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International Journal of Current Research
Vol. 4, Issue, 03, pp.281-285, March, 2012

INTERNATIONAL JOURNAL OF CURRENT RESEARCH

ISSN: 0975-833X

RESEARCH ARTICLE

OBSERVATION ON OOGENESIS AND EGG SHELL FORMATION IN *HAEMONCHUS CONTORTUS* (NEMATODA)

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ARTICLE INFO

Article History:
Received 20th December, 2011
Received in revised form
05th January, 2012
Accepted 19th February, 2012
Published online 31st March, 2012

Key words:
Oogenesis,
Nematoda,
Histology,
Haemonchus contortus,
Female genital tract.

ABSTRACT

The process of oogenesis and egg shell formation in *Haemonchus contortus*, a blood sucking nematode parasite of small ruminants was studied. In the germinative zone, the oogonia having prominent nuclei and clear cytoplasm, divide mitotically and are arranged around a central anucleate tube like rachis which is not directly connected to the oogonia by their cytoplasmic extensions but sends out various branches in inter-oogonial spaces. In the growth zone of the ovary, the primary and secondary oocytes attain a substantial increase in size. This region of ovary is tightly coiled around the intestine suggesting trans-membrane flow of nutrients from the gut to the gonads. The secondary hexagonal shaped oocytes detach from the rachis and pass through the narrow oviduct and enter into seminal receptaculum. The process of fertilization initiates egg shell formation. The layers of the egg shell being an indiscernible vitelline layer, a chitinous layer and an outer uterine layer. Uterine lipids 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. The ova lying in the proximal part of the uterus have already completed the first segmentation division before their expulsion.

Citation: Jatinderpal Singh, 2012. "Observation on oogenesis and egg shell formation in *Haemonchus contortus* (Nematoda)", *International Journal of Current Research*, 04, (03), 281-285.

INTRODUCTION

Morphologically Nematoda is an exceedingly variable group and there hardly exists any common statement that could be made regarding their histomorphology and histochemistry, which would apply to all forms (Chitwood and Chitwood, 1950). Pawlowski (1987) while addressing the 6th International Congress at Brisbane, Australia stated that there is a renewed interest in basic research which can fill the hitherto unexplained gaps. The studies on oogenesis has been made by a number of workers in different groups of free living as well as parasitic nematodes. Wharton (1980) reviewed the literature on oogenesis and shell formation and stated that the structure of ova varied with taxon but the variations are largely a result of the needs of developing ova. Oogenesis has also been discussed in detail by Chitwood and Chitwood (1950), Maggenti (1981), Adiyodi and Adiyodi (1983) and Bird and Bird (1991). The histomorphology and histochemistry of various organ systems of *Haemonchus contortus* has been discussed by Singh and Johal (1997), Singh (2000), Singh and Johal (2001a, 2001b, 2001c and 2004). The present research paper describes the process of oogenesis and egg shell formation in female *Haemonchus contortus*, which can fill the hitherto existing gaps in information regarding this aspect. This microscopic study will be of significance to understand the metabolic activities and fundamental functional aspects. It can also form the basis in evolving chemotherapeutic measures against this pathogenic parasite.

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MATERIALS AND METHODS

The adult female *Haemonchus contortus* extracted from the abomasum of sheep (*Ovis aries*) were washed in 0.85% NaCl solution to remove debris. For whole mount preparation, after fixation in 70% alcohol at 60 ^{0}C, the nematode worms were cleared and mounted in lectophenol. For histomorphological studies, each worm was fixed in alcoholic Bouin's fixative for 12-24 hours, 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. The serial sections arranged on slides were stained with haematoxylin and eosin. Some slides were also stained with Sudan Black B Staining (McManus, 1946), Best's Carmine Staining (Best, 1906) and Alcian blue (Steedman, 1950) for the detection of lipids, glycogen and acid mucopolysaccharides in the egg shell. The slides were examined under the microscope and photo micrographed.

