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Electron and Light Microscopic Investigations of Follicular Epithelium in Vitellogenic Oocyte of Zebrafish (*Danio rerio*)

Nazan Deniz Koç (Yön)* and Cansu Akbulut
Department of Biology, Faculty of Arts and Letters, Sakarya University, Sakarya, Turkey

Abstract.- The zebrafish (*Danio rerio*) is an extremely dynamic organ in which follicles undergo asynchronous development. The oocytes of zebrafish ovary are observed in various phases. The oocyte development of zebrafish was divided into four stages (primary growth, cortical alveolus-previtellogenic, vitellogenic and mature oocyte). Zebrafish follicles contain only a single layer of granulosa cells that are separated from the oocyte by the vitelline envelope (zona radiata). The follicular epithelium and theca of oocytes in zebrafish differentiates during the primary growth phase. Histological analysis revealed that the zona radiata is formed during the vitellogenic growth stage. Specializations associated to the outher layer of the zona radiata may be related to the egg's adherence to the substrata. We conclude that cytological characteristics of follicular cell and oocytes don't differ from those described in other teleosts species.

Key Words: Follicular epithelium, oocyte, growth, zebrafish, ultrastructure.

INTRODUCTION

The basic pattern of oocytes growth is similar in teleosts (Taylor and Sumpter, 1996). The morphological characteristic of oocytes are important for an understanding of the dynamics of oogenesis, including oocyte final maturation and ovulation.

The structural basis of the local regulatory network in the ovary is the follicle, which consists of three major cellular compartments: the oocyte, the inner granulosa cells and the outer thecal cells. In all vertebrate groups, the development of the oocyte is accompanied by significant morphological and functional changes of the follicle (Ge, 2005). Teleost oocytes as in other vertebrates are surrounded by two major cell layers as an outher thecal layer and an inner granulosa. As the oocytes grow, the follicle cells multiply and form a continuous follicular layer called the granulosa cell layer (Andrade *et al*., 2001). Fish oocyte development can be divided into oocyte growth and oocyte maturation. Vitellogenesis plays an important role in oocyte growth. Germinal vesicle migration and breakdown, coalescence of lipid droplets and yolk globules, and release of the 1st polar body are the characteristic event in the precess of maturation (Nagahama, 1983, Yueh and Chang, 2000). In all vertebrate groups, it has been well documented that the somatic garnulosa and thecal cells of the follicle provide an appropriate yet dynamic microenvironment that supports and nurtures the development of the oocyte from the begining to the end (Ge, 2005).

Most our knowledge about the regulation of ovarian development and function comes from studies in mammals, and such information remains relatively limited in non-mammalian vertebrates. In the past decade, the zebrafish has quickly become one of the top vertebrate models for genetic and developmental studies due to its advantageous attributes, such as small body size, fast development, short generation time.

In this paper, histological and ultrastructural techniques were used to study the vitelline envelope, follicular layer of ovary of zebrafish. We aimed that cytological characteristics of follicular cell don't differ from those described in other fish species.

MATERIALS AND METHODS

Model organism

The zebrafish, *Danio rerio* (Hamilton-Buchanan, 1822) (Teleostei, Cyprinidae) is a widely used laboratory model species, especially in developmental biology (Nüsslein-Volhard and Dahm, 2002). The zebrafish has a relatively short

* Corresponding author: ndy31@hotmail.com, nkoc@sakarya.edu.tr
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life cycle of about 4 months. Zebrafish were under standardised conditions at 28±1°C. The light/dark cycle was 12h/12h. Fish were fed daily with nauplia lavae of the crustacean Artemia sp.

Light microscope

For the histological analysis, the fishes were anaesthetised in the ice water and fixed as a whole Bouin's fluid for 24 h. Fixed tissue was dehydrated and embedded in the parafin wax and sectioned transversely at 6-7 µm thickness and stained with Hematoxylin Eosin and Toluidin blue. The Periodic Acid Schiff reaction was applied. The samples were evaluated by examining under the light microscope.

