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Histological evaluation of *Rhipicephalus (Boophilus) microplus* tick ovaries subjected to cobalt-60 ionizing radiation

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Abstract Females of *Rhipicephalus (Boophilus) microplus* ticks ingurgitated and in the prepost phase were submitted to doses of 0, 5, 10, 15, 20 and 25 Gy of Cobalt-60 with the aim of evaluating their effects on ovarian morphohistology with consequent establishment of the degree of damage caused to the reproductive organs of this species. For this purpose, 24 hours after exposure to Co-60 (142 Gy/h) the ectoparasites were dissected and had their ovaries removed and prepared for histology with haematoxylin and eosin staining. The results obtained showed that the 5 Gy dose radiation had a morphological aspect similar to the control standard adopted. The histological sections related to doses of 10 and 15 Gy did not cause many changes in the ovaries, except that changes were observed in the calf granules (size, distribution and staining pattern), as well as the extensive presence of cytoplasmic vacuoles in the ovocytes, especially in the region that makes contact with the ovocyte/pedicel, suggesting the occurrence of changes also in the physiology of the organ. In the ovaries exposed to doses of 20 and 25 Gy, severe alterations were observed in the organ as a whole, as well as in the germ cells (oocytes) which suffered alterations in size and shape, distribution of calf granules, involvement of the DNA present in the germinal vesicle (oocyte nuclei), besides the extensive cytoplasmic vacuolization, alterations which made the maturation of these cells impossible and consequently inhibited the production of new individuals.

Keywords: cobalt-60, histology, ovaries, tick

1. Introduction

Rhipicephalus (Boophilus) microplus belongs to the *Acari* subclass and *Ixodidae* family. It is a species of ticks that can be found in tropical climate countries and parasitizes several zootechnical interest animals, mostly bovine. In Brazil, the industrial agricultural market invested 5.954 billion of Brazilian reais in 2018, with 29% of this amount related to the use of antiparasitic products, and ruminants demanded over 55% of this share (Sindan 2018).

The Brazilian Ministry of Agriculture, Livestock, and Supply (MAPA), through the Normative Instruction - In no. 13 dated 29/May/2014, suspended the use of macrocyclic lactones (avermectins), long-term treatment endectocides in the Brazilian market, which directly affected beef cattle, dairy cattle of juvenile animals, and caprine/ovine raising; subsequently, this prohibition was revoked, on 27/Mar/2015, but the international market does not accept meat and dairy products and derivatives with avermectin residues. Therefore, alternative control methods for the parasites of these animals should be researched.

The prophylaxis methods used for hemoparasites are vector control, pasture rotation, chemoprophylaxis, vaccines, and, for some insect species, the sterile insect technique (SIT) has already been used. SIT is a method that breeds male target specimens, which are then sterilized using radiation and are subsequently released into the environment to mate wild females; as a consequence, there will be no offspring, resulting in a gradual decrease of the wild population without polluting the environment (Arthur et al 2015).

Accordingly, this paper aimed to provide additional information on the ionizing radiation effects on the tissues of *R.(B) microplus* tick species, particularly in those that are part of the ovaries, in which engorged females in the pre-egg-laying phase were exposed to different dose levels of Cobalt-60 and, subsequently, through the routine histological techniques applied, it was evaluated whether damages occurred or not; further, if damages occurred, what was the most aggressive dose level to

render the germ cell development (oocyte) unfeasible. In parallel with the histological results, evaluation of the genotoxicity of ionizing effects in these organs and egg-laying and hatchability rates was also performed, for reference and sterile insect technique development purposes.

2. Material and Methods

At the Vocational School *Escola Técnica ETEC Dr. Carolino da Motta e Silva*, in the city of Espírito Santo do Pinhal, State of São Paulo, latitude 22º11'27" south and longitude 46º44'27" west, 25 adult and engorged *R.(B.) microplus* females, in the pre-egg-laying phase, obtained from a natural infestation in dairy cows without treatment with acaricides for over 30 days were washed, dried, and selected from a morphological pattern as of their physical integrity, motility, and vivacity, with an average weight of 0.2g. At the Food Radiation and Radiation Entomology Lab [*Laboratório de Irradiação de Alimentos e Radioentomologia*] CENA-USP, in the city of Piracicaba, State of São Paulo, these females were subjected to radiation from a Co - 60 source at GAMMACELL® 220 Excel (MSD-Nordion), then from a Co - 60 source at GAMMACELL® 220 Excel (MSD-Nordion), in the dose levels of 0, 5; 10; 15; 20; and 25 Gy (142 Gy/h.) (5 females/dose level) at room temperature in the presence of oxygen.

