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Original article

Comparative impact of coumaphos, amitraz and plant extract of *Ageratum conyzoides* on the oogenesis of *Rhipicephalus microplus*

Ajith Kumar K.G.[a,1], Ashutosh Fular[a], Gajanan Chigure[a,2], Anil Kumar Sharma[a], Gaurav Nagar[a], Francinea F. Souza[b], Gervasio H. Bechara[b], Srikant Ghosh[a,*]

[a] Entomology Laboratory, Division of Parasitology, ICAR-Indian Veterinary Research Institute, Izatnagar, 243122, UP, India
[b] Graduate Program in Animal Science, Pontifícia Universidade Católica do Paraná, Curitiba, 80215-901, PR, Brazil

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ABSTRACT

The present experiment was conducted to evaluate and compare the impact of *Ageratum conyzoides* plant extract (ACE) with routinely used synthetic acaricides i.e., amitraz and coumaphos on the oogenesis of engorged adult females of *Rhipicephalus microplus* tick. On the day of dropping from the host, panoistic ovary of *R. microplus* appeared white in colour, horseshoe shaped, hollow tubular organ with immature oocytes predominantly in dorsal groove. Different developmental stages of oocytes (I–V) proceed simultaneously and asynchronously. Oocytes showed gradual increase in size, deep brown colored with accumulation of eggs in oviduct during 24–72 hours of development.At LC_{90} concentration a highly significant ($p < 0.001$) cessation of egg laying after exposure to amitraz and ACE while significant reduction ($p < 0.01$) of egg laying in coumaphos treated ticks was observed. Upon dissection of treated ticks, uterus and oviduct packed with eggs, which failed to pass out was observed. The histo-architectural alterations including presence of extensive vacuolation, alteration of oocyte morphology, deformation of chorion and disorganization of yolk granules were observed in the treated ovaries. Histochemically, low level of storage or synthesis of essential elements viz., proteins, polysaccharides and lipids in treated oocytes responsible for reduction of fertility and inhibition of progress of vitellogenesis was observed.

1. Introduction

The cattle tick, *Rhipicephalus microplus* (Canestrini, 1888) is the most economically important tick species of tropical and sub-tropical countries of the world, causing estimated global annual losses of US$ 22 to 30 billion (Lew-Tabor and Rodriguez Valle, 2016) and US$3.4 billion during 2012 in Brazil (Grisi et al., 2014). In India, the cost of management of ticks and tick borne diseases (TTBDs) has been estimated as US$ 498.7 million/annum (Minjauw and McLeod, 2003). The *R. microplus* is reported from almost all the states of India and is responsible for transmission of babesiosis and anaplasmosis in cattle (Ghosh and Azhahianambi, 2007), low productivity (Rodrigues and Leite, 2013) and reduction in value of leather (Biswas, 2003). It is estimated that each engorging female of *R. microplus* is responsible for a loss of 8.9 ± 2.1 ml milk and 1.0 ± 0.38 g live weight gain which is equivalent to 0.081 MJ metabolisable energy losses per tick infestation (Jonsson et al., 1998, 2001).

In spite of some well-known drawbacks of use of chemicals viz., environmental pollution, residues in milk and meat; selection and establishment of acaricide resistant ticks; acaricides are still remaining a primary tool in tick management platform. Currently, use of four classes of chemical acaricides viz., organophosphates, pyrethroids, formamidines and macrocyclic lactones, is the mainstay of tick management programme in India. However, in recent years use of amitraz and ivermectin is comparatively increased due to inefficiency of OP and SP group of acaricides. The use of amitraz has been reported since 1971 (Palmer et al., 1971) for the control of ticks.

Amitraz is a triazapentadiene compound belongs to formamidine pesticide family and is used in veterinary medicine, agricultural and in horticultural practices throughout the world since 1974. It hydrolyzes

* Corresponding author.
E-mail address: sghoshtick@gmail.com (S. Ghosh).
[1] Department of Veterinary Parasitology, College of Veterinary and Animal Sciences, Pookode, Kerala Veterinary And Animal Sciences University, Wayanad -673576, Kerala, India.
[2] Department of Veterinary Parasitology, College of Veterinary and Animal Sciences, Parbhani, Maharashtra Animal and Fishery Sciences University, Maharashtra, India.

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into two metabolites such as 2, 4-dimethylformamide (DMF) and N-(2, 4-dimethylphenyl)-N–methylformamidine (DMPF). These metabolites are further metabolized to 2, 4-demethylaniline (DMA) and ultimately to 4-amino-3-methylbenzoic acid, which is the major metabolite found in the urine and liver (Aronson et al., 1988; Jones, 1990). In arthropods, it acts as an octopaminergic agonist resulting in hyperpolarisation (Evans and Gee, 1980). Recently, through RNA sequencing a relationship between octopamine receptor and ionotropic glutamate receptor in establishing amitraz resistance was proposed. In the presence of amitraz in susceptible ticks, reduced Ca^{+2} entry inhibits glutamate release which in turns maintains Mg^{+2} block thus further reduce Ca^{+2} entry via NMDA receptors, leading to constant excitation and paralysis Baron et al. (2018).

Coumaphos is an organophosphorus pesticide having the chemical name of o-ester of 3-chloro-7-hydroxy-4-methylcoumarin and o,o-diethylphosphorothioate (Martin, 1973). The inhibition of AChE by organophosphates occurs by means of a chemical reaction in which the serine hydroxyl moiety at the active site of the enzyme is phosphorylated in a manner analogous to the acetylation of AChE. The serine hydroxyl moiety, once blocked by the phosphorylated fraction, is no longer able to participate in the hydrolysis of acetylcholine and continuous signaling leads to paralysis and death (Fukuto, 1990). Resistance to organophosphates in insects showed relatively rapid revision upon cessation of selection pressure (Yu, 2008). In a recent study, coumaphos, which was practically withdrawn from veterinary use in India around 20 years ago due to its ineffectiveness, was found effective against ticks (Chigure et al., 2018).

As Indian *R. microplus* has developed resistance to multiple acaricides (Singh et al., 2010; Sharma et al., 2012; Singh and Rath, 2014; Kumar et al., 2011, 2014; Chigure et al., 2018; Nandi et al., 2018), identification of new molecules having different mode of action alone or in synergy in the presence of additives/inhibitors is one of the key research areas.