RESULTS AND DISCUSSION

The process of oogenesis in *Haemonchus contortus* starts in the germinal zone at the tip of the ovaries and includes four phases: multiplication, growth, maturation and fertilization of ovum which is completed in the uterus. The oogonia proliferated in the germinal zone at the tip of ovaries multiply by several mitotic divisions (Fig. 1). Thin protoplasmic strands extending from the oogonia pass inbetween the inter-oogonial spaces (Fig. 1 and 2), join to form thick branches before merging with a central anucleate cytoplasmic cord called the rachis (Fig. 3 and 4). The oogonia are rounded in shape measuring 8.5-10 µm, having indistinct walls and a lightly staining clear cytoplasm. The oogonial nucleus is darkly staining compact mass without any distinct nucleolemma (Fig 1). The oogonial divisions finally result in the formation of primary oocytes. Each primary oocyte is like an elongated triangle in shape, with its nucleus placed at the base. The prominent large vesicular nucleus is covered by a distinct thick nucleolemma and contains condensed chromatin granules in the centre (Fig. 5). In the growth zone, the primary oocytes grow in size and divide mitotically to form secondary oocytes. Simultaneously, their darkly staining cytoplasm transforms into a granular one (Figs.4 and 6).

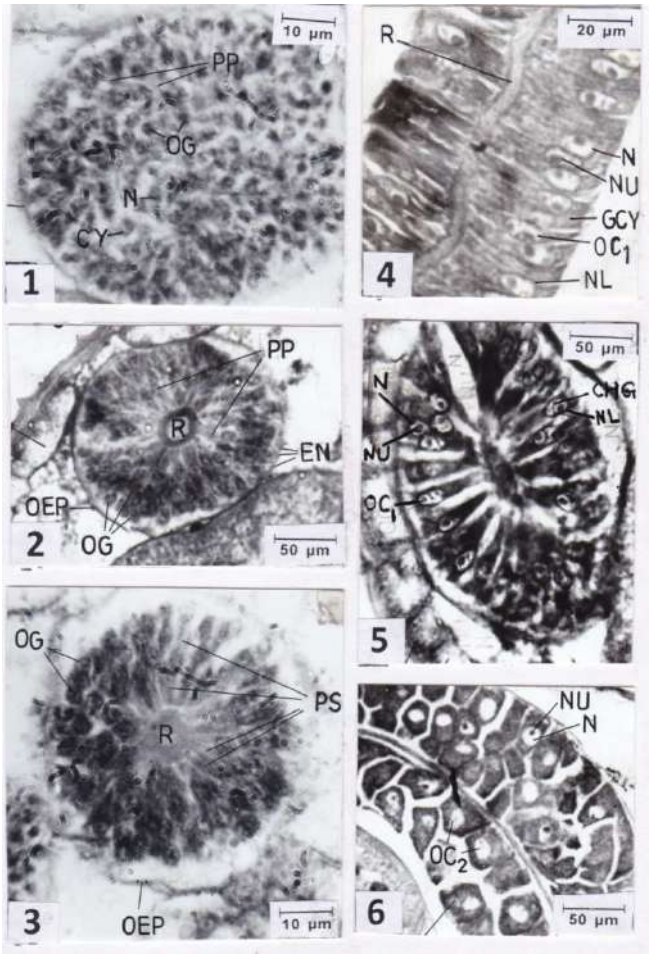

Fig.1-6: *Haemonchus contortus*
Fig.1, 2 and 3. T.S. through the germinal zone of ovary.
Fig.4. L.S. of ovary showing dividing oocytes.
Fig.5. T.S. of female showing dividing oocytes in ovary.
Fig.6. A portion of L.S. of female showing growth zone of ovary.

Abbreviations used: **CHG**: Condensed Chromatin Granules; **CY**: Cytoplasm; **EN**: Epithelial Cell Nuclei; **GCY**: Granular Cytoplasm; **N**: Nucleus; **NU**: Nucleolus; **NL**: Nucleolemma; **OEP**: Ovarian Epithelium; **OC_1**: Primary Oocytes; **OC_2**: Secondary Oocytes; **OG**: Oogonia; **PP**: Protoplasmic Processes; **PS**: Protoplasmic Strands; **R**: Rachis**.**

