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ULTRASTRUCTURAL STUDY ON SPERMATOGENESIS IN RUMEN AMPHISTOME *ORTHOCOELIUM SCOLIOCOELIUM* (TREMATODA: DIGENEA), A PARASITE OF *BUBALUS BUBALIS* IN UDAIPUR

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ABSTRACT
Spermatogenesis in rumen amphistome species *Orthocoelium scoliocoelium* (Trematoda: Digenea, Paramphistomidae) a parasite of *Bubalis bubalis* has been examined by transmission electron microscopy. Observations demonstrated that the each testis is covered by tunica with fibrous connective tissues. Below the tunica different stages of spermatogonia, spermatocytes, spermatid cells and spermatozoa were observed. The spermatozoon of *O. scoliocoelium* are elongate, similar to the pattern described in other digenea, showing nuclei, mitochondria and two axonemes with the 9+1 configuration. On the basis of present investigations, it may be concluded that the ultrastructure of spermatogenesis in amphistome *O. scoliocoelium* basically follows the common digenean plan.

INTRODUCTION

The ultrastructural studies on spermatogenesis have previously been used as characters for phylogenetic studies within the Platyhelminthes (Grant *et al.,* 1976; Justine, 1991a and 1991b, 1995, 1999 and 2001).

Important investigations were carried out on the ultrastructure of spermatogenesis of monogenean in the past decade (Halton and Hardcastle, 1976; Sharma and Rai, 1995; Watson and Rohde, 1995 and Watson *et al.,* 1995).

In recently published papers, the ultrastructure of spermatogenesis of digenea has been dealt with only a few of the existing species, such as *Haematoloechus medioplexus* (Burton, 1972), *Schistosoma mansoni* (Kitajima *et al.,* 1976), *Cryptocotyle lingua* (Rees, 1979), *Corrigia vitta* (Robinson and Halton, 1982), *Bucephaloides gracilescens* (Erwin and Halton, 1983), *Didymozoon* (Justine and Mattei, 1983 and 1984). *Fasciola hepatica* (Stitt and Fairweather, 1990), *Dicrocoelium dendriticum* (Cifrian *et al.,* 1993), *Hypoderaeum conoideum* (Chen *et al.,* 1996), Postorchigenes *gymnesicus* (Gracenea *et al.,* 1997), *Mesocoelium monas* (Iomini *et al.* 1997), *Opecoeloides furcatus* (Miquel *et al.,* 2000), *Saccocoelioides godoyi* (Baptista-Farias *et al.,* 2001) and *Didymocystis wedli* (Pamplone-Basilio *et al.,* 2001), *Schistosoma japonicum* (Yang *et al.,* 2003) and *Fasciola gigantica* (Ndiaye *et al.,* 2004).

Ultrastructural studies of spermiogenesis of paramphistomidae family only four species have been studied by transmission electron microscopy. These are *Basidiodiscus ectorchus* and *Sardonia sudanensis* (Ashour *et al.,* 2007, *Paramphistomum microbothrium* (Seck *et al.,* 2007), and *Cotylophoron cotylophorum* (Seck *et al.,* 2008). But none of the scientist paid attention on the ultrastructural studies on spermatogenesis in Paramphistomidae.

The aim of present study is to expose the characteristics of Ultrastructural study on spermatogenesis in rumen amphistome *Orthocoelium scoliocoelium* (Trematoda: Digenea, Paramphistomidae) a parasite of *Bubalis bubalis* in Udaipur.

MATERIALS AND METHODS

Live amphistomes were collected from the rumen of the freshly slaughtered buffalo (*Bubals bubalis*) at local Zoo abattoir in Udaipur. Amphistomes removed from rumen and washed several times in tap water to make them free from debris and mucous. Then they were transferred into physiological saline solution for their maintenance. Small fragments of mature amphistome (*Orthocoelium scoliocoelium*) were fixed at 4ºC for one hour in 4% glutaraldehyde in 0.1M phosphate buffer at pH 7.2. and post fixed for one hour 1% osmium tetraoxide in 0.1M phosphate buffer. Then dehydrated, through acetone ascending series and embedded in durcupan resin. The blocks were sectioned using glass or diamond knives on a LKBIII ultramicrotome. The ultrathin sections were mounted on uncoated 200 mesh copper grids. Stain with uranyl acetate and lead citrate. The grids were examined under Philips

Electron Microscope.

