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Research article

Oocyte development and maturation in the sea cucumber, *Holothuria scabra*

Nittiya Nontunha[a], Supakant Chaichotranunt[b], Tanate Poomtong[b], Yotsawan Tinikul[a], Prasert Sobhon[a], Worawit Suphamungmee[a,]*

[a] *Department of Anatomy, Faculty of Science, Mahidol University, 272 Rama VI Road, Ratchathevi, Bangkok 10400, Thailand*
[b] *The Coastal Fisheries Research and Development Center, Klong Wan, Muang, Prachuap Khiri Khan 77000, Thailand*

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Abstract

The sea cucumber, *Holothuria scabra*, has economic value for tropical countries in the Indo-Pacific region. Currently, fertilization success in aquaculture is limited due to the unpredictable period of spawning that results in inadequate amounts and quality of the naturally-released eggs that could be harvested. Mature female gametes from the ovary may be an alternative source for artificial fertilization, although its practical outcome remains unverified. Thus, the development and morphological characteristics were investigated of early, developing and mature oocytes of *H. scabra* during oogenesis. The results of light and transmission electron microscopy indicated that the ovary of *H. scabra* is composed of multiple tubules joined together at the short gonadal duct. Each ovarian tubule is surrounded by a thick wall comprising collagen fibers and smooth muscles that are lined by the epithelium. The outer epithelium comprises clear and granulated cells which contain numerous vesicles in the cytoplasm having the appearance of hormone-producing cells. The inner wall of the ovarian tubule is lined by flat follicular cells which anchor early-stage oocytes, while subsequent stages of developing oocytes are localized toward and freely released into the lumen. Female germ cells comprise oogonia and developing oocytes. The early pre-vitellogenic oocyte (Oc1) contains partially decondensed chromatin and synaptonemal complexes. The late previtellogenic oocyte (Oc2) exhibits mitochondrial aggregations in cytoplasmic poles. The early vitellogenic oocyte (Oc3) becomes enlarged due to the presences of cytoplasmic granules. The late vitellogenic oocyte (Oc4) has numerous cytoplasmic granules, a thick jelly coating and germinal vesicle breakdown, implicating its complete maturation. Thus, Oc4 may be a potential candidate for artificial fertilization in aquaculture.

Introduction

Sea cucumbers have been consumed by people around the world since ancient times, especially in Asia, due to their reputation for promoting health and their medicinal properties (Conand and Byrne, 1993). The sand fish, *Holothuria scabra,* is a tropical sea cucumber that thrives in shallow coastal water around the Indo-Pacific region from east Africa to western Pacific areas (Dabbagh et al., 2012). *H. scabra* has become a well-known species that is in high demand not only for dietary and medicinal purposes, but also for ecological

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* Corresponding author.
E-mail address: worawit.sup@mahidol.ac.th (W. Suphamungmee)

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reasons due to its ability in cleaning the sea of organic detritus (Purcell et al., 2016). Over harvesting and exploitation have reduced the natural, wild populations of *H. scabra* (Demeuldre and Eeckhaut, 2012). Aquaculture of this species has been carried out, but the success rate and productivity are low due to limitations in gamete production, the low fertilization rate and the low larval survival rate and settlement (Indriana et al., 2017). One major problem is the unpredictability of the spawning period of the broodstock and the failure of male and female parent stocks to spawn simultaneously that results in inadequate amounts of released male and female gametes for successful fertilization. The use of fully-developed oocytes in the ovary may be an alternative way to obtain sufficient amounts of eggs for fertilization, although the actual outcome has not yet been proven. Understanding of the process of oogenesis and morphological characteristics of oocytes in the ovary may assist in developing indices for determining the quality and maturation of oocytes in *H. scabra* as well as for evaluation of ovarian maturation in mature female broodstock prior to spawning.

The process of oogenesis and the stages of female gametogenic cells are similar in all sea cucumber species (Smiley, 1984; Eckelbarger, 2005); however, there are several variables among species (Smiley and Cloney, 1985; Eckelbarger, 1992). Purwati (2006) used a light microscope to demonstrate the four developmental phases of the *H. scabra* ovary consisting of the proliferative, maturation, spawning and spent phases. Of these phases, the maturation phase contains an abundance of mature oocytes which are readily released almost at once during the spawning phase. However, detail on the morphological characteristics of the oocytes and the associated structures of the ovarian tubules may provide valuable information on oocyte maturation that is still lacking, especially at the ultrastructural level. Therefore, the present study investigated the ultrastructural characteristics of the different stages of the oocytes and ovarian tubules that house the process of oogenesis in *H. scabra*. Valuable key indices were defined to determine the stage of fully mature oocytes, as these cells may be fertilizable if collected from the ovary.