2.1. Histology slide preparation

At the Histology Laboratory of the Biology Department [*Departamento de Biologia*] at UNESP, in the city of Rio Claro, State of São Paulo, the 25 females subjected to radiation received anesthesia by thermal shock in a refrigerator, for 5 minutes. Next, they were dissected with ophthalmic scissors and tweezers, in a Petri dish containing saline solution (NaCl - 7.5 g/L, Na_2HP_4 - 2.38 g/L, and KH_2PO_4 - 2.72 g/L in 1000 mL of distilled water), for ovary extraction. The material was then placed into Eppendorf tubes containing buffer, 4% paraformaldehyde 4% solution (pH 7.4) and remained there for 48 hours. Then the material was dehydrated with alcohol (70, 80, 90 and 100%) increased in 30-minute intervals, to be subsequently included into embedding resin (Historesina®, Leica, USA) for 48 hours. At the end of this period, the material was placed into resin plus polymerizer plastic molds and remained for a period in the oven (at 37 ºC) so the blocks could be hardened. They were then sectioned at LEICA® RM 2255 microtome, each section with 3 µm thickness, which were collected in glass slides previously cleaned (Figure 1).

The slides containing the sections were stained using Harris hematoxylin for 8 minutes. After washing them in running water, they were stained using aqueous eosin for 10 minutes, and washed once again in running water. After obtaining and staining the sections, the material slides were placed into wood support to be dried at room temperature. After that, they were covered with Canada balsam and coverslips (Junqueira and Carneiro 2000), then once again placed into the oven to dry for approximately 72 hours to be subsequently analyzed and documented under the brightfield microscope system LEICA® DM750.

Figure 1 A Pool of ovaries removed from females of each treatment to be processed for histology; **B** Wood blocks in which the materials already placed on resin were included; **C** Photo taken with the microtome model Leica RM2255, from which the histology sections were obtained.

3. Results

The histology results showed full preservation of the aspect in *R.(B) microplus* female ovaries not subjected to radiation, which were named here as control, as shown in Figure 2 (adapted from Saito et al 2005). Figure 2 shows several oocytes in different development stages (II to VI), attached to the ovary wall by a pedicel, which also features epithelial cells and the ovary itself. Some oocytes show a cell nucleus, which is referred to as a germinal vesicle in these cells.

3.1. Females subjected to radiation

3.1.1. 5 Gy Dose Level

The ovaries of engorged females exposed to 5 Gy dose levels had their histology aspect similar to the one observed in the ovaries of females allocated to the control group, that is, no significant changes were observed.

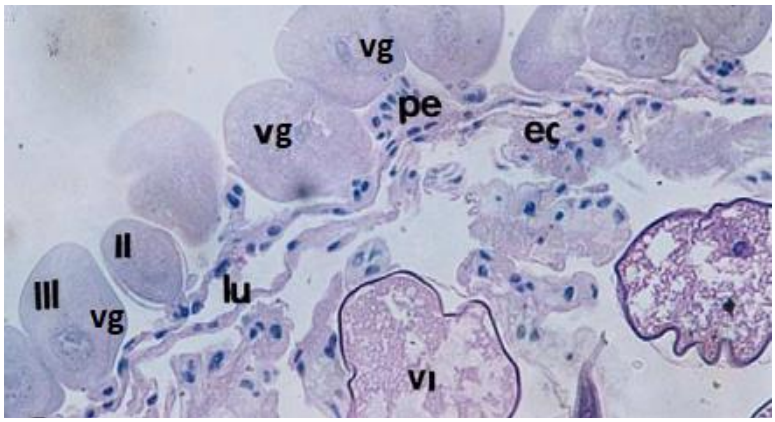

Figure 2 Histology section of a *R. (B) microplus* control ovary (female not subjected to radiation) featuring oocytes in different development stages (II-V), including stage VI, oocytes being reabsorbed and the respective remains to be reused by the own tick. **pe**: pedicel; **lu**: ovary lumen; **ec**: ovary wall epithelial cells; **vg**: germinal vesicle. Source: Adapted from Saito et al., 2005.