RESULTS

Transmission electron microscopic observations demonstrated that the testes of amphistome species *Orthocoelium scoliocoelium* are covered by fibrous connective tissue or tunica, which forms a continuous layer around each testis. Below the tunica a uniform layers of different stages of spermatogonial cells were observed and cytoplasm of this cells are contain clusters of ribosome's, numerous electron lucent vesicles, granular endoplasmic reticulum and oval to elongate mitochondria with a slightly dense matrix. The spermatogonia constitute three cell generations, primary spermatogonia, secondary spermatogonia and tertiary spermatogonia after successive mitotic divisions (Fig. 1).

Spermatogonia

In testes of amphistome *O. scoliocoelium*, spermatogonia are cells which have a central nucleus, usually spherical, occupying almost all the cytoplasm. Spermatogonia form a peripheral layer, appeared as irregular- shaped cells, characterized by a prominent nucleus, showing a low cytoplasmic-nucleus ratio that did not contain a typical cytoplasmic structures that are characteristic for non-differentiated cells. Their nucleus-to-cytoplasm volume ratio was high. Heterochromatic nucleus was surrounded by a narrow perinuclear space. The cytoplasm matrix is electrondense due the presence of ribosome's, disposed in rosettes or polysome-like. Mitochondria and cisternae of granular endoplasmatic reticulum are present in cytoplasm but Golgi complex was not observed. Primary spermatogonia following the first mitotic division, two secondary spermatogonia are formed and both cells remain in close proximity to each other (Fig. 1).

Little morphological differences were observed between the primary and secondary spermatogonia, both having a large round nucleus surrounded by a small amount of cytoplasm and standard organelles. Secondary spermatogonia divide mitotically to produce four tertiary spermatogonia. Spermatogonia in the tertiary stage present mitochondria joined at the cell apex with the nucleus displaced to the base (Fig. 2).

Spermatocytes

Spermatocytes cells are the largest of the process, and show an increase cyto-plasmic-nucleus ratio. The nucleus presents sparse chromatin, arranged in small groups. The cytoplasm presents numerous mitochondria dispersed throughout it, granular endoplasmatic reticulum and free ribosome's, disposed in groups or rosettes (Fig. 3, 4, 5 and 6).

Primary Spermatocytes

The spermatogonia produce eight primary spermatocytes through three consecutive mitotic divisions and they were largest cells to occur during spermatogenesis. The primary spermatocytes were clearly identified by the presence of synaptonemal complex within their nuclei. This indicated the pairing of homologous chromosomes in meiotic prophase. In the karyoplasm, condensed chromatin was sparse, presenting either in small aggregation adjacent to the nuclear membrane or dispersed throughout the karyoplasm. The perinuclear space in the spermatocytes was smaller than that in the spermatogonia. The cytoplasm presents numerous mitochondria with short cristae, granular endoplasmatic reticulum and numerous free ribosome's (Fig. 3).

Secondary Spermatocytes

The eight primary spermatocytes undergo synchronous

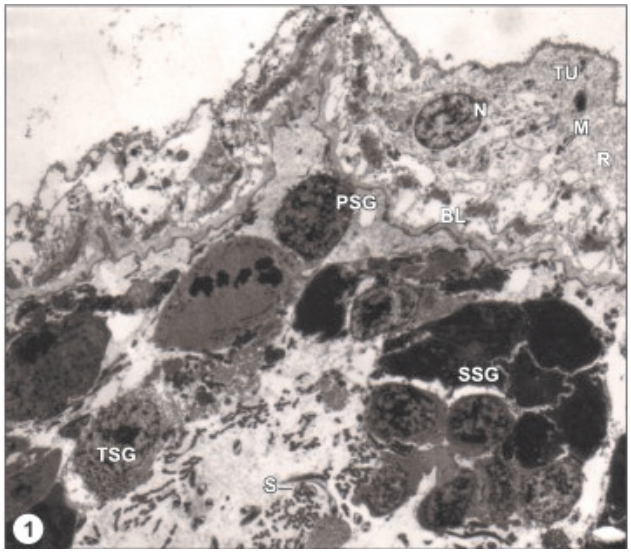

Figure 1: Ultrastructural photograph through peripheral of testes of amphistome species Cotylophoron cotylophorum exhibiting tunica (Tu) which forms a continuous layer around the testis with germinal cell, mitochondria (M), ribosome's (R) and basal lamella (BL). The germinal tissue of testis is separated from the tunica by Primary Spermatogonia (PSG), Secondary Spermatogonia (SSG) and Tertiary Spermatogonia (TSG) appeared as irregular- shaped cells with a prominent heterochromatic nucleus and sperm (S). X 10000