Materials and Methods

Animals and specimen collection

H. scabra female broodstock was provided by the Coastal Aquaculture Research and Development Center, Department of Fisheries, Prachuap Khiri Khan province, Thailand. The females were rested in a concrete tank filled with filtered seawater (33 parts per million salinity, 26–30°C). The sampling period was during April–June 2019 when the sample had a maturation period with a high gonadal index. Prior to specimen collection, five sea cucumbers (250–300g body weight) were anesthetized by immersing in seawater containing 3% magnesium chloride. Then, the ventral surface of the body wall was cut open and the ovaries were dissected out.

Preparation of tissues for light microscopy and transmission electron microscopy

Ovarian tubules were separated and each was diced into small pieces (approximately 2 mm^3 in size) and immediately placed in a fixative solution (2.5% glutaraldehyde, 4% paraformaldehyde in phosphate buffer, pH 7.2) for 20 hr at 4°C. Samples were further fixed in 1% osmium tetroxide for 1 hr at 4°C, followed by several washes in phosphate buffer saline. Then, they were dehydrated with increasing concentrations of ethanol before being embedded in Araldite 502 resin (Electron Microscopy Sciences; USA). Resin polymerization was carried out in an oven at 60°C. The tissue blocks were sectioned to a thickness of 700–800 nm for semithin sections, and the same blocks were also cut for 70–90 nm thin sections in an ultramicrotome (EM UC6; Leica GmbH; Germany). The semithin sections were stained with methylene blue and basic fuchsin dyes prior to the histological observation. Large areas of ovarian tubules containing gametogenic cells were observed and photographed using a light microscope Eclipse E600; (Nikon; Japan). Thin sections were placed on 300-mesh copper grids and stained with uranyl acetate and lead citrate, respectively. Ultrastructural organization of the tubular wall and cellular components in the ovarian tubules were observed using a transmission electron microscope (H-7100 TEM; Hitachi Co. Ltd.; Japan) operating at 100 kV. The developing oocytes were identified based on the ultrastructural characteristics of the nucleus, the chromatin pattern and the cytoplasmic organelles.

Results

General morphology and histology of ovary

A single ovary of *H. scabra* is located in the anterior part of the coelomic cavity (Fig. 1A). It consists of multiple long tubules that are joined together into a single gonadal duct that opens to the exterior near the mouth (Fig. 1B). Upon reaching maturation, the ovarian tubules appear creamy-to-yellowish and increase in length and number. As its volume increases, the mature ovary occupies most of the anterior part of the coelom.

Each ovarian tubule typically branches into a secondary order and frequently also a tertiary order. During the early developing period, the general morphology and color of the ovary in the female are hardly distinguishable from the testis in the male. However, the ovary is distinguished by its intense yellowish color upon its maturation. Histologically, at maturation, an ovarian tubule contains five stages of the female germ cells (Fig. 1C): the first stage is the oogonium (Og) which contains the smallest cells (approximately 6–8 µm in diameter). Each Og has a small dense nucleus surrounded by a thin rim of cytoplasm that aggregate in groups of 2–4 cells. The second and third stages are previtellogenic oocytes (Oc1, Oc2) which are larger cells (approximately 7–10 µm and 15–40 µm in diameter, respectively) with larger, clearer nuclei and cytoplasm that contain few dense granules (Fig. 1D). They are attached to the inner layer of the wall of the ovarian tubule which appears as a very thin basement membrane lined by flat cells. The early vitellogenic oocyte (Oc3) is approximately 50–70 µm in diameter. The plasma membrane of Oc3 is surrounded completely by flat follicular cells on the adluminal surface, while its basal surface remains attached to the inner layer of the wall (Figs. 1C–D). Oc3 has a larger and clearer nucleus than Oc2 and its cytoplasm exhibits an increasing number of dense granules (Fig. 1E). The late vitellogenic oocyte (Oc4) is the largest cell (approximately 150–200 µm in diameter) and is surrounded by a thick jelly coating. It has a large and very pale nucleus and cytoplasm that is fully packed with numerous dense granules. Oc4 is the fully mature stage that has freed itself from the tubular wall (Figs. 1C and 1F).

In addition to the germ cells detailed observations were made on the wall of the tubule which provides the anchoring and supporting structure and perhaps also plays a nurturing role in the proliferation and differentiation of germ cells. The three compartments of the tubular wall comprise the outer epithelium consisting of cuboidal-shaped, granulated and clear cells, the middle compartment comprising a thin muscular and thick collagenous connective tissues, and the inner compartment which is composed of a very thin basement membrane lined by flat follicular cells (Figs. 1C–1E). The thickness of the tubular wall varies according to the cycle of ovarian maturation. The wall is relatively thick (approximately 40–50 µm) during the early stage of the ovarian cycle when the tubule contains mostly Og, Oc1 and Oc2. When the ovary becomes fully mature and the tubule lumen is filled with Oc4, the thinnest wall at 10–15 µm is more apparent (Figs. 1C–D).