To develop a sustainable tick management strategy in the present complicated scenario, attempts have been made to identify plants with strong antitick activity with success (Srivastava et al., 2008; Ghosh et al., 2015; Adenubi et al., 2016; Avinash et al., 2017). *Ageratum conyzoides* Linn. (Family: Asteraceae), is a common annual herbaceous weed widely distributed. A number of medicinal properties of this weed have been reported earlier viz. as analgesic, antioxidative, hepatoprotective, blood booster, antioxidant and used for the treatment of high blood pressure, fever, diabetes, pneumonia, wounds and burn, microbial infections, arthrosis, headache, inflammation, dyspnea, pain, asthma, spasms, gynecological diseases, leprosy and numerous infectious and skin diseases (Xuan et al., 2004; Menut et al., 1993; Jagetia et al., 2003; Kamboj and Saluja, 2008; Ita et al., 2007, 2009). It is reported that the multi-functional activities of the plants are possibly be due to synergistic properties of phyto-constituents present in the solvent guided extracts (Rudolf, 1969; Durodola, 1977; Sultana et al., 2012). In our earlier study, the ethanolic extract of the plant was found 76.7–90% efficacious against acaricides-resistant field ticks and its active fraction and subfraction contained 6,7-dimethoxy-2,2-dimethyl-2H-1-benzopyran (Precocene II, ageratochromene) as the major chemical component (Kumar et al., 2016). The antitick activity of Precocene II has previously been demonstrated using engorged females (Booth et al., 1986; Ribeiro et al., 2011).

A great deal of literature documented inhibition of egg-laying (Estrada-Pena and Ascher, 1999; Cossío-Bayúgar et al., 2015) and hatching (Haque et al., 2014) properties of amitraz and coumaphos (Ravindran et al., 2018). However, until now limited or no information is available on impact of these compounds on cellular alteration and cytochemical changes in ovarian developmental process. With an objective to establish the possible mode of action of the identified anti-tick plant extract of *A. conyzoides* (ACE), the present study was conducted to evaluate and compare the impact of the ACE with routinely used synthetic acaricides on the oogenesis of *R. microplus*.

2. Material and methods

2.1. Ticks

The colony of reference acaricide-susceptible IVRI line-I of *R. microplus* (Reg. No. NBAII/IVRI/BM/1/1998) maintained in the Entomology laboratories, Division of Parasitology, ICAR- IVRI were reared on disease-free cross-bred male calves as per the protocol standardized in the laboratory (Ghosh and Azhahianambi, 2007). A total of 120 freshly dropped, fully engorged adults were collected. The collected ticks were washed thoroughly with tap water, dried on soft absorbent paper and divided into six groups each consisting of 20 ticks. Necessary permission (F.25/8/2016-CPCSEA) from Committee for the Purpose of Control and Supervision of Experimentation on Animals (CPCSEA), the regulatory authority monitoring animal experimentation was taken.

2.2. Treatment of ticks

Technical grade amitraz 99.98% and coumaphos 99.8% pure were purchased from AccuStandard® Inc. U.S.A. The extract of aerial parts of *A. conyzoides* (ACE) was prepared in 95% ethanol (Kumar et al., 2016). The LC_{90} concentration of amitraz, coumaphos (Kumar et al., 2014) and ACE (Kumar et al., 2016) were determined earlier using reference IVRI-I strain and the values were 0.038%, 0.007% and 8.91% respectively.

The adult immersion test (AIT) as per the protocol standardized in the laboratory was adopted for treatment of ticks (Sharma et al., 2012). Briefly, the ticks (n = 20 for each drug) were exposed to LC_{90} concentration of amitraz, coumaphos and the extract while control ticks (n = 20 for each group) were exposed to 10% methanol for synthetic acaricides and 10% ethanol plus 2% tween 20 for ACE. All the ticks were immersed for 2 min and then transferred to the petri dishes padded with Whatman filter paper no 1. After 24 h, ticks were transferred to the glass vials capped with cotton cloth and were kept in BOD incubator maintained at 28 °C and 85 ± 5% RH. The biological parameters were monitored up to 14 days and the ticks that did not oviposit were considered as dead. The mortality of ticks was recorded regularly by observing loss of motility and pedal reflex after exposing to light. The ticks that survived were reared subsequently for generating the data on inhibition of oviposition. The inhibition of oviposition was determined (Stendel, 1980).

Reproductive Index = Egg mass/Engorged tick weight

$$\text{Percentage inhibition of oviposition (\%IO)} = \frac{\text{RI(control)-RI(treated)} \times 100}{\text{RI(control)}}$$

The entomological data were statistically analysed using GraphPad Prism 5 software. To compare experimental data with control set, one way ANOVA using Tuckey's test was performed

2.3. Processing of ticks for histological and histochemical studies

The group of ticks treated with amitraz, coumaphos and ACE were dissected after 48 h of treatment to collect the ovaries as per the laboratory standardized protocol. Briefly, ticks were anesthetized using thermal shock by keeping at 4 °C for 10 min and then fixed on the dissection plate filled with paraffin. Dissection was done under dissecting microscope (Olympus microscope BX53). After cutting of both sides, dorsal cuticle was gently lifted from the anterior end and detached the attachments from visceral organs with a teasing needle. Then ovary was gently lifted and transferred to chilled phosphate buffered saline, washed twice and then transferred to the relevant fixative for sectioning and staining.

Three fixatives viz., 4% paraformaldehyde for 24 h (H&E and Bromo phenol blue staining for cellular architecture and detection of protein,

respectively), 10% calcium formaldehyde or formal calcium and Bouin's solution for 12 h (for the detection of lipids and neutral polysaccharides, respectively), were used. Prior to dehydration in alcohol, tissues were cut into small pieces of 4 mm size and then dehydrated in increasing strength of ethanol (70, 80, 90 and 95%) for 15 min. each at room temperature while tissues fixed in formal calcium were dehydrated in chilled ethanol at 4 °C to prevent the loss of lipid during dehydration. Infiltration and embedding of ovarian tissue were done with JB-4 Embedding Kit (Sigma-Aldrich, USA). The tissues were then transferred to polyethylene embedding BEEM® (Polysciences, Inc., USA) capsules previously filled with resin containing a catalyzer. After closing the cap of embedding capsule, transferred to vacuum desiccators fixed at not more than 15 psi, filled with ice to provide a temperature of 2–8 °C required for exothermic reaction during resin polymerization. After polymerization, tissues were sectioned (3 µm) using ultra-microtome (Leica EM UC7) and stained with hematoxylin and eosin, following routine staining procedures.

For histochemical study, tissue fixed in 4% paraformaldehyde was stained with Bromophenol Blue for detection of protein. For lipids, Bakers staining was adopted. Polysaccharides were stained simultaneous with Periodic acid–Schiff (PAS)/ Alcian Blue.

2.4. Bromophenol blue staining (Pearse, 1985) for detection of protein

The 4% paraformaldehyde fixed ovary sections were stained with bromophenol blue for 2 h at room temperature (RT) and then, immersed in 0.5% acetic acid for 5 min. After washing with running water for 15 min, sample was quickly immersed in tertiary butyl alcohol for 5 s and then left to dry at RT, mounted in DPX and examined under microscope (Olympus BX53).

2.5. Baker's staining (Baker, 1946) for detection of lipids

Lipids content was detected as per the protocol of Sampieri et al. (2013). The formal calcium fixed tissue sections were immersed in calcium dichromate for 18 h, washed in distilled water and dipped in hematin solution for 5 h. After giving a final wash in distilled water the slides were dried on a hot plate set at low temperature and immediately mounted with glycerin jelly, examined under light microscope and photographed.