Initially in the growth zone, the oocytes are attached to the rachis (Fig. 5), as they increase in size, their connection with rachis weakens and ultimately disrupts, thus leaving them free in the lumen (Fig. 6). The secondary oocytes attain a substantial increase in size (46.6 µm in size), their cytoplasm becomes densely granulated and their vesicular nuclei show ill-defined nuclear walls and each measure 16.6 µm. A prominent nucleolus about 5 µm in size is seen in the nucleus. The region of ovary, containing developing oocytes, is tightly coiled around the intestine (Fig. 7). Some drastic morphogenetic changes are seen in the developing oocytes at that time. An increase in size accompanied by accumulation of cytoplasmic granules is observed. Subsequently, the shape of the mature secondary oocyte changes first to form a rough hexagon measuring 46.6 µm (Fig. 8) and then to a spheroid one (Fig. 9). Simultaneously a change in the nuclei is also witnessed. Early stages of meiotic division are seen in which the chromosomes separate out and the nucleoli start dividing (Fig. 9) and the reduction division is completed after the fertilization. The subspherical secondary oocytes lying in the lumen of the ovary pass through the narrow muscular oviduct to enter the seminal receptaculum where these are fertilized by the spermatozoa accumulated in large number at that place. Each ovum is seen to be surrounded by a large number of sperms (Fig. 10). After fertilization, a spurt of ribosomal granules is observed in ovum which completely camouflages the nucleus (Fig. 11). The fertilized egg becomes oval in shape and measure 43.3x30 µm.. The egg shell of *Haemonchus* ova is a transparent, three layered structure. The layers being an indiscernible vitelline layer, a chitinous layer and an outer uterine layer. The process of fertilization initiates shell formation around the mature ova. A vitelline membrane is laid around the fertilized ovum (Fig 11), underneath which concentration of cytoplasmic granules is observed (Fig. 12).

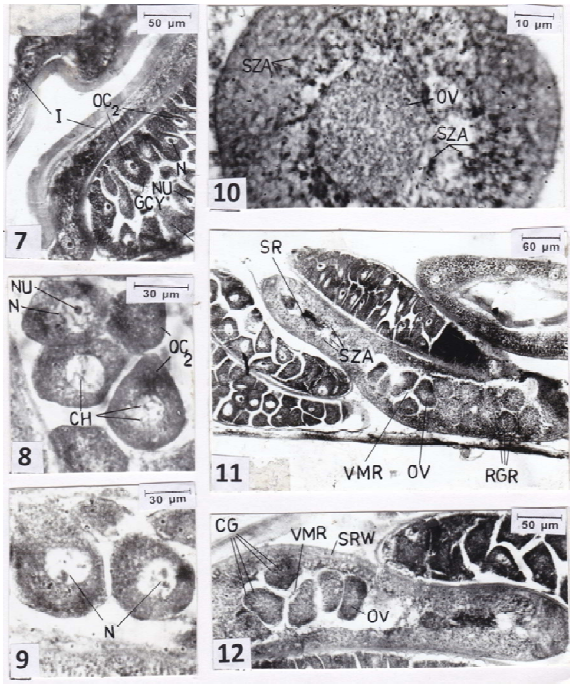

Fig.7-12: *Haemonchus contortus*.
Fig.7, 8 & 9. A portion of L.S. through the growth zone of ovary.
Fig.10. T.S. of seminal receptaculum.
Fig.11 and 12. L.S. of female showing seminal receptaculum.

Abbreviations used: **CH**: Chromosomes; **CG**: Cytoplasmic Granules; **GCY**: Granular Cytoplasm; **I**: Intestine; **N**: Nucleus; **NU**: Nucleolus; **OC_2**: Secondary Oocytes; **OV**: Ovum; **R**: Rachis**; RGR:** Ribosomal Granules; **SR:** Seminal Receptaculum; **SRW**: Seminal Receptaculum Wall; **SZA**: Spermatozoa; **VMR**: Vitelline Membrane.

Subsequently, this concentration gets demarcated in the form of a second chitinous layer, which is formed endogenously from the glycogen reserves of the egg cytoplasm (Fig. 13). As the ova roll down in the uterus these are surrounded by granules of the uterine secretions (Fig. 14) lying in the lumen which aggregate to form loose envelops around them (Fig. 15). Subsequently this loose envelope transforms into a compact outer lipoproteinaceous layer of the egg shell (Figs. 16 and 17).