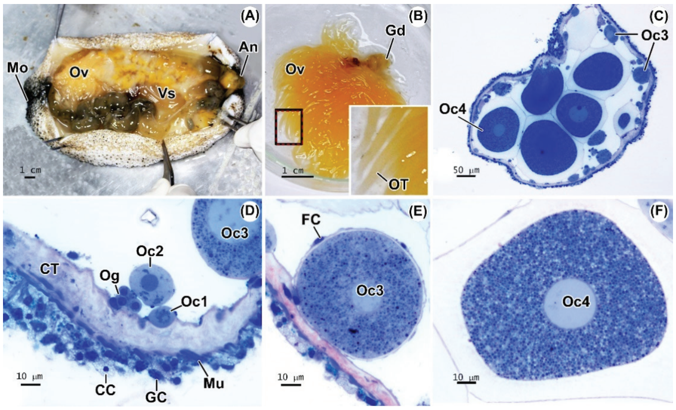

Fig. 1 General morphology of female gonad of *Holothuria scabra*: (A) ventral dissection revealing ovary located on left superior of body; (B) fully mature ovary organized by ovarian tubules (inset) eventually joining up at a gonadal duct; (C) histological section of single ovarian tubule at maturation phase showing fully mature oocytes in the lumen; (D) groups of oogonia and the early-stage oocytes; (E) mid-stage oocyte; (F) fully mature oocyte, where An = anus; CC = clear cell; CT = connective tissue; FC = follicular cell; GC = granulated cell; Gd = gonadal duct; Mo = mouth; Mu = smooth muscle; Oc1 = stage 1 oocyte; Oc2 = stage 2 oocyte; Oc3 = stage 3 oocyte; Oc4 = stage 4 oocyte ;Og = oogonia; OT = ovarian tubule; Ov = ovary; Vs = visceral organs

Ultrastructural characteristics of female germ cells

Oogonium

The oogonium (Og) is located on the inner surface of the wall of the ovarian tubule (Figs. 2A–2B). The cell has a diameter of 6–8 µm with a round nucleus containing clumps of heterochromatin blocks attached to the nuclear envelope and scattered throughout the nucleus. There is a thin rim of cytoplasm around the nucleus; thus the nuclear-cytoplasmic ratio is large. Few mitochondria, a rough endoplasmic reticulum and lipid droplets are scattered in the cytoplasm while there are still no granules. The Og aggregate in groups of two and four cells which suggests that they are dividing and some are differentiating into early previtellogenic oocytes (Oc1).

Early previtellogenic oocyte (stage 1 oocyte)

As the result of Og meiotic division, the stage 1 oocyte (Oc1) is derived and represents the early prophase stage. Oc1 has a diameter of 7–10 µm and is partly adhered to the inner surface of the tubular wall (Fig. 2C). Its centrally located nucleus contains a few blocks of heterochromatin that are mostly attached to the nuclear envelope, while the central area of the nucleus contains mainly decondensed euchromatin.There are synaptonemal complexes among the euchromatin and some have one arm attached to the nuclear envelope (Fig. 2D).The cytoplasm of Oc1 is slightly larger than for Og and there are increasing numbers of free ribosomes, a rough endoplasmic reticulum, mitochondria, Golgi complexes and a few electron-dense granules.Oc1 may be wrapped by a common follicular cell (Figs. 2C–D).

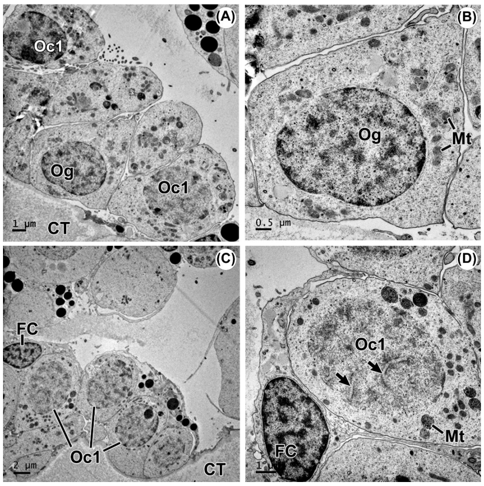

Fig. 2 Transmission electron microscopy image of oogonia and early-stage oocytes in ovarian tubule: (A) Og and Oc1 presenting on basal lamina of the tubular wall; (B) high magnification of Og showing scattered heterochromatin blocks throughout the nucleus; (C) Oc1 with surrounding follicular cells; (D) high magnification of Oc1 showing dispersed chromatin and synaptonemal complexes (arrows) in the nucleus., where CT = connective tissue layer; FC = follicular cell; Mt = mitochondria; Oc1 = stage 1 oocyte; Og = oogonia