2.6. Alcian blue/PAS staining (Junqueira and Junqueira, 1983) for acid and neutral polysaccharides

Sections prepared from Bouin's fixed tissue were rehydrated by immersing in distilled water, transferred to 1% Alcian blue in 3% acetic acid for 30 min. After washing in distilled water, slides were transferred to 1% PAS solution for 5 min and again washed for 10 min in distilled water. The slides were immersed in Schiff reagent for 15 min., washed in running water and allowed to dry at RT, examined under light microscope and photographed.

3. Results

3.1. Gross architecture of ovary of untreated and treated R. microplus ticks

Ovary of *R. microplus* was a hollow horseshoe shaped tubular organ (Fig. 1a, b) opening distally into oviducts and was of panoistic type. At the time of dropping from the host, ovary appeared white in colour (Fig. 1a). Most of the immature oocytes were present in dorsal groove, which was situated along the entire longitudinal axis of ovary (Fig. 1b). Ovarian wall was lined by a single layer of epithelial cell to which oval to round oocytes of various sizes and shapes are attached. Different developmental stages of oocytes are processed simultaneously and asynchronously. Oocytes area is attached to ovary by a stalk called pedicel or funicle formed by a group of epithelial cells. Pedicel cells were cylindrical or pyramidal in shape. Gradual increase in size and deep brown colour was noticeable during 24 to 48 h of development with the accumulation of eggs in the oviduct towards the end of preoviposition (Fig. 1c, d).

After treatment of engorged females with amitraz, coumaphos and ACE, a significantly high percentage of ticks were not able to lay eggs on fourth day of treatment while normal laying pattern was observed in untreated ticks. A significantly ($p < 0.001$) low egg masses production (17.2 ± 4.0 mg) in the survived ticks was recorded with 90.7% inhibition of oviposition as compared to control (Table 1). Upon dissection of treated ticks, packed uterus and oviduct with eggs, which failed to pass out (Fig.1e–g) was noticed. Slower development of oocytes (more number of immature oocytes in treated ticks in comparison to untreated ticks) was observed at 24 and 48 h of oviposition.

3.2. Cellular profile of untreated ovary (H&E staining)

Type I oocytes: smallest, round to elliptical shaped with a mean size of 42.92 ± 2.4 µm x 28.1 ± 2.7 µm. Germinal vesicles (23.93 ± 0.67 µm) were at the center with ring shaped nucleoli. Frequently, two nucleoli with maximum of four nucleoli (4.1 ± 0.41 µm) were seen in some cases. Cytoplasm was agranular and basophilic in nature and was delimited by a thin plasmic membrane. Nucleus to cytoplasmic ratio was highest compared to other type of oocytes (Fig. 2a).

Type II oocytes: oocytes were elliptical, larger (mean 61.0 ± 4.8 µm x 44.4 ± 2.6 µm) than type I, with fine granulation dispersed throughout the cytoplasm. A central germinal vesicle (21.64 ± 1.84 µm) with one or two nucleoli (3.95 ± 0.63 µm) was evident. Nucleus to cytoplasmic ratio was decreased greatly as compared to type I (Fig. 2b).

Type III oocytes: round or roughly elliptical, mean size of 93.53 ± 6.8 µm x 67.34 ± 4.02 µm was observed with higher dominancy at 48 h of dropping. Cytoplasm contained coarser granulation distributed homogenously. Germinal vesicle moved towards the ovarian wall at a pole next to the oocyte pedicel junction. Germinal vesicle frequently contained one or two nucleoli. Chorion deposition was evident with mean thickness of 0.7 ± 0.06 µm at the external surface of plasmic membrane surrounding the oocyte (Fig. 2c).

Type IV oocytes: predominant at 48 h of dropping having the mean size of 113.0 ± 9.94 µm x 98.50 ± 9.3 µm. Cytoplasm contained both large and small yolk granules. Larger yolk granules were seen at the periphery while small yolk granules at central region. Germinal vesicle was evident however, due to presence of large amount of yolk granules distributed throughout the cytoplasm it was not easily visible and if it is present, located at pole next to oocyte pedicel junction. Thick chorion (1.28 ± 0.1 µm) was present around the oocyte (Fig. 2d).

Type V oocytes: Oocytes with largest germinative cell (228.10 ± 13.8 µm x 197.2 ± 7.2 µm), rounded in shape and germinal vesicle is not visible due to the presence of big and numerous, rounded or hexagonal cytoplasmic granules. The chorion was thick (2.55 ± 0.08 µm) and fully deposited surrounding the oocyte (Fig. 2e). The oocyte V began to appear at 48 h of dropping.

3.3. Cellular profile of treated ovary

Amitraz: damaged plasmic membrane with vacuolations at the periphery of type I and II oocytes was observed (Fig. 2f, g). A large area of vacuolations occupying periphery as well as oocyte pedicel junction was present in type III oocytes (Fig. 2h). Some of the yolk granules of type IV oocyte were negative to haematoxylin and vacuolations were seen at oocyte pedicel junction (Fig. 2i). Morphological alteration i.e. wavy chorion and vacuolations around almost all yolk granules were seen in type V oocytes (Fig. 2j).

Coumaphos: in type I oocytes vacuolations were observed at the periphery (Fig. 2k) while in type II oocytes these vacuolated areas were prominent near germinal vesicles (Fig. 2l). Most of the yolk granules of