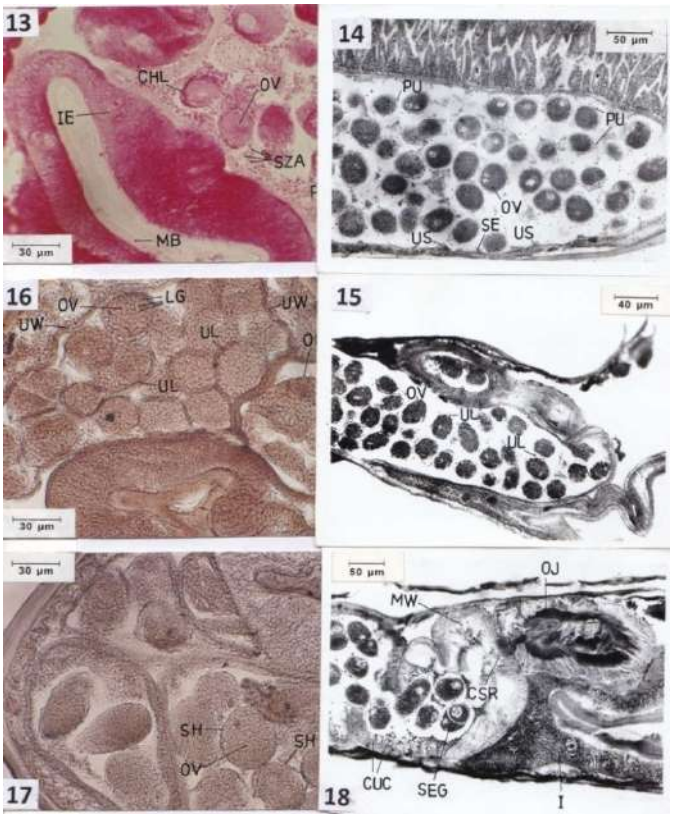

Fig.13-18: *Haemonchus contortus*
Fig.13, 14 and 15. T.S. of female through different regions of uterus.
Fig.16, 17 & 18. L.S. of female through different regions of uterus.

Abbreviations used: **CH**: Chitinous Layer Of Fertilized Ovum; **CSR**: Constriction between uterus and ovijector; **COC**: Oblique Columnar Cells; CSR: Constriction between uterus and Ovijector; **CUC**: Cuboidal Cells; **LG**: Lipid Granules; **MW**: Muscular Wall of Uterus; **OJ**: Ovijector: **OV**: Ovum; **PU**: Primordium of Uterine Layer; **SE**: Secretory Epithelium; **SEG**: Segmented ova; **SH**: Egg shell of the fertilized ova; **SZA**: Spermatozoa; **UL**: Uterine Layer of egg shell; **US**: Uterine Secretion; **UW**: Uterine Wall.

The ova lying in the proximal part of the uterus have already completed the first segmentation division before expulsion. These are expelled out through vagina at two-celled stage measuring 50 to 63 µm long and 22.6 to 33 µm wide (Fig. 18). The studies on oogenesis performed by a host of workers reveal that a number of morphological differences occur in the oogonia and developing ova of different species of nematodes. In *Aspiculuris tetraptera*, the newly proliferated oogonia at the proximal end of the ovaries are enveloped by a thin membrane. They increase considerably in size as a result of the accumulation of food reserves, especially glycogen. Two types of inclusions namely the hyaline spheres and the large refringent bodies fill most of the cytoplasm (Anya, 1964). In *Dipetalonema viteae*, irregularly shaped oogonia, developing from the syncytium, quickly become more cuboidal in shape, contain large nuclei, ribosomes, small mitochondria and a few golgi bodies (McLaren, 1973). According to Wharton (1979), the oogonia of *Aspiculuris tetraptera* have a granular cytoplasm, some mitochondria and large nuclei with 1 or 2 nucleoli in each. In *Gyrinicola batrachiensis*, Adamson (1983) has described a spherical oogonium with most of its volume filled with dark spherical nucleus. The cytoplasm is rich in ribosomes and contains a few mitochondria, occasional lipid droplets and irregular aggregations of electron dense material. Brunanska (1991) has reported that in *Syngamus trachea* cell organelles and inclusions like mitochondria, ribosomes, lysosomes, glycogen and lipid granules increase in number with the maturation of oogonia. Joshi (1991) and Takahashi *et al*. (1993) have observed a very small quantity of clear cytoplasm without any evident granulation in the oogonia of *Trichuris ovis* and *Trichinella spiralis* respectively. In the present study on *Haemonchus contortus*, numerous oogonia undergoing mitotic divisions are seen in the germinal zone. Each oogonium is rounded in shape, comparatively larger in size i.e., 10 µm with a lightly staining clear cytoplasm and an indistinct cell wall. The oogonial nucleus is a darkly staining compact mass without any distinct nucleolemma. The ill defined bounding walls may be due to the rapid divisions going on at this state.

