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Follicular epithelium, theca and vitelline envelope formation and structure in vitellogenic oocyte of zebrafish (*Danio rerio*)

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ABSTRACT

The zebrafish 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. Follicular cell and oocyte cytological characteristics don't differ from those described in other teleosts species.

Introduction

The basic pattern of oocytes growth is similar in teleosts (Tyler & 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. 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 et al., 1983; Yueh & 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. The zebrafish *(Danio rerio)* is a member of the family Cyprinidae and is native to India and Pakistan. It is awidely used laboratory model species, especially in developmental biology. The zebrafish model is becoming more and more popular because it is easy to produceA model organism should offer technical and practical advantages for studying principal biological processes, effects and mechanisms. In addition, it needs to have traits that can be generalized, i.e. the model organism has to be representative for a larger group of organisms. Both arguments come true for the zebrafish—it is a species convenient and cost-effective to work with from a technical and methodological point of view, and it provides conceptual insights into many aspects of vertebrate biology, genetics, toxicology and disease (Segner, 2009). Whereas information on many aspects of zebrafish biology and ecology in the field is surprisingly limited (Spence et al., 2008), considerable knowledge exists with respect to optimum breeding and maintenance conditions in the laboratory (Westerfield, 2000). The relatively short generation time of three to four months is an advantage. The principal advantages of the zebrafish model discussed above make this species also a suitable model for toxicological purposes.

In this paper, histological and ultrastructural techniques were used to study the vitelline envelope, follicular layer of ovary of zebrafish.

Material and Methods

Model Organism

The zebrafish (*Danio rerio*) is a small fish about 6cm in length, characterized by a series of five pigmented stripes running the entire length of each side of its body. The zebrafish's hardiness makes them excellent stress test subjects, as they can survive fairly severe environmental changes without succumbing, surviving long enough to show developmental defects. Finally, zebrafish are easy and inexpensive to raise, requiring only filtered water, and a minimal investment in fish food, making them an ideal animal model for research labs with limited funding. All of these characteristics have contributed to making zebrafish the model of choice in this study.

Zebrafish were obtained from Petsmart (Lynchburg, Virginia) and raised in a computer controlled incubation chamber. Ideal breeding conditions were maintained to ensure a maximum yield. The zebrafish received fourteen hours of daylight and ten hours of darkness every night. The temperature and humidity were kept at 28.5°C and 61%, respectively.

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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

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. 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.

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 were not become thicker completely in the growth phase. In cortical alvelolus stage, the zona radiata begin to form, the follicle epithelium became thicker. In vitellogenic stage (Vitellogenesis), the vitellin membrane began to develop at this stage (Fig. 1c and 1d). 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. 1b 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 outher layer of the zona radiata. The cytoplasmic process of follicular cells extended toward the oocyte, often reaching the zona radiata pore canals. In atretic oocyte, openings in the outer areas of the vitellus membrane were observed(zona radiata breakdown and yolk resorpsion).

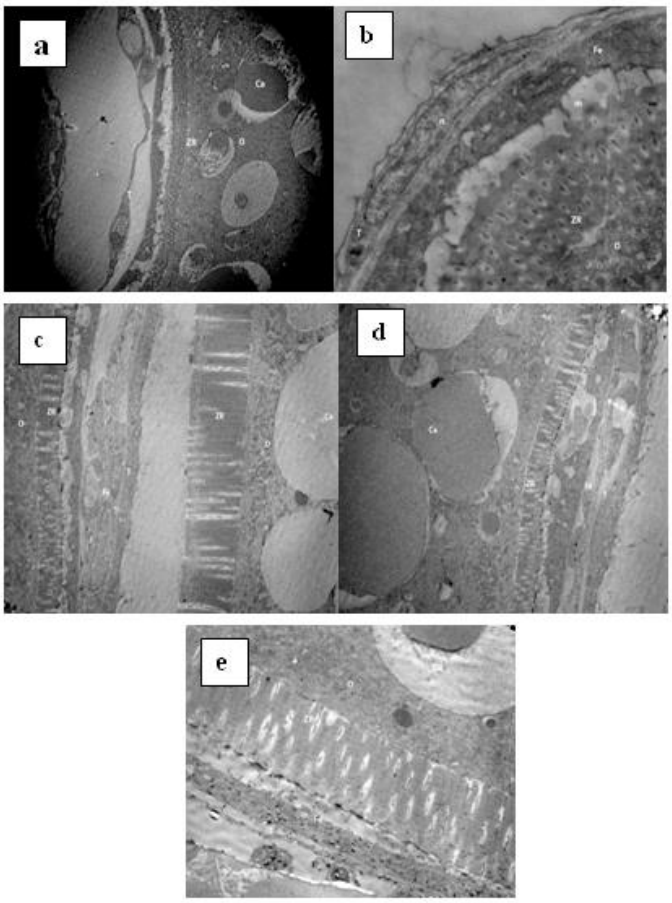

Fig. 1a) General overview of vitelline envelope of zebrafish oocytes; b)The electron micrograph of vitellogenic oocyte c) The electron micrograph of previtellogenic oocyte and vitellegenic oocyte. d-e)The electron micrograph of zona radiata of oocyte in vitellogenic phase.(ZR)Zona radiata, (O)Ooplazma, (Ca)Cortical alveoli, (Fe)Folicular epithelium, (T)Teca cell, (n)nucleus, (m)microvillus.a)x2000 b)x12000 c)x3000 d)x3000 e)x5000.

Discussion

Reproductive studies of fishes require knowledge of the stage of gonad development in teleosts. 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. In this study, the vitellin membrane began to development at the vitellogenic growth stage.The zona radiate 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 (Guraya, 1996). In Bryconops affinis, the inner layer is thick and outher layer is thin,. As in other teleosts, the zona radiate 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 (Abraham *et al*.,1984; Rizzo & Bazzoli, 1991). Electron microscopic and histochemical studies have suggested that the follicular cells would be inveolved 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 (Hamlett *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 founf that the vitellin 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. 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 by Örn *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 protruding its pit inside the cytoplasm. Oocyte microvilli and follicular cells should be linked by different types of junction, gap, tight (Selman & Wallace, 1989).

When no formation between oocyte and follicle cells surrounding oocyte in the primary growth phase (previtellogenic phase), microvilluses developed in oocyte membrane were extend towards follicle cells and these formations were observed in entire oocyte surface ın zebrafish. In *C. tarichi*, the microvilli began to form on the oocyte surface in cortical alveolus phase (Ünal, 2005). Zona radiata was striated in early vitellogenesis in *Liza aurata*. In the zona radiata of L. aurata, each striated line represented a canal with pores opening at both ends described by Shabanipour and Heidari (2004). In addition, a perivitelline space was noted between the zona radiata and oolemma, as also seen in *Crenicichla johanna* (Cruz-Höfling & Cruz-Landim, 1993). 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.

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