Late previtellogenic oocyte (stage 2 oocyte)

The stage 2 oocyte (Oc2) is presumably derived from Oc1. While continuing to develop, it becomes larger in size (from 15 to 40 m in diameter). The cytoplasm is also enlarged; thus, the nuclear-cytoplasmic ratio decreases (Fig. 3A). Chromatin becomes decondensed into euchromatin throughout the nucleus, while only a few heterochromatin blocks still persist. The nuclear pores also increase in number on the nuclear envelope. In the cytoplasm, there are aggregates of mitochondria localized in one or twosites of the cell (Fig. 3B). A rough endoplasmic reticulum also appears to be abundant throughout the cytoplasm. There are only a few dense granules in the cytoplasm. The periphery of Oc2 is surrounded by a single layer of flat follicular cells.

Early vitellogenic oocyte (stage 3 oocyte)

The stage 3 oocyte (Oc3) is partially attached to the inner layer of the tubular wall through a string of follicular cells, while in a later stage, the developing cell moves toward the lumen. The flat follicular cells also surround the plasma membrane of Oc3. The first notable change of Oc3 is a rapid increase in the cell diameter up to 50–70 µm (Fig. 3C). The nucleus is surrounded by a well-defined nuclear envelope. The oocyte in this stage represents the totally decondensed chromatin to become finely dispersed euchromatin with the presence of a prominent nucleolus. The second notable change is the substantial increase in cytoplasmic granules and yolk platelets. Based on the electron density of the matrix, it size and location, the cytoplasmic granules of Oc3 can be categorized into three types (Figs. 3C–D): type 1 granule (Gr1) is the smallest and contains a very dense matrix; type 2 granule (Gr2) is larger in size with a moderately dense matrix; and type 3 granule (Gr3) is similar size to Gr2 and contains the lightest matrix. Small lipid droplets appear throughout the cytoplasm. Additionally, a thin layer of jelly coating is apparent around the outer surface of Oc3 as well as extensions of short microvilli from the plasma membrane into the jelly layer, which is in turn wrapped by flattened follicular cells.

Late vitellogenic oocyte (stage 4 oocyte)

The stage 4 oocyte (Oc4) is the maturation stage that becomes fully grown (150-200 µm in diameter). It has a large nucleus with a very fine euchromatin that is evenly dispersed throughout the nucleus (Fig. 4A). A thick layer of jelly coating (10–40 µm in width) surrounds the plasma membrane with outwardly extending microvilli (Fig. 4C). The jelly coating is in turn surrounded by an incomplete lining of flat follicular cells (Figs. 4B–4C). The cytoplasmic granules are numerous and densely packed together (Figs. 4A–4E). Gr1 are aligned in a row at the cell cortex with some lying just underneath the plasma membrane (Figs. 4B and 4D). A mixture of Gr2, Gr3 and lipid droplets appears throughout the cytoplasm. There appear to be type 4 granules (Gr4), which are characterized by a mottled matrix with tiny dense dots in the clear background. Gr4 appear to be sparse in the central cytoplasm, and most are highly presented in the cytoplasmic cortex. Furthermore, annulated lamellae are present that are similar to a crystal lattice (Figs. 4E–4F) in the cytoplasm.

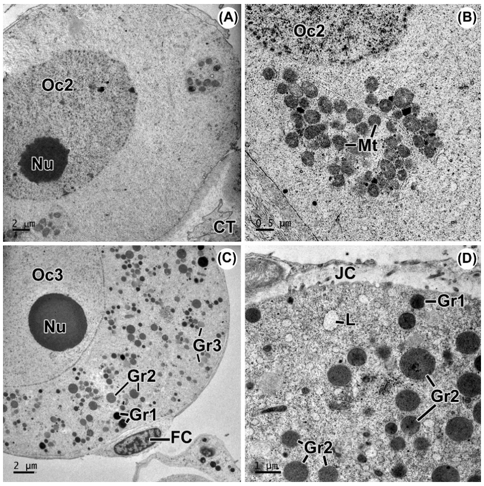

Fig. 3 Transmission electron microscopy image of early-stage and mid-stage oocytes in ovarian tubule: (A) Oc2 with large nucleus and more dispersed chromatin; (B) high magnification of Oc2 showing mitochondrial aggregation on one side of cytoplasm and numerous free ribosomes; (C) large cell of Oc3 containing fine euchromatin and increasing amount of the cytoplasmic granules with lipid vesicles; (D) thin jelly coating presenting around Oc3, where CT = connective tissue; FC = follicular cell; Gr1 = type 1 granule; Gr2 = type 2 granule; JC = egg jelly; L = lipid vesicle; Mt = mitochondria; Nu = nucleolus; Oc2 = stage 2 oocyte; Oc3 = stage 3 oocyte