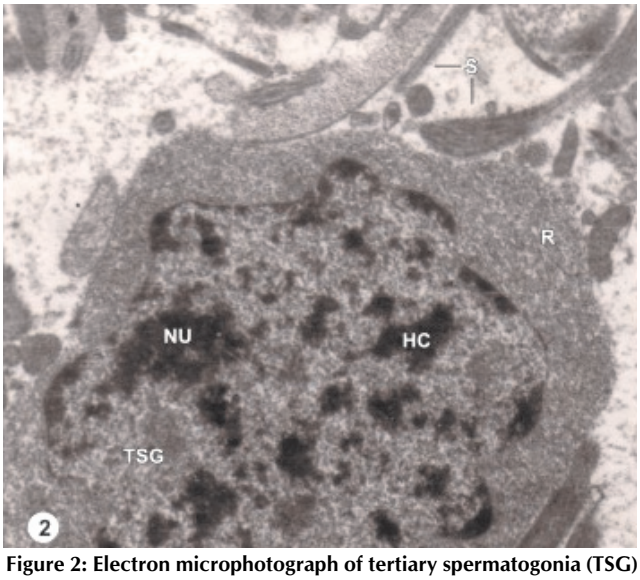

Figure 2: Electron microphotograph of tertiary spermatogonia (TSG) containing prominent heterochromatin material (HC) and Nucleolus (Nu) in nucleus and cytoplasmic matrix of this cell is electrondense due the presence of ribosome's (R). Note the sperm (S) present outside of the spermatogonial cell X 18000

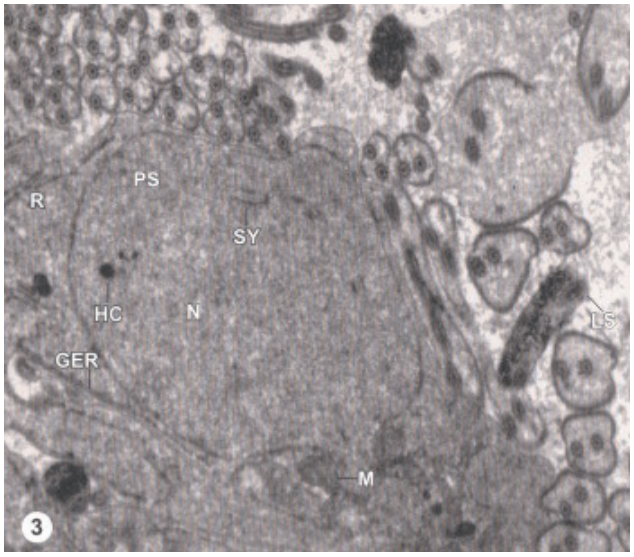

Figure 3: Ultrathin section through primary spermatocyte (PS) showing synaptonemal complex (SY) and spars hetero chromatin granules (HC) presenting either in small aggregation adjacent to the nuclear membrane and dispersed throughout the karyoplasm within nucleus (N). The cytoplasm of this cell contains numerous mitochondria (M), granular endoplasmatic reticulum (GER) and free ribosome's (R). Note transverse and longitudinal (LS) sections of sperms. X 14000

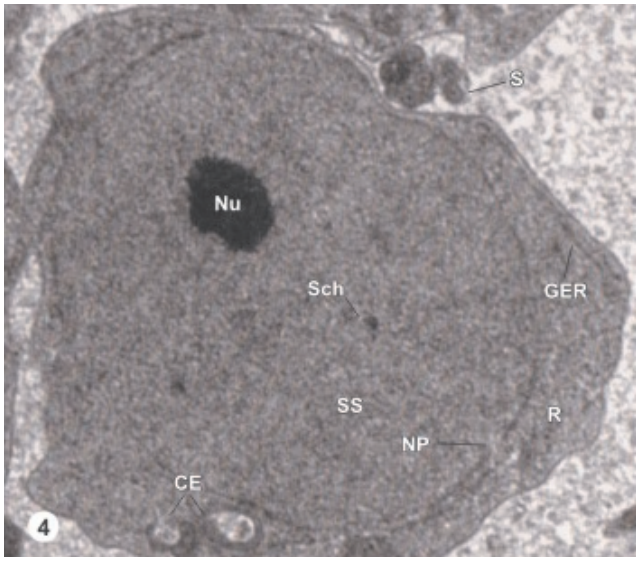

Figure 4: Ultrastructure photograph through secondary spermatocytes (SS) showing rounded big size nucleus with spars chromatin material (S Ch), nuclear pores (NP) and nucleolus (Nu) and cytoplasm of this cell contains granular endoplasmatic reticulum (GER), free ribosome's (R) and a pair of centrioles (CE). X 14000