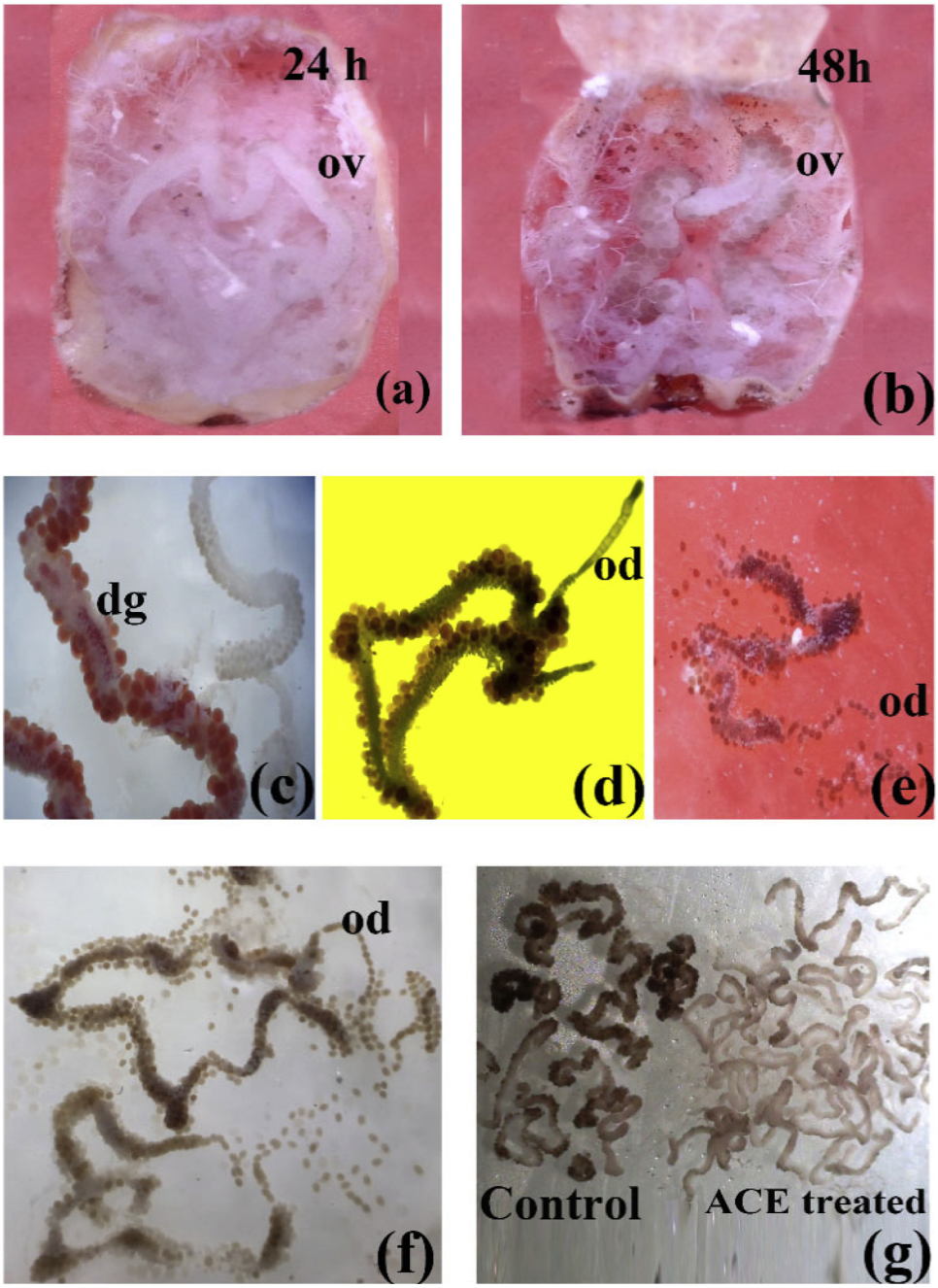

Fig. 1. Gross view of ovary of normal and treated *R. microplus* ticks. a- ovary after 24 h of dropping; b- ovary after 48 h; c- ovary showing dorsal groove; d- ovary with oviduct; e- fully packed ovary and oviduct with eggs which failed to pass out in amitraz treated ticks at 72 h; f- fully packed ovary and oviduct with eggs that failed to pass out in coumaphos treated ticks at 72 h; g- presence of large number of immature oocytes in ACE treated ticks at 48 h in comparison to control ticks at 48 h. ov- ovary; od- oviduct; dg- dorsal groove.

type III oocytes were weakly stained with H&E and vacuolations were present around some yolk granules (Fig. 2m). A large areas of vacuolations at the oocyte pedicle junction and near the germinal vesicle was a characteristic feature of type IV oocytes (Fig. 2n). The cell boundaries of type V oocytes became irregular and chorion exhibited many infoldings (Fig. 2o).

ACE : Oocyte with irregular margins and decrease in nuclear cytoplasmic ratio was noted in some primary oocytes (Fig. 2p). Vacuolations near the germinal vesicles in both type II and III oocytes and more prominently pedicle cells of type III were observed (Fig. 2q, r). Extensive vacuolations around each of yolk granule and near germinal vesicle were seen in type IV oocytes (Fig. 2s). Deformed and vacuolated oocytes were the major impact in all ACE treated tick ovary (Fig. 2t). The comparative drug induced alteration in different stages of oocytes is presented in Table 2.

3.4. Comparative localization of proteins, lipid and polysaccharides in untreated and in treated ovaries

3.4.1. Proteins

Untreated oocytes

Type I oocytes : cytoplasm was weakly stained; germinal vesicle was negative to the test while nucleolus was strongly positive (Fig. 3a).

Type II oocytes : strongly positive fine yolk granules distributed homogenously throughout the cytoplasm. Similar to type I oocytes, germinal vesicle was weakly positive while nucleolus was strongly

Table 1
Comparative analysis of reproduction parameters of *R. microplus* after treatment with amitraz, coumaphos and ACE.

Drug	LC90 (%)	Egg masses (mg) (Mean ± SE)	Reproductive Index (Mean ± SE)	% Inhibition of oviposition
Amitraz	0.038	32.0 ± 3.2[b]	0.223 ± 0.03[c]	51.7
Coumaphos	0.007	57.7 ± 1.7	0.472 ± 0.02[b]	14.0
ACE	8.91	17.2 ± 4.0[c]	0.04 ± 0.01[c]	90.7
Control	–	60.5 ± 6.2	0.591 ± 0.02	–

[a]significant at $p < 0.05$.
[b] significant at $p < 0.01$.
[c] significant at $p < 0.001$.

positive (Fig. 3b).

Type III oocytes : **s**trongly positive coarse granulation distributed throughout the cytoplasm, germinal vesicle and cytoplasm was weakly positive while nucleolus was strongly positive (Fig. 3c).

Type IV & V oocytes: like type III, strongly positive large and small yolk granules with strongly positive chorion was visible, while germinal vesicle was moderately stained (Fig. 3d, e).

Treated oocytes

Amitraz: no alterations in protein profile in cytoplasm of type I oocytes (Fig. 3f). The yolk granules of type II and III oocytes were moderately positive in comparison to strongly positive untreated oocytes (Fig. 3g, h). Unlike in untreated ticks, in advanced developmental stages, majority yolk granules were weakly positive to the test (Fig. 3i, j), indicating loss of protein content after treatment with amitraz.

Coumaphos: no changes in type I oocytes in comparison to control was noticed (Fig. 3k). A small cytoplasmic area negative to the test was located especially around the germ vesicle and next to pedicel was observed in type II and III oocytes (Fig. 3l, m). As observed in amitraz treated ticks, loss of intense granulation in comparison to strongly stained untreated type IV and V oocytes was observed in this group of ticks (Fig. 3n, o).

ACE: like amitraz and coumaphos treated group of ticks, cytoplasm of different developmental stages of oocytes stained weakly (Fig. 3p, q). Yolk granules of type III oocytes were weakly stained in comparison to corresponding control oocytes indicating less protein content in vitellogenic oocytes (Fig. 3r). A large area of weakly stained yolk granules near germinal vesicle and completely negative stained area at periphery of type IV oocytes was found as observed in amitraz and coumaphos treated groups. The chorion was weakly positive compared to strongly positive in untreated group (Fig. 3s). Yolk granules and chorion were weakly positive compared to strongly positive control group (Fig. 3t).

3.4.2. Lipids

Untreated oocytes

Type I oocytes: moderately positive cytoplasm was the characteristic feature (Fig. 4a).

Type II oocytes: fine and moderately positive, homogenously distributed granulations were detected. Germinal vesicle was strongly positive (Fig. 4b).

Type III oocytes: coarse yolk granules distributed throughout the cytoplasm were moderately positive while germinal vesicle was strongly positive (Fig. 4c).

