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Ultrastructure of Oocytes During Oogenesis and Oocyte Degeneration Associated with Follicle Cells in Female *Sinonovacula constricta* (BIVALVIA: PHARIDAE) in Western Korea

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Abstract: The ultrastructure of oocytes during oogenesis and oocyte degeneration associated with follicle cells in female *Sinonovacula constricta* (Lamarck, 1818) were investigated by electron microscope observations. Ovarian follicles are surrounded by a matrix of vesicular connective tissue cells (VCT cells). VCT cells contain large quantities of glycogen particles and several lipid droplets in their cytoplasm. It is suggested that VCT cells act as a source of nutrients for vitellogenesis during oogenesis. In early vitellogenic oocytes, several coated vesicles, which appear at the basal region of the oocyte, lead to the formation of membrane-bound vesicles via endocytosis. The uptake of nutritive materials in coated vesicles formed by endocytosis appears through the formation of coated pits on the oolemma during vitellogenesis. During the late stage of oogenesis, yolk precursors (yolk granules), mitochondria and lipid droplets are present in the cytoplasm of late vitellogenic oocytes. In particular, proteinaceous yolk granules containing several different components are intermingles and form immature yolk granules. In the mature oocyte, small immature yolk granules are intermingled and form large mature yolk granules. Vitellogenesis occurs through a process of autosynthesis, involving combined activity of the Golgi complex, mitochondria and rough endoplasmic reticulum in the cytoplasm of vitellogenic oocytes. The process of heterosynthesis is where extraovarian precursors are incorporated into oocytes by endocytosis at the basal region of early vitellogenic oocytes before the formation of the vitelline coat. Follicle cells appear to play an important role in vitellogenesis and oocyte degeneration. The functions of attached follicle cells to the oocyte during oocyte degeneration are phagocytosis and digestion of phagosomes originating from oocyte degeneration. After digestion of phagosomes, it is assumed that the function of follicle cells can permit a transfer of yolk precursors necessary for vitellogenesis and allows for the accumulation of glycogen and lipid during oocyte degeneration, which can be employed by vitellogenic oocytes. Follicle cells of *S. constricta* may possess a lysosomal system for induction of oocyte breakdown and might resorb phagosomes in the cytoplasm for nutrient accumulation during oocyte degeneration.

Key words: *Sinonovacula constricta*, ultrastructure, oogenesis, oocyte degeneration, follicle cell

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INTRODUCTION

The razor clam, *Sinonovacular constricta* (Bivalvia: Pharidae), is distributed along the coasts of Korea, China, and Japan (Yoo, 1976; Kwon et al., 1993). In Korea, this clam is primarily found in silty sand of the intertidal zone along the south and west coasts, and is a commercially important species. Understanding reproductive biology parameters such as reproductive mechanisms associated with oocyte development during oogenesis, and induction of oocyte degeneration by follicle cells is important for propagation and reproduction.

To date, comprehensive ultrastructural studies of bivalve oogenesis have been limited, mainly restricted to economic importance species such as *Mytilus edulis* (Pipe, 1987a,b), *Brachidontes virgiliae* (Bernard et al., 1988), *Pecten maximus* (Dorange and Le Pennec, 1989a,b; Le Pennec et al., 1991), *Cyclina sinensis* (Chung et al., 2007), *Pinna nobilis* (Gaulejac et al., 1995), *C. virginica* (Eckelbarger and Davis, 1996), *Bathymodiolus childressi* (Eckelbarger and

Young, 1999), *Mactra veneriformis* (Chung and Ryou, 2000) and *Patinopecten yessoensis* (Chung et al., 2005).

Previously, there have been many studies in Korea, Japan and other countries on aspects of reproduction, including reproductive cycle and first sexual maturity (Kim and Lee, 2008). On aspects of ecology (Yoo, 1976; Kwon et al., 1993; Wu and Lin, 1987; Yoshimoto and Shutou, 1989, 1990; Lin and Tianming, 1990; Yoshimoto et al., 1990; Wang and Xu, 1997; Ko et al., 1997), and on aspects of physiology (Yoshimoto, 1989; Lee, 2002; Han et al., 2005) of *S. constricta*.