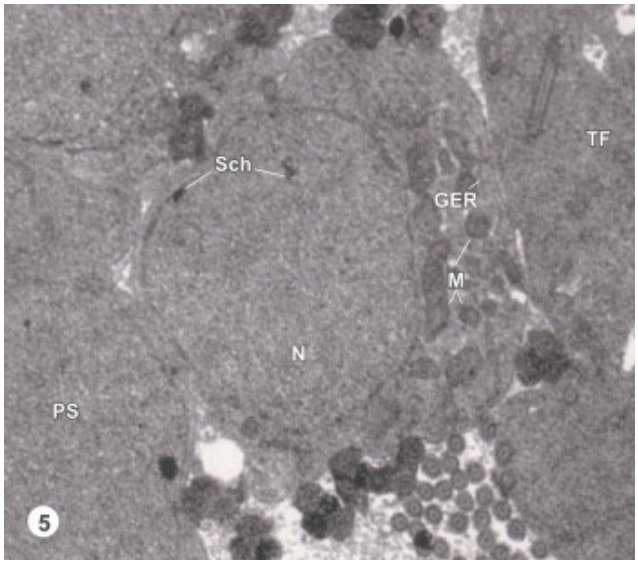

Figure 5: Electron microphotograph exhibiting testes follicles (TF), primary spermatocyte (PS), secondary spermatocytes contains rounded big size nucleus (N) with spars chromatin(S Ch) material. Cytoplasm of secondary spermatocytes contains granular endoplasmatic reticulum (GER) and numerous mitochondria (M) with short cristae in the apical region. X 19000

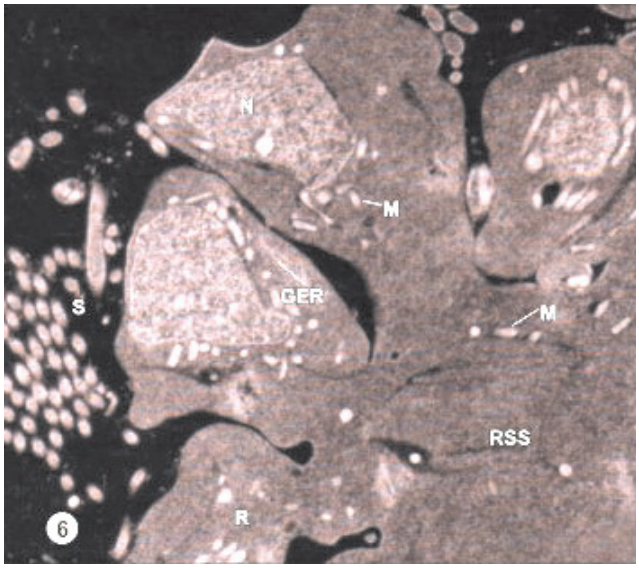

Figure 6: Electron micrograph showing secondary spermatocytes arrange in a rosette form (RSS) with large nucleus (N), granular endoplasmatic reticulum (GER), free ribosome's (R) and numerous mitochondria (M). Note the sperm(S) present outside of the rosette form secondary spermatocytes X 18000

meiotic divisions to form sixteen secondary spermatocytes arranged in a rosette. This appearance to be relatively short lived stage, as it has been observed only infrequently in the section examined. These cells are characterized by the presence of mitochondria aggregates at the apical pore and nucleoprotein in the form of electron dense thread in the cytoplasm. The nucleopores are clearly visible; there is also a reduction in heterochromatin (Fig. 6).

The secondary spermatocytes were separated by interspaces between the irregularly shaped cell surfaces. These cells are characterized by: large rounded nucleus with spars chromatin material, nuclear pores and nucleolus. Cytoplasm of secondary spermatocytes contains coated vesicles, granular endoplasmatic reticulum and free ribosome's, occasionally, a pair of centrioles was observed near the nuclear membrane

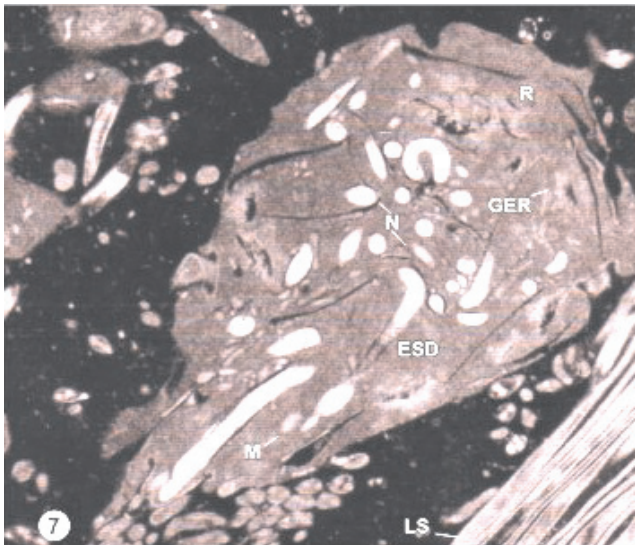

Figure 7: Transmission electron photomicrograph of early spermatid (ESD) exhibiting small elongated nucleus (N), granular endoplasmatic reticulum (GER), free ribosome's (R) and numerous mitochondria (M). Note the longitudinal sperm (LS). X 18000

(Fig. 4) and numerous mitochondria with short cristae in the apical region (Fig. 5).