3.1.2. 10 and 15 Gy Dose Levels

The histology results of ovaries from females exposed to 10 and 15 Gy dose levels demonstrated changes in the ovaries, especially with the 15 Gy dose level (Figure 3). Changes in the size of oocytes, in their form, and in the cytoplasmatic constitution were observed, considering that an extensive vacuolization was detected inside the oocytes and were often found in the contact area between the oocyte cell limit and the pedicel cells. The latter also suffered changes, mainly cytoplasmatic vacuolization. In some ovary wall cells, it was possible to detect the presence of several pyknotic nuclei, that is, those with totally condensed chromatin, which indicates cell death.

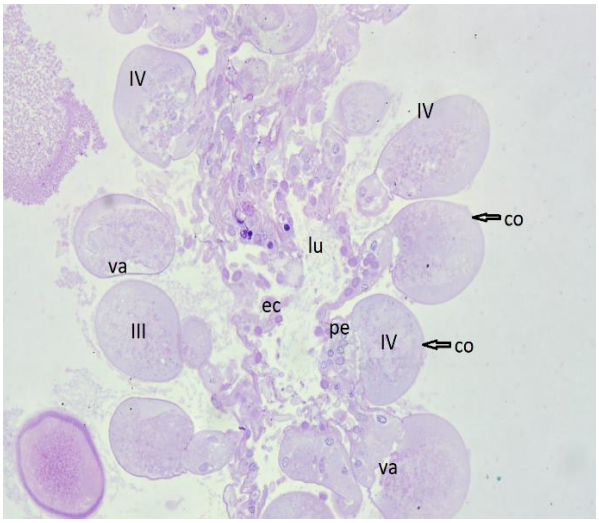

Figure 3 Ovary histology of females subjected to Co-60 radiation with a 15 Gy dose level. Oocytes in several development stages (I-IV) featuring deformations and different sizes can be observed. Extensive vacuolization can be seen in the cytoplasm, as well as ovary wall epithelial cells with pyknotic nuclei. **pe**: pedicel; **lu**: lumen; **ec**: ovary epithelium; **co**: chorion; **va**: vacuoles. Magnification 200 X.

3.1.3. 20 Gy Dose Level

For females subjected to radiation with 20 Gy (Figure 4), it was observed that the ovaries underwent changes immensely similar to those observed with the 15 Gy dose level (Figure 3). It is also noticeable that a great number of oocytes featured morphological change represented by median contrition. Another change found was that not all development stages occurred in these ovaries, with most of them in immature stages, in which the yolk granules were not totally deposited yet, as well as their essential components, namely, proteins, lipids, and carbohydrates, which suggest that radiation prevented the development of these cells and their advance to the subsequent stages.

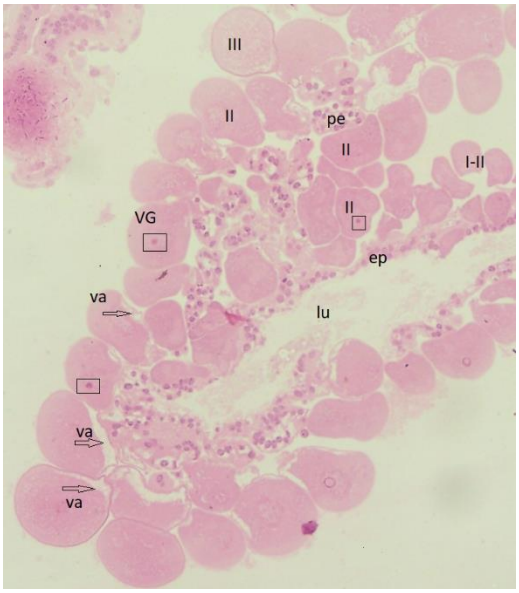

Figure 4 Histology section of an ovary from a female subjected to a 20 Gy dose level of Co-60. Notice that some oocyte development stages are not featured. Pedicel cells show vacuolization around their nuclei. **pe**: pedicel; **lu**: lumen; **ec**: ovary epithelium; **co**: chorion; **va**: vacuoles; **vg**: germinal vesicle. Magnification 200X.

