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RESEARCH ARTICLE

Observation on the histochemistry of the developing ova in *Haemonchus contortus* (Nematoda)

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ABSTRACT

In *Haemonchus contortus*, the concentration of various metabolites differs in various stages of oogenesis. Though an adequate quantity of carbohydrates is evidenced in ovarian epithelium, perinuclear spaces of oogonia, oocytes and rachis but the protoplasmic processes connecting the oogonia to the rachis are completely devoid of the same. Developing oocytes imbibe large concentrations of glycogen from the ovarian epithelium and subsequently use it for the formation of chitinous layer of the egg shell. The mature ovum gets surrounded by an additional resistant layer of acid mucopolysaccarides. High nucleic acid activity has been detected in both oogonia as well as oocytes and fertilized ova show a spurt of ribosomes in them. The secondary oocytes are full of proteinaceous granules and show an intense activity of RNA indicating the occurrence of rapid protein synthesis at this stage. The lipids seem to be a major constituent of the egg shell envelope of the fertilized ova.

Keywords: Histochemistry, oogenesis, egg shell, Nematoda, *Haemonchus contortus*.

INTRODUCTION

Haemonchus contortus is a serious nematode parasite of sheep (*Ovis aries*) and goat (*Capra hircus*) of cosmopolitan distribution. It causes severe anaemia resulting in weight loss, poor milk yield and wool production. Medium infection causes sheep to lose condition and heavy infection may result into death. Thousand of worms may occur in a single ruminant stomach and it has been estimated that 4000 worms suck about 63 cm^3 of blood per day (Smyth, 1996). Baker *et al*. (1956) have estimated that a single worm causes an average daily loss of 0.08 ml. of blood.

Previously, the histomorphology and histochemistry of various organ systems of *Haemonchus contortus* was studied by Singh and Johal (1997), Singh (2000), Singh and Johal (2001a; 2001b; 2001c), Singh and Johal (2004) and Singh (2015a; 2015b; 2015c, 2015d; 2015e). The present research paper describes the histochemical aspect of developing ova in female *Haemonchus contortus*, which can fill the hitherto existing gaps in information regarding this aspect. The earlier literature on histochemistry of nematode parasites reveals that both the reproductive tract as well as the developing gametocytes shows a variable spectrum of distribution of metabolites viz. glycogen, proteins and lipids. The present histochemical localization of macromolecules will be of significance in understanding the metabolic activities and fundamental functional aspects of the organs of this nematode. This study will fill the hitherto existing gaps in information regarding this aspect in nematodes and also form a basis for the development of effective chemotherapeutic measures against this serious pathogenic parasite of domestic ruminants.

MATERIALS AND METHODS

The adult female worms were extracted from the abomasum portion of stomach of sheep (*Ovis aries*). In order to remove debris, the nematode worms were washed in 0.85% NaCl solution. For histochemical studies, the worms were fixed in alcoholic Bouin's fixative and Carnoy's fixative, dehydrated in a graded series of alcohol, cleared in methyl benzoate and embedded in paraffin wax. The sections were cut at 7µm in transverse and longitudinal planes by using rotary microtome. The serial sections arranged on albuminised slides were stained.

General carbohydrates were studied by Periodic acid Schiff's staining technique (McManus, 1948). Glycogen was detected histochemically by Best's carmine staining (Best, 1906) and acid mucopolysaccharides by Alcian blue (Steedman, 1950). Nucleic acids were detected by Gallocyanin chromalum (Einarson, 1951) and Methyl green pyronin Y (Kurnick, 1955) techniques. For the localization of proteins, Mercuric bromophenol blue staining (Bonhag, 1955) and Ninhydrin Schiff's staining (Yasuma and Ichikawa, 1953) were used. The histochemical presence of lipids was detected by Sudan black B staining (McManus, 1946) and Oil red O in isopropanol (Lillie and Ashburn, 1943). The slides were examined under the microscope and photo micrographed.

RESULTS AND DISCUSSION

In female *Haemonchus contortus*, carbohydrates are localized in all the developing stages of germ cells. An adequate quantity of carbohydrates is found in the oogonia and oocytes present in the ovary as evidenced by Periodic acid Schiff's staining (Figs. 1 and 2). Glycogen is seen in the perinuclear spaces of oogonia, oocytes and the wall of rachis, whereas the protoplasmic processes connecting the two are devoid of it (Fig. 4). In the secondary oocytes, the carbohydrates get more concentrated in the cytoplasmic portion (Figs. 2 and 3). The spermatozoa lying in the seminal receptacle show a substantial amount of glycogen, whereas in the fertilized ova glycogen is concentrated in the chitinous layer and the egg yolk has only a lesser amount of it (Fig. 5 and 6).

