TENCH OOGENESIS: ULTRASTRUCTURAL ASPECTS OF THE OVARIAN FOLLICLES

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OVOGENEZA U LINJAKA: ULTRASTRUKTURNI ASPEKTI OVARIJALNIH FOLIKULA

Abstrakt

Ovaj rad predstavlja studiju folikula linjaka upotrebom transmisione elektronske mikroskopije (TEM), skening elektronske mikroskopije (SEM) i biohemijskih metoda. Studija ja fokusirana na morfološke aspekte previtelogenih, vitelogenih, postvitelogenih i atretičnih folikula ovarijuma. U folikularnoj fazi razvoja ovocit je okružen sa zona radiata, slojem folikularnih (granulosa) ćelija koje naležu na izraženu bazalnu membranu, vaskularizovani sloj theca folliculi sa brojnim kapilarima u mreži vezivnog tkiva i tankim epitelom na površini. Zabeležene su promene u folikulima uporedo sa pojavom proteina žumanceta. Antitela vitelogenina linjaka su pokazala prisustvo proteina žumanceta u krvnoj plazmi i vitelogenim folikulima.

Ključne reči*: linjak, ovarijum, folikul, ultrastruktura, vitelogeneza*

INTRODUCTION

The ovary structure and the stages of oocyte development in tench are well known. As in other teleosts, the oocytes grow within the ovarian follicles covered by cellular and acellular layers. Oocyte structure during oogenesis in tench has fragmently been studed by light microscopy (Epler et al., 1981; Horoszewicz, 1983; Pimpicka, 1986, 1989, 1990, 1995, 1997; Linhart, Billard, 1995). However ultrastructure of the ovarian follicles in tench appears unknown and it is upon this topic that we focused our research.

MATERIAL AND METHODS

Adult female tench were obtained from a fish farm. Blood and follicle samples were taken twice a month from April to October.

For scanning electron microscopy (SEM) the follicles were fixed on ice for one hour in 2% glutaralaldehyde in 0.1M sodium cacodylate buffer, dehydrated in an ethanol series, followed by critical point drying, mouting and gold sputtering. The samples were analyzed with a SEM LEO 420.

For transmission electron microscopy (TEM) and semi-thin sections, the follicles were embedded individually, after fixation in 2% glutaralaldehyde and in 1% O_sO_4 sodium cacodylate buffer, and in 1% agarose for orientation. Dehydration, embedding in LR-White and sectioning was done according to standard methods. Examination of ultra-thin sections stained with uranyl acetate was conducted with a Philips EM 208.

The electrophoretical separation of yolk proteins from follicles at various stages of ovarian development and from blood was done by SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

The immunological identification of the yolk proteins in both follicles and blood was conducted with antibodies raised against vitellogenin from vitellogenic follicles. The yolk proteins were detected on ultra-thin sections from vitellogenic follicles using immunohistochemical techniques (rabbit anti-tench vitellogenin and goat anti-rabbit IgG, gold conjugate 10 nm) according to Liddell, Weeks (1996). Standard PAP-techniques were applied for immunological determination of the yolk proteins in blood plasma and follicle homogenate.

RESULTS

It is known that two stages are observed in oocyte development in teleosts: pre-follicle and follicle. This study only examines the latter, follicle stage of development or folliculogenesis. Nucleus changes accompanying cell divisions are not discussed either.

The follicle stage begins with the separation of each of the primary oocytes and their covering by the follicle, thecal and serosa cells, zona radiata and ends with ovulation or atresion. The folliculogenesis can be divided into several phases if the most important event in the oocyte development - the vitellogenesis - serves as a base. The following stages are distinguished: previtellogenic, vitellogenic, postvitellogenic and atretic.

A) Previtellogenic follicles

Before the beginning of vitellogenesis, previtellogenic follicles are built as a complex structure. They contain the oocyte in which the cortical alveoli (CA) develop, along with the adjoining cellular and acellular layers of the follicle wall which consist of the vitelline envelope or zona radiata (ZR), follicle cells (FC), basal lamina (BL), thecal cells (TC) and theca serosa (TS) (Fig.1).

