposited between basal lamina and plasma membrane (Fig. 1G–H).

Oocyte IV: Large number of yolk droplets, few lipid droplets and chorionic plates were the peculiarities in stage IV oocytes. Few mitochondria with short stubby cristae were seen (Fig. 1I).

Oocyte V: Lipid droplets were absent. The yolk droplets were merged at the center. Fusion of chorionic plates occurred during this stage (Fig. 1J).

3.1.1. Day wise changes of the ovary of R (B.) annulatus during postengorgement period

First and second stage oocytes were more prominent in the day zero and day one after engorgement. Day three of engorgement was characterized by the stage three and four oocytes. Mature oocytes, *viz.,* stage IV and V were prominent in the days four and five, after engorgement. Degenerative changes were observed in the oocytes since day two after engorgement, whereas, more prominent degenerative changes were evident from day five onwards. These changes appeared initially in the oocytes followed by germinal epithelium. Vacuolations, elongation and degenerative changes of the cytoplasm were the major changes observed in the germinal epithelium (Fig. 1K–L).

Limited early stage oocytes and many mature oocytes with deteriorating changes were seen abundantly during day five to day nine after engorgement. Swelling and cristolysis of mitochondria were the