Although sexual maturation of *S. constricta* has been examined using light microcopy, there are still gaps in our knowledge regarding other reproductive biology of this species. In particular, little information is available on oocyte development and vitellogenesis during oogenesis, and oocyte degeneration associated with follicle cells of this species. Above all, it is necessary to study them. To understand the reproductive mechanism, in particular it is necessary to study the development and degeneration of oocytes associated with follicle cells of this species. The aim of this study is to describe the ultrastuructures of oocytes and vitellogenesis during oogenesis and oocyte degeneration associated with follicle cells for the study of the reproductive mechanism in female *S. constricta*, using cytological method.

MATERIALS AND METHODS

Sampling

Specimens of *S. constricta* were collected monthly in the intertidal zone of Simpo, Korea, for one year from January to December, 2006. A total of 126 female clams ranging from 80.6 mm to 94.7 mm in shell length were used for the studies of oogenesis and oocyte degeneration by electron microscopic observations.

Ultrasructural studies of oogenesis, oocyte degeneration and the follicle cells

For transmission electron microscopy, excised samples of the gonads were cut into small pieces and fixed immediately in 2.5% paraformaldehyde-glutaraldehyde in 0.1 M phosphate buffer solution (pH 7.4) for 2 hours at 4°C. After prefixation, the specimens were washed several times in the buffer solution, and then postfixed in a 1% osmium tetroxide solution in 0.2 M phosphate buffer (pH 7.4) for 1 hour at 4°C. Specimens then were dehydrated in increasing concentrations of ethanol, cleared in propylene oxide and embedded in an Epon-Araldite mixture. Ultrathin sections of Epon-embedded specimens were cut with glass knives on a Sorvall MT-2 microtome and LKB ultramicrotome at a thickness of about 80-100 nm. Tissue sections were mounted on collodion-coated copper grids, doubly stained with uranyl acetate, followed by lead citrate, and observed with a JEM 100 CX-II (80-KV) electron microscope (Chung et al., 2000).

RESULTS

Position and structures of the ovary

The razor clam, *Sinonovacula constricta*, is a dioecious. The ovary is located between the subregion of the mid-intestinal glands in the visceral cavity and the reticular connective tissues of the foot. The ovary is a diffuse organ that composed of highly branching follicles, in which germ cells develop. The ovarian follicles are surrounded by a matrix of the vesicular connective tissue cells (VCT cells), which are encircled by a connective tissue compartment and hemocoel. In this study, as shown in Fig. 1C, early vitellogenic oocytes are distributed along the inner wall of each follicle in all stages of differentiation, whereas the outer wall of the follicle is characterized by a discontinuous layer of thin squamous myoepithelial cells that forms a partial barrier between vitellogenic oocytes and the hemocoel. More specifically, VCT cells, which are attached to the outer wall of the follicle, appear near the vitellogenic oocyte. The ultrastructure of VCT cells in this species is bag-like in shape, with an irregularly-shaped nucleus.

Ultrastructure of Germ Cells and the Follicle Cells During Oogenesis

Oogenesis occur in the ovarian follicles of the ovary. Germ cells in four phases of oogenesis could be recognized: (1) oogonia, (2) previtellogenic oocytes, (3) vitellogenic oocytes, and (4) mature oocytes.

Oogonia

The primary oogonia (10-11 μm in diameter) are round or oval in shape. They possess a large ovoid nucleus, in which the chromatin is reticular and marginal. The primary oogonia divide mitotically to produce the secondary oogonia. Several mitochondria and vacuoles appear in the cytoplasm of the primary and secondary oogonia (Fig. 1A).

Previtellogenic oocytes

In the first prophase of meiosis, the secondary oogonia develop into the previtellogenic oocytes. These oocytes are slightly pedunculated, and the diameters of the nucleus and the previtellogenic oocytes are approximately 4-5 μm and 20-25 μm in diameter, respectively. A number of the mitochondria, vacuoles and the rough endoplasmic reticulum are concentrated around the nucleus, but microvilli are not yet on the oolemma. At this stage, the previtellogenic oocytes are partially surrounded by follicle cells that maintain intimate contact with the smooth oolemma of the oocyte. Follicle cells, which measure from 7 to 8 μm in