3.1.4. 25 Gy Dose Level

The ovaries removed from females subjected to radiation with a 25 Gy dose level (Figure 5) suffered more morpho-histological changes when compared with ovaries from the control group females. Oocytes IV and V featured extensive and significant cytoplasmatic vacuolization, often concentrated in the contact area between pedicel and oocyte limit. Pedicel cells had hypertrophy and their nuclei showed an extensive vacuolization around them. It was clear enough that the germinal vesicle of several oocytes presented changes, mainly those in immature stages, including ring-shaped nucleolus, which is typical in cells with DNA damages.

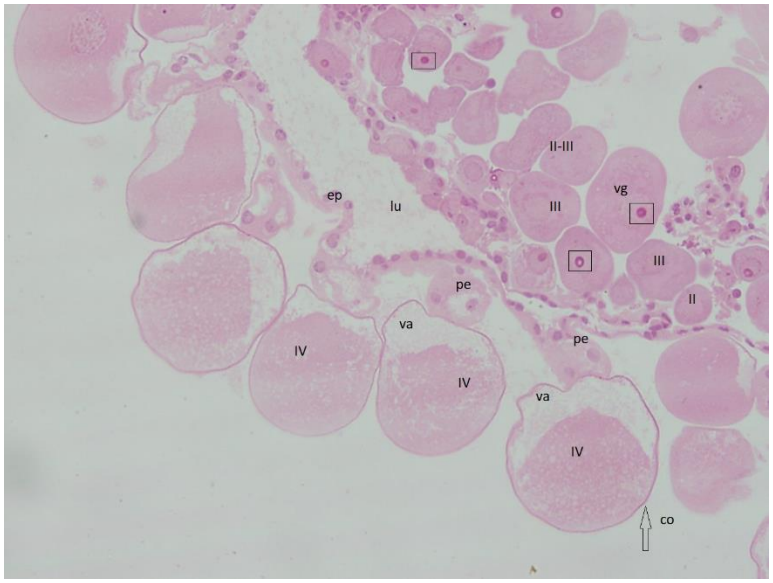

Figure 5 ovaries from females subjected to the 25 Gy dose level of Co-60, in which it is observed that the radiation effects were much more aggressive when compared with the effects of lower dose levels. Notice an extensive vacuolization in oocyte cytoplasm (II to IV stages), particularly in the oocyte/pedicel cells contact. Hypertrophy was identified in pedicel cells and extensive vacuolization around their nuclei. Magnification 200X. **pe**: pedicel; **lu**: lumen; **ec**: ovary epithelium; **co**: chorion; **va**: vacuoles; **vg**: germinal vesicle.

4. Discussion

Studies under development on the reproduction of ticks have been showing that there are morphological and histological differences in the ovaries of the species of *Argasidae* and *Ixodidae* families, and including among ixodidae/hard ticks (Denardi et al 2004; Oliveira et al 2006). Tick ovaries, just like in some insects, are panoistic, that is, they have no nurse

cells (Denardi et al 2004; Oliveira et al 2005; Oliveira et al 2006) and, specifically for ticks, no follicular cells are present as well. This organ has an epithelial cell wall and, where oocytes are attached to this wall, these cells are divided and form the pedicels, which are structures responsible not only for keeping the oocytes attached to the ovary wall, but also for capturing elements (proteins, carbohydrates, and lipids) from the hemolymph and place them inside the oocyte, where the yolk granules will be formed (Denardi et al 2004; Oliveira et al 2006; Ricardo et al 2007); since panoistic ovaries have no nurse cells, pedicel cells would replace them for this specific function.