Spermatids

Synchronous divisions of the secondary spermatocytes produce a rosette of thirty- two (32) spermatid. The spermatid nuclei were significantly smaller than those of the spermatocytes. Small nuclei of spermatid contain small nucleolus and chromatin with equally distributed in clumps or short and dense filaments. Free ribosome's in compact groups are observed in the cytoplasm of the spermatids.

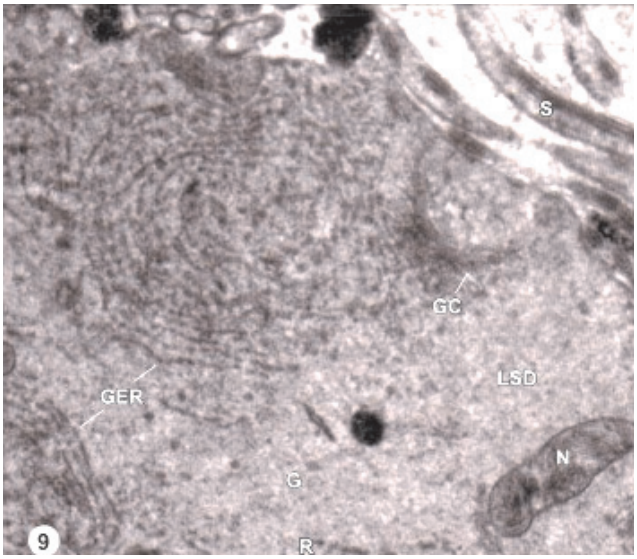

Figure 9: High magnification photomicrograph of a part of cytoplasm of late spermatid (LSD) showing, large rounded form grouped granular endoplasmic reticulum (GER), Golgi complexes (GC), numerous free ribosome's (R), elongation of the nucleus (N) and glycogen granules(G). Note the sperm(S) present outside of the cytoplasm of late spermatid. X 20000

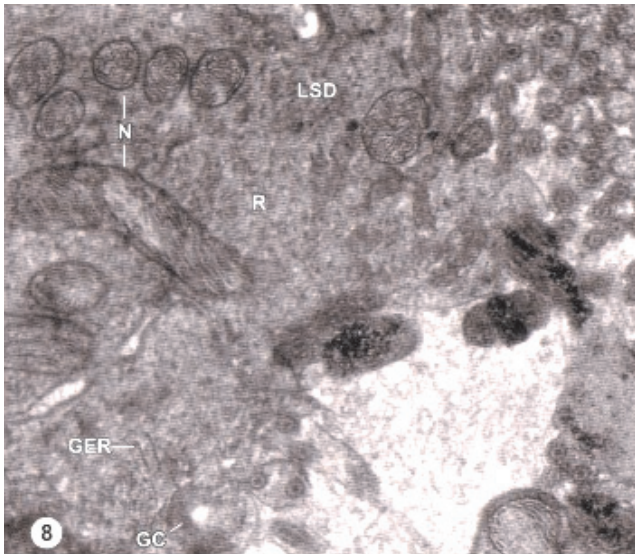

Figure 8: High magnification Electron micrograph of a part of cytoplasm of late spermatid (LSD) showing syncytial masses with the chromatins condensed, helicoidally twisted lamellae in the elongated nuclei (N) moved into the cytoplasmic projections, granular endoplasmic reticulum (GER), Golgi complexes (GC) and numerous free ribosome's (R) in the cytoplasm. X 25000

Early spermatids

The nucleus of early spermatid cell migrates to the peripheral cytoplasm, it elongates through the longitudinal axis of the cellular projection and changing its shape to cylindrical. The chromatins of this cell become condensed and lamellar. In transverse sections of early spermatids shows; the lamellae