Both structural as well as cytoplasmic proteins are found in abundance in the developing stages of ova of *Haemonchus contortus*. The proliferating oogonia in germinal zone of ovaries show a profuse and rachis a moderate concentration of proteins (Figs. 7 and 8). In the developing oocytes, the protein granules are spread over in the cytoplasmic region, whereas the nuclear spaces are clear of it except the presence of some condensed chromatin granules (Fig. 9). The mature ova lying in the uterus are full of proteinaceous ribosomes and the outer uterine layer of egg shell has protein as its main constituent (Figs. 10, 11 and 12). Near the vaginal region the two-celled stage of fertilized ova have an intense concentration of protein granules in the egg yolk. The chromosomes of dividing nuclei and outer shell wall are also positive for proteins (Fig. 13).

A substantial amount of nucleic acids is observed in the developing stages of the ova. The nucleic acids are seen restricted to only the nuclear region of the oogonia indicating DNA activity at this stage, as evidenced by Gallocyanin chromalum staining (Fig. 14). In the secondary oocytes, the nucleus as well as cytoplasm stains intensely with Gallocyanin chromalum revealing the presence of both DNA and RNA activity in these stages of ova (Figs. 15, 16 and 17). The absence of cytoplasmic RNA in rapidly dividing oogonia indicate the absence of protein synthesis at this site and on the contrary the high amount of protein in the cytoplasmic area along with the presence of cytoplasmic RNA in the growing oocytes suggests that intense protein synthesis is taking place in this area of the developing ova (Figs 9, 14 and 16).

The oolemma surrounding the secondary oocytes is lipid positive and the lipid granules are seen distributed throughout the cytoplasmic region in high concentration (Fig. 18). Furthermore, the lipid seems to be the major constituent of the egg shell envelope of fertilized ova, whereas its content is comparatively less in the cytoplasm (Figs. 19 and 20). The presence of acid mucopolysaccharides is only seen the egg envelope of the mature ova (Fig. 21). No evidence of this constituent is seen in earlier developing stage of oogonia and oocytes.