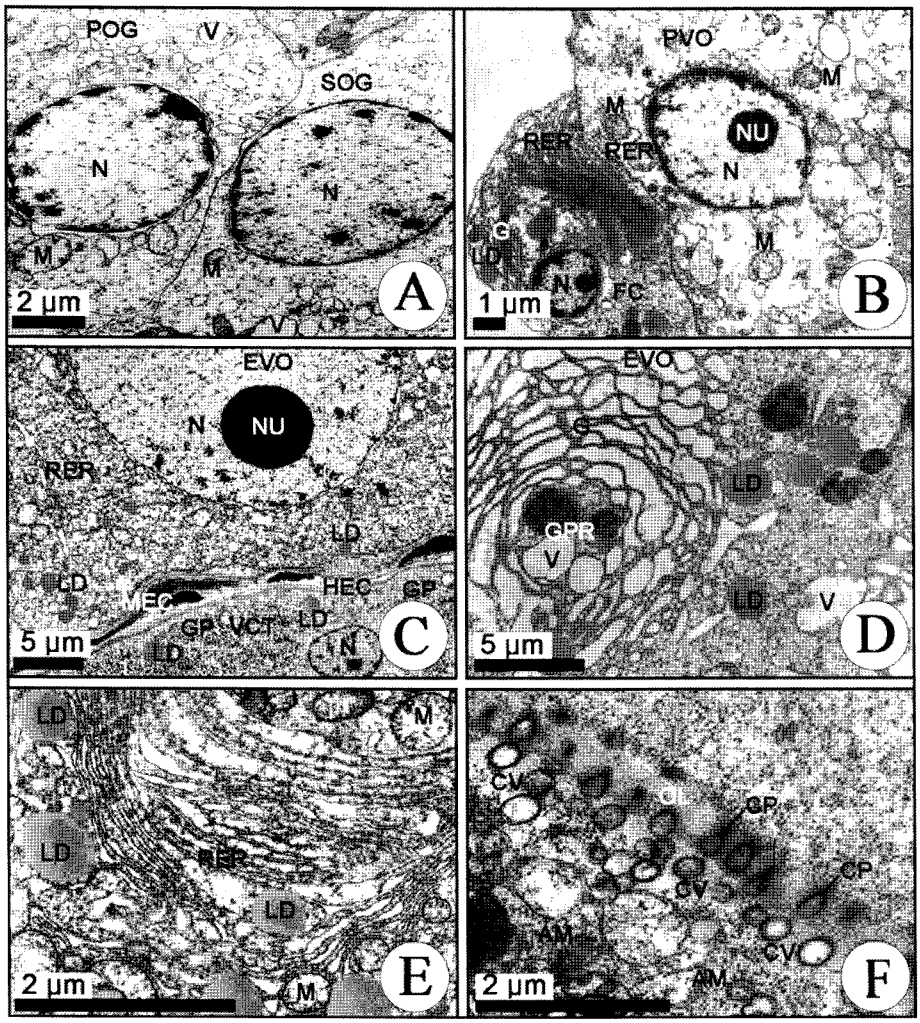

Fig. 1. Electron micrographs of oocytes and follicle cells during oogenesis in female *Sinonovacula constricta* (A-F). A, The primary oogonia and secondary oogonia, with a large nucleus, several mitochondria, and Golgi complexes in the cytoplasm; B, a previtellogenic oocyte and the follicle cell containing the rough endoplasmic reticulum; C, an early vitellogenic oocyte and the VCT cell containing a nucleus and large quantity of glycogen particles and several lipid droplets; D, the early vitellogenic oocyte, with several lipid droplets, the Golgi products in the Golgi complex, and a number of vacuoles; E, The early vitellogenic oocyte, with lipid droplets between well-developed rough endoplasmic reticulum and the mitochondria; F, the early vitellogenic oocyte, with coated vesicles at the basal region through the coated pits (upper part) by endocytosis, and amorphous materials formed by exocytosis at the cortical region. Abbreviations: AM, amorphous material; CP, coated pit; CV, coated vesicle; EVO, early vitellogenic oocyte; FC, follicle cell; G, Golgi complex; GP, glycogen particle; GPR, Golgi product; HEC, hemocoel; LD, lipid droplet; M, mitochondrion; MEC, myoepithelial cell; N, nucleus; NU, nucleolus; OL, oolemma; POG, primary oogonium; PVO, previtellogenic oocyte; RER, rough endoplasmic reticulum; SOG, secondary oogonium; V, vacuole; VCT, vesicular connective tissue.

diameter, possess a dense chromatin and marginal chromatin in the nucleus and contain characteristically parallel arrays of the rough endoplasmic reticulum, the Golgi complex, mitochondria, and lipid droplets in the cytoplasm (Fig. 1B).