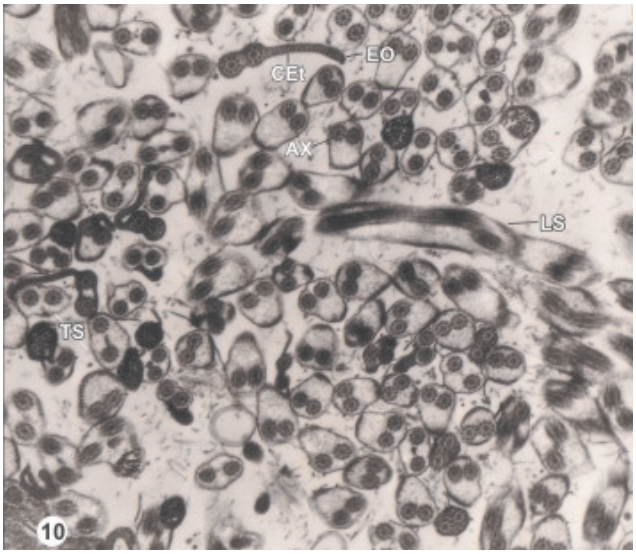

Figure 10: Ultrastructural photomicrograph showing, longitudinal (LS) and transverse (TS) sections of mature spermatozoa. The posterior portion of the terminal region of spermatozoa revealed the cytoplasmic expansion (CEt) and the external ornamentation (EO) of cell membrane, two axonemes (AX) and peripheral microtubules; each axoneme is composed of a central unit, surrounded by nine doublets of microtubules, characterizing the 9 + 1 configuration. X 20000

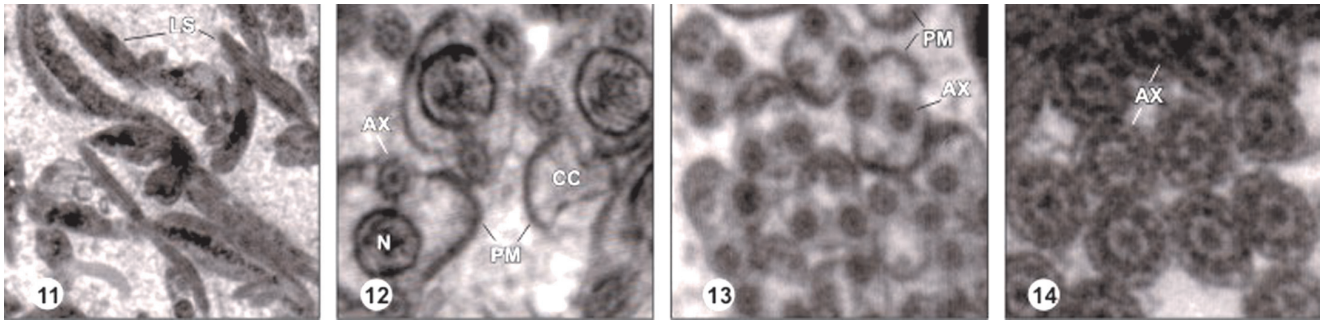

Figure 11: Ultrastructural photomicrograph showing, longitudinal section of mature spermatozoa (LS). X 22000

Figure 12: Transmission electron micrograph through transversal section of the apex of the spermatozoa showing cytoplasmic cap (CC) with peripheral microtubule (PM), the nucleus (N), two axonemes (AX) with the traditional configuration 9 + 1 and peripheral microtubules (PM) situated beneath the dorsal and ventral membranes. X 18000

Figure 13: High power photomicrograph through transversal section, through the beginning of the terminal region of the spermatozoa, showing two axonemes (AX) with the traditional configuration 9 + 1 and peripheral microtubules (PM) situated beneath the dorsal and ventral membranes. X 22000

Figure 14: High power photomicrograph through transversal sections of the terminal region of spermatozoa showing, single axoneme (AX) with composed of a central unit, surrounded by nine doublets of microtubules, characterizing the 9 + 1 configuration. X 22000

present a honeycomb- like appearance, the nucleolus remains for a short time. In early spermatids, the mitochondria occupy a perinuclear position with dense matrix and longitudinal cristae. Central bodies and basal bodies were occasionally seen. Golgi Complexes, endoplasmic reticulum and ribosome's were observed in the cytoplasm of spermatid (Fig. 7).

Late Spermatid

The late spermatid clustered formed syncytial mass. The syncytial mass of late spermatid contains; conspicuous multivesicular bodies, electron-lucent vesicles of various sizes, large sized dense bodies, various strands of large grouped granular endoplasmic reticulum, Golgi Complexes, free ribosome's and glycogen granules. The chromatins were present condensed in long, helicoidally twisted lamellae. In the transverse section, the chromatin material gives a typical honeycomb appearance. The nucleus continues to elongate and migrates to the distal position of the median process. Some of the large electron-lucent vesicles contain small granules, indicating that they are forerunners of the multivesicular bodies. The mitochondria, which are in a perinuclear position of early spermatids, also migrate to the median process of late spermatids, forming a long cylindrical body (Figs. 8 and 9).

