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Thymol action on cells and tissues of the synganglia and salivary glands of *Rhipicephalus sanguineus* sensu lato females (Acari: Ixodidae)

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ABSTRACT

Thymol is a monoterpene present in plants of the families Lamiaceae, Verbenaceae and Apiaceae. Despite its proven acaricidal activity, little is known about the mechanism of action of thymol in ticks. Thus, the aim of this study was to perform a morpho-histochemical analysis of the synganglion and salivary glands of partially engorged females of the brown dog tick, *Rhipicephalus sanguineus* sensu lato (s.l.), exposed to thymol at different concentrations. Five groups were established: Control Group I (distilled water), Control Group II (ethanol 30%), Group III (thymol 1.25 mg/mL), Group IV (thymol 2.5 mg/mL) and Group V (thymol 5.0 mg/mL). The females were exposed to the treatments by the immersion method and subsequently kept in a climatic chamber (27 ± 1 °C and relative humidity 80 ± 10%) for five days. After this period, the synganglion and salivary glands were removed, and the hematoxylin/eosin morphological technique was applied. The von Kossa staining method with counterstaining neutral red was performed on the salivary glands. The results showed that females exposed to thymol had damaged synganglia, with pyknotic nuclei and vacuoles in the cortex and subperineurial regions, as well as rupture of the neural lamellae. The salivary glands showed type I acini with a dilated lumen. Cells with extremely vacuolated cytoplasm and fragmented nuclei were observed in type II and III acini. Type II acini of the females exposed to thymol revealed different calcium staining when compared to the Control Groups I and II. We therefore conclude that the salivary glands and synganglion are subject to changes in morphology and calcium levels when exposed to thymol at concentrations of 1.25, 2.5 and 5.0 mg/mL, demonstrating that this monoterpene has acaricidal potential on partially engorged females of *R. sanguineus* (s.l.)

1. Introduction

Rhipicephalus sanguineus sensu lato (s.l.) (Acari: Ixodidae), commonly called the brown dog tick, poses a threat to animal health because it can act as a vector of pathogens for dogs, among them *Ehrlichia canis, Babesia vogeli* and *Hepatozoon canis* (Aguiar et al., 2007; Baneth et al., 2007; Souza et al., 2010; Araújo et al., 2015a). This arthropod is also an important public health risk as a vector of *Rickettsia rickettsii* in areas of the United States, Mexico and Brazil, and *Rickettsia conorii* in the Mediterranean Basin (Wikswo et al., 2007; Cunha et al., 2009; Pacheco et al., 2011; Costa et al., 2011; Serra-Freire et al., 2011; Dantas-Torres et al., 2012; Szabó et al., 2013; Dantas-Torres and Domenico, 2015; Araújo et al., 2015b; Silva et al., 2017).

Control of *R. sanguineus* (s.l.) is normally performed by applying synthetic acaricides (Borges et al., 2007), however, constant use has caused selection of resistant ticks, causing a need for new control technologies. In this sense, control of ticks with essential oils, extracts and compounds isolated from plants may be a promising alternative (George et al., 2014; Araújo et al., 2015c; Rosado-Aguilar et al., 2017).

Thymol, a monoterpene found in plants of the families Lamiaceae, Verbenaceae, and Apiaceae, has already had its acaricidal activity demonstrated in different tick species and life stages, such as *Amblyomma sculptum* Berlese, 1888; *Dermacentor nitens* Neumann, 1897; *Rhipicephalus microplus* (Canestrini, 1888) and *R. sanguineus* (s.l.)

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(Mendes et al., 2011; Daemon et al., 2012a,b; Senra et al., 2013a,b; Novato et al., 2015; Araújo et al., 2015c). In addition, thymol has low dermal toxicity when tested on rats, mice and rabbits, reinforcing its potential for the development of acaricides (Rosado-Aguilar et al., 2017). Although the acaricidal activity of thymol has already been demonstrated, little is known about the places and forms of action on ticks (Mendes et al., 2011; Daemon et al., 2012a,b; Senra et al., 2013a,b; Novato et al., 2015; Araújo et al., 2015c). One exception is the work of Matos et al. (2014b), who demonstrated that thymol causes damage in oocytes of the *R. sanguineus* (s.l.) Thus, there is a need for histopathological investigations about its effects on other organs and tissues to better understand the modes of action.