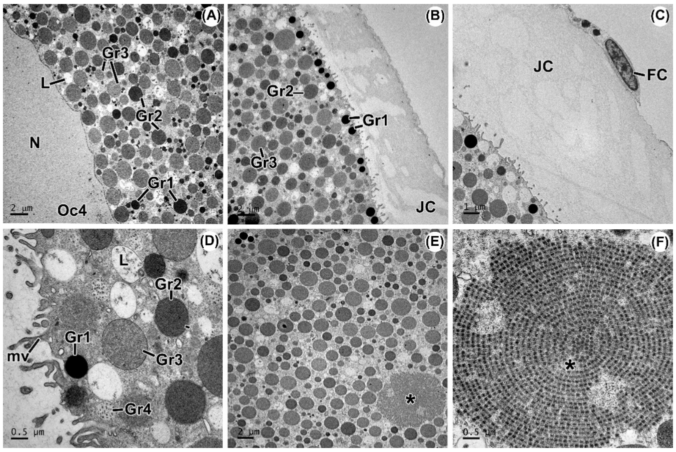

Fig. 4 Transmission electron microscopy image of late-stage oocyte in ovarian tubule: (A) large cell of Oc4 containing very fine euchromatin in the nucleus and abundant cytoplasmic granules with lipid vesicles; (B) Gr1 presenting the densest matrix aligning underneath the plasma membrane; (C) thick jelly coating at periphery of Oc4; (D) high magnification showing various electron densities of cytoplasmic granules and lipid vesicles; (E–F) occasional appearance of annulated lamellae (asterisk) in the Oc4 cytoplasm, where FC = follicular cell; Gr1 = type 1 granule; Gr2 = type 2 granule; Gr3 = type 3 granule; Gr4 = type 4 granule; JC = egg jelly; L = lipid vesicle; mv = microvilli; N = nucleus; Oc4 = stage 4 oocyte

Follicular cells

There are two forms of follicular cells (FCs). The first form surrounds the adluminal surfaces of developing and maturing oocytes which appear flattened, and their cytoplasm contains a high number of dense granules. They also have an ellipsoidal nucleus with large blocks of heterochromatin attached to the inner surface of nuclear envelope (Fig. 5A). The second form are those that attach the early-stage oocytes (Oc1–Oc3) to the inner layer of the tubular wall, which appear rather triangular or spherical in shape, and their cytoplasm contains much fewer granules (Fig. 5B).

Ultrastructure of ovarian tubule wall

The wall of the ovarian tubule consists of the outer epithelium comprising cuboidal-shaped, granulated cells and clear cells. The granulated cell has a pleomorphic-shaped nucleus with dense chromatin, and its cytoplasm contains a few granules with a dense matrix and more granules with a moderately dense matrix (Figs. 5C–5D). The clear cell has a spherical nucleus with blocks of dense heterochromatin and its cytoplasm is packed with vesicles containing a clear matrix (Figs. 5C–5E). Both types of outer epithelial cells have numerous microvilli. The outer epithelium is supported in turn by a thin layer of smooth muscles and a thick layer of dense, irregular, collagenous connective tissue which together form the middle compartment of the wall (Fig. 5D). There are nerve bundles between the outer epithelium and the underlying muscle. A high magnification of the middle compartment of the tubular wall shows interlacing thick bundles of collagen fibers covered on the outside by a thin layer of smooth muscles (Fig. 5F and Fig. 6). The inner compartment of the wall is made up of thin cytoplasmic flaps of follicular cells which are separated from the middle compartment by a thin basement membrane (Fig. 5C).