Electron microscopy

For transmission electron microscopy individual tissue were fixed by immersion in a solution containing 4% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M phosphate buffer at pH 7.4 for 4 h. The ovaries were fixed further overnight at 4°C using 2% glutaraldeahyde in 0.1 M phosphate buffer at pH 7.4. After an additional fixation with 1% OsO_4 and pre-embedding staining with 1% uranyl acetate, ovaries were dehydrated and embedded in Embed 812 resin. The sectioning was performed using a Leica Ultracut ultramicrotome. Thick sections were stained with tolouidine blue and visualized in a Olympus light microscope to select the area of interest. Thereafter, thin sections were collected and counterstained with 1% uranyl acetate and lead citrate and examined transmission electron microscope.

RESULTS AND DISCUSSION

Oogenesis

Zebrafish ovary is an extremely dynamic organ in which the follicles undergo asynchronous development. The development of zebrafish oocytes is divided into four stages, based on morphological features (Koç *et al*., 2008). First stage is primary growth. Primary oocyte, identifiable by a few peripherally located nucleoli as well as by small, localised areas of intense basophilia in the cytoplasm. Second stage is cortical alveolus (previtellogenic) stage. In this stage is identifiable by the appearance of cortical alveoli marks (yolk vesicles). This stage is begining the formation of a vitelline envelope. During vitellogenic stage, the oocytes increases in size, due to accumulation of yolk. In mature oocytes, the nucleus is dissolved and the ooplasm consists of yolk bodies (Fig. 1F, G).

Zona pellucida and follicular cells

Zebrafish follicles contain only a single layer of granulosa cells that are separated from the oocyte by the vitelline envelope (zona radiata). External to granulosa cells are a thin vascularized theca layer containing both fibroblast and thecal cells (Fig.1A).

In primary growth stage, the layers (zona radiata) around the follicle do not become thicker completely in the growth phase. In cortical alvelolus stage, the zona radiata forms, the follicle epithelium became thicker. In vitellogenic stage (Vitellogenesis), the vitelline membrane began to develop at this stage (Fig. 1C,D). In mature oocytes, Vitelline membrane which constitutes the inner zone of the zona radiata was started to disintegrate by leaving void spaces from the exterior parts. Outside of the membrane, the follicle epithelium cells were screened with their uniformly arranged nuclei.

The structure of the zona radiata was monitored clearly by using optic and electron microscope. Vitelline envelope appears on the surface of oocytes subsequent to the formation of microvilli. The envelope cannot be described apart from the specialized surface of the oolemma. The oolemma of oocyte is closely associated with the plasmalemma of follicle cells. The surface of the oocyte is completely covered with microvilli (Fig. 1E and 1e). These microvilli are long and project into the space which is formed between the oocyte and follicle cells (Fig. 1E). This space is produced when the follicle cells move away from the surface of the oocyte. Some microvilli are so long that they project into the intercellular space of the follicle cells (Fig. 1E). Follicle cells that partially surround oogonia and completely encircle oocytes are squamous. Each cell contains a rather spindle-shaped nucleus. Ultrastructural analysis showed that the zona radiata was formed in the cortical alveoli phase, after which globose specializations gradually attached to the outer layer of the zona radiata. The cytoplasmic process of follicular cells extended toward the oocyte, often reaching the zona