Among the organs that deserve to be investigated, we can mention the synganglia (= central nervous system) of ticks, responsible for the control of all vital activities, and the salivary glands, which act in the synthesis and release of bioactive substances essential for the parasitic phase of ticks, attachment and feeding, control of host immune responses and transmission of pathogens (Lees and Bowman, 2007; Šimo et al., 2012; Roma et al., 2012; Sonenshine, 2013).

To investigate the action of thymol on the synganglia and salivary glands of ticks, a good alternative is to use histological and histochemical techniques. The action of *Ricinus communis* oil esters, permethrin and deltamethrin on the nervous system and salivary glands has already been demonstrated through the use of histological and histochemical techniques (Arnosti et al., 2011; Nodari et al., 2011, 2012; Roma et al., 2013; Pereira et al., 2017).

Therefore, this study had the objective of analysing the changes caused to the nervous system (synganglion) and the salivary glands of partially engorged *R. sanguineus* (s.l.) females exposed to different concentrations of thymol.

2. Material and methods

2.1. Obtaining R. sanguineus (s.l.) females

In order to obtain partially engorged females (four days of feeding), adults (males and females) were fed on rabbits of the Botucatu genetic group, with weight range of 3.0 to 3.5 kg, at the Department of Biology of the Rio Claro Institute of Biosciences, UNESP, São Paulo, Brazil. Fifty males and 100 females were placed inside each feeding chamber, which was divided into quadrants with a marker pen, allowing observation of the females' attachment area on the back of the hosts (Bechara et al., 1995). The first observation of the attachment area was made 8 h after releasing the ticks. Subsequent observations were performed every 3 h, from 8:00 a.m. until 7:00 p.m., until the females completed four days of feeding. Afterwards, the females were removed from the rabbits, washed in distilled water and submitted to the immersion test. The study design was approved by the Animal Ethics Committee (CEUA-UNESP), under protocol no. 005094-2/2.3.

2.2. Thymol

The thymol, obtained from the company Henrifarma Ltd., São Paulo, SP, Brazil, with purity ≥99.9%, was solubilized using 30% ethanol (ethanol and distilled water) (Matos et al., 2014a).

2.3. Immersion test

The immersion test was performed according to Drummond et al. (1973). Partially engorged females, after four feeding days, were divided into five groups, each with 10 individuals, and immersed in thymol solutions at different concentrations for five min. Two control groups were also formed, for a total of five groups: Control Group I (distilled water); Control Group II (30% ethanol); Group III (thymol 1.25 mg/mL); Group IV (thymol 2.5 mg/mL); and Group V (thymol 5.0 mg/mL) according to Matos et al. (2014a,b).

After immersion, all individuals were placed for 3 min on paper towel to remove excess liquid, transferred to Petri dishes (5.5 cm x 1.3 cm) and kept in a climatic chamber at 27 ± 1 °C and relative humidity (RH) of 80 ± 10% for five days (Eletrolab El 202 BOD-Biological Oxygen Demand – Eletrolab, São Paulo, Brazil). The ticks from the control groups and the treated groups were kept in different chambers to avoid interference caused by monoterpene volatility.

2.4. Histology and histochemistry

After five days of exposure, the synganglia and the salivary glands were dissected, fixed in 4% paraformaldehyde and dehydrated in an increasing ethanol series (70, 80, 90, 95 and 100%), at 15-minute intervals. The infiltration was carried out with Leica resin. Subsequently, the material was placed in plastic moulds and kept at 4 °C to retard prepolymerisation. The samples were sectioned (3 µm) and placed on glass slides for hematoxylin-eosin (HE) staining (Junqueira and Junqueira, 1983). The von Kossa method with counterstaining neutral red was applied to detect calcium in the cytoplasm of salivary gland cells, so they were fixed in neutral buffer at 10% (Tolosa et al., 2003).