1. Follicle wall formation

Follicle wall formation begins with the separation of the primary oocytes from the nest. Initially the follicle cells form a thick monolayer around the oocyte and are interconnected by desmosomes. They are separated from the thecal cells by the basal lamina, which is made up of multilayered lamellae, the most interior what serves as a base. Over the basal lamina are the thecal cells organized in two layers – theca interna (TCI) and theca externa (TCE). The cells of the TCI lie on the outer lamella of the basal lamina. They are large, polygonal cells, forming long overlapping outgrowths on the periphery (Fig.2). The TCE is made of connective tissue components, which provide mechanical support for the follicle, and blood capillaries. The theca serosa composed

of thin polygonal cells, is the external cover of the follicles and it separates one follicle from another (Fig.3).

The zona radiata is an acellular layer of the follicle wall situated between the follicle cells and the oocyte (Fig.4). Its formation and the formation of microvilli are synchronous. With the appearance of follicle cells (FC) around the oocyte, many microvilli appear on the surface of the oocyte and the follicle cells (Fig.5). Initially there is a large perivitelline space between the follicle cells and the oocyte where the microvilli are situated. Electron-dense envelope material begins to accumulate between the microvilli that extend from the surface of the oocyte towards the overlaying follicle cells (Fig.6). This accumullation leads to the formation of canal pores in the zona radiata (Fig.7a,7b). Microvillar processes (microvilli) from both the oocyte and follicle cells are apparent within patent canals that traverse the developing zona radiata (Fig.4).

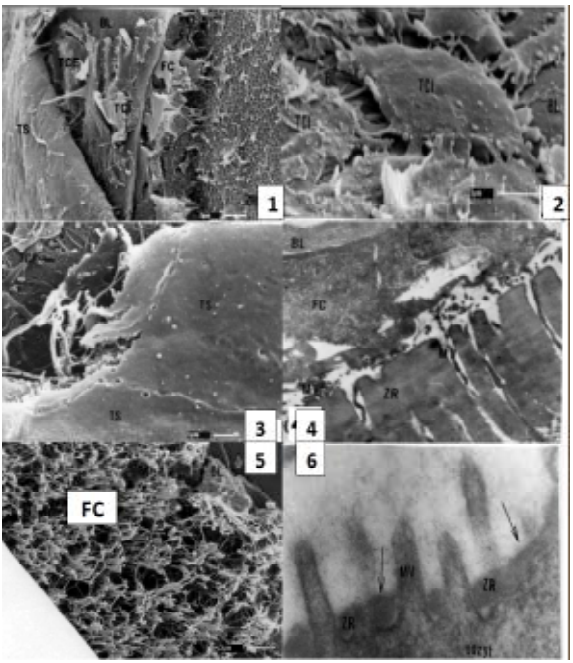

Fig.1. SEM of a portion of the previtellogenic follicle wall. Note the different cellular and acellular components of the wall. TS-theca serosa; TCE-thecal cells external; TCI-thecal cells internal; BL-basal lamina; FC-follicle cells; MV-microvilli; ZR-zona radiata (vitelline envelope).

Fig.2. SEM of a few thecal cells internal (TCI), which lie on the outer lamella of the basal lamina (BL).

Fig.3. SEM of part of the theca external (TCE) and theca serosa (TS). The theca externa comprises connective tissue components and the theca serosa comprises thin polygonal cells.

Fig.4. TEM of a section of the previtellogenic follicle wall. Elongate microvilli (MV) from both the oocyte (O) and overlying follicle cells (FC) are visible within patent pore canals of the developing zona radiata (ZR). x8000.

Fig.5. SEM of the apical side of the follicle cells (FC). The FC produce numerous microvilli (MV) on their apical side that traverse the pore canals of the zona radiata (ZR).

Fig.6. TEM of a section of the previtellogenic follicle wall. Note the zona radiata (ZR) formation. Electron-dense material (arrows) begins to accumulate between the microvilli (MV) that extend from the surface of the oocyte towards the overlying follicle cells. x31500.

2. Cortical alveoli formation

The first cortical alveoli are observed by light microscopy, within the periphery of the oocyte. Electron microscopy shows that their formation starts simultaneously with the formation of zona radiata. When zona radiata building material begins to accumulate around the microvilli, the first cortical alvaoli appear individually or in small groups in the ooplasm. Their size and number increase towards the oocyte cortex. In the immediate proximity of larger cortical alveoli, numerous small alveoli are observed, some of them in the process of fusion (Fig.8). Cortical alveoli comprised two different structures: medial, fine granulated, and lateral, coarse granulated.