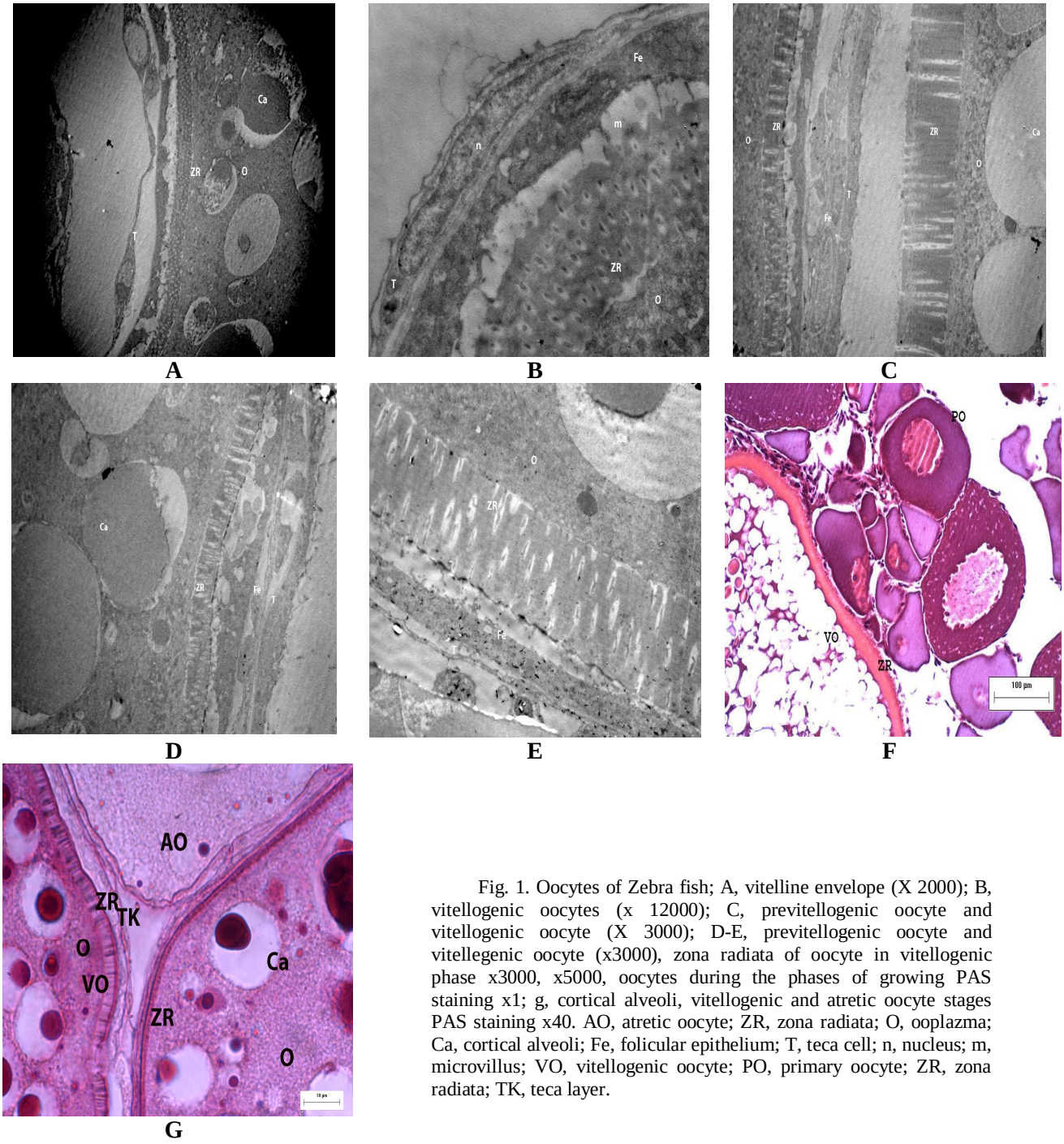

Fig. 1. Oocytes of Zebra fish; A, vitelline envelope (X 2000); B, vitellogenic oocytes (x 12000); C, previtellogenic oocyte and vitellogenic oocyte (X 3000); D-E, previtellogenic oocyte and vitellegenic oocyte (x3000), zona radiata of oocyte in vitellogenic phase x3000, x5000, oocytes during the phases of growing PAS staining x1; g, cortical alveoli, vitellogenic and atretic oocyte stages PAS staining x40. AO, atretic oocyte; ZR, zona radiata; O, ooplazma; Ca, cortical alveoli; Fe, folicular epithelium; T, teca cell; n, nucleus; m, microvillus; VO, vitellogenic oocyte; PO, primary oocyte; ZR, zona radiata; TK, teca layer.

radiata pore canals. In atretic oocyte, openings in the outer areas of the vitellus membrane were observed (zona radiata breakdown and yolk resorpsion).