In order to analyse the results, the samples were photographed under a photomicroscope (Leica DM750, São Paulo, Brazil) with built-in ICC50 HD camera in the Laboratory of the Department of Biology at the Biosciences Institute, UNESP, Rio Claro, SP, Brazil.

3. Results

3.1. Synganglia

3.1.1. Control groups

Individuals from Control Group I (distilled water) had the morphological characteristics of this tissue preserved, showing neurilemma, the external acellular membrane that covers the synganglion and also the perineurium, which is more internal and consists of the glial cells (Fig. 1A–C). The cells in the cortical region of the synganglion remained intact (Fig. 1A–C). The neuropil remained intact, as did the subperineurium, which is composed of glial cells and located between the cortex and the neuropil (Fig. 1A–C). In Control Group II (30% ethanol), the cortex and neuropil revealed alterations, showing small vacuoles in their cytoplasm (Fig. 1D–F).

3.1.2. Thymol treatments

Females from Group III (thymol 1.25 mg/mL) showed many cells with pyknotic nuclei in the cortex region, suggesting they were undergoing death (Fig. 1G–I). The cortex showed vacuolation in some cells and tissue regions in Groups IV and V, where the females were exposed to thymol at 2.5 mg/mL (Fig. 1J–L), and 5.0 mg/mL (Fig. 1M–O), respectively.

The subperineurium showed vacuolated cytoplasm, and part of the neural lamella revealed intense vacuolation in Group V. Some areas were difficult to visualise due to vacuole rupture (Fig. 1N–O).

3.2. Salivary glands

3.2.1. Control groups

Histological samples from Control Group I (distilled water) showed intact tissues. Type I, II, III acini maintained their original shape, which is regular and rounded. The ducts of the acini were dilated and intact (Fig. 2A–C).

Type I acini (Fig. 2A) (agranular) showed the largest and most heavily stained central cell. Peripheral cells were smaller and had smaller nuclei (Fig. 2A). The granular types II and III (Fig. 2B–C) showed all the cell types preserved. Type III acini (Fig. 2C), naturally larger than type II acini, were preserved.

Samples collected from Control Group II (30% ethanol) revealed type I acini (Fig. 2D) with a hypertrophied central cell, and in type II