B) Vitellogenic follicles

With the start of vitellogenesis, changes occur in the oocyte itself as a place of yolk protein accumulation, as well as in the follicle wall.

The first yolk granules appear between the cortical alveoli in the oocyte cortex. Intercellular spaces appear among the follicle cells (Fig.11) and between the cells of theca interna. Their sizes increase in proportion to intensity of the yolk accumulation. Such spaces are not seen in the previtellogenic follicles.

Antivitellogeninantibody marks selectively yolk proteins (vitellogenin) in the oocyte ooplasm (Fig.12), the cortical ooplasm close to the oolemma, the basal lamina, and the zona radiata, the latte was exceptionally strongly marked. The antibody did not detect vitellogenin in follicle cells

The SDS-PAGE showed that in the vitellogenic follicles numerous protein bands are present with molecular weights of 122 to 12 kDa (Fig.9). Antibodies against vitellogenin detect vitellogenic protein bands (P_1,P_2,P_3,P_4,P_5 and P_6) in the follicle homogenate from mature females during the reproductive season (Fig.10, lane 4). In the blood serum of mature females in the pre-spawning period (May) the antibody detects only one protein band P_0 with molecular weight 203 kDa (Fig.10, lane 1). In blood serum of mature females during the autumn-winter period the antibody does not detect vitellogenic bands (Fig.10, lane 3). In the blood serum of mature females with atretic follicles in the ovary during the reproductive season the antibodies detect vitellogenin protein fractions also, which results from the resorption of the vitellins from the vitellogenic follicles (line 2).

C) Postvitellogenic follicle

With the end of vitellogenesis in postvitellogenic follicles, withdrawal of follicle cell and oocyte microvilli from the zona radiata pore canals is observed and the canals are left empty (Fig.13). The follicle cells, which are cubic and bear numerous microvilli on their apical side during vitellogenesis become rounded and have no more microvilli during that period of follicle development. As a result of that, the intercellular spaces between them, typical for the period of vitellogenesis, have become very narrow or are absent.

The apex of the follicle cells shows exocytotic activity. A substance (mucopolysaccharide) is accumulated on the zona radiata and partially penetrates into its canal pores, forming the chorion layer of the follicle wall (Fig.14). The micropyle is the only area where this substance is absent (Fig.15).