Type IV oocytes : the cytoplasm with small and strongly positive yolk granules in the center and larger granules were located mainly in the peripheral region (Fig. 4d).

Type V oocytes: the entire cytoplasm of oocytes V stages were filled with strongly positive stained granules (Fig. 4e).

Treated oocytes

Amitraz: the cytoplasm of type I oocytes and yolk granules of type II oocytes were strongly positive in comparison to moderately positive control (Fig. 4f, g). No changes in staining pattern in type III oocytes of treated and control group of ticks were observed. Germinal vesicle was strongly positive (Fig. 4h). Most of the type IV oocytes were highly

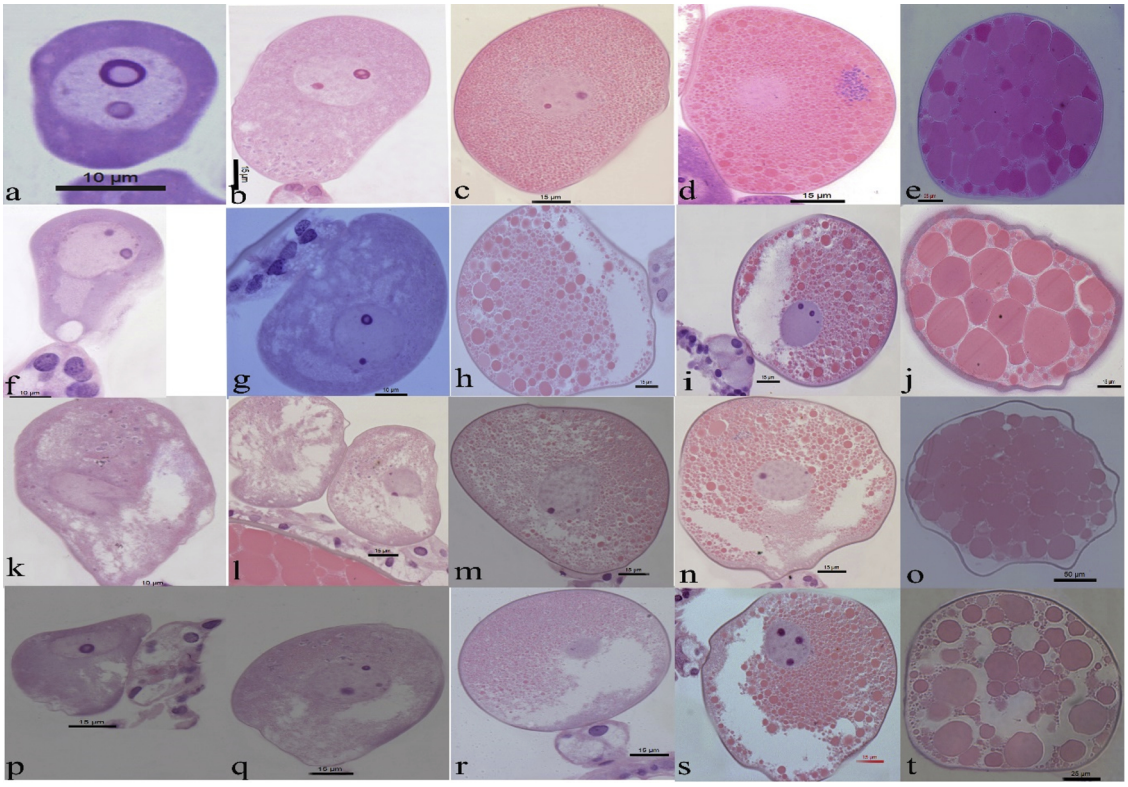

Fig. 2. Drug-induced comparative cellular changes in the oocytes I–V of ticks. Figs. a–e: untreated, f–j: amitraz treated, k–o: coumaphos treated and; p–t: ACE treated *R. microplus* following H&E staining.

Table 2
Summary of cellular alterations in different stages of *R. microplus* oocytes after 48 h treatment with amitraz, coumaphos and ACE in comparison to untreated oocytes.

Oocytes	Untreated	Amitraz	Coumaphos	ACE
I	Smallest, round to elliptical, highest nucleus to cytoplasmic ratio	Damaged plasmic membrane, vacuolations at oocytesperiphery	Vacuolations at the periphery of oocytes	Vacuolations at periphery, oocyte pedicle junction
II	Fine granulation in cytoplasm, a central germinal vesicle with 1 or 2 nucleoli, no cytoplasm vacuolation	Damaged plasmic membrane, vacuolations at oocytes periphery	Vacuolations near germinal vesicle	Vacuolations mainly at the periphery of oocytes
III	Coarse granulation, evident chorion deposition, no cytoplasm vacuolation	Vacuolations at oocytes periphery, oocyte pedicel junction	Vacuolations around some yolk granules	Vacuolations at periphery, oocyte pedicle junction
IV	Cytoplasm with large and small yolk granules, larger yolk granules at the periphery, small yolk granules at central region.	Vacuolations at oocyte pedicel junction	Vacuolations at the oocyte pedicel junction and near the germinal vesicle	Extensive vacuolation around each of yolk granules and near germinal vesicle
V	Largest germinative cell, larger and numerous rounded or hexagonal cytoplasmic granules, complete deposition of thick chorium.	Wavy chorion and vacuolations around almost all yolk granules	Irregular cell, chorion exhibited many infoldings	Deformation with fold and bulges of chorion

deformed and were largely negative to Bakers staining consequent to the loss of granules (Fig. 4i). The type V oocytes were moderately positive with negatively stained vacuolated areas in comparison to the strongly positive untreated oocytes (Fig. 4j).

Coumaphos: moderately positive cytoplasm in type 1 oocytes (Fig. 4k) and fine, strongly positive, homogenously distributed granulation was detected in type II oocytes (Fig. 4l). In both type I and II oocytes, nucleolus was strongly positive. In type III oocytes, yolk granules were weakly positive compared to moderately positive control and amitraz treated group (Fig. 4m) while in type IV oocytes peripheral granules were either weakly or moderately positive but central granules were strongly positive (Fig. 4n). The type V oocytes with weakly positive yolk granules as detected in amitraz treated group but contrast to strongly positive control group were observed (Fig. 4o).

ACE: a large strongly negative vacuolated area around germinal vesicle of type I oocytes was observed compared to moderately stained in control and coumaphos treated and strongly positive amitraz treated groups (Fig. 4p). Moderate to strongly positive granulation was found in type II oocyte. The nucleolus of type II oocytes was strongly positive as seen in the control, amitraz and coumaphos treated groups (Fig. 4q). The yolk granules of type III oocytes were moderately positive compared to strongly positive granules of control and amitraz treated groups (Fig. 4r). Most of the peripheral granules of type IV oocytes were moderately positive, while in the control group strongly positive granules were observed (Fig. 4s). The yolk granules of type V oocytes presents moderate to strongly positive granulation with large negatively stained vacuolated areas (Fig. 4t).