Vitellogenic oocytes

As the further development of previfellogenic oocytes proceeds, they develop into the early vitellogenic oocytes. In the early stages of oogenesis, the early vitellogenic oocytes are about 25-30 μm in diameter. The Golgi complex is present in the perinuclear region and vacuoles are scattered in the cytoplasm. The early vitellogenic oocyte is still attached to the follicular wall, and its apex bulge into the lumen of the acinus. At this time, the oocytes contain a number of the mitochondria, rough endoplasmic reticulum, and a few lipid droplets in the cytoplasm. More specifically, the vesicular connective tissue cells (VCT cells), which are attached to the outer wall of the follicle, appeared near the early vitellogenic oocyte. In particular, during vitellogenesis, the cytoplasm of VCT cells is filled with a large quantity of glycogen particles, a few mitochondria and a small number of lipid droplets (Fig. 1C).

When their oocytes are 30-35 μm in diameter, a number of lipid droplets, vacuoles and the Golgi products in the

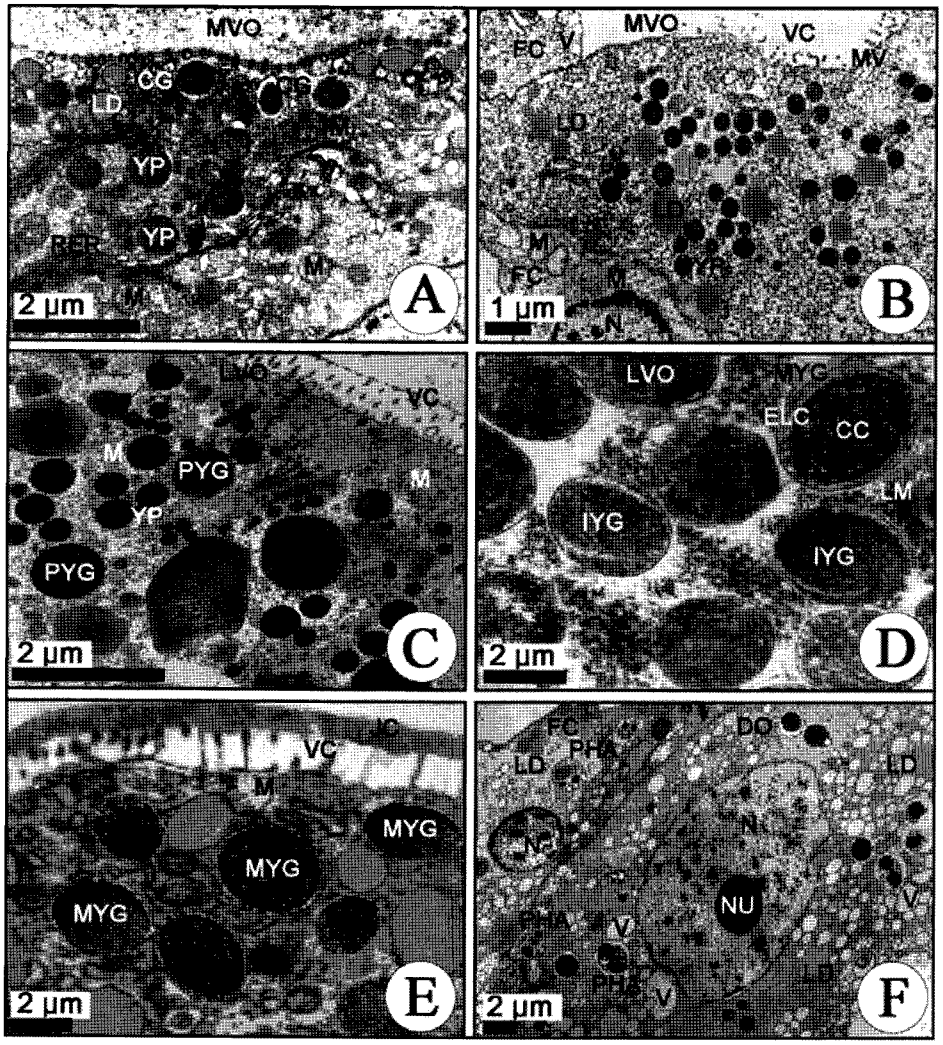

Fig. 2. Electron micrographs of oocytes and follicle cells during oogenesis and degeneration in female *Sinonovacula constricta* (A-F). A, A mid-vitellogenic oocyte, with yolk precursors among lipid droplets and well-developed rough endoplasmic reticulum, the mitochondria, and cortical granules in the cortical layer; B, follicle cells containing myeline-like organelles and a mid-vitellogenic oocyte, with the microvilli, yolk precursors and lipid droplets; C, a late vitellogenic oocyte, with the microvilli, proteinaceous yolk granules and several yolk precursors; D, late vitellogenic oocyte, with immature yolk granules and a few mature yolk granules being composed of three parts: 1) crystalline core, 2) electron lucient cortex, and 3) a limiting membrane; E, a mature oocyte, with mature yolk granules and the vitelline coat surrounded with the jelly coat; F, a