Ticks and Tick-borne Diseases 10 (2019) 766–774

Contents lists available at ScienceDirect

Ticks and Tick-borne Diseases

journal homepage: www.elsevier.com/locate/ttbdis

Sublethal concentrations of acetylcarvacrol strongly impact oocyte development of engorged female cattle ticks *Rhipicephalus microplus* (Canestrini, 1888) (Acari: Ixodidae)

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ARTICLE INFO

Keywords:
Acetylcarvacrol
Morphology
Acaricide

ABSTRACT

Rhipicephalus microplus, commonly known as southern cattle tick, causes huge economic losses in the cattle industry. Its infestation affects the production of meat and milk and causes discomfort to hosts. In addition, it is the vector of *Babesia* spp. and *Anaplasma* spp. The most frequent method used to control these parasites is through synthetic acaricides. However, their indiscriminate use can be toxic for hosts and environment as well as cause selection of resistant ticks. Plant extracts and essential oils emerge as promising alternatives to manage tick infestation. Carvacrol, an aromatic monoterpene extracted from plants, has recognized antimicrobial, antioxidant, insecticidal, repellent and acaricidal activities. Acetylation of carvacrol is believed to enhance its nematicidal and acaricidal activities and to decrease its toxicity to hosts. Thus, the aim of this study was to evaluate the effect of different concentrations of acetylcarvacrol in the morphology of ovaries of engorged *R. microplus* ticks. The most remarkable morphological alterations found in the female germ cells were irregular and thicker chorion, decreasing in size and irregular shape of female germ cells (oocytes), cytoplasmatic vacuolization as well as ring-shaped nucleoli. These alterations were analyzed through a semi-quantitative method proposed in this study for ixodid ticks. Treatment group IV, which was exposed to 4.5 µL/mL of carvacrol acetate, showed the most significant alterations, and it was also statistically different when compared to control groups. Therefore, sublethal concentrations of acetylcarvacrol demonstrated to impact the reproductive system of *R. microplus* by causing several damages in the female germ cells. This would hinder the generation of new individuals, probably contributing for a long-term control of tick infestation.

1. Introduction

Ticks are widely spread hematophagous parasites divided into three families: Ixodidae, Argasidae and Nuttalliellidae. Several members of the Ixodidae family are known for their capacity to transmit various pathogens to humans and other animals, mainly due to their feeding habits (Sonenshine and Roe, 2014). Among over 60 species of the Brazilian Ixodidae fauna, *Rhipicephalus microplus*, known as southern cattle tick, is considered to be of great veterinary importance (Dantas-Torres et al., 2009; Nava et al., 2014). *R. microplus* infestation causes discomfort to animals and affects the development of herd and the production of meat and milk. In Brazil, it is estimated that cattle tick infestation accounts for an economical loss of US$ 968 million dollars per year (Rodrigues and Leite, 2013). In addition, this tick transmits *Babesia* spp. and *Anaplasma* spp., which occur even simultaneously and cause the "bovine parasitic sadness" (Pascoeti et al., 2016). Severe economic losses caused by this parasite around the world as well as the increasing number of reports of resistance to acaricides currently available on the market has led to the search of cost-effective control methods (Banumathi et al., 2017; Klafke et al., 2017).

Carvacrol is a phenolic monoterpene found in plant essential oils, especially oregano and thyme (*Origanum* sp. and *Thymus* sp.) (Kulisic et al., 2004). Besides its biological activities, such as antimicrobial, antioxidant, insecticidal and antifungal properties, carvacrol has already demonstrated a recognized acaricidal activity (Mechergui et al., 2016; Novato et al., 2015, 2018). Also, chemical modification of

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https://doi.org/10.1016/j.ttbdis.2019.03.010
Received 12 November 2018; Received in revised form 20 February 2019; Accepted 13 March 2019
Available online 16 March 2019
1877-959X/

carvacrol can enhance its biocidal effect (Cacciatore et al., 2015; Nesterkina et al., 2018). Acetylation, in particular, confers greater stability to carvacrol by conversion of the phenolic hydroxyl group, more susceptible to oxidation, into an ester group (Solomons et al., 2016). Moreover, acetylcarvacrol, also known as carvacrol acetate, exhibited antibacterial (Myangar and Patel, 2011) as well as acaricidal effect (Ramírez et al., 2016), with reduced toxicity to the host when compared to carvacrol (Andre et al., 2016).

The ovary of *R. microplus* is composed of a single tubular structure, continuous and delimitated by a wall of small epithelial cells, to which the oocytes in different developmental stages (I–VI) are attached through a pedicel (Saito et al., 2005). The mature oocytes are released into the ovary lumen and from there to the exterior. A single female cattle tick is able to lay up to three thousand eggs, which contributes immensely to the perpetuation of this parasite (Senbill et al., 2018). Therefore, control methods based on the vitellogenesis stand out as highly recommended to mitigate tick infestation.

Several studies over the last decade were based on morphological analysis to qualitatively demonstrate various alterations in the ovary of ticks, such as cytoplasmic vacuolization, irregular shape of the oocytes, folds in the cell membrane, as well as many ultrastrcutural alterations (Camargo-Mathias et al., 2017; Denardi et al., 2011; Oliveira et al., 2009, 2017; Remedio et al., 2014). Notwithstanding the great contribution of these studies in trying to find a cost-effective chemical compound to control tick infestation, they were based on qualitative analysis, which makes it difficult to compare the effectiveness of different substances evaluated. Barbosa et al. (2016) firstly introduced morphometric analysis in the study of tick's ovary by measuring cytoplasmic and germinal vesicle diameters. However, several other alterations found in their study remained as qualitative analysis.