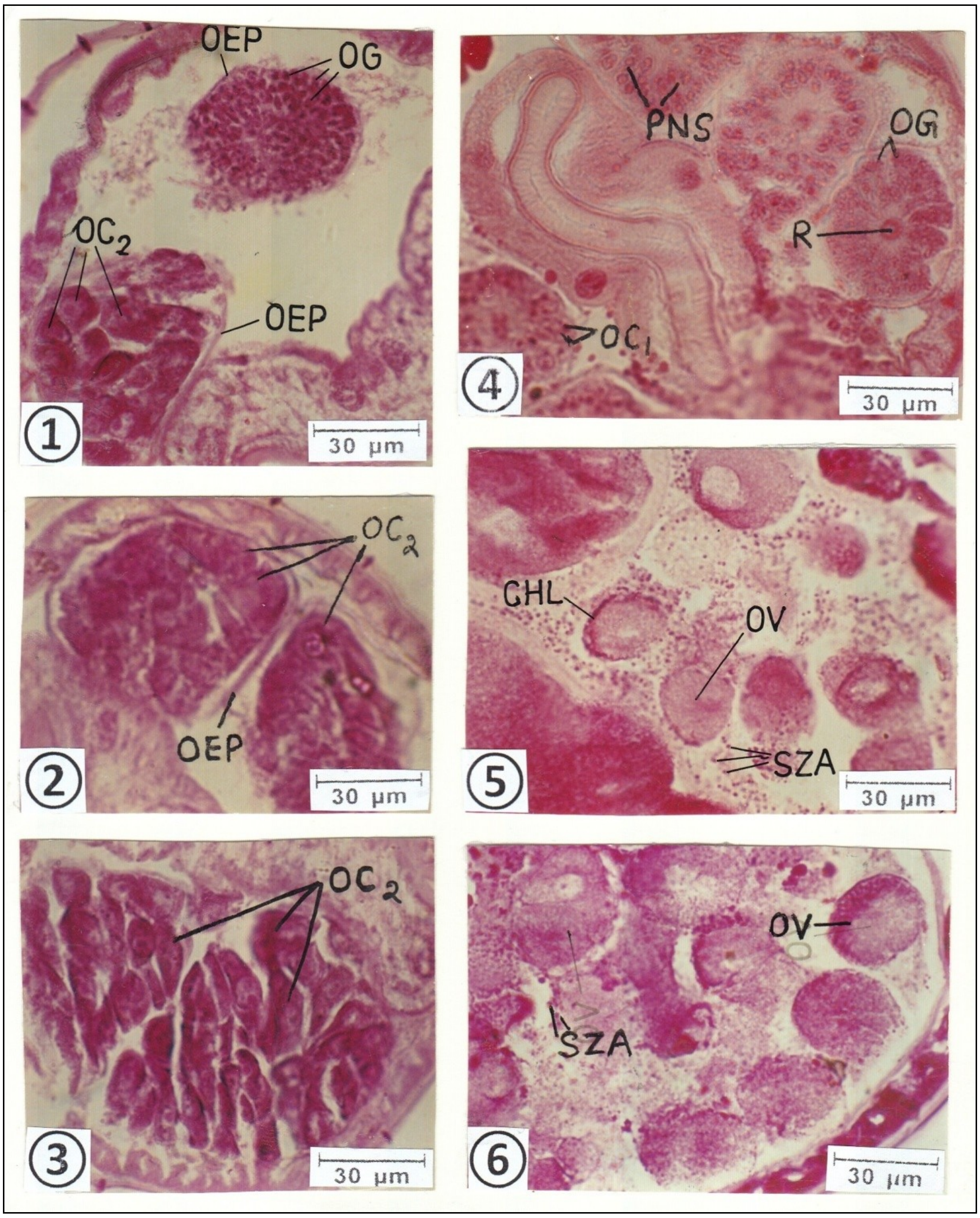

Fig. 1 - 6: *Haemonchus contortus.*

Fig. 1, 2 and 3: A Portion of Transverse Sections (T.S.) of female through ovary showing concentration of carbohydrates in the various stages of oogonia and oocytes (Periodic acid Schiff's staining);
Fig. 4, 5 and 6: A Portion of Transverse Sections (T.S.) of female showing concentration of glycogen in the developing stages of ova (Best's carmine staining).

Abbreviations used: PNS: Perinuclear Spaces of Oogonia; OEP: Ovarian Epithelium; OC_1: Primary Oocytes; OC_2: Secondary Oocytes; OG: Oogonia; OV: Fertilized Ova; R: Rachis; SZA: Spermatozoa fertilizing ova; CHL: Chitinous layer of the egg shell.