degenerating oocyte and follicle cells, with degenerating yolk granules, lipid droplets and phagosomes. Abbreviations: CC, crystalline core; CG, cortical granule; DO, degenerated oocyte; ELC, electron lucient cortex; FC, follicle cell; IYG, immature yolk granule; JC, jelly coat; LD, lipid droplet; LVO, late vitellogenic oocyte; M, mitochondrion; MO, mature oocyte; MV, microvillus; MVO, mid-vitellogenic oocyte; MYG, mature yolk granule; N, nucleus; NU, nucleolus; PHA, phagosome; PYG, proteinaeceous yolk granule; RER, rough endoplasmic reticulum; V, vacuole; VC, vitelline coat; YP, yolk precursor.

Golgi complex appear in the cytoplasm of the early vitellogenic oocytes (Fig. 1D). At this time, a number of lipid droplets appear between the mitochondria and well-developed rough endoplasmic reticulum (Fig. 1E). When the oocytes begin to form the microvilli on the oolemma, the initial contours of the microvilli are ovoid shape or slightly long. During the early stages of oogenesis, several coated vesicles, which appear at the basal region of the oocyte, lead to the formation of membrane-bound vesicles via endocytosis. The uptake of nutritive material in the coated vesicle formed by receptor-mediated endocytosis appears through the formation of coated pits on the oolemma during vitellogenesis. At this time, amorphous materials that comprise the vitelline coat are deposited near the cortical region by exocytosis, and the microvillous borders then form on the oolemma (Fig. 1F). From this point on, the mid-vitellogenic oocytes contain cortical granules at the cortical region near the oolemma (Fig. 2A). In the mid-vitellogenic oocytes, as oocyte volume increases, the ooplasm of the stalked region is filled with a number of lipid droplets, numerous yolk precursors (or yolk granules), the mitochondria, and rough endoplasmic reticulum. At this time, the follicle cells gradually lose their intimate association with the oocyte surface, and the microvilli