Thus, the present study aimed to evaluate morphological changes in the ovary of *R. microplus* engorged ticks exposed to sublethal concentrations of carvacrol acetate, in order to demonstrate the interference of this compound in the reproduction of this parasite. Moreover, a novel method for semi-quantitative analysis to measure these alterations is proposed.

2. Material and methods

2.1. R. microplus ticks

Engorged *R. microplus* females weighing 160 mg in average were manually collected on naturally infested cattle without recent acaricide treatment, in the municipality of Piau (21°32′05.9″S 43°16′40.5″W), state of Minas Gerais, in the southeastern of Brazil. The ticks were washed in a sieve with tap water, dried on soft absorbent paper and selected under a stereomicroscope (Zeiss/Stemi 2000C/AxioCamERc 5 s) according to external morphological conditions, using10x eyepiece lens.

2.2. Synthesis of carvacrol acetate

Acetylcarvacrol was obtained by acetylation of carvacrol, 5-isopropyl-2-methylphenol, purchased from Sigma-Aldrich Co. (St Louis Mo, USA) in 99% purity (Fig. 1). For the reaction, 5 mL of carvacrol was added to a volumetric flask containing 25 mL of 10% sodium hydroxide solution at room temperature. Subsequently, 5.5 mL of acetic anhydride was added to the flask under cooling. The reaction mixture was left under stirring for 15 min. The oil obtained was separated from solution and characterized according to its melting point and by infrared (IR) spectroscopy (Moraes et al., 2013; Solomons et al., 2016).

2.3. Adult immersion test

The adult immersion test was performed according to Drummond et al. (1973). Sixty ticks were distributed in six groups of 10 individuals, among treated and control groups. The control group I (C1) was exposed to placebo (distilled water) and the control group II (C2) was exposed to 3% DMSO solution. The treated groups contained increasing concentrations of acetylcarvacrol diluted in 3% DMSO solution: group T1 (3.0 µL/mL), T2 (3.5 µL/mL), T3 (4.0 µL/mL), T4 (4.5 µL/mL). For each group, the ticks were immersed for 5 min in beakers containing the above concentrations of acetylcarvacrol as well as the control solutions. Afterwards, ticks were dried on soft absorbent paper and placed in Petri dishes at room temperature for 7 days, as suggested by Oliveira et al. (2008). Subsequently, five ticks of each group were randomly selected for histological analysis.

2.4. Histology

All the ticks were maintained in refrigerator for thermal shock anesthesia, then dissected on Petri dishes containing phosphate buffered saline solution (NaCl 0.13 M, Na_2HPO_4 0.017 M, KH_2PO_4 0.02 M, pH 7.2), under a stereomicroscope for collection of ovaries. The samples were then fixed in 4% paraformaldehyde solution for 72 h, dehydrated in an alcoholic series (70, 80, 90, and 95%) for 20-minute intervals, embedded in *Leica* historesin for 24 h at 4 °C, and transferred to plastic molds. After resin polymerization, 16 histological sections were obtained from each block at a thickness of 3.0 µm using a Lupetec MRP09 microtome and distributed on two glass slides. Sections were then stained with hematoxilin and eosin. The slides were dried and covered with Entellan® and a coverslip. Afterwards, sections of the ovary were examined and photographed using a capture system and image analysis, consisting of trinocular Olympus CX31 microscope (Olympus Optical Ltd. Brazil, São Paulo, SP, Brazil) and camera (SC30 Color CMOS Camera for Light Microscopy, Olympus Optical Ltd. Brazil, São Paulo, SP, Brazil).

2.5. Semi-quantitative and morphometric analysis

Semi-quantitative analysis of tick ovaries was based on work published by Marinho et al. (2014). From information available in literature, the main alterations described in the ovary of ticks subjected to acaricidal treatment were displayed in Table 1. Each alteration received an importance factor (w), ranging from 1 to 3, according to their relevance for development and surviving of ovarian cells: (1) minimal importance, when easily reversible after interruption of exposure to the compound; (2) moderate importance, when mostly reversible; and (3) high importance, when usually irreversible. After histological analysis, and based on information present in Table 1, the morphological changes found in this work were classified in scores (α), varying from 0 to 5. Zero represents features similar to control groups, and 5 means that the morphological changes are present in all cells evaluated. The individual index ($Index_{ind}$) was calculated by the sum of each alteration index (w x α), as follows: $Index_{ind} = \Sigma(w \times \alpha)$. The individual indexes were compared statistically by means of the Kruskal-Wallis test,

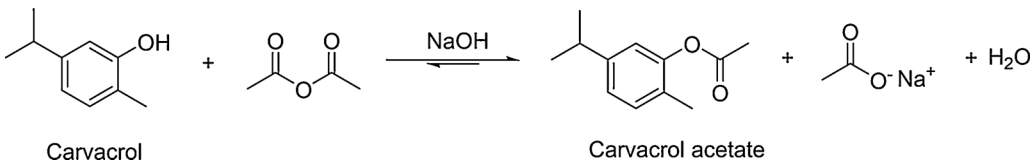

Fig. 1. Synthesis of acetylcarvacrol by the acetylation of carvacrol.

Table 1
Histological and ultrastructural changes observed in ovaries of ixodid ticks and their respective importance factors (w) for semiquantitative analysis.