Discussion

A female *Holothuria scabra* has a simple form of ovary consisting of multiple tubules which individually contain mainly oogonia (Og) and early-stage oocytes (Oc1 and Oc2) in the early phase of the ovarian cycle, and maturing (Oc3) as well as fully developed (Oc4) oocytes with Og and few early oocytes in the maturation phase of the ovarian cycle. The present study has shown that most features of the developing oocytes in the *H. scabra* ovary resemble those of other marine holothuroids (Smiley and Cloney, 1985). The germinal epithelium resting on an inner wall of the ovary is composed of Og and early developing oocytes, whereas the late-stage oocytes are freed from the tubular wall and become loosely packed in the lumen of the ovarian tubule. During the early stages of oocyte development, the cells exhibit a dynamic process of protein synthesis as indicated by the presence of abundant ribosomes in the cytoplasm of Oc1. Abundant free ribosomes in the early-stage oocytes implicate their active role in protein synthesis during the initial period of oocyte development (Wallace and Selman, 1981; Wassarman, 1983; Schultz, 1986; Patiño and Sullivan, 2002; Jalabert, 2005). Similar characteristics of the early-stage oocytes have also been reported in other echinoderms (Holland, 1976; Aisenshtadt and Vassetzky, 1986; Byrne, 1989). Beside the abundance of polyribosomes, early-stage *H. scabra* oocytes (Oc1) exhibit a stage-specific distribution of mitochondria which are found initially in one side of the cytoplasm and then scatter throughout the cytoplasm in the fully-grown oocytes (Oc3 and Oc4). Being derived from Og, Oc1 immediately undergoes the first meiotic division as evidenced by the presence of synaptonemal

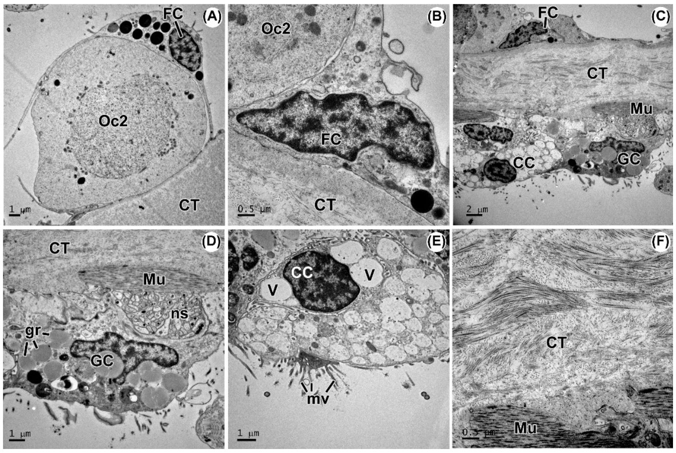

Fig. 5 Transmission electron microscopy image of follicular cells and wall of ovarian tubule: (A) flat follicular cell over periphery of developing oocyte; (B) triangular-shaped follicular cells located between basal part of developing oocytes; (C) ovarian tubule organizing into outer epithelial cells, muscular layer and thick collagenous connective tissue; (D-E) high magnification of outer epithelial cells comprising granulated cell and clear cell; (F) transitional area between thin, smooth muscle and thick bundles of collagen fibers, where CC = clear cell; CT = collagenous connective tissue; FC = follicular cell; GC = granulated cell; gr = electron dense granule; Oc2 = stage 2 oocyte; V = vacuolated vesicle; Mu = smooth muscle; mv = microvilli; ns = nerve bundle

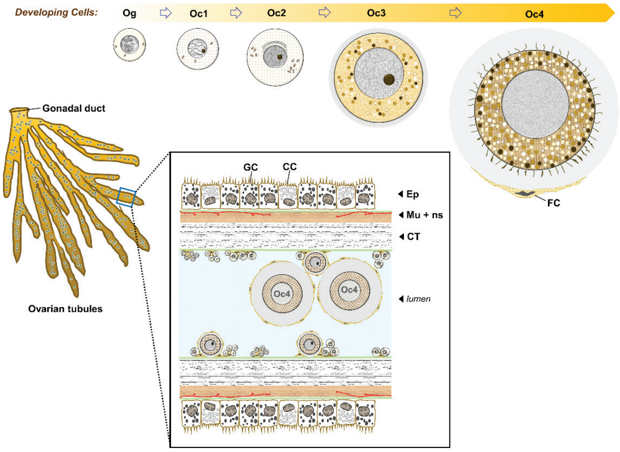

Fig. 6 Summary diagram depicting wall of ovarian tubule and germ cell development, showing increasing distribution of cytoplasmic granules, including cortical granules, yolk granules and lipid droplets that appear prominently in Oc3 and Oc4, respectively, where, CC = clear cell; CT = collagenous connective tissue; Ep = outer epithelium; FC = follicular cell; Gc = granulated cell; Mu+ns = smooth muscles with nerve bundles; Oc1 = stage 1 oocyte; Oc2 = stage 2 oocyte; Oc3 = stage 3 oocyte; Oc4 = stage 4 oocyte; Og = oogonia

complexes resulting in the crossing-over of homologous chromosomes. Later, in Oc2, the mitochondria are relocated to the bipolar areas along the microtubule arrays that radiate from the microtubule organizing center. A stage-specific redistribution of mitochondria may facilitate the energy dispensing process in Oc1 and Oc2during the active cellular processes, in this case for spindle formation and chromosomal segregation (Van Blerkom and Runner, 1984). A similar feature of mitochondrial redistribution has been observed in the developing oocytes of sea urchin which later becomes segregated into the oral pole of the embryo (Coffman et al., 2004).