3.4.3. Polysaccharides

Untreated oocytes

Type I oocytes: cytoplasm was weakly positive (slight magenta in colour) for neutral polysaccharides while germinal vesicle, nucleoli and pedicel cells were alcianophilic (bluish**–green)**, positive for acid polysaccharides (Fig. 5a).

Type II oocytes: in comparison to type I oocyte, the cytoplasmic granulation was strongly positive for neutral polysaccharide while germinal vesicle, nucleoli and pedicel cells were alcianophilic (Fig. 5b).

Type III, IV, V oocytes: strongly PAS positive granules were distributed throughout the cytoplasm and chorion was moderately alcianophilic (Fig. 5c–e).

Treated oocytes

Amitraz: The staining characters were almost similar to that of control except in areas around the vacuolation. The cytoplasm of type I oocytes was weakly positive while type II oocytes showed moderate positivity compared to strongly positive control group (Fig. 5f, g). There were large PAS negative areas around the vacuolation in type III oocytes compared to control group (Fig. 5h). The type IV and V oocytes presented similar reactions pattern as observed in the control group except few yolk granules showed negative reaction in vacuolated areas in type IV oocytes (Fig. 5i, j).

Coumaphos: The type I oocytes appeared weakly positive to PAS as seen in control and in amitraz treated group of ticks (Fig. 5k). The type II oocytes showed moderately positive with thin granulation as seen in amitraz treated group in contrast to strongly positive control group (Fig. 5l). The type IV and V oocytes exhibited vacuolated areas negative to the test near the germ vesicle and towards the oocytes pedicle junction (Fig. 5n, o). The carbohydrate deficient areas were mainly concentrated around the vacuolated areas.

ACE: the cytoplasm of type I and II oocytes were moderately positive compared to the control group. The vacuolated areas especially near the germ vesicle and pedicel were negative to the test (Fig. 5p, q). Largest cytoplasmic areas negative to the test was due to the presence of vacuoles in type III oocytes (Fig. 5r). The type IV oocytes presented yolk granules with moderate to strongly positive reaction and small vacuoles that were negative to the test in a region near the cell periphery

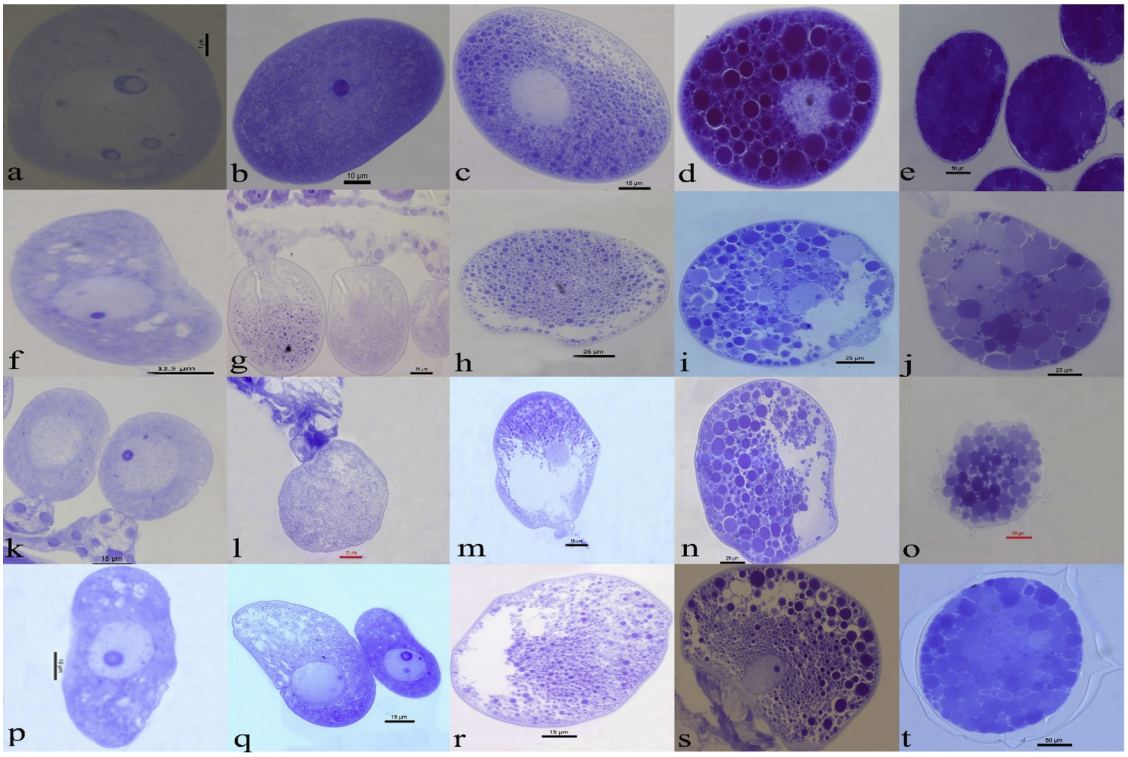

Fig. 3. Drug-induced comparative cellular changes in the oocytes I–V of ticks. Figs. a–e: untreated, f–j: amitraz treated, k–o: coumaphos treated and; p–t: ACE treated *R. microplus* following BPB staining.