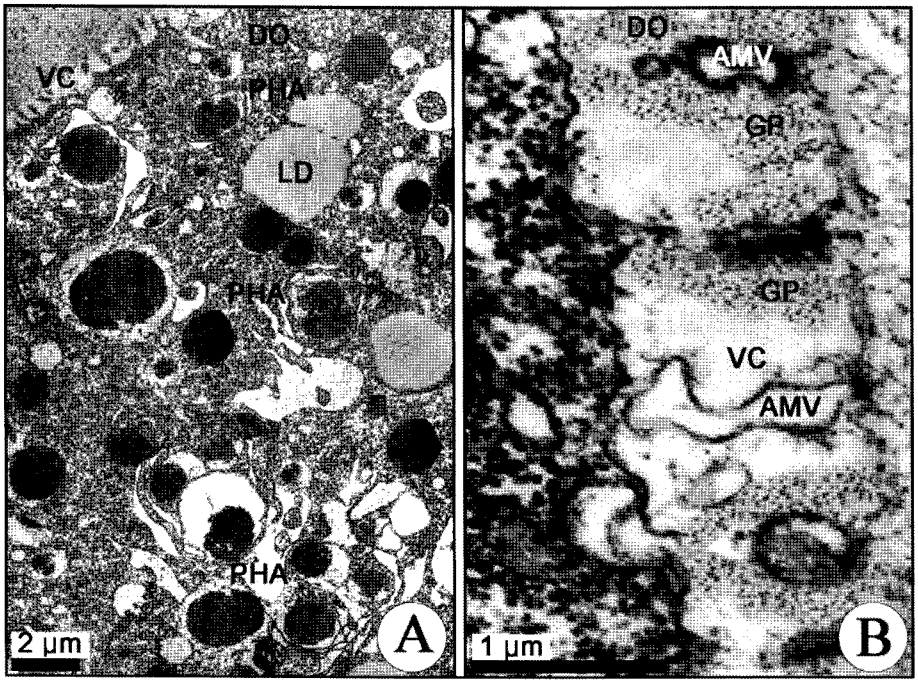

Fig. 3. Electron micrographs of degenerated oocyte in female *Sinonovacula constricta* (A-B). A, A degenerated oocyte, with distended endoplasmic reticulum, a number of vacuoles, lipid droplets, degenerated yolk granules, and various phagosomes (lysosomes) in the cytoplasm; B, the vitelline coat of the degenerated oocyte, with abnormal microvilli and large quantity of glycogen particles on the vitelline coat. Abbreviations: AMV, abnormal microvillus; DO, degenerated oocyte; GP, glycogen particle; LD, lipid droplet; PHA, phagosome; VC, vitelline coat.

appear along the vitelline coat, where the follicle cells withdraw. The cytoplasm of the follicle cell is filled with a myelin-like organelle, the mitochondria and vacuoles (Fig. 2B). In the late vitellogenic oocytes, yolk precursors (yolk granules), the mitochondria and lipid droplets are present in the cytoplasm, especially, a number of proteinaceous yolk granules appear among the mitochondria, lipid droplets, and yolk precursors near the vitelline coat (Fig. 2C). Proteinaceous yolk granules containing several different components are intermingled and form immature yolk granules (approximately 2.0 to 2.5 μm in diameter) in the cytoplasm of the oocyte (Fig. 2D).

Mature oocytes

As the further development of late vitellogenic oocytes proceeds, they develop into mature oocytes. In the mature oocyte, small immature yolk granules are intermingled and form larger mature yolk granules (2.7 to 3.0 μm). A mature yolk granule in the cytoplasm of a mature oocyte is composed of three parts: (1) a crystalline core, (2) an electron lucient cortex, and (3) a limiting membrane (Fig. 3D). Upon reaching maturity, mature oocytes are separated from the acinus wall (germinal epithelium) and protrude into the lumen of the acinus. At this stage, the tips of the microvilli protrude and extend just beyond the outer border of the vitelline coat. The vitelline coat of the mature oocye is covered with a jelly coat. Mature oocytes that contain numerous mature yolk granules in the cytoplasm measure approximately 60-67 μm in diameter. Finally, the mature oocyte is separated from the follicular wall (germinal epithelium) (Fig. 2E).

Ultrastructure of Degenerated Oocytes

The degenerating oocytes are irregular and deformed by compression in the follicle. In degenerating oocytes, several phagosomes (lysosomes) appear among degenerating yolk granules, lipid droplets, and a number of vacuoles in the cytoplasm. At this time, a few phagosomes (lysosomes) also appear among a number of vacuoles and lipid droplets in the cytoplasm of the follicle cells, while glycogen particles decrease in the cytoplasm of the follicle cells that are attached to the degenerating oocyte (Fig. 2F). In particular, the endoplasmic reticulum is involved in the degenerative process during the gradual disintegration of the oocytes: the smooth or rough endoplasmic reticulum become distended, which leads to vacuolation of ooplasm. As characteristics of oocyte degeneration, the mitochondria and yolk granules disintegrate in the cytoplasm, and lysis is initiated at the periphery of the oocyte: numerous heterogenous, dense granules that appear similar to phagosomes (lysosomes) are present in the ooplasm. At this time, many disintegrated granules are visible at the periphery of the oocyte (Fig. 3A). The perivitelline space of degenerated oocyte increases, and numerous glycogen particles can be

seen in the vitelline coat. Finaly the vitelline coat shows abnormal microvillus structures and glycogen particles breakdowns, and these components are then released into the ooplasm (Fig. 3B).