Studies with tick ovaries have been intensified, since the reproduction system is responsible for providing the germ cells that will be developed and originate new individuals, therefore preserving the species (Camargo-Mathias et al 2017). Consequently, seeking alternatives able to inhibit the development of the reproduction system may represent a strategy to control these ectoparasites, which cause great damage to their hosts and the country and world economy.

Hitherto, ionizing radiation has been used as a strategy for population control of some insect species through SIT. In Brazil, the literature consulted reports the use of this technique in several insect species, such as the studies performed by Rocha et al (2014) to control fruit flies (*C. Capitata; A. fraterculus*); the study on the gamma radiation effects on sugarcane leaf cicadas (*Malhanarva posticata*) in the Brazilian northeast region (Rodrigues et al 1978); the study of moth sterilizations (*Stenoma catenifer*) (Silva et al 2007), and ongoing researches performed at MOSCAMED® with *Aedes aegypti*, are examples of this technique as another option for the biological pest control.

Therefore, morphological and biological parameters were consulted in other trials that used protocols with acaricides and ionizing agents in insects and mites of other species, to try to establish a relationship between the morpho-physiological findings of engorged *R.(B) microplus* females in the pre-egg-laying phase subjected to ionizing radiation for the development of SIT.

From the previously developed works that sought means to control ticks, we can mention Sousa et al (2013), which evaluated the morpho-histology of engorged *R. (B) microplus* female ovaries exposed to *Melia azedarach* green fruits hexanic extracts through histology techniques, which found that the ovaries had weight decrease, a predominance of oocytes in the immature stages, cell and nuclei decrease, extensive cytoplasmatic vacuolization, and disorganization of yolk granules, which certainly prevented these oocytes to continue developing, therefore resulting in an inhibition of offspring.

Another work developed by Oliveira (2010) exposed *Rhipicephalus sanguineus* females to the acaricide fipronil in 1, 5, and 10 ppm in vitro concentrations, obtaining results showing that the highest dose level used provoked morphological changes like the presence of few and small vacuoles and interruption of vitellogenesis, and cell death.

Camargo-Mathias et al (2017) reported that the use of pyrethroid deltamethrin (Butox®) in 25-200 ppm concentrations in *R. sanguineus* resulted in the highest concentrations used, morphological changes in ovaries, with effects throughout the oocyte development stages, disorganization of cytoplasm, deep vacuolization processes, chorion structure and secretion process changes, and chromatin segmentation causing damages in the germinal vesicle, therefore resulting in damages to the cell development and, as a consequence, unfertilized eggs.

The tissue injuries shown in the histological findings related to ovaries and their attachments in insects and mite exposed to pyrethroid agents and fipronil can be described as autophagic processes that would result from the acid hydrolysis activity (acid phosphatase), attested by extensive and numerous autophagic vacuoles and consequent cell destruction, as well as from apoptotic processes, whereas endonuclease actions would cleave the genetic material, resulting in chromatin condensation and cell death (Castanha-Zanoli et al 2012).

Other processes that would also describe the occurrence of hydrolysis and apoptosis in eukaryotic cells resulting from the exposure to pyrethroid agents and fipronil were described by Schimith et al (2017) and Palma (2014), respectively, as due to the mitochondrial respiratory dysfunction secondary to increased permeability of its membranes and, as a consequence, impairment of the electrochemical proton gradient and hence of the production of triphosphate adenosine (ATP). When the mitochondrial function is impaired, it leads to oxidative metabolism dysfunction, responsible for the control of apoptotic and necrotic cell pathways.

Therefore, if these insecticides did not promote the death of the target specimens, their reproductive functions would be impaired by the severe damages to the ovary and oocyte tissues.

The use of ionizing radiation through SIT aims at establishing the population control or eradication of a target species by sterilizing the females, aspermia or azoospermia in males, and inability to mate or dominant lethal mutation in both males and females, without any risks of environmental contamination or poisoning of natural predators due to the improper use of insecticides (Silva and Arthur 2004).

In this context, Cantwell and Henneberry (1964) performed a trial using male and female *Drosphila melanogaster meigen* flies, 3 to 4 days of age under exposure to Co-60 radiation between 80 and 160 Gy. Female ovaries were removed and, when analyzed under optical microscopy, a significant reduction of the organ's size was found, as well as full rupture of oocytes, follicle cells, and structural breakdown of adjacent tissues; also, no egg-laying occurred with the females subjected to the 160 Gy dose level.