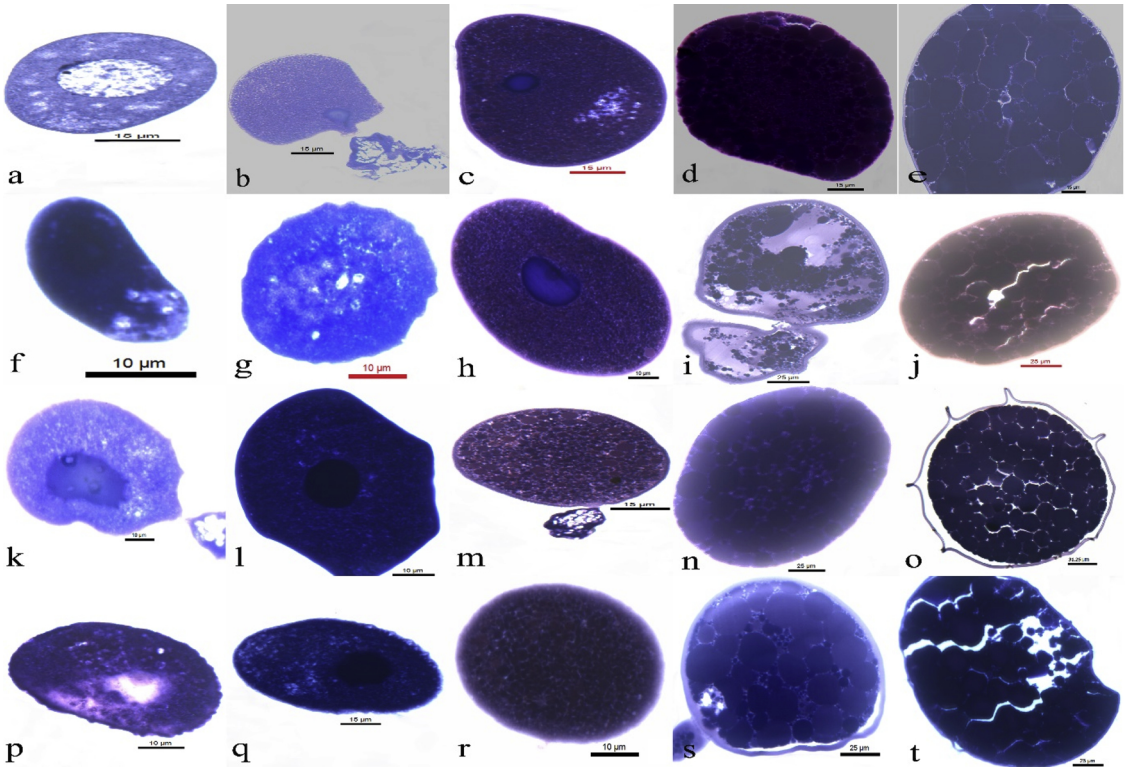

Fig. 4. Drug-induced comparative cellular changes in the oocytes I–V of ticks. Figs. a–e: untreated, f–j: amitraz treated, k-o: coumaphos treated and; p–t: ACE treated *R. microplus* following Baker staining.

(Fig. 5s) were detected. Yolk granules of type V oocytes were weakly positive and large area negative to the test was seen in more than half portion of the oocytes as seen in coumaphos treated group (Fig. 5t) indicating severe reduction in carbohydrate content in yolk granules.

The comparative profile of proteins, lipid and polysaccharides of different stages of ovary treated with amitraz, coumaphos and ACE is given in Table 3.

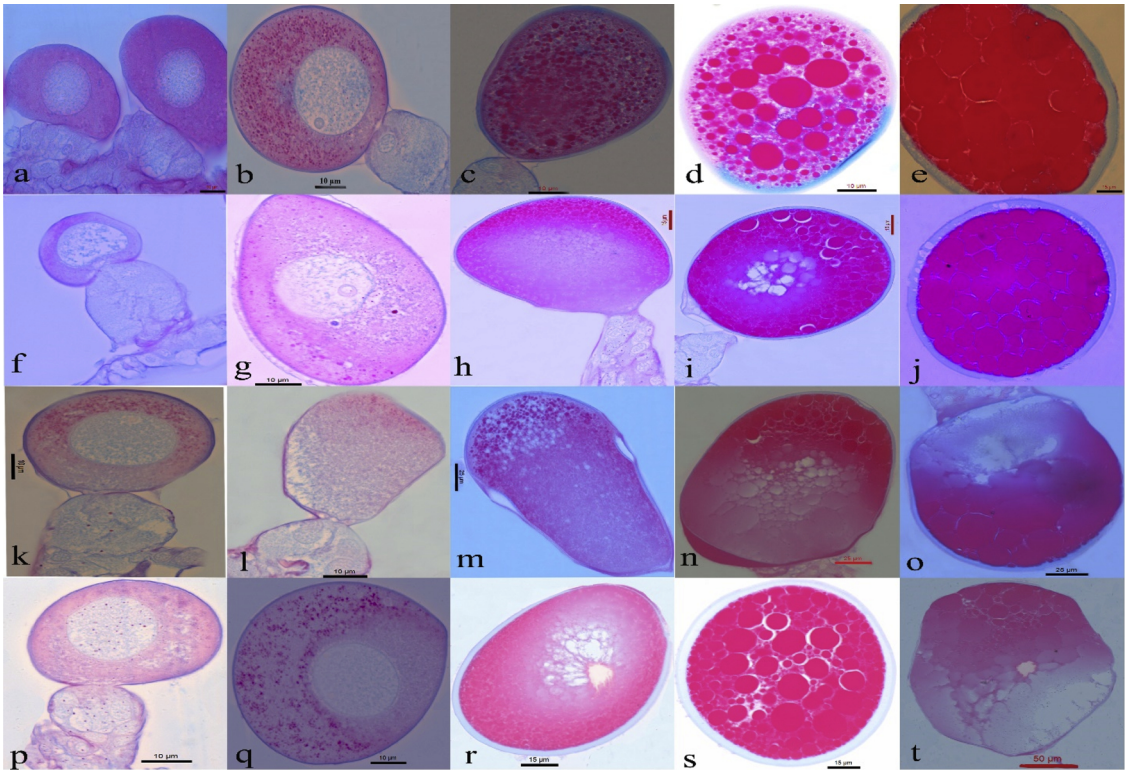

Fig. 5. Drug-induced comparative cellular changes in oocytes I–V of ticks. Figs. a–e: untreated, f–j: amitraz treated, k-o: coumaphos treated and; p-t: ACE treated *R. microplus* following PAS staining.

4. Discussion

A female *R. microplus* oviposits approximately 2500–3000 eggs. Complete oviposition and viability of eggs are critical components to ensure larval establishment on pastures to continue their biological cycle. In ixodid ticks, ovarian development reached at peak in response to nervous system stimulation, which in turn promotes an increase in release of ecdysteroids, mainly 20-hydroxyecdysone (vitellogenesis hormone) in the haemolymph (Seixas et al., 2008). Contrary to this, Booth (1989) and Cossío-Bayúgar et al. (2012) demonstrated that α and β adrenergic ligands are able to inhibit oviposition in *R. microplus in vivo* and arthropod ovaries require the combined effect of contraction and relaxation in order to push mature eggs into oviducts for fertilization and oviposition (Rodríguez-Valentín et al., 2006; Fuchs et al., 2014). In the present study, a highly significant ($p < 0.001$) cessation in egg laying after exposure to amitraz and ACE while significant reduction ($p < 0.01$) in coumaphos treated ticks was observed (Table 1). The dissection of the treated ticks revealed that the uterus and oviduct were packed with eggs that failed to pass out. Majority of oocytes from treated ovaries were in the initial developmental stages compared to larger quantity of mature oocytes in the ovaries and oviducts of untreated ticks. Similar effects on the egg structure and reproductive organs of two *R. microplus* strains treated with carbamates, ethyl-(4-bromophenyl) carbamate and ethyl-(4-chlorophenyl) carbamate were reported (Prado-Ochoa et al., 2014). Earlier, Broglio-Micheletti et al. (2010) observed *in vitro* inhibition of total oviposition in females and Pazinato et al. (2016) reported treatment of *R. microplus* with 1% oil of *Juniperus communis* led to lower egg hatchability in addition to lower oviposition. A significant decrease in oviposition of *Rhipicephalus* spp. was observed after exposure to essential oils of *Lippia triplinervis* (Lage et al., 2013), *Pelargonium graveolens* (Pirali-Kheirabadi et al., 2009) and *Hesperozygis ringens* (Ribeiro et al., 2010), suggesting that sub-lethal doses of essential oils may have an effect on tick fecundity. In the present study, the observed effects on reproductive organs of engorged females indicated direct action of these acaricides on some critical ovarian cells.