McLaren (1973) in his study on oogenesis in *Dipetalonema viteae* has described an enormous number of primary oocytes in the ovary. Each oocyte contains a large rounded nucleus with a dense nucleolus, elongate mitochondria, little endoplasmic reticulum and a few golgi bodies, along with some lamellar bodies. In the growth zone of the ovary, the oocytes increase in size and become elongated in shape. A marked growth in size is observed in the oocytes of *Aspiculuris tetraptera* and *Syphacia obvelata* by Wharton (1979) in the growth region of ovary. During this period the oocytes separate off from the rachis and show an increase in the number of mitochondria, electron-lucent hyaline granules, electron-dense shell granules and glycogen granules. This is also accompanied by an appearance of strands of endoplasmic reticulum in the cytoplasm. In *Ascaris suum*, Wu and Foor (1983) have described the immature oocyte as elongate bipolar cells with an apical region containing numerous lobate cytoplasmic processes and a slender opposite pole corresponding to the basal region previously attached to the central cytoplasmic core or rachis, during its development. In *Heligmosomoides polygyrus,* the mature oocyte prior to fertilization becomes oval in shape, has a distinct nucleus, its cytoplasm gets packed with 3 types of granules and the plasma membrane acquires a delicate flocculent coat (Mackinnon, 1987). In the growth zone of the ovary of *Haemonchus contortus*, the primary oocytes connected to the rachis are thin and elongate in shape have large vesicular nuclei, are covered by a thick oolemma and divide to form secondary oocytes which ultimately detach from the rachis and come to lie free in the lumen of the ovary. Each young secondary oocyte is an elongated club shaped structure with its vesicular nucleus placed more near to the apical end. The nucleolemma as well as the cell membrane of secondary oocytes becomes indistinct contain chromatin granules in the centre. The cytoplasm shows intense granulation. Subsequently in the last portion of the ovary, the densely granulated secondary oocytes undergo a substantial increase in size and assume a rough hexagonal shape measuring 46.6 µm. The nucleolemma of vesicular nuclei is still indistinct but the cell membrane begins to appear. In some oocytes, the initiation of meiotic division is observed.

In *Dipetalonema viteae*, while the oocytes are still in the growth zone, a surface coat appears around them and by the time they reach the fertilization chamber, the surface coat

becomes more conspicuous and is seen to be very closely applied to the outer leaflet of the oolemma (McLaren, 1973). After fertilization, in the ova of *Trichuris muris*, the first visible change observed is the redistribution of the two types of granular cytoplasmic inclusions. Prior to and immediately after sperm entry, the external vitelline layer is seen in close association with the oolemma (Preston and Jenkins, 1984). In various other nematodes, a new oolomma is synthesized beneath the original membrane (Lee and Lestan, 1971; Mclaren, 1973; Wharton, 1979 and Perry *et al*., 1982). Structurally, the vitelline membrane has a unit membrane appearance in *Aspiculuris tetraptera* (Anya, 1964), *Capillaria hepatica* (Grigonis and Solomon, 1976) and *Trichuris muris* (Preston and Jenkins, 1984). In the present study on *Haemonchus contortus,* the hexagonal shaped oocytes from the posterior part of the ovary while passing through the narrow oviduct acquire a slight change in shape and get fertilized by the sperm accumulated in large numbers in the fertilization chamber. Although an actual entry of the sperm nucleus into the ovum is not seen but a spurt of ribosomal granules completely camouflaging the nucleus is observed. By the time the uterine layer is formed, the nucleus again becomes distinct.

During 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 egg shell, 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), Chitwood (1930), Jacobs (1950) and Anya (1964) 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 envelope 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.

Acknowledgements

Author is thankful to Prof. (Dr.) Manjit Johal for her guidance, encouragement and constant support for this research work. Author is also indebted to the Professor and Head, Department of Zoology, Punjabi University, Patiala and Principal, Baring Union Christian College, Batala for providing necessary facilities to carry out the research.

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