Morphological alterations	Importance Factor (w)	References
Irregular chorion	1	(Arnosti et al., 2011; Barbosa et al., 2016; Denardi et al., 2010, 2011; Matos et al., 2014; Oliveira et al., 2016; Roma et al., 2010a,b, 2011;)
Non existent chorion	3	(Oliveira et al., 2009; Roma et al., 2010a)
Fragmented chorion	3	(Camargo-Mathias et al., 2017; Denardi et al., 2011; Oliveira et al., 2009, 2016; Sreelekha et al., 2017)
Thicker chorion	1	(Sampieri et al., 2012)
Changes in the size of oocytes	1	(Barbosa et al., 2016; Matos et al., 2014; Oliveira et al., 2017; Roma et al., 2010b)
Irregular oocyte shape	2	(Camargo-Mathias et al., 2017; Denardi et al., 2010, 2011; Matos et al., 2014; Oliveira et al., 2017; Sampieri et al., 2012; Vendramini et al., 2012)
Oocyte disappearance	3	(Oliveira et al., 2016)
Irregular basal lamina	2	(Oliveira et al., 2009, 2016, 2017; Roma et al., 2010a)
Reduction in the amount of microvilli	1	(Oliveira et al., 2009, 2017)
Absence/alteration of microvilli	1	(Oliveira et al., 2009; Sampieri et al., 2012)
Cytoplasmic vacuolization	2	(Arnosti et al., 2011; Barbosa et al., 2016; Camargo-Mathias et al., 2017; Denardi et al., 2010, 2011; Matos et al., 2014; Oliveira et al., 2009, 2016, 2017; Remedio et al., 2014; Roma et al., 2010a,b, 2011; Vendramini et al., 2012)
Cytoplasmic disorganization	2	(Oliveira et al., 2016; Remedio et al., 2014; Sreelekha et al., 2017)
Presence of acidophilus areas in the cytoplasm	1	(Remedio et al., 2014)
Increase of yolk granules size	1	(Arnosti et al., 2011; Barbosa et al., 2016; Camargo-Mathias et al., 2017; Denardi et al., 2010; Matos et al., 2014; Oliveira et al., 2016)
Decrease in size/amount of yolk granules	1	(Arnosti et al., 2011; Oliveira et al., 2017; Vendramini et al., 2012)
Breaching/fusion of yolk granules	2	(Matos et al., 2014; Sreelekha et al., 2017)
Presence of myelinic figures	2	(Denardi et al., 2012; Oliveira et al., 2017; Roma et al., 2010a)
Decreasing in the amount of lipid droplets	1	(Oliveira et al., 2009, 2017)
Degenerating/Irregular/Fragmented Protein Granules	1	(Denardi et al., 2011; Oliveira et al., 2009; Roma et al., 2010a)
Irregular mitochondria	1	(Denardi et al., 2012; Oliveira et al., 2009)
Absence/alteration of mitochondrial inner membrane	2	(Denardi et al., 2012; Oliveira et al., 2009; Remedio et al., 2014; Roma et al., 2010a; Sampieri et al., 2012; Sreelekha et al., 2017)
Strongly electrondense mitochondrial matrix	1	(Remedio et al., 2014)
Decrease in the amount of mitochondria	1	(Oliveira et al., 2017; Sampieri et al., 2012)
Disorganized/dilated/vesiculated RER	2	(Denardi et al., 2011, 2012; Oliveira et al., 2009; Remedio et al., 2014)
Disorganized cytoskeletal elements	2	(Denardi et al., 2012)
Irregular nuclear envelope	1	(Arnosti et al., 2011; Denardi et al., 2010,2012; Oliveira et al., 2009; Remedio et al., 2014; Roma et al., 2011; Sampieri et al., 2012; Sreelekha et al., 2017)
Picnotic/degenerated nucleus	3	(Arnosti et al., 2011; Oliveira et al., 2016; Vendramini et al., 2012)
Disorganized/vacuolized/fragmented nucleolus	3	(Arnosti et al., 2011; Denardi et al., 2010,2011; Oliveira et al., 2009; Remedio et al., 2014; Roma et al., 2010a; Vendramini et al., 2012)
Disappearance of the nucleus	3	(Denardi et al., 2012; Oliveira et al., 2016)

followed by Dunn's multiple comparison *post-hoc* test ($\alpha < 0.01$), using GraphPad Prism software (version 7.00).

Morphometric analysis of oocytes was performed on ImageJ software (NIH). The oocytes were classified in accordance with the developing stages described for *R. microplus* (I to VI) (Saito et al., 2005). Then, measurements of cytoplasmic area of 10 oocytes for each stage for each animal were taken. Analysis of oocytes I, II and III was performed only in cells in which germinal vesicle was evident. Because the germinal vesicle is rarely observed in stages IV and V, these cells were randomly selected for the measurements (Barbosa et al., 2016). Type VI oocytes were not found. The cytoplasmic area of the oocytes were compared statistically by means of the one-way ANOVA test, followed by Tukey's *post-hoc* analysis ($\alpha < 0.05$), using GraphPad Prism software (version 7.00).

3. Results

3.1. Histopathological analysis

3.1.1. Control group I (distilled water)

The results obtained in the analysis of the ovaries of ticks exposed to distilled water corroborate with the description of the female reproductive system of *R. microplus* (Saito et al., 2005). Below, a summary of the morphological characteristics of the oocytes in this group (Fig. 2A–B):

Oocytes I represent the smallest cells of the line, increasing progressively throughout the vitellogenesis. These cells presented a rounded shape, slightly irregular, with homogeneously basophilic cytoplasm. The nucleus was more basophilic than the cytoplasm, presented a round shape and was located in the central region of the cell. The nucleolus was poorly evident, but stained more intensely in purple than the nucleus.

Oocytes II presented an elliptical shape, with fine and weakly basophilic homogeneous granulations. The nucleus exhibited a slightly irregular shape and evident nucleolus. Externally, involving the oocyte, a basal membrane stained strongly in purple was observed. Oocytes with intermediate characteristics between stages II and III presented an elongated region in contact with the pedicel, without reactivity to the dyes.