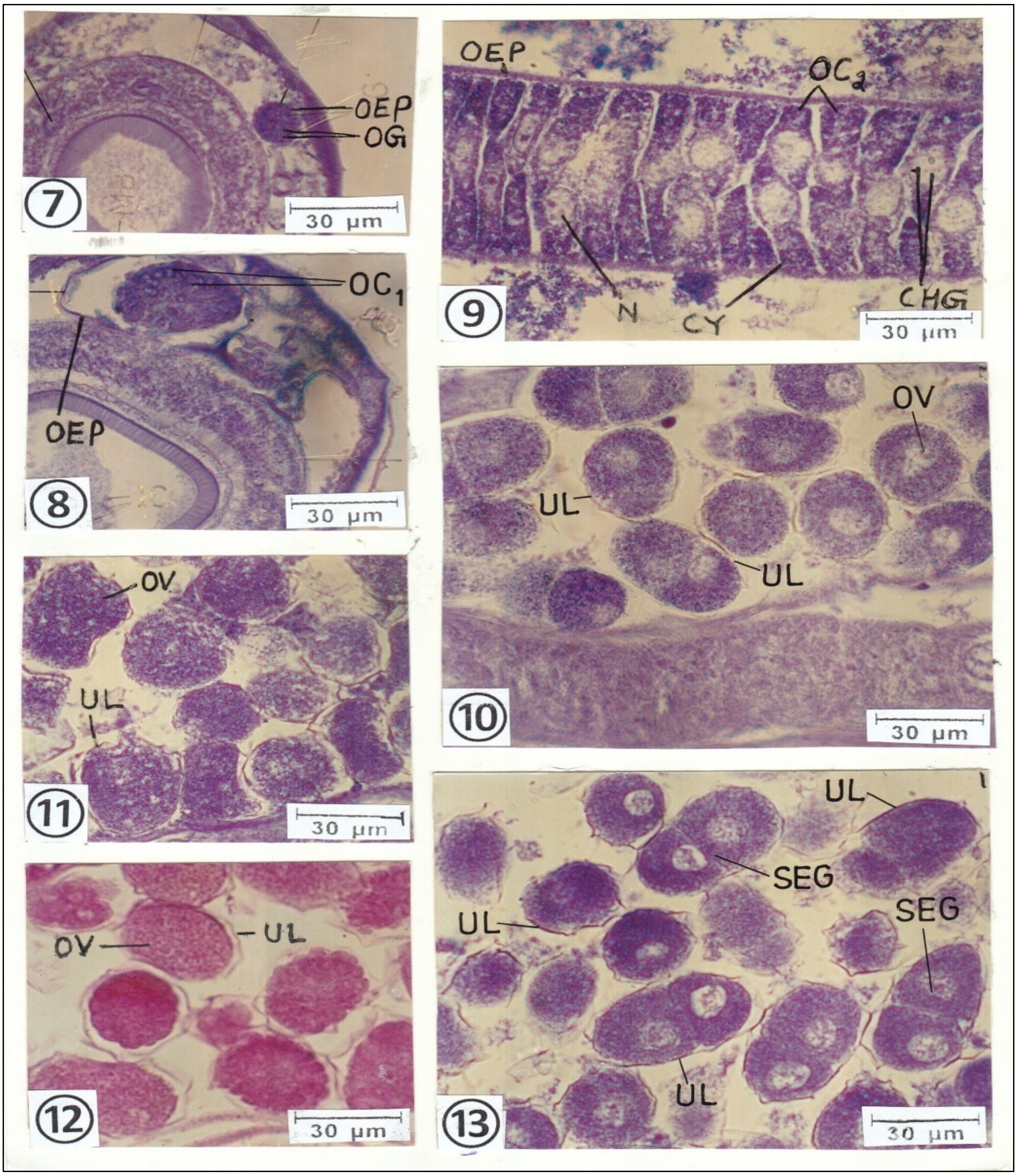

Fig. 7 - 13: ***Haemonchus contortus.***

Fig. 7 and 8: A Portion of T.S. of female through germinal zone of ovary showing concentration of proteins in oogonia and oocytes (Mercuric bromophenol blue staining);

Fig. 9: A Portion of L.S. of female through growth zone of the ovary showing distribution of proteins in secondary oocytes (Mercuric bromophenol blue staining);

Fig. 10 and 11: A Portion of L.S. through the uterus of the female showing concentration of proteins in the uterine layer of the ovum (Mercuric bromophenol blue staining);

Fig. 12: A Portion of L. S. of female showing concentration of $-NH_2$ proteins in the uterine layer of the ovum (Ninhydrin Schiff's staining);

Fig. 13: A Portion of L. S. of female showing distribution of proteins in the segmented stage of the fertilized ova (Mercuric bromophenol blue staining).

Abbreviations used: CHG: Chromatin Granules; CY: Cytoplasm; OEP: Ovarian Epithelium; OC_1: Primary Oocytes; OC_2: Secondary Oocytes; OG: Oogonia; OV: Fertilized Ova; SEG: Segmented Stage of fertilized ova; UL: Uterine Layer of the egg shell.