In Oc3 and Oc4, a large amount of ATP is required for the syntheses of various types of cytoplasmic granules and the yolk. Thus, mitochondria are scattered within the large volume of the ooplasm, while the oocytes undergo meiotic arrest at Oc4 which could correspond to the end of prophase I as reported in *A. japonnicus* (Maruyama, 1985; Wakai et al., 2012; Kishimoto, 2018). The mid-stage oocyte of the Echinoidea starts to exhibit the yolk protein precursors which are members of the transferrin superfamily. In the present study there was the clear presence of yolk in Oc3 which thus is named the early vitellogenic stage. To date, there has been no vitellin or vitellogenin protein identified in sea urchin and sea cucumber. However, the major yolk protein in the holothurians is assumed to be vitellogenin-like, and so its production during oogenesis is also termed vitellogenesis (Fujiwara et al., 2010). Unlike the vitellogenin in many vertebrates, a major yolk protein of sea cucumber is largely presented in the gastrointestinal tract, both male and female gonads and in the coelomic fluid, suggesting that a major yolk protein may be transferred from external origins to the gonads via the coelomic fluid (Shyu et al., 1986). This protein is eventually metabolized to support sperm production and oocyte development, and in the latter case may also support the larval development after fertilization.

In the present study, late-stage oocytes of *H. scabra* exhibit a remarkable increase in the cytoplasmic volume and the accumulation of cytoplasmic granules, including scattered yolk granules and lipid droplets. A most distinctive structure is the presence of secretory granules with electron-dense material, the so-called cortical granules (Gr 1) that appear throughout the cytoplasm of Oc3 and become well-aligned in a row underneath the plasma membrane in Oc4. The roles of cortical granules have been studied in various animals for their proteolytic action and cortical reaction in response to activation by sperm receptors upon first contact of sperm with the egg surface (Foltz and Lennarz, 1993; Wessel et al., 2001). Sea urchins and many amphibians contain a vitelline layer with the presence of a glycoprotein-rich coating attached to the plasma membrane. Once a single sperm enters the egg coating during fertilization, the cortical granules exocytose and cause a vitelline layer elevation to form the fertilization envelope which aims to block polyspermy (Anderson, 1968; Wong and Wessel, 2004). Secretory product released from the cortical granules also triggers a calcium influx which initiates the signal transduction cascades that activate the fertilized eggs. However, a fertilization phenomenon in the Holothuroidea regarding a potential function of the cortical granule may be slightly different as their oocytes do not exhibit a distinct region of the vitelline layer (Holland, 1981). Thus, future study is required to investigate the role of cortical granules in *H. scabra* and whether they perform a similar function as in other echinoderms.

Proteinaceous materials in certain granules, Gr3, are exocytosed at the periphery of the plasma membrane and supposedly form the egg jelly. Thickening of the egg jelly has been observed in many species of echinoderms, especially sea urchins, whose gametogenesis has been well elucidated (Anderson, 1968; Aisenshtadt and Vassetzky, 1986). The egg jelly of the sea urchin is composed of sulfated polysaccharides that are species-specific inducers for sperm acrosome reaction. Long chains of polysaccharides (sulfated α-L-fucan) are also present in the egg jelly of the sea cucumber, *Ludwigothurea grisea*, but with a different sulfation pattern (Ribeiro et al., 1994). In *H. scabra*, the formation of a thick jelly coating in Oc4 is definitely the distinguishable characteristic that marks the full maturation of oocytes. Additionally, the thickness and texture of the Oc4 jelly coating are very similar to the jelly coating of the naturally-spawned eggs.

During the formation of the jelly coating, the projection of microvilli from the plasma membrane into the jelly layer is also noted in the *H. scabra* oocyte beginning from Oc3 and they become more numerous in Oc4. These microvilli may act to facilitate the absorption of nutrients and small molecules from the follicular cells that surround the maturing oocytes. In starfish oocytes they spike out of actin filaments to form microvilli that extend from the plasma membrane of the fertilized egg in response to calcium intake (Chun et al., 2010; Tsai et al., 2014). In addition, the extension of the actin-based microvilli may provide mechanical supports for the elevated fertilization envelope and perhaps also the increased surface membrane following cortical granule exocytosis.