Spermatozoa

The mature spermatozoa of amphistome species *O. scoliocoelium* is characterized by two axonemes, a nucleus, a mitochondrion, two set of parallel arranged peripheral cortical microtubules (Figs. 10, 11, 12, 13 and 14).

A mature spermatozoon may be differentiated into proximal part contain head and middle regions and distal or terminal regions because of the length of the nucleus which occupies about two third of the total length of the spermatozoon from the proximal end (Fig. 11).

The apex of the sperm is made up of the cytoplasmic cap only; however, the microtubule below the plasma membrane may be seen arranged peripherally (Fig. 12). A little behind the cytoplasmic apex begins the electron-dense nucleus and glycogen granules.

Anterior region of spermatozoa exhibits nucleus with honeycomb appearance of the chromatin and origin of two axonemes (Fig. 12).

The median region of the spermatozoon contains the nucleus, mitochondria, two axonemes with the traditional configuration 9 + 1, abundant glycogen granules and a row of peripheral microtubules, located just below the dorsal and ventral membranes, which support the plasma membrane.

The terminal region extends from the distal extremity of the nucleus to the posterior end of mature spermatozoa of amphistome species O. *scoliocoelium*. The anterior portion of the terminal region of spermatozoa observed a nucleus, two axonemes, glycogen granules and peripheral microtubules. The posterior portion of the terminal region of spermatozoa revealed the cytoplasmic expansion and the external ornamentation of cell membrane, two axonemes and peripheral microtubules; each axoneme is composed of a central unit, surrounded by nine doublets of microtubules, characterizing the 9 + 1 configuration and surrounded by situated beneath the dorsal and ventral membranes (Figs. 10 and 13) and in the final portion; the cell is narrower and presents just one or two remaining microtubules in the dorsal and ventral membranes. The terminal region of a mature spermatozoon contains single axoneme (AX) with the traditional configuration 9 + 1 (Fig. 14). Large amounts of glycogen granules were observed in mature sperm.

DISCUSSION

The ultrastructural organization of the testis and cytological transformations during the spermatogenesis in amphistome species *O. scoliocoelium* are in agreement with those reported for other trematodes species including *Haematoloechus medioplexus* (Burton, 1972), *Cryptocotyle lingua* (Rees, 1979), *Corrigia vita* (Robinson and Halton, 1982), *Bucephaloides gracilescens* (Erwin and Halton, 1983), *Fasciola hepatica* (Stitt

and Fairweather, 1990), *Ganeo tigrinum* (Sharma and Rai, 1995), *Concinnocotyla australensis* (Watson *et al.,* 1995), *Multicotyle purvisi* (Watson and Rohde, 1995), *Hypoderaeum conoideum* (Chen *et al.,* 1996), *Postorchigenes gymnesicus* (Gracenea *et al.,* 1997), *Mesocoelium monas* (Iomini *et al.,* 1997). *Opecoeloides furcatus* (Miquel *et al.,* 2000) and *Didymocystis wedli ariola* (Pamplone-Basilio *et al.,* 2001) .

Present investigations are agreements of spermatogenesis of *Fasciola hepatica* (Stitt and Fairweather, 1990) and *Dicrocoelium dendriticum* (Cifrian *et al.,* 1993) found that the primary spermatogonia engaged the periphery of the testis; undergo three mitotic divisions to give rise to eight primary spermatocytes. Each primary spermatocyte again undergoes meiotic division, first to form a cluster of sixteen secondary spermatocytes and subsequently the rosette of thirty-two spermatids which develop into fully mature spermatozoa by the process called spermiogenesis. The division in primary and secondary spermatogonia is partial, so their resulting cell population remains connected at the base by cytoplasmic bridges, In the present amphistome like other digenetic trematodes there are fixed number of spermatozoa (32) formed from a single spermatogonia, but in monogenean genera viz. Megalocotyle and Diplectanum instead of thirty two, sixty four spermatozoa are formed (Justine and Mattei, 1983 and 1984).

The process of spermatogenesis in *O. scoliocoelium* is similar as previously observed in *Saccocoelioides godoyi* (Baptista-Farias *et al.,* 2001) that the irregular-shaped spermatogonia form a peripheral layer, and show a prominent nucleus. Spermatocytes are larger than spermatogonia, and in the early stage present synaptonemal complex.