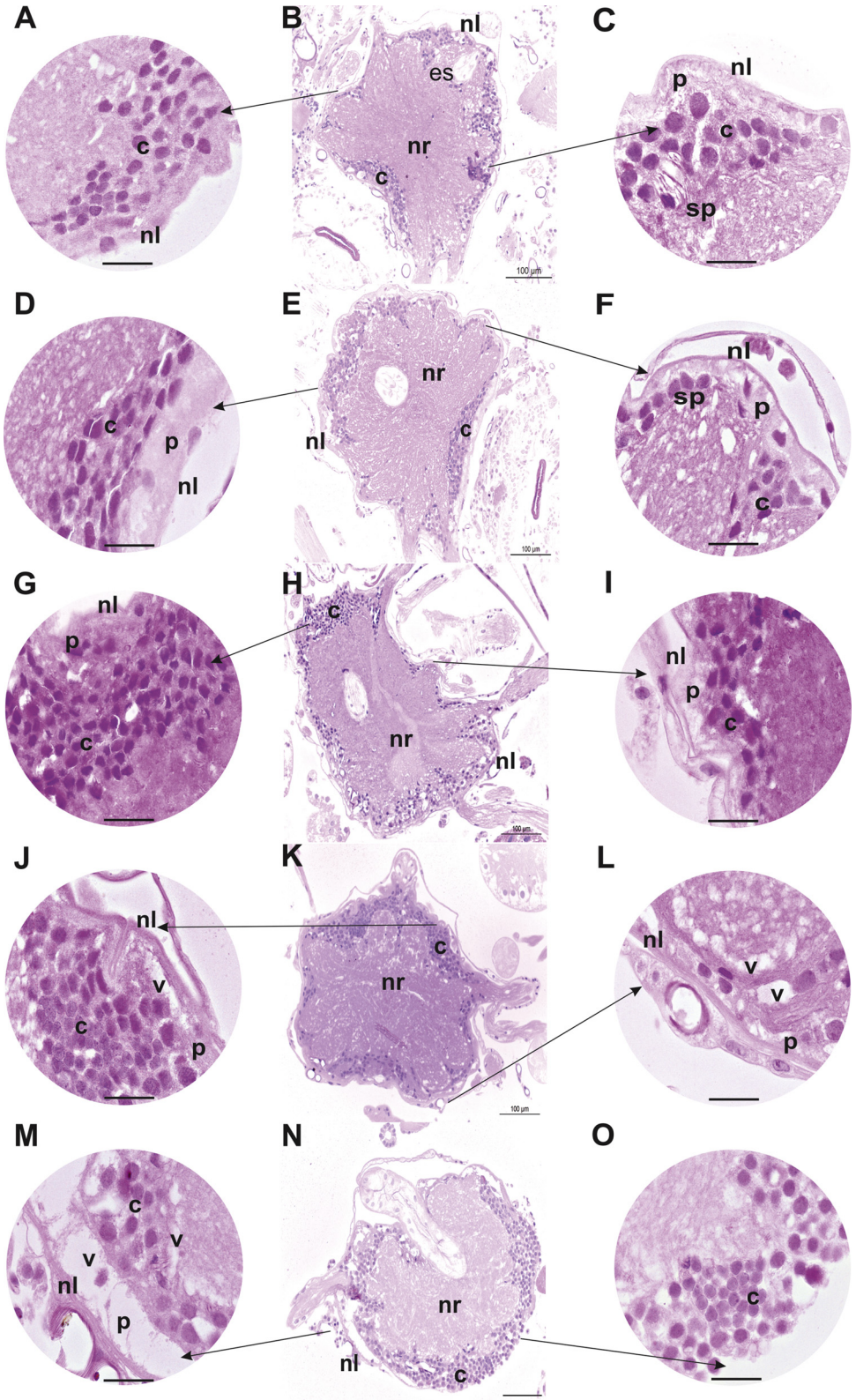

Fig. 1. Histological sections of the synganglion of partially engorged females (four days of feeding) of *Rhipicephalus sanguineus* (s.l.), stained with hematoxylin and eosin (HE). Control Group I (distilled water): A–C. Control Group II (ethanol 30%): D–F. Group III (thymol 1.25 mg/mL): G–I. Group IV (thymol 2.5 mg/mL): J–L. Group V (thymol 5.0 mg/mL): M–O. nl: neural lamella; np : neuropil; c: cortex; p: perineurium; sp: subperineurium; es: esphagus; v: vacuoles; Scale bar: 20 µm (Fig. 1: A, C, D, F, G, I, J, L, M, O); 100 µm (Fig. 1: B, E, H, K, N).