In the present study, we classified the oocytes into five stages as proposed by Denardi et al. (2004) in *Amblyomma cajennense* opposing the view of Saito et al. (2005) who classified oocytes of *R. microplus* into six stages with additional features of oocyte degeneration. None of the ticks from control group showed degenerating type of oocytes after 48 h of dropping while Sreelekha et al. (2015) reported oocytes with degenerative changes in *R. annulatus*. The presence of basophilic cytoplasm of type I oocytes in *R. microplus* was reported first time in the present study and corroborates the report of Sreelekha et al. (2015) in *R. annulatus*. This basophilic nature of the cytoplasm may be due to increased contents of ribosomes.

Extensive vacuolation of oocytes was the most common effect of the studied compounds in the present study. According to Carvalho and Recco-Pimentel (2007) and Junqueira and Carneiro (2013) autophagic vacuoles are mainly seen in cells where degradation or recycling of damaged portions or organelles are taking place. This occurs for reabsorption of some mass and/or the remaining cell portions and to reutilize components of the cell. Both endogenous and exogenous acquisition of yolk components is a common feature in ticks. According to Oliveira et al. (2007) pedicel cells play a role in synthesizing and transferring yolk components into the oocytes. Hence, the location of vacuoles in the ACE, coumaphos and amitraz treated groups were mainly at the oocyte-pedicel junction suggesting that the toxicant circulating in the haemolymph was reaching to the oocyte through pedicel cell. Similar results were obtained in ticks exposed to permethrin (Roma et al., 2010) aqueous extract of neem leaves (Denardi et al., 2010) and ricinoleic acid ester (Sampieri et al., 2013).

The type V oocytes of ACE treated group of ticks lost original shape as evidenced by folding of chorion which may be due to shrinkage caused by the loss or disruption of the yolk granules. Similar observation was reported previously in fipronil treatment ticks (Oliveira et al., 2008).

Table 3
Comparative histochemical characteristics of different stages of *R. microplus* oocyte after 48 h treatment with amitraz, coumaphos and ACE in comparison to untreated oocyte.

	I	II	III	IV	V
Protein					
Control	Cy.: weakly positive, GV: negative, Nu: strongly positive	Yg (fine): strongly positive, GV: negative, Nu: strongly positive	Cy. and GV: weakly positive, Nu: strongly positive, Yg: strongly positive	Yg (large & small): strongly positive	Yg (large & small) and chorion: strongly positive
Amitraz	Cy: weakly positive, GV: negative, Nu: strongly positive	Yg (fine): moderately positive	Yg (fine): moderately positive	Yg (large & small) and chorion: weakly positive	Yg (large & small) and chorion: weakly positive
Coumaphos	Cy: weakly positive, GV: negative, Nu: strongly positive	Small cytoplasmic negative area around the GV and next to the pedicel	Small cytoplasmic negative area around the GV and next to the pedicel	Yg (large & small) and chorion: weakly positive	Yg (large & small) and chorion: weakly positive
ACE	Small vacuolated areas negative to test	Small vacuolated areas negative to test	Yg: weakly positive	Weakly stainedYg and large vacuolated areas	Yg (large & small) and chorion: weakly positive
Lipid					
Control	Cy: moderately positive,	Yg: fine, moderately positive; Gv: strongly positive	Yg: coarse, moderately positive; Gv: strongly positive	Yg (large & small): strongly positive	Yg (large & small) and chorion: strongly positive
Amitraz	Cy: strongly positive	Yg: fine, strongly positive	Yg: coarse, moderately positive; Gv: strongly positive	Highly deformed, large vacuolated areas negative to test	Yg: Moderate positive, vacuolated areas negative to test
Coumaphos	Cy: moderately positive	Yg: fine, moderately positive; Gv: strongly positive	Yg: coarse, weakly positive	Yg: weak / moderately positive at periphery, strongly positive at central	Yg: weakly positive
ACE	Cy :Negative, vacuolated area around Gv	Yg: fine, moderate to strongly positive	Yg: coarse, weakly positive	Yg: weak / moderately positive at periphery	Large vacuolated areas negative to test
Polysaccharides					
Control	Cy: strongly positive ; Gv& Nu: strongly alcianophilic	Cy: strongly positive ; Gv& Nu: strongly alcianophilic	Yg: strongly PAS positive	Yg: strongly PAS positive	Yg: strongly PAS positive
Amitraz	Cy: weakly positive	Yg: weakly positive	Yg: weakly positive at Centre	Yg: negatively stained vacuolated areas	Yg: weakly positive
Coumaphos	Cy: Weakly positive	Weakly positive	Weakly positive	Weakly positive	Large negatively stained vacuolated areas
ACE	Weakly positive	Weakly positive	Weakly positive	Yg: negative to test at centre	Large negatively stained vacuolated areas

Cy = Cytoplasm, Yg = Yolk Granules, GV = Germinal Vesicle.

In arthropods, lipids, followed by proteins and polysaccharides are deposited in the oocyte in sequence in the form of yolk granules (Ramamurty, 1968). In all the treated ovaries, reduced lipid contents was seen mainly at advanced stages while in ACE treated ovaries it was more prominently in type III–V oocytes. The reduced level of carbohydrates and lipids along with other components inhibited the hatching of treated eggs. On the contrary, Sampieri et al. (2013) reported a compensatory increase in lipid content when there was a reduction in carbohydrate in ticks treated with ricinoleic ester. However, in the present study, compensatory increase of lipids was observed in type I and II oocytes of ACE treated ticks only.

The major yolk protein vitellin (Vn) is processed from vitellogenin (Vg). The Vg is produced in both fat body and midgut in a number of tick species. Following synthesis, Vg is released into the haemolymph and taken up into the developing oocytes by receptor-mediated endocytosis, where Vg is further processed and stored (Smith and Kaufman, 2013). This conversion of vitellogenin into vitelline probably occurs due to enzyme action, as well as other processes that require more energy (Sampieri et al., 2013).

In the present study, cytoplasm of both control and treated type I oocytes was weakly positive for protein as opposed to Sampieri et al. (2013) who reported type I oocytes were negative to the test. However, they reported positivity in type I oocytes when ticks treated with ricinoleic acid ester owing to the increased activity of these compounds to neutralize the toxic components arising from esters. In all the treatment groups, protein contents of the yolk granules in advanced stages of oocytes (type IV and V) was reduced with highest reduction was noted in amitraz treated group.

Ricardo et al. (2007) reported that the incorporation or production of carbohydrates started in type II oocytes having pedicel cells and hemolymph as exogenous sources. In the present study, processing of the type II – IV oocytes from ACE, amitraz and coumaphos treated individuals indicated that the compounds might have acted on hydrolysis of polysaccharides and subsequent delayed the synthesis and/or incorporation of carbohydrates. Similar inhibition of carbohydrate was observed in oocytes of *R. sanguineus* treated with ricinoleic ester from castor oil (Sampieri et al., 2013).

In conclusion, the presence of malformed oocytes with extensive vacuolation and reduced yolk components due to ACE, amitraz and coumaphos treatment was noticed. Thus, the present study showed that ACE can be used in suitable delivery mode for the management of multi-acaricide resistant tick population.

Conflict of interest

Authors have no conflict of interest.

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