DISCUSSION

Vitellogenesis of the Oocyte and the Functions of Follicle Cells and VCT Cells during Oogenesis

From the ultrastructural study of *Sinonovacula constricta*, vitellogenesis during oogenesis can be classified into two separated processes: autosynthetic and heterosynthetic yolk formations (Eckelbarger and Davis, 1996). Vitellogenesis occurs through a process of autosynthesis, which involves the combined activity of the Golgi complex, mitochondria and rough endoplasmic reticulum. In contrast, Pipe (1987a) reported endocytotic activity in the oocytes of *M. edulis*, and Eckelbarger and Davis (1996) suggested an evidence for heterosynthetic yolk formation in the oocytes of *Crassostrea virginica*. In the present study, extraovarian precursors were found to be incorporated into oocytes by endocytosis at the basal region of the early vitellogenic oocytes which acts as evidence for heterosythetic yolk formation. Beside the process of heterosynthetic yolk formation by endocytosis, it is assumed that the VCT cells and the follicle cells may be involved in supplying nutrients to vitellogenic oocytes in the process of heterosynthetic yolk formation.

Regarding the relationship between the follicle cells and the formation of microvilli on the oolemma of the oocyte, Pipe (1987a) reported that once the follicle cells withdraw in *M. edulis*, the microvilli appear along the oolemma of the oocyte. In this study, the same phenomenone was observed as that shown in *M. edulis* (Pipe, 1987a) and *Meretrix lusoria* (Chung, 2007). Therefore, it is suggested that the follicle cells, which are attached to the oolemma of the oocyte, play a role in the formation of the microvilli on the oolemma (Pipe, 1987a).

With reference to the physiological functions of the ovarian follicle cells and the vesicular connective tissue cells (VCT cells), Eckerbarger and Davis (1996) described that the follicle cells are not likely to be the primary source of yolk precursors in *C. virginica*, while they suggested that the VCT cells are more likely to be a major source of yolk precursors (Pipe, 1987b). In the present study, large VCT cells were found at the extra-follicle near the oocyte. In particular, these cells contain a large quantity of glycogen particles and several lipid droplets in the cytoplasm. However, the follicle cells contain relatively small amount of glycogen and a few lipid droplets in the cytoplasm. Accordingly, it is assumed that the VCT cells (reported by Eckelbarger and Davis, 1996) are more likely to be the major sources of yolk precursors for vitellogenesis. So far, the descriptions associated with the functions of the VCT cells during oogenesis, which was reported in bivalves, could not find in gastropods, such as *Rapana venosa* (Chung et al., 2002) and *Neptunea (Barbitonia) arthritica cumingii* (Chung et al., 2006). Henceforth, the presence or lack of the structure of the VCT cell-like organ in gastropods should be investigated in further detail in future.

Induction of Oocyte Degeneration and Resorption by the Follicle Cells

During the period of degenerating oocytes, a number of degenerating yolk granules showed characteristics of a functional role for hydrolytic enzyme activity, and lipid droplets also appeared in the ooplasm of the degenerating oocytes in *S. constricta.* At this time, a few phagosomes (lysosomes) and lipid droplets increased in number in the cytoplasm of the follicle cells, which are attached to the degenerating oocyte, however, glycogen particles decreased in the cytoplasm of the follicle cells, as seen in *M. edulis* (Pipe, 1987a) and *P. maximus* (Dorange and Le Pennec, 1989b). Therefore, it is assumed that this function can permit a transfer of yolk precursors necessary for vitellogenesis and allows for the accumulation of glycogen and lipids during oocyte degeneration, with can be employed by vitellogenic oocytes (Gaulejac et al., 1995).

In this study, more specifically, morphologically similar phagosomes (lysosomes), which were easily observed also in the cytoplasm of degenerated oocytes, also appeared in the follicle cells of *S. constricta*, as seen in *C. sinensis* (Chung et al., 2007) and *M. lusoria* (Chung, 2007). Thus, the follicle cells appeared to play an integral role in vitellogenesis and oocyte degeneration: the follicle cells function in phagocytosis and intracellular digestion of products originating from oocyte degeneration through a lysosomal system (Chung, 2007), as those seen in *Pinna nobilis* (Gaulejac et al., 1995), *C. sinensis* (Chung et al., 2007) and *M. lusoria* (Chung, 2007).

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