Oocytes III exhibited rounded shape and small uniform acidophilic granulations in the cytoplasm. The nucleus displayed characteristics similar to those observed in the previous stage, although it was eccentric and close to the pedicel. It was observed the beginning of the chorion deposition by the appearance of a thin acidophilus layer externally to the basal membrane.

Oocytes IV presented a rounded shape, with a slightly irregular surface and a thicker chorion than the previous stage. The nucleus, rarely observed, was located at the basal pole of the cell. In the cytoplasm, yolk granules with rounded or irregular shape were visualized, the smaller ones being located in the central region. In addition, distinct reactivity of the yolk granules was observed, some more basophilic and others more acidophilc, with the presence of small spaces between them without reactivity to the dyes.

Oocytes V, the largest cells of the vitellogenesis, exhibited a fully deposited chorion and thicker than in the previous stage. The yolk granules were larger, with uniform size and round shape. The same distinct staining pattern of the yolk granules presented in oocyte IV was observed in this stage.

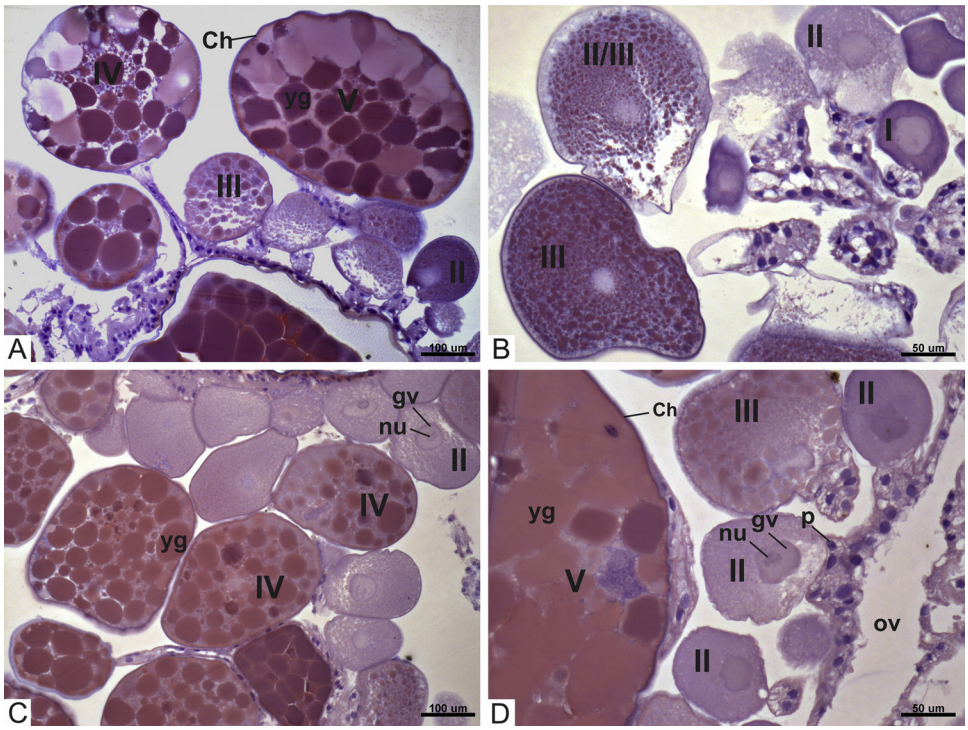

Fig. 2. Histological sections of ovaries of *Rhipicephalus microplus* engorged ticks (control groups). (A–B) Control Group I (distilled water); (C–D) Control Group II (3% DMSO solution). Legends: (I–V) stages of oocyte development, (Ch) chorion, (gv) germinal vesicle, (nu) nucleolus, (ov) oviduct, (p) pedicel, (yg) yolk granules. Bars: (A, C) 100 µm; (B,D) 50 µm.

3.1.2. Control group II (3% DMSO)

This group (Fig. 2C–D) showed no differences in comparison to the control group I. However, one specimen had cytoplasm regions slightly more vacuolated than the others, especially in oocytes III and IV.

3.1.3. Treatment group I (3.0 µL/mL)

In this group (Fig. 3A–B), oocytes I and II showed small vacuolated regions in the cytoplasm and the nucleus was weakly stained. The presence of vacuolated nucleolus was observed in two specimens. In stages III and IV, the cells were slightly vacuolated. In stage V, irregular cell shape and chorion detachment were observed. In general, the changes were observed in small extensions of the tissue.

3.1.4. Treatment group II (3.5 µL/mL)

In this group (Fig. 3C–D), oocytes I and II exhibited characteristics similar to the previous treatment, although more vacuolated. In the transition stage between stages II and III the vacuolization was more evident than in the controls, occupying, in some cases, about half of the cell. Some oocytes in these stages presented a ring-shaped nucleolus. In the subsequent stages, the chorion was thicker in two specimens, evidenced by the presence of a thick basophilic region around the cell. Chorion detachment and irregular oocyte shape were also observed in this treatment.

Treatment Group III (4.0 µL/mL)

Ovaries of the individuals exposed to this treatment presented similar alterations to the previous treatment (Fig. 3E–F). Vacuolization was observed at all stages of the vitellogenesis. Oocytes V exhibited irregular and detached chorion as well as apparent decrease in cell size, which was confirmed by morphometric analysis.

Treatment Group IV (4.5 µL/mL)

Ovaries of the individuals in this group showed the most significant alterations when compared to the other groups (Fig. 4A–F). Oocytes I and II presented irregular shape and less intense staining of the nucleus in comparison to controls. Most of the cells exhibited a ring-shaped nucleolus. In addition, early deposition of the chorion was observed in this treatment. In stages IV and V, the cells presented great vacuolization, with irregular shape and chorion thicker than the controls. Also, it was observed the fusion of yolk granules (Fig. 3D) and vacuoles around them (Fig. 3E) in oocytes V. An inverse relationship between chorion thickening and vacuolization of oocytes was observed in this treatment. In other words, the more vacuolated cells showed a similar chorion to the controls and the ones that presented thicker chorion were less vacuolated. In addition, oocytes in all development stages were visibly smaller when compared to controls.