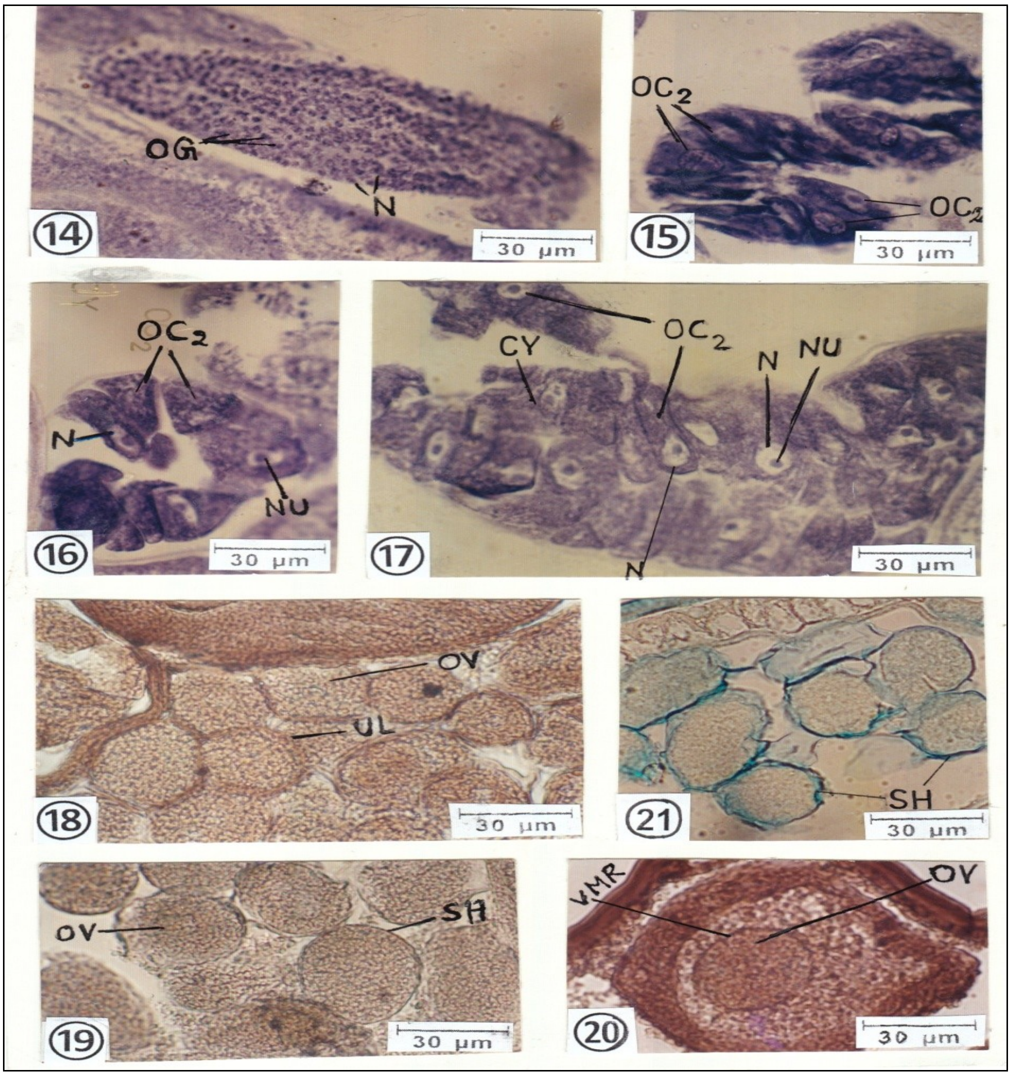

Fig. 14- 21: *Haemonchus contortus.*

Fig. 14: A Portion of L.S. of female through germinal zone of ovary showing concentration of nucleic acids in oogonia (Gallocyanin chromalum staining);
Fig. 15, 16 and 17: A Portion of L.S. of the female through the growth zone of ovaries showing concentration of nucleic acids in the oocytes (Gallocyanin chromalum staining);
Fig. 18, 19 and 20: A Portion of T. S. of female showing concentration of lipids in the fertilized ova (Oil red O in isopropanol staining);
Fig. 21: A Portion of L. S. of female showing concentration of acid mucopolysaccharides in the mature and fertilized ova (Alcian blue staining).
Abbreviations used: CY: Cytoplasm; N: Nucleus; NU: Nucleolus; OC_2: Secondary Oocytes; OG: Oogonia; OV: Fertilized Ova; SEG: Segmented Stage of fertilized ova; SH: Egg Shell of ovum; UL: Outer Uterine Layer; VMR: Vitelline Membrane of the fertilized ovum.