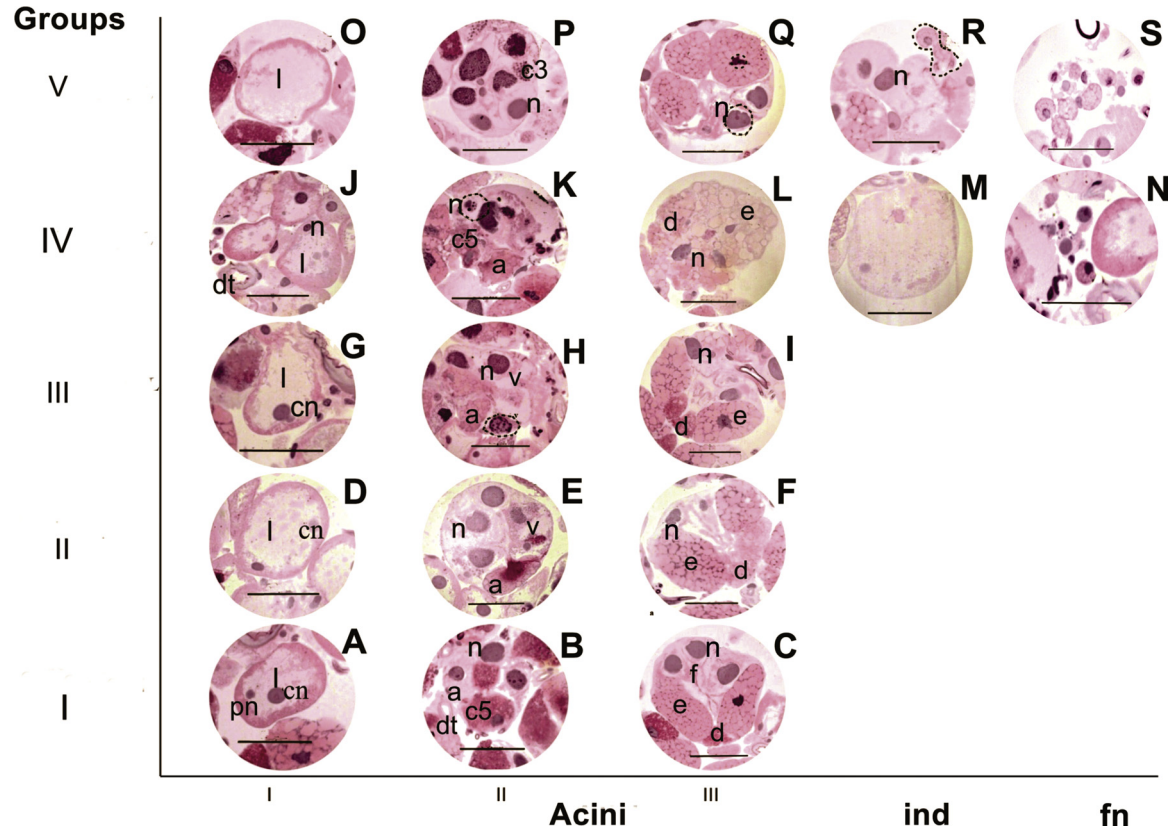

Fig. 2. Histological sections of the salivary glands of partially engorged females (four days of feeding) of *Rhipicephalus sanguineus* (s.l.), stained with hematoxylin and eosin (HE). Control Group I (distilled water): acini I (A), II (B) and III (C). Control Group II (ethanol 30%): acini I (D), II (E) and III (F). Group III (thymol 1.25 mg/mL): acini I (G), II (H) and III (I). Group IV (thymol 2.5 mg/ml): acini I (J), II (K), III (L) and undetermined (M), fragmented nuclei (N). Group V (thymol 5.0 mg/mL): acini I (O), II (P), III (Q) and undetermined (R), fragmented nuclei (S). l: lumen. nu: nucleus. *v*: vacuole. (—) fragmented nuclear material and fragmented nuclei. dt: duct. pn: peripheral nucleus. cn: central nucleus. fn: fragmented nuclei. Scale bar: 50 µm.

acini (Fig. 2E) all cells had a heterogeneous cytoplasm and areas not stained by eosin. Type III acini (Fig. 2F) preserved their histological characteristics.

3.2.2. *Thymol treatments*

The salivary glands had significant histological changes in all acini from Group III (thymol 1.25 mg/mL) (Fig. 1G–I). Type I acini (Fig. 2G) showed the central cell hypertrophied, and it was possible to observe irregular cell limits, causing alteration in shape and consequently in acinus morphology. Type II acini (Fig. 2H) showed cytoplasmic vacuolation near the nucleus and in the peripheral cytoplasm. Type III acini had the secretory granules ruptured, giving the cytoplasm an amorphous aspect (Fig. 2I).

Group IV (thymol 2.5 mg/mL) showed more intense histological alterations compared to Group III. Most type I acini were intact and had the central cell hypertrophied, although a few showed irregularities in their original form (Fig. 2J). Type II acini (Fig. 2K) revealed intense degeneration, as well as characteristics related to cell death processes, such as severe vacuolation in the cytoplasm and nuclei with chromatin marginalisation and fragmentation. Type III acini (Fig. 2L) were also undergoing advanced degeneration, with only the **d** and **e** cells being identifiable. Some acini could not be identified due to this intense degeneration (Fig. 2M–N).

Samples from Group V (thymol 5.0 mg/mL) suffered drastic alterations in their histology (Fig. 2O–S). The intensity of glandular cell degeneration and death was similar to that of Group IV. However, the presence of fragmented nuclei indicated more intense degeneration in Group V (Fig. 2S).