3.2. Morphometric analysis

Measurements of cytoplasm area of oocytes (I–V) showed decreased values in all treatment groups in comparison with the control groups (Table 2). Control groups I and II did not show statistical differences. The oocytes I were equally affected by carvacrol acetate, showing no difference between treatments. For stages II to V, it was observed that treatment group IV had the most significant diminution of cytoplasm area of oocytes. This group, which was exposed to the highest concentration of carvacrol acetate, had the cytoplasm area heavily reduced when compared to the other groups ($p < 0.05$).

3.3. Semi-quantitative analysis

The morphological changes found in ovaries of *R. microplus* ticks with their respective alteration indexes are shown in Table 3. Control group I did not exhibit any alterations while one specimen of control group II showed slight changes in the size and shape of oocytes. The sum of the alteration indexes for each animal in each group was used to calculate the individual index, which is shown in Table 4. Control groups I and II did not show statistical differences in the values. Treatment groups I to IV are statistically equal. However, treatment group IV presented a higher (15.20 ± 4.21) and statistically different individual index when compared to the control groups ($p < 0.01$).

4. Discussion

Many plant species synthesize secondary metabolites to protect them against pathogens and pests (Benelli, 2015; Erb and Robert, 2016; Shitan, 2016). These self-defense mechanisms can have fungicidal (Tayel et al., 2016), bactericidal (Tian et al., 2018) and/or antioxidative

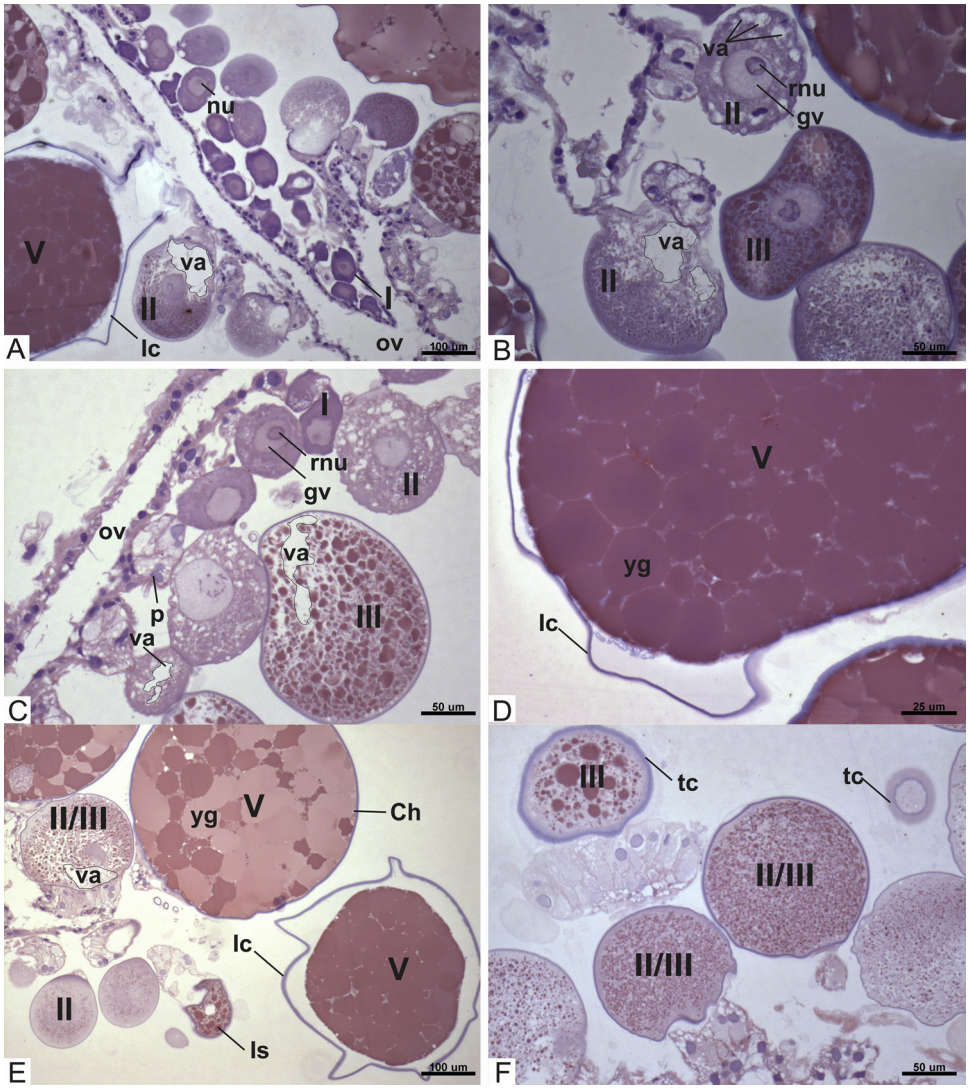

Fig. 3. Histological sections of ovaries of *Rhipicephalus microplus* engorged ticks exposed to acetylcarvacrol. (A–B) Treatment Group I (3.0 µL/mL); (C–D) Treatment GroupII (3.5 µL/mL); (E–F) Treatment Group III (4.0 µL/mL). Legends: (I–V) stages of oocyte development, (Ch) chorion, (gv) germinal vesicle, (Ic) irregular chorion, (Is) irregular oocyte shape, (nu) nucleolus, (ov) oviduct, (p) pedicel, (rnu) ring-shaped nucleolus, (tc) thicker chorion, (va) cytoplasmic vacuolization, (yg) yolk granules. Bars: (A,E) 100 µm; (B,C, F) 50 µm; (D) 25 µm.