The primary spermatocytes are the largest cells formed during spermatogenesis in amphistome species *O. scoliocoelium*. These cell contain large nucleus with synaptonemal complexes, nucleolus like body (NLB), coated invaginations and associated vesicles were observed, as previously noted in *C. vitta* (Robinson and Halton, 1982) and they suggested their role in the cellular uptake of exogenous proteins by diverse tissues (Halton and Hardcastle, 1976), abundant free ribosome's present in the cytoplasm of primary spermatocytes are generally associated with rapid protein synthesis and cytoplasmic growth. Mitochondria are numerous and provide necessary energy for their division and differentiation, whereas the Golgi complex contributes membrane components to the plasma membrane to accommodate growth and differentiation of the cells during spermatogenesis as suggested by Halton and Hardcastle (1976) for *Diclidophora merlangi*. In this present amphistome the exogenous proteins along with other nutrients are perhaps supplied by lymph vessels and partly by parenchyma.

The ultrastructure of secondary spermatocytes exhibits the presence of ribonucleoprotein in the cytoplasm of the cell presumably derived by transport of nuclear material through nuclear pores. The ribonucleoprotein is a significant component for germ cell differentiation. It can therefore be conjectured that this stage provides functional RNA for protein synthesis needed for spermiogenesis. In this respect, Present observation differ from those other monogenetic and digenetic trematodes where occurrence of nucleolar like bodies responsible to provide functional RNA for protein synthesis have been reported to be present in spermatogonia (Burton, 1972).

Spermatids show nuclei smaller than the spermatocytes. Spermiogenesis is characterized by outgrowth of the zone of differentiation, presenting basal bodies, separated by an intercentriolar body. At the end of this process, the spermatozoa are released into the residual cytoplasmic mass. The spermatozoa of *O. scoliocoelium are* elongate, similar to the pattern described in other digenea, showing nuclei, mitochondria and two axonemes with the 9 + 1 configuration. The peripheral cortical microtubules on the dorsal and ventral faces are laterally interrupted.

Ultrastructure of mature spermatozoon in amphistome species *O. scoliocoelium* is shows ultrastructural features similar to those described in the majority of the digeneans (Miquel *et al.,* 2000; Baptista-Farias *et al.,* 2001; Justine, 1991a, 1991b and 2001; Yang *et al.,* 2003; Ndiaye *et al.,* 2004; Seck *et al.,* 2008). Transversal sections of the anterior region of the mature spermatozoa exhibits apex of the sperm is made up of the cytoplasmic cap only; however, the microtubule below the plasma membrane may be seen arranged a continuous row of peripherally microtubules. A little behind the cytoplasmic apex begins nucleus. While the middle region with nucleus and mitochondria presents two axonemes with the configuration 9 + 1 and peripheral cortical microtubules in the ventral and dorsal faces laterally interrupted. Justine (1995) recognized that the structure found in the non-nuclear region presents a great variety among the axon, and would be of great value for phylogenetic considerations within the group.

The migration of the nucleus, mitochondria and axial filaments from anterior to middle region of the spermatozoa was observed in amphistome species *O. scoliocoelium*. However, similar observations were reported in *H. medioplexus*, by Burton (1972), *C. vitta* by Robinson and Halton (1982) and *B. gracilescens* by Erwin and Halton (1983). The elongations of the nucleus in the longitudinal axis of the spermatid and the condensation of the chromatin into lamellae have already been observed in other Digenea (Burton, 1972; Grant *et al.,* 1976; Rees, 1979).

The majority of the Platyhelminthes exhibit a homogeneous pattern for the spermatozoa with the nucleus, mitochondrion, two axonemes with a 9 + 1 configuration and cortical microtubules (Justine, 1999). The spermatozoa of amphistome *O. scoliocoelium* follow this classic pattern. Discrepancies occur within the group: the two axonemes of the spermatozoon in *Didymozoon* sp. (Justine and Mattei, 1983) and the single axoneme of *Schistosoma mansoni* (Kitajima *et al.,* 1976) present a 9 + 0 configuration, representing the only exceptions within the Platyhelminthes with this configuration. Another species of Didymozoidae already studied, *Gonapodasmius* sp., shows the basic pattern for the structure of the spermatozoon.

The posterior region of this spermatozoon is characterized by the presence of a lateral expansion after the appearance of the second axoneme in *O. scoliocoelium*. This lateral expansion in the spermatozoon was already observed in other digenean species such as *Basidiodiscus ectorchus* and *Sandonia sudanensis* (Ashour *et al.,* 2007), *Opecoeloides furcatus*, (Miquel

et al., 2000) *Paramphistomum microbothrium* (Seck *et al.,* 2007) and *Cotylophoron cotylophorum* (Seck *et al.,* 2008).

On the basis of foregoing discussion, it may be concluded that the ultrastructure of spermatogenesis in amphistome basically follows the common digenean plan.

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