Histochemical studies reveal that the concentration of various metabolites varies during different stages of oogenesis. Faure-Fremiet (1913) has negated the presence of glycogen in the dividing oogonia of *Parascaris equorum*. Lee (1960) has described that in *Thelastoma bulhoesi,* the amount of glycogen in oogonia increases along the length of the ovary. Anya (1964a), Weber (1987) and Johal and Joshi (1993) confirm the findings of the first author and have noted a complete absence of glycogen in the oogonia of *Aspiculuris tetraptera, Loa loa and Trichuris ovis* respectively. Fairbairn (1957), Anya (1964b), Adamson (1983) and Mackinnon (1987) have accounted for a progressive increase in carbohydrate contents in the developing oocytes with a simultaneous depletion of glycogen from the ovary. In *Oesophagostomum columbianum* too, Johal (1995) has reported an increase in the amount of carbohydrates in the developing oocytes lying in the ovary. In present study on *Haemonchus contortus,* the oogonia possess an adequate quantity of carbohydrate especially in their perinuclear spaces. In the secondary oocytes, a rich concentration of carbohydrate is seen in the cytoplasmic portion. The glycogen aggregated in the oocytes is later used in the endogenous formation of the chitinous layer of the fertilized ova. Thereafter, the ova reveal a little amount of glycogen in the egg yolk.

Fairbairn (1957), Anya (1964a), Wharton (1979) and Adamson (1983) have recorded the complete absence of proteins in the proliferating nematode oogonia. They also reported that the occytes accumulate large quantities of protein during their migration down the ovary. In the oocytes the protein is present in the form of hyaline granules. In the present study on *Haemonchus contortus*, the proliferating oogonia show a profuse quantity of protein and even the cytoplasmic strands connecting them are moderately proteinaceous and the protein is seen spread over whole of the cytoplasmic region of the developing oocytes in the form of granules.

In *Trichuris ovis* (Johal and Joshi, 1993) and *Oesophagostomum columbianum* (Johal, 1995), the proliferating oogonia show a significant concentration of DNA as well as RNA in the nucleus. In present study on *H. contortus*, the nucleic acids are restricted to the nuclear region of the oogonia indicating an intense DNA activity in their rapidly dividing stages. Both the nuclei as well as cytoplasm reveal intense nucleic acid activity in the developing oocytes. Their cytoplasm also reveal a rich amount of protein along with RNA indicating that this area is metabolically active.

Adamson (1983) has reported a large amount of lipid droplets in the developing oocytes of *Ascaris lumbricoides*, Mackinnon (1987) too, demonstrated that the large granules located in the cytoplasm of developing oocytes are lipoidal in nature. In the oocytes of *Toxocara canis*, lipid in the form of drops is described by Brunanska (1997). In *H. contortus*, a high concentration of the lipid granules is found in the cytoplasmic region of the secondary oocytes and the oolemma surrounding them is also lipoidal in nature

In egg shell formation in *Haemonchus contortus* the first vitelline layer gets demarcated in the fertilized ova, this is accompanied by a simultaneous shift of glycogen granules towards the periphery which get concentrated to form the second or the chitinous layer of the eggshell, endogenously. All the previous authors working on oogenesis are in consonance about the endogenous formation of the chitinous layer.

About the outer coat of the egg shell it was earlier established by Faure-Fremiet (1913), Wharton (1915), Chitwood (1931), Jacobs (1950) and Anya (1964a; 1964b) that it is proteinaceous in nature and is formed from the secretions of the uterine cells. The research work of Johal (1995) and Johal and Joshi (1993) reveals that the deposition and composition of the outer uterine layer differs in different species. In *Oesophagostomum columbianum*, thick jelly like lipoproteinaceous stands emerge out from the uterine wall and form a loose network around the fertilized ova. Later their interconnections are broken down, resulting in the formation of loose envelops around the ova which become compact as the ova roll down the uterus (Johal, 1995). In *Trichuris ovis* (Johal and Joshi, 1993), the uterine wall secretes a granular secretion and the fertilized ova press to the uterine epithelium to get coated by the secretion which condenses to form a thick layer. The uterine layer is present only on the sides of the ova leaving the polar plugs uncoated. In the present study on *Haemonchus contortus*, an enormous quantity of secretory granules are shed into the lumen of the uterus which align around the fertilized ova in loose granular envelops. The granules subsequently condense to form regular outer wall of the egg shell which is lipoproteinaceous in nature.

The above facts indicate that the oogonia possess protein and lipid in their active phase of division. The secondary oocytes accumulate large quantities of carbohydrates which are later used up in the formation of chitinous layer, whereas the protein and lipid imbibed, mainly from the yolk granules. The uterine lipid and proteins contribute to the formation of outer layer of egg shell which gets coated by acid mucopolysaccharides in the last portion of the uterus.

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