properties (Shah et al., 2014). Certain plants used within the medical and food industries are known to be acaricidal, or toxic against ticks (Benelli et al., 2016). Among the various acaricidal compounds that have been isolated, carvacrol, which is used as a food preservative, stands out as a promising research candidate in the search for more efficacious strategies to manage tick infestations (Mahian and Sani, 2016; Novato et al., 2015). The promise of carvacrol, as in many other research chemicals, arises from the ability to chemically alter its functional groups to create semi-synthetic analogs that may be more biologically active than the parent molecule itself (Kim et al., 2015). Several semi-synthetic carvacrol derivatives have already been shown to exhibit antihelminthic (Moraes et al., 2013), insecticidal (Bagul et al., 2018) and acaricidal properties (Novato et al., 2018; Ramírez et al., 2016). Additionally, acetylcarvacrol presented a greater mortality rate on *R. microplus* larvae when compared to carvacrol (Ramírez et al., 2016). Besides mortality itself, sublethal concentrations of many chemicals are able to affect fundamental biological processes associated with the survival of ticks (Camargo-Mathias et al., 2017).

The reproductive system of ticks has been the target of several studies aiming at inhibiting the ovary development by using sub lethal concentrations of various drugs (Camargo-Mathias et al., 2017; Matos et al., 2014; Oliveira et al., 2016; Remedio et al., 2014). These chemicals frequently act slowly on the tick's physiology, and that is the reason of an observation period of seven days established in this study (Roma et al., 2010a). Thus, this research brings relevant data on the impact of acetylcarvacrol on the female germ cells of *R. microplus*, crucially important in a scenario in which reports of tick resistance to acaricides are increasing (Klafke et al., 2017).

The morphological alterations found in *R. microplus* ovary exposed to acetylcarvacrol were dose-dependent, as evidenced in treatment group IV, which was exposed to the highest concentration of the drug (4,5 µL/mL). Morphological alterations found in the female germ cells were irregular and thicker chorion, decreasing in size and irregular shape of oocytes, cytoplasmatic vacuolization as well as ring-shaped nucleolus. Similar results were found in germ cells of semi-engorged *Rhipicephalus sanguineus* sensu lato female ticks using 50 µL/mL carvacrol (Souza et al., 2019). It is worth pointing out that this concentration of carvacrol tested by Souza et al. (2019) is over 10 times greater than the concentration tested in treatment group IV in this study. This indicates that acetylation of carvacrol enhances enormously its capacity to impair oogenesis.