Fish egg blankets are very adaptable to the environmental conditions within which they develop. Fish develop complex and thick blankets to their surrounding conditions. The structures, components and developmental patterns of follicle barriers differ significantly according to fish types. Reproductive studies of fishes require knowledge of

the stage of gonad development in teleosts. As the oocyte grows, the follicle cells increase in number. The development phases of the follicle epithelium cells were found in accordance with the follicle alterations as well as vitelline envelope in zebrafish. Structural alterations were observed in zebrafish oocytes during oocyte development in the histological studies performed. In this study oocyte development of zebrafish divided to four stages. According to Casadevall *et al.* (1993), ovarian development is asynchronous. Six stages are described in the oogenesis from the oogonia to the mature egg, using different histological techniques and based on differences of staining, of size and on the nucleus and cytoplasm structure, as viewed through a light microscope. Three of them correspond to the previtellogenie phase and the other three to the vitellogenic phase. In *Maja brachydactyla*, Four stages of gonad maturity were originally determined based on macroscopic criteria and histological examinations.Vitellogenesis is divided into two successive phases that clearly differentiate the oocyte growth period. Primary vitellogenesis is characterized by endogen protein yolk accumulation, while in secondary vitellogenesis extracellular protein substances are incorporated through pinocytosis (Rotllant *et al*., 2007). According to Bardakcı *et al*. (2000) oogenesis was classified according to size, appearance of nucleus and nucleoli and distribution of cytoplasmic inclusions in *Garr rufa* females from both localities studied. Piefish *Syngnathus scovelli* (Begowac and Wallace, 1988) and mudfish, *Labeo capensis* (Van Der *et al*., 1988), *P. japonicus* (Yano, 1988) were divided to six, six and ten stages, respectively.

In this study, the vitelline membrane began to development at the vitellogenic growth stage. The zona radiata of oocytes of teleosts is a complex structure, generally consisting of two layers crossed by pores or canals containing oocyte microvilli and follicular cell processed (Cruz-Höfling and Cruz-Landim, 1993; Guraya, 1996). In *Penaeus japonicus* prematuration stage was characterized by the formation of cortical crypts and vitelline envelope. After spawning, the cortical crypts release their contents which produce the cortical layer around the oocyte and which appear to enhance sperm penetration into the oocyte (Yano, 1988).

In *Bryconops affinis* (Abraham *et al*., 1984; Andrade *et al*., 2001), the inner layer is thick and outher layer is thin,. As in other teleosts, the zona radiata of zebrafish begins to be formed in previtellogenic oocyte, with its outer layer being formed through electron-dense material deposition between microvilli of the oocyte and follicular cells (Rizzo and Bazzoli, 1991). Electron microscopic and histochemical studies have suggested that the follicular cells would be involved in the synthesis of different proteins and lipids during oocyte growth. Part of these proteins would be used by the oocyte for its development as well as for the formation of the vitelline envelope (Hamlet *et al*., 1999).

The origin of the zona radiate of teleosts is still a controversial issue, follicular cell may play a role in the formation of this structure (Oppen-Berntsen *et al*., 1992). The remarkable thickness of the zona radiate layer and the large size of vitellogenic oocytes of *Hemiodus ternetzi* distinguished this species from the others. In the pipefish *Syngnathus scovelli*, Begovac and Wallace (1988) have found that the vitelline envelope is structured in three different layer, called Z1, Z2 and Z3, being the Z1 the outer one. In the black scraper, *Navodon modestus* (Hosokawa, 1985) the development process of the vitelline envelope is a little different, but it is still structured in three distinct layers, being the inner the one with fibrilar structure. In *Heterandria formosa,* transmission electron microscopy reveals that the ultrastructure of the egg envelope and the follicular epithelium that invests vitellogenic oocytes is typical of that described for teleosts. The egg envelope is a dense matrix, penetrated by microvilli of the oocyte. The follicular epithelium consists of a single layer of cuboidal cells that lack apical microvilli, basal surface specializations, and junctional complexes. The egg envelope, which remains intact throughout gestation and lacks perforations, becomes progressively thinner and less dense as gestation proceeds (Grove and Wourms, 2005). According to Chung *et al*. (2007) the process of heterosynthesis involved endocytotic incorporation of extraovarian precursors into the basal region of the early vitellogenic oocytes prior to the formation of vitelline envelope. The follicle cells appear to play