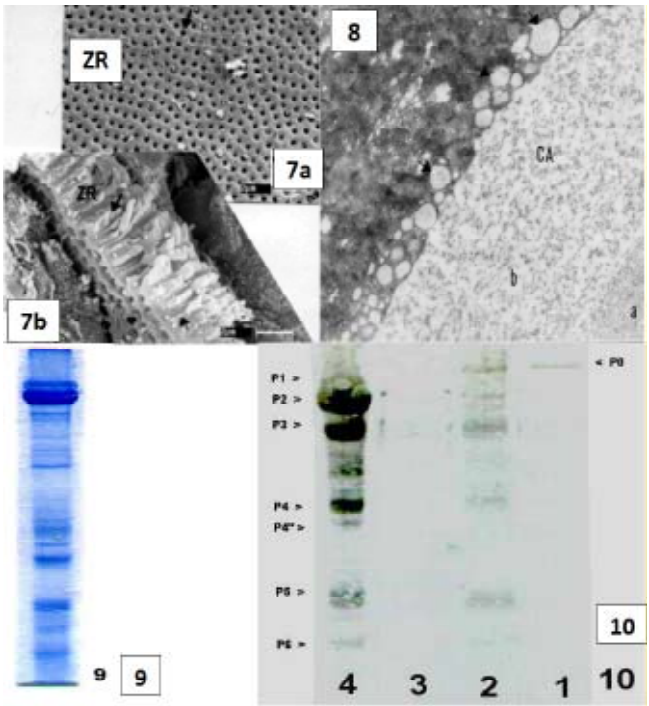

Fig.7a. SEM of a part of the zona radiata (ZR). Microvillar processes (microvilli) (arrows) are apparent within patent pore canals of the zona radiata (ZR).
Fig. 7b. SEM of a part of the outer side of the zona radiate (ZR). Pore canals (arrow) traverse zona radiata.
Fig. 8. TEM of a part of a cortical alveolus (CA) situated in the periphery of a previtellogenic follicle. Numerous small cortical alveoli (arrows) of variable size are closely associated with the surface of the larger alveolus. Note the two different structures in the CA: (a) medial, fine granulated, and (b) lateral, coarse granulated. x10000.
Fig.9. SDS-PAGE of extract of vitellogenic follicles. Note numerous protein bands with molecular weights from 122 to 12 kDa.
Fig.10. Western Blot. Antibodies against vitellogenin detect vitellogenic protein bands (P_1,P_2,P_3,P_4,P_5 and P_6) in the follicle homogenate from mature females during the reproductive season (lane 4). In the blood serum of mature females in the pre-spawning period (May) the antibody detects only one protein band P_0 with molecular weight 203 kDa (lane 1). In blood serum of mature females during the autumn-winter period the antibody does not detect vitellogenic bands (lane 3). In the blood serum of mature females with atretic follicles in the ovary during the reproductive season the antibodies detect vitellogenin protein fractions, which results from the resorption of the vitellins from the vitellogenic follicles (line 2).

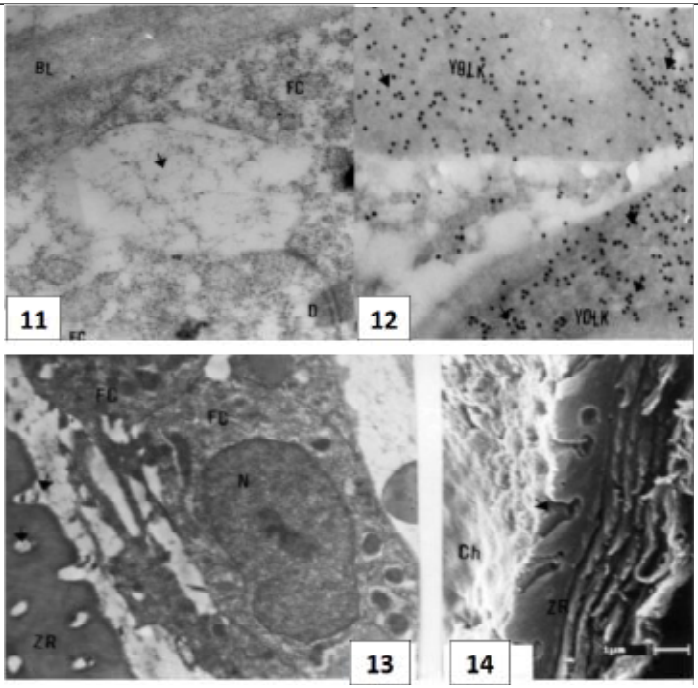

Fig.11. TEM of part of a vitellogenic follicle. Note the intercellular spaces (arrow) that appear between the follicle cells (FC) during vitellogenesis.(D) - desmosomes; (BL) – basal lamina. x25000.
Fig.12. TEM of parts of two yolk globuli in the ooplasm of a vitellogenic follicle. The antibodies mark vitellogenin substance (arrows) in the yolk globuli. (N) –nucleus from a FC. x40000.
Fig.13. TEM of part of the postvitellogenic follicle wall. Note the withdrawal of follicle cells (FC) and oocyte microvilli from zona zadiata (ZR) pore canals. x63000.
Fig.14. SEM of part of the postvitellogenic follicle wall. Note the empty pore canals (arrows) of the zona radiata (ZR) and the chorion layer (Ch) of the follicle wall.

D) Atretic follicle

After oocyte ovulation, only a part of the follicle wall remains in the ovarian stroma when the oocyte leaves the follicle with the zona radiata and the chorion layer. In this case the atretic follicle is built of follicle, thecal and serosa cells only. If ovulation does not occur, the destructive processes affect the whole follicle. Zona radiata loses its homogenous structure and numerous small "cracks" form in it (Fig.16). The follicle wall cells become hypertrophic, cell membranes perforate and become fragmented. Cortical alveoli lose their typical form and their composition mixes with cortical ooplasm and small yolk granules (Fig.17). Destructive processes occur in ooplasm and yolk also. Yolk proteins and cellular and acellular parts of follicle decompose very quickly and are conveyed through the blood vessels (Fig.18).