3.3. *The von Kossa technique (detection of calcium)*

In Control Group I (distilled water), the cytoplasm of type I acini (Fig. 3A) was not marked for calcium, whereas type II acini (Fig. 3B) showed a weakly stained cytoplasm. Type III acini did not show calcium deposits (Fig. 3C). The same was observed in type I, II and III acini from Control Group II (30% ethanol) (Fig. 3D–F).

In Groups III (thymol 1.25 mg/mL) and IV (thymol 2.5 mg/mL), the cytoplasm of type I acini (Fig. 3G–J) did not show calcium deposits, differing from what was observed in type II acini of both groups, which had moderate deposits (Fig. 3H–K). The cytoplasm of type III acini was also not marked for calcium in these groups (Fig. 3I–L).

The cytoplasm of type I acini (Fig. 3M) was negative for calcium in Group V (thymol 5.0 mg/mL), whereas type II acini (Fig. 3N) were strongly positive, although only in some regions. Type III acini did not show calcium deposits (Fig. 3O).

4. Discussion

Thymol and essential oils containing this compound have strong acaricidal activity for different species of ticks, presenting potential for the development of new acaricides (Gomes et al., 2012; Lage et al., 2013; Novato et al., 2015; Araújo et al., 2016d). However, little is known about the action of thymol in different organs and tissues of ticks (Matos et al., 2014b). In this study, we demonstrate the action of thymol on synganglia and salivary glands of *R. sanguineus* (s.l.) ticks.

In the control group I (distilled water), it was possible to verify that the synganglia and the salivary glands were intact, presenting morphological characteristics according to the description of Roma et al. (2012) and Furquim et al. (2008b), respectively. Thus, we can affirm