an integral role in vitellogenesis and oocyte degeneration, functioning in phagocytosis and digestion of products originating from the degenerated oocytes: these functions can permit the transfer of yolk precursors needed for vitellogenesis. Follicle cells might have a lysosomal system for breakdown and might also resorb phagosomes in the cytoplasm for nutrient storage during oocyte degeneration. From the ultrastructural study of this species, vitellogenesis can be classified into two processes: autosynthetic and heterosynthetic yolk formations. The follicle cells initially appeared close to the previtellogenic oocyte, and thereafter, progressively surrounded the oocyte. At this stage, a small number of vacuoles was visible in the cytoplasm of the follicle cells near the adherence zone. The attached follicle cells also showed cytological modifications. Pipe (1987) reported that endocytotic figures appeared between vitellogenic oocytes and the follicle cells, indicating a transfer nutrients in *Mytilus edulis*. In *Cyclina sinensis*, a few follicle cells attached to previtellogenic and early vitellogenic oocytes during the early stages of oogenesis (Lee and Cho, 1985).

The period of gonad transformation and to evaluate the impact of estrogenic androgenic model substances on sex differentiation and vitellogenin induction in juvenile zebrafish analysised (Orn *et al*., 2003). Degeneration was characterized by granulation of the cytoplasm, appearance of large vacuoles and irregularity in the shape of the oocytes. Surrounding follicular epithelial cells contained degradation products. Although the follicle epithelium cells became more distinctive according to the growth of the egg, their existence in primary growth phase were also observed.

During oocyte development the vitelline envelope is regularly crossed by thousand of microvillar process from oocyte and the follicular cell. In *S. marmoratus*, as was observed in the chum salmon (Kobayashi, 1985), the microvilli from the oocyte surface extend through the vitelline envelope and project deeply into the extracellular spaces of the overlying follicular cells. In some cases they lie in close proximity to short follicular microvilli within the sub-follicular space or they can enter in direct contact with the follicular cell surface, normally their pits inside the cytoplasm. Oocyte microvilli and follicular cells should be linked by different types of junction, gap, tight (Selman and Wallace, 1989).

When there is no oocyte and follicle cells surrounding oocyte in the primary growth phase (previtellogenic phase), microvilli and several others developed in oocyte membrane were extend towards follicle cells and these formations were observed in entire oocyte surface ın zebrafish. In *C. tarichi* (Unal *et al*., 2005), the microvilli began to form on the oocyte surface in cortical alveolus phase.

Zona radiata was striated in early vitellogenesis in *Liza aurata* (Shabanipour and Heidari, 2004). In the zona radiata of *L. aurata*, each striated line represented a canal with pores opening at both ends described. In addition, a perivitelline space was noted between the zona radiata and oolemma, as also seen in *Crenicichla johanna*. Its development was completed in vitellogenic phase and its dissolution was observed in atretic oocyte phase. Development phases of the follicle epithelium cells were found in accordance with follicle alterations as well as zona radiata in zebrafish.

In conclusion, the oocytes follicular cells were similar in the teleosts. The oocytes development in the zebrafish was manifested in a series of changes. During the oocytes development, the oocyte enlarged in fishes. The vitelline envelope began to form and develop in the different stages in teleost fish. Results of the present study hopefully would contribute knowledge to the research on the process of the follicular epithelium's development of the zebrafish.

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