After mating, ovary asynchronously engages in oogenesis to

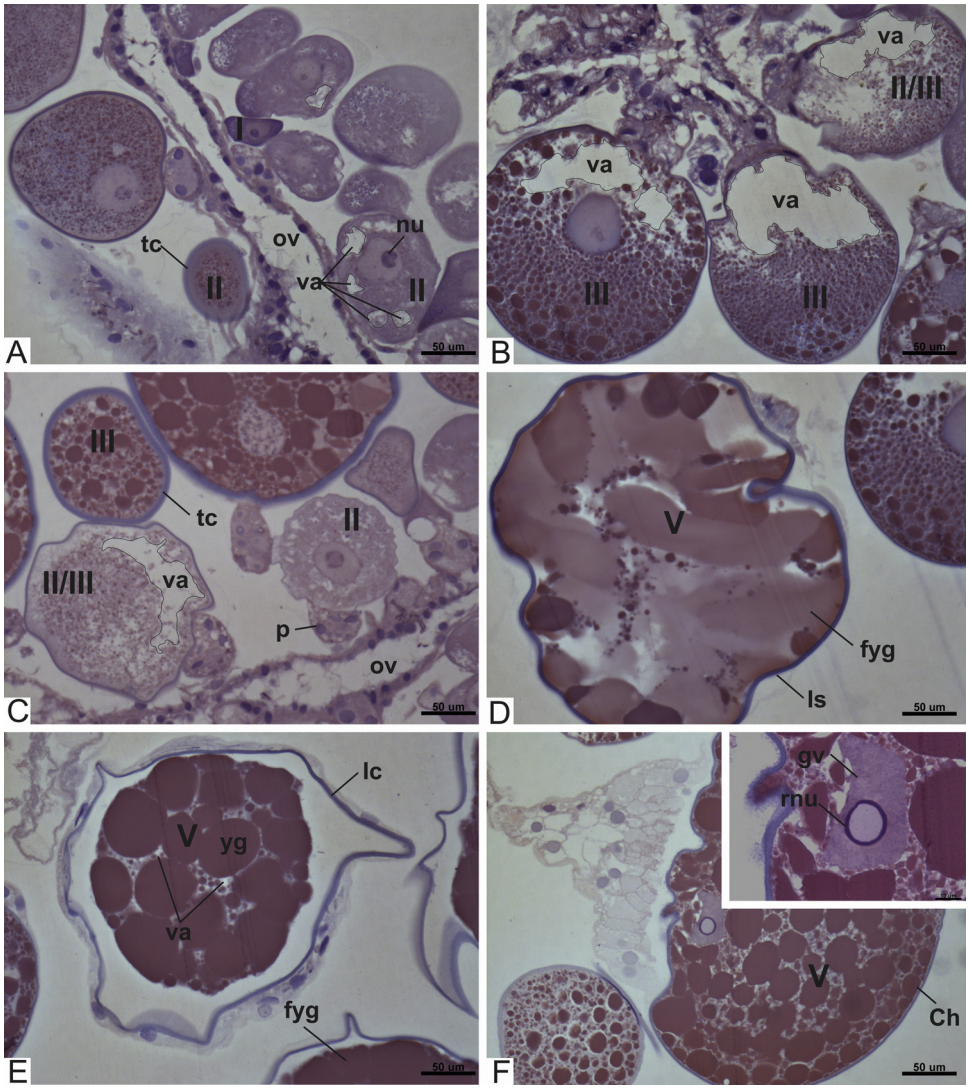

Fig. 4. (A–F) Histological sections of ovaries of *Rhipicephalus microplus* engorged ticks exposed to the highest concentration of acetylcarvacrol (4.5 µL/mL). Legends: (I–V) stages of oocyte development, (Ch) chorion, (fyg) fused yolk granules, (gv) germinal vesicle, (Ic) irregular chorion, (Is) irregular oocyte shape, (nu) nucleolus, (ov) oviduct, (p) pedicel, (rnu) ring-shaped nucleolus, (tc) thicker chorion, (va) cytoplasmic vacuolization, (yg) yolk granules. Bars: (A–F) 50 µm.

produce eggs, being possible to find oocytes in different development stages (Saito et al., 2005). During the vitellogenesis, a process by which protein precursors and other molecules are produced by ovarian and extraovarian tissues and accumulated inside the germ cells, the size of the oocytes increases considerably (Estrela et al., 2010; Xavier et al., 2018). Our results showed that oocytes I presented smaller cytoplasm area when compared to the control groups, but they were equally affected by different concentrations of carvacrol acetate. Interestingly, for stages II to V, it was observed that treatment group IV had the most significant diminution of cytoplasm area of oocytes. Therefore, acetylcarvacrol seems to impact not only the oogenesis itself by damaging the oocytes morphology, but also the vitellogenesis by impairing the synthesis of macromolecules. As the intake of the precursors that are responsible for increasing in size of oocytes is more significant after stage I (Xavier et al., 2018), the dose-dependent diminution of cytoplasm area of the germ cells in stages II to V indicates that it is probably caused by the drug. Thus, acetylcarvacrol may affect the endocytosis of macromolecules or their synthesis or both. Consequently, the reduction in size of oocytes may impair *R. microplus* reproduction by generating eggs with reduced amount of nutrients.

According to Saito et al. (2005), the chorion of *R. microplus* is composed of an electron-dense material produced by the oocyte. As the chorion is produced, it fuses to the plasmatic membrane of the oocytes, starting at stage III of oogenesis (Coons and Alberti, 1999). This material serves as a protective barrier against adverse situations, such as desiccation, mechanical shocks, predation, and changes in humidity and temperature. In addition, the chorion has micropores that allows the oxygenation of the embryo (Hinton, 1981; Sampieri et al., 2012). We observed that the chorion of treatment groups II, III and IV was considerably thicker when compared to control groups. Also, it exhibited irregular format in all of the treatment groups. These findings corroborate reported data by various authors (Arnosti et al., 2011; Barbosa et al., 2016; Matos et al., 2014; Sampieri et al., 2012). We believe that chorion thickening is probably due to a protective response against carvacrol acetate. However, as the chorion allows the input of oxygen inside the egg, a thicker layer would prejudice the survival of the embryo by interfering in gas exchange.

Among all morphological alterations described in the ovary of ticks subjected to acaricidal treatment, cytoplasmic vacuolization is the most frequent one. Cytoplasmic vacuoles were present in oocytes of all treatment groups. Several authors describe this vacuolization process as an attempt of the cell to isolate toxic compounds or even as an autophagic process of degradation or recycling of damaged cytoplasmic components (Arnosti et al., 2011; Roma et al., 2011). Interestingly, the

Table 2
Mean ± SD (μm^2) of the oocyte cytoplasm area of the cattle-tick *Rhipicephalus microplus* exposed to different concentrations of acetylcarvacrol.

Oocytestages	H_2O	DMSO	T1	T2	T3	T4
I	1,304.89 ± 149.57[a]	1,236.64 ± 117.22 [a]	1,016.37 ± 164.35[b]	900.87 ± 150.56 [b]	940.49 ± 83.66 [b]	884.81 ± 162.52 [b]
II	4,985.78 ± 792.10 [a]	5,041.06 ± 524.84 [a]	4,132.32 ± 120.97 [b]	3,696.93 ± 350.54 [b, c]	4,088.76 ± 140.58 [b]	3,552.11 ± 746.77 [c]
III	8,932.36 ± 820.38 [a]	9,378.39 ± 547.56 [a, b]	8,981.27 ± 777.48 [a, b, c]	8,439.11 ± 856.64 [a, c, d]	7,796.12 ± 433.80 [d,e]	7,236.52 ± 731.40 [e]
IV	21,397.01 ± 2,245.67 [a]	20,526.02 ± 2,172.63 [a, c]	18,045.09 ± 2,306.30 [b]	18,343.87 ± 1,785.75 [b, c]	18,390.64 ± 703.43 [b, c]	15,478.15 ± 3,324.35 [c]
V	36,059.08 ± 3,786.75 [a]	34,773.20 ± 3,130.71 [a]	32,344.72 ± 3,350.21[a, b]	29,947.19 ± 1,668.20[b]	29,206.97 ± 1,825.82[b]	25,111.81 ± 6,550.86[c]

Means followed by different lowercase letters in lines differ significantly (One way ANOVA; $P < 0.05$).