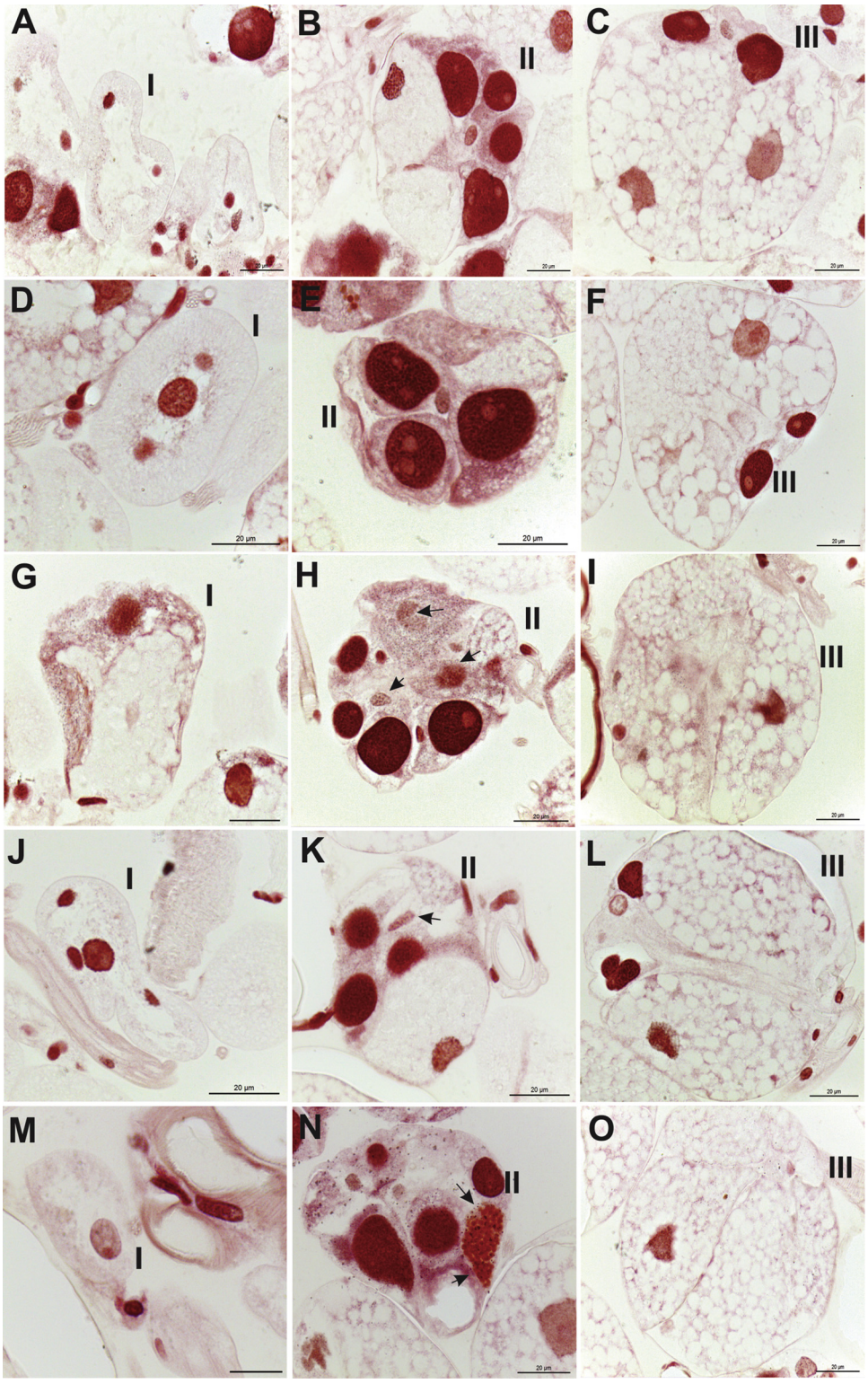

Fig. 3. Histological sections of the salivary glands of partially engorged females (four days of feeding) of *Rhipicephalus sanguineus* (s.l.), by the von Kossa technique with counterstaining neutral red. Control Group I (distilled water): acini I (A), II (B) and III (C). Control Group II (ethanol 30%): acini I (D), II (E) and III (F). Group III (thymol 125 mg/mL): acini I (G), II (H) and III (I). Group IV (thymol 2,5 mg/mL): acini I (*J*), II (K) and III (L), undetermined (M–N). Group V (thymol 5,0 mg/mL): acini I (O), II (P) and III (Q), indeterminate. (R–S) →: Calcium deposits in the cytoplasm. cn: central nucleus; pn: peripheral nucleus. ind: indeterminate. n: nucleus; fn: fragmented nucleus Scale bar: 20 µm.

that the deleterious changes observed in the other groups were correlated with the action of thymol.

The results of this study indicated morphological alterations in the synganglion of *R. sanguineus* (s.l.) females, as verified by Roma et al. (2013) for action of permethrin in the synganglia of this same tick, with cell agglomerates with pyknotic nuclei, in addition to nuclear marginalisation of cell chromatin, especially in the cortical region of the organ. Changes in cell nuclei, such as those reported in these studies, may indicate an apoptotic process, which could be triggered by the toxic action of permethrin (Roma et al., 2013), and thymol in the present study.

The females in group V (5.0 mg/mL thymol) showed displacement and rupture of the neural lamella, indicating severe damage. The neural lamella or neurilemma encloses the synganglion, thus being the first layer to have contact with the elements present in the haemolymph, which includes acaricides. Thus, this structure is the first protective barrier of ticks' nervous system, acting selectively in the permeability to the different compounds (Marzouk et al., 1985; Roma et al., 2013).

According to Sonenshine (2013), the neural lamella is composed of glial cells, and any modification in its structure, including rupture, leads to organ dysfunction.

The presence of vacuoles in the synganglion, mainly in the cells and tissue of the cortex region, also resulted from exposure to thymol at different concentrations. This can indicate that cells are self-defending from the action of thymol by seizing cytoplasmic remains or destroyed organelles within vacuoles. Some studies have demonstrated this mechanism as a form of protection to guarantee cell functionality (Furquim et al., 2008; Denardi et al., 2011). The synganglion impairment will affect the functionality of all other organs.