Completion of germinal vesicle breakdown in Oc4 also indicates that these mature oocytes are ready for fertilization. The fully mature Oc4 are delivered into a single large gonadal duct that opens close to the mouth prior to release into seawater. Because of its length, the gonadal duct probably acts only as a passive passage way for the released eggs without any modification of the oocytes in transit, as is the case for the higher animals. Therefore, the mature oocytes in the final stage of the ovarian cycle could possess the same characteristics as the naturally-released eggs, and they could be activated directly by seawater and capable of fertilization. However, most holothurian oocytes do not have full competent fertilizability until they are spawned and subsequently make contact with seawater, which is needed for the hydration of the egg jelly and activation of the eggs themselves (Hamel and Mercier, 2007). It would be interesting to test whether fully mature Oc4, taken directly from the ovary, can exhibit full fertilizable potential upon contact with seawater. If this potential is realizable, then the oocytes from culled mature females or broodstock could be used in lieu of the released eggs, which would expedite fertilization and larval production in a farm setting.

Regarding the influence of hormonal treatments and chemical inducers, the oocytes become triggered to germinal vesicle breakdown which lets them advance to metaphase I of the maturation period or metaphase II after fertilization. Several reports using *Apostichopus japonicus*, *H. leucospilota*, *H. pardalis*, *H. moebi* and *H. pervicax* have demonstrated a potential effect of the oocyte maturation inducer, the so-called gonad stimulating substance-like peptide (GSS), which is presented in the radial nerve and the wall of ovarian tubules. This peptide is expected to act either on oocytes in the ovary or on the isolated oocytes with follicular cells (Ahmed et al., 2011).

Follicular cells may act as nurse cells as they initially serve to anchor young oocytes to the inner layer of the tubular wall, while later, they completely surround the early-stage and middle-stage oocytes. In addition to providing a protective coating for the developing oocytes, they probably deliver nutrients and hormonal factors from the wall to groups of oogonia and young oocytes attached to the wall. Since the follicular cells form the outermost covering of the oocytes before the appearance of the jelly coating, they may also participate in the synthesis of the latter. However, this proposition needs to be confirmed by future study using molecular labelling methods. In the late-stage oocyte, Oc4, follicular cells become highly flattened and their cytoplasmic flaps may be fragmented resulting in an incomplete layer at the periphery of the fully mature oocytes. Thus, follicular cells may cease their function just before the mature oocytes are released into the gonadal duct (Fig. 6).

The wall of the ovarian tubule has been described in several sea cucumber species (Atwood, 1973; Eckelbarger and Young, 1992; Demeuldre and Eeckhaut, 2012). The present study has provided detailed observations of the ovarian tubular wall of *H. scabra* using transmission electron microscopy. The outermost compartment of the wall is composed of two types of columnar epithelial cells (granulated cells and clear cells). According to their histological and ultrastructural characteristics, the granulated cells are expected to produce hormonal factors required for germ cell proliferation, while the actual role of the clear cells remains unknown. The presence of steroid-synthesizing cells with similar characteristics and location has also been reported in the ovaries of the starfish, *Asterias rubens* (Schoenmakers et al., 1981). However, both the granulated and clear cells in sea cucumber likely have additional function in absorbing the nutrients from peri-visceral fluid and transferring them to the developing oocytes, as in the case for yolk precursors from coelomic fluid during oogenesis (Smiley and Cloney, 1985). The middle compartment of the tubular wall is composed of thick interlacing bundles of collagen fibers and smooth muscles, while the innermost compartment is composed of a thin basement membrane overlaid by very thin follicular cells on which Og, Oc1 and Oc2 are resting.

Gonad maturation in the echinoderm is controlled by several neuropeptide hormones which are effectively released from the central nervous system as well as produced locally in the wall of the ovarian tubules. Neuroendocrine pathways of the gonad-stimulating and gonad-inhibiting peptides of sea cucumber have been investigated (Ahmed et al., 2011; Chieu et al., 2019). One key neuropeptide presented in sea cucumber is GSS, a neuropeptide hormone that primarily stimulates the ovarian tubules to reach maturation (Mita, 2013). As mentioned earlier, GSS has been detected in the neurosecretory cells of the radial nerve and in morula cells in the wall of the ovarian tubules (Ahmed et al., 2011). Thus, the present study has demonstrated that the wall of the *H. scabra* ovarian tubule has cellular constituents that may promote it having synergistic controls in providing certain hormonal factors and nutrient uptake for germ cell proliferation and maturation (the outer compartment of the ovarian wall) as well as facilitating the contraction of muscular bands during spawning (the middle compartment of the ovarian wall).

In summary, the oogenetic process of *H. scabra* in the ovarian tubules is composed of germ cells in five stages, namely: Og and Oc1–Oc4. Oc4 is the fully developed oocyte with several key characteristics that resemble those of the spawned eggs. Thus, Oc4 taken directly from the ovary may be an alternative source for artificial fertilization for the production of larvae in aquaculture. However, further elucidation is required regarding their fertilizability upon contact with seawater, as well as on their binding mechanisms with sperm.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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