inverse relationship between chorion thickening and vacuolization of oocytes observed in treatment group IV suggests that acetylcarvacrol entered more considerably in cells that were vacuolated. The uptake of the drug possibly occurred through the pedicel, which is one of the main entrance routes of chemicals (Oliveira et al., 2009; Sampieri et al., 2012). A fact that supports this idea is that more vacuolated regions appeared closer to the pedicel. Furthermore, the presence of small vacuoles in the cytoplasm of oocytes with intermediate characteristics between stages II and III of control groups demonstrates that permeability of these cells is probably increased, as the input of macromolecules starts to rise considerably in this stage of oogenesis (Saito et al., 2005). Matos et al. (2014) also observed the presence of vacuoles in the cytoplasm of type II oocytes of *Rhipicephalus sanguineus* exposed to 30% ethanol as well as in treatment groups exposed to different thymol concentrations. In fact, it is well known that besides the increase of permeability of the germ cells in earlier stages, which could lead to a greater intake of carvacrol acetate, oocytes in the beginning of development are more vulnerable to chemicals due to the absence of the chorion (Remedio et al., 2014). We believe that the lipophilic chain of acetylcarvacrol could destabilize the membrane of pedicel cells in contact with oocytes and increase even more the permeability of these cells. Then, once the drug is inside the germ cells, it causes several damages as shown in our results. In addition, as acetylcarvacrol possibly affects permeability of oocytes, larger vacuoles generated with greater concentrations of the drug could be due to the loss or degradation of cytoplasm components. This could lead to a decreasing of cytoplasm contents, which explains the size diminution of oocytes in treatment group IV. The increase of permeability could also lead to a greater intake of liquids. However, the cytoplasm area might still decrease due to a reduction of yolk granules, since all cell metabolism may have been impaired.

Vacuolated regions in the nucleolus of oocytes of ticks exposed to acetylcarvacrol were also a remarkable morphological alteration. These nucleoli exhibited a compact ring-shaped mass with a central vacuole, which was also reported by numerous authors (Denardi et al., 2010, 2011; Remedio et al., 2014; Vendramini et al., 2012). These authors suggest the occurrence of genetic material degeneration leading to the death of the oocytes.

The semi-quantitative analysis was proposed for the first time in this study, to the best of our knowledge, to evaluate morphological alterations in ovaries of ixodid ticks. It demonstrated to be a straightforward and more reliable method to determinate the efficiency of any chemical based on extension and impact factor of morphological alterations. Our results showed that treatment group IV exhibited the individual index statistically different from control groups. This demonstrates the impact of acetylcarvacrol in the reproductive system of *R. microplus* by causing several damages in the oocytes.

Control methods using sublethal concentrations of natural based chemical compounds are usually less harmful to the environment and also safer for hosts. The morphological alterations observed in the oocytes of ticks exposed to sublethal concentrations of acetylcarvacrol may affect the viability of the embryos and probably contribute to mitigate tick infestation. Additionally, further evaluation of the reproductive performance of these ticks, through estimation of the egg production indexes and hatching rates, may provide complementary information that will allow the understanding of the effects of acetylcarvacrol in *R. microplus* offspring. Finally, we would like to propose the use of the semi-quantitative analysis performed in this research in further studies in order to facilitate the comparison of the effects of new acaricidal compounds in the reproductive system of ticks.

Financial support

Fundação de Amparo a Pesquisa de Minas Gerais (FAPEMIG, Brazil), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES, Brazil) and Conselho Nacional de Desenvolvimento Científico e

Table 3
Mean ± SD of the alteration indexes observed in oocytes of *Rhipicephalus microplus* exposed to different concentrations of acetylcarvacrol.

		Alteration index (w x α)					
Morphological changes	Importance factor (w)	CI	CII	TI	TII	TIII	TIV
Irregular chorion	1	0 ± 0	0 ± 0	0.80 ± 0.45	0.80 ± 0.45	1.40 ± 0.55	1.20 ± 0.84
Thickerchorion	1	0 ± 0	0 ± 0	0 ± 0	0.60 ± 0.55	0.60 ± 0.55	1 + 00 ± 0.71
Changes in the size of oocytes	1	0 ± 0	0.20 ± 0.45	0.60 ± 0.55	1.60 ± 0.55	1.40 ± 0.55	2.00 ± 0.71
Irregular oocyteshape	2	0 ± 0	0.40 ± 0.89	1.20 ± 1.10	1.60 ± 0.89	2.00 ± 0.00	2.80 ± 1.10
Cytoplasmaticvacuolization	2	0 ± 0	0 ± 0	1.60 ± 0.89	3.20 ± 1.10	2.40 ± 0.89	2.80 ± 2.28
Disorganized/vacuolized/fragmented nucleolus	3	0 ± 0	0 ± 0	1.20 ± 1.64	1.20 ± 1.64	0.60 ± 1.34	5.40 ± 2.51

Table 4
Mean ± SD of the individual indexes calculated for each cattle-tick exposed to sublethal concentrations of acetylcarvacrol.

	Individual index Σ(w x α)					
Individual	CI	CII	TI	TII	TIII	TIV
I	0	3	5	9	10	14
II	0	0	4	10	8	20
III	0	0	5	10	8	19
IV	0	0	6	9	8	10
V	0	0	7	7	8	13
Mean ± SD	0 ± 0[a]	0.60 ± 1.34[a]	5.40 ± 1.14[a, b]	9.0 ± 1.23[a, b]	8.40 ± 0.89 [a, b]	15.20 ± 4.21[b]

Means followed by different lowercase letters in lines differ significantly (Kruskal-Wallis; $P < 0.01$).

Tecnológico (CNPq, Brazil, Grant number 430327/2018-8).

Conflict of interest statement

No conflicts of interest to declare.

Acknowledgments

This research was financially supported by CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior), CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico) and FAPEMIG (Fundação de Amparo à Pesquisa de Minas Gerais).

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