We chose the 4-day feeding time (partially engorged female), since it is the ideal period to evaluate the effect of acaricidal substances on the salivary glands of ticks, based on other studies, such as Nodari et al. (2011) and Nodari et al., (2012). In addition, the feeding period of females of the species *R. sanguineus* (s.l.) when maintained on host rabbits, can last from 7 to 12 days according to Troughton and Levin (2007), so the salivary glands of the females on the fourth day of feeding would be fully functioning, differing from the glands of females after 2 days of feeding, which would be in an early stage and could present undifferentiated cells, or females after engorgement, which could present glands already at the beginning of the degeneration process (which begins at the end of total engorgement, and intensifies after the end of feeding) (Amoreaux et al., 2003; Furquim et al., 2008; Sonenshine, 2013).

The exposure to different concentrations of thymol (1.25, 2.5 and 5.0 mg/mL) also caused tissue damage in the salivary glands, with presence of fragmented nuclei indicating more intense degeneration in Group V. The lumen of type I acini was dilated in samples from all the treated groups. Nodari et al. (2011) also observed dilation of the lumen of type I acini, when *R. sanguineus* (s.l.) fasting females were exposed to permethrin (206 ppm). According to Balashov (1972) and Coons et al. (1973), type I acini have an osmoregulatory function in ticks, and this luminal dilatation could be a strategy to eliminate the toxic products circulating in the haemolymph. Thus, in the present study, this dilation could be related to the attempt to eliminate thymol.

Morphological alterations were also observed in type II and III acini, in samples exposed to all thymol concentrations. Cell and tissue damages were seen, including: cytoplasmic vacuoles, loss of the original form in the acini and their cells, rupture of the cell limits and nuclear fragmentation, with the presence of cellular debris in the tissue. The natural degeneration of *R. sanguineus* (s.l.) salivary glands occurs by atypical apoptosis and results in intense cellular vacuolation (Furquim et al., 2008). However, although thymol accelerated the degeneration process of glandular tissue, it was still possible to observe the occurrence of typical morphological features of apoptosis, such as pyknotic nuclei and fragmented nuclei.

In the current study, the acaricidal effect of thymol was also evidenced in the salivary glands by histochemical analysis for the presence of calcium. This element was weakly detected in type II acini in the Control Group I and II, moderately in Groups III and IV (1.25 and 2.5 mg/mL), and with a strong positivity in Group V (5.0 mg/mL). Calcium has an important role in tick physiology, especially when feeding in the parasitic phase, because it signals the calcium-dependent pathways that act in immunosuppression and vasodilation of the host (Sauer et al., 2000; Furquim et al., 2014). Matos et al. (2014b) evaluated the presence of calcium in oocytes of *R. sanguineus* (s.l.) exposed to different concentrations of thymol, reporting an increase in the positivity of this mineral in oocytes I, II, III and IV. Calcium increase in the treated individuals could be related to the toxic action of thymol. Blenau et al. (2012) observed high calcium levels in cells of other thymol-treated arthropods, linking this response to the toxicity of this monoterpene, they suggested that such an increase is related to the alterations caused at the membrane receptors of the target cells.

In addition, increased calcium levels may also be related to the regulation of events during cell death, such as necrosis, which may be associated with the intracellular overload of these ions, and in cell death by apoptosis and autophagy, these events of cell death have already been demonstrated in cells of the salivary glands of ticks (Kumar et al., 1994; Amoreaux et al., 2003; Furquim et al., 2008; Zhivotovskya and Orrenius, 2011). Thus, the increase in calcium deposits may be related to the characteristics of the different death processes observed in cell morphology, such as the presence of vacuoles and nuclear fragmentation (Kumar et al., 1994; Zhivotovskya and Orrenius, 2011).

We observed that type II acini are the most affected by thymol. These acini contain c1 and c3 cells, which are responsible for synthesising proteins, lipids and polysaccharides, involved in the formation of the cement cone, and anticoagulant activities, necessary for attachment and feeding (Binnington, 1978; Walker et al., 1985; Fawcett et al., 1986; Nodari et al., 2012; Sonenshine, 2013). Damage caused by thymol in these cells can seriously impair ticks during the parasitic phase.

We can conclude that both the synganglia and the salivary glands underwent morphological changes when exposed to thymol at 1.25, 2.5 and 5.0 mg/mL, revealing the potential of this monoterpene for tick control, as well as elucidating some of the deleterious effects on organs and tissues.

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