El Naggar et al (2010) studied the morphology and histology of *Rhynchophorus ferrugineus* red palm weevil ovaries submitted to the 5, 10, and 15 Gy dose levels of gamma radiation and subsequently mated with non-irradiated males. The

gamma irradiation effect on ovary morphology revealed elongation of the terminal filament, rupture, separation, or shrinking of the outer follicle epithelium edge, degeneration or absence of nurse cells, and ruptured oocytes, as well as different grades of vacuolizations inside the oocytes at 15 Gy.

Walder and Calkins (1992) evaluated pre-emerging (*Anastrepha suspensa*) Caribbean fruit fly pupae irradiated with Cs-137 in the 0, 15, 20, 25, 30, 50, and 70 Gy dose levels and, subsequently, dissected the females born in the 1-, 5-, 10-, 15-, 20-, 25-, and 30-day intervals to evaluate the radiation effects on ovaries. They reported that, in the 15 Gy dose levels, the ovaries already presented significant conformation changes; with the 20 Gy dose levels, the ovaries presented a decreased number of oocytes inside them, and for dose levels equal to, or higher than 25 Gy, severe atrophy of ovaries and irreversible damages to germinal tissues occurred, without any apparent tissue regeneration.

When using any type of ionizing, electromagnetic (X and gamma rays), or particulate (neutrons and alpha/beta particles) radiation, the energy absorbed by a biological material can interact directly with cell targets and, among them, the DNA, resulting in changes in its structure or by direct action, through the breakdown of single and double molecule strands, between DNA and proteins or even in purine and pyrimidine bases, or by indirect action, when the radiation energy is transferred to DNA circulating molecules, particularly water, leading to the outset of chemical and biochemical reaction chains that result in the production of free radicals able to affect the cell structure and function. Both the direct and indirect effects produce several DNA molecule damages. However, the biological effects are closely related to the linear energy transfer (LET), which is defined as the measure of the average energy amount deposited on the tissue per distance traversed unit (keV/µm) (Hall 2000).

Currently it is not totally understood yet, how cells respond to oxidative stress resulting from irradiated cells, that is, if they die due to apoptosis or necrosis, or if they survive and proliferate.

Bakri et al (2005) gathered several publications about sterilizing dose levels by ionizing radiation in arthropods with insects and mite: from 130 to 400 Gy in Lepidoptera, from 30 to 280 Gy in Acari, from 40 to 200 Gy in Coleopteros, from 10 to 180 Gy in Hemipteros, from 20 to 160 Gy in Dipteros, from 20 to 150 Gy in Araneae, from 5 to 140 Gy in Dictyoptera, 100 Gy in Thysanoptera, and 4 Gy in Orthoptera.

Since there is a more appropriate development stage for the sterilization for each insect and mite species (Silva and Arthur 2004), aiming at the possibility of mating with their peers, the histology findings of this trial demonstrated that the ovary tissues and attachments exposed to dose levels of up to 15 Gy presented stochastic effects, which may result in effects applied to SIT, while the exposure to dose levels from 20 to 25 Gy presented deterministic effects represented by tissue denaturation.

5. Conclusions

In the analysis of histology slides of *R.(B) microplus* female ovaries in the pre-egg-laying phase, dissected and stained with H&E, 24 hours after the exposure to the Co-60 gamma radiation, and subjected to the 20 and 25 Gy dose levels, acute effects on ovary tissues and attachments were observed, with significant denaturation of oocytes, which would result in cell death and reduced egg-laying feasibility. The histology sections related to the 10 and 15 Gy dose levels did not show effective denaturation of pedicels or epithelial of ovaries; however, breakdowns of yolk granules and presence of numerous vacuoles in oocytes, mainly found in the proximal pedicel area, may support the cell degeneration or autophagy, therefore impairing the feasibility of laying a great number of eggs. The histology findings of tissues exposed to the 5 Gy dose level had morpho-histology aspects similar to the control pattern adopted.

Conflict of Interest

There was no conflict of interest.

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