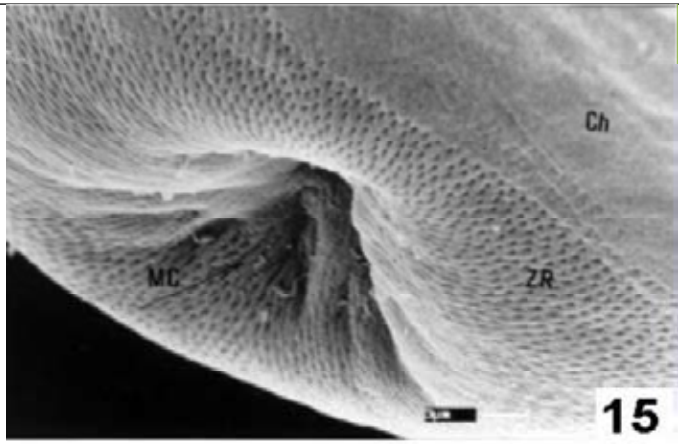

Fig.15. SEM of the micropyle area (arrow) of a postvitellogenic follicle after ovulation. (Ch) – chorion; (MC) – micropyle.

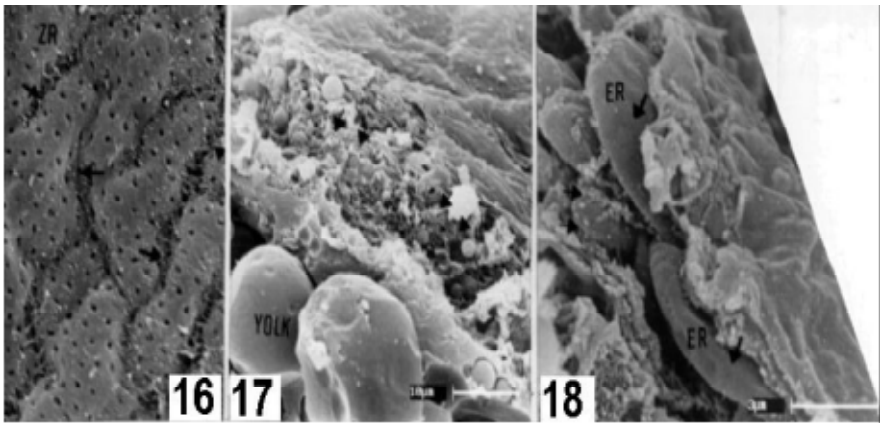

Fig.16. SEM of part of zona radiata (ZR) of an atretic follicle. Because of destructive processes zona radiate (ZR) loses its homogenous structure and small "cracks" (arrows) form in it.
Fig.17. SEM of part of the atretic follicle wall. Small yolk granules (arrows) mixes with cortical ooplasm and cellular and acellular parts of the follicle wall. Bigger yolk granules are seen in the periphery of the atretic oocyte.
Fig.18. SEM of part of the atretic follicle wall. Note that yolk proteins and other parts of the follicle wall are conveyed through the blood vessels (arrows: ER-erythrocyte).

DISCUSSION

The ultrastructural study has shown that the zona radiata is already differentiated as a structural element in the previtellogenic follicle. Through formation of numerous microvilli situated in the canal pores of the zona radiate, the oocyte surface is increased many times. The large area of the microvilli allows fast and intensive endocytosis of vitellogenin (Wallace et al., 1981).

When the oocyte increases during vitellogenesis, the zona radiata area should also increase at the same time. By using immunological methods, it has been shown that the basic proteins from which the zona radiate is assembled like vitellogenin, are synthesized in the liver and through the blood vessels they reach the follicles (Hamazaki et al., 1987, 1992; Nagahama et al., 1989). The authors propose that vitellogenin incorporation and zona radiata formation are parallel processes.

When the oocyte and zona radiata grow within the follicle, the follicle cells have to cover a larger area. We did not observe an increase in their number through mitotic division, thus we assume oocyte covering is achieved by means of follicle cells moving apart and forming relatively big intercellular spaces.

There is evidence of yolk protein synthesis in liver of teleost females (Ng, Idler, 1983; Wallace, 1985; Mommsen, Walsh, 1988; Wallace and Selman., 1990; Ding et al., 1994; Spannhof, 1995;) and its transport through the blood to the follicles (Wallace, Selman, 1981; Byrne et al., 1989). Our study showed that the final localization of blood capillaries in the follicle is the outer part of the basal lamina, between the TCI. This is where transition of vitellogenic macromolecules from blood capillaries should take place. That is possible for substances with a diameter of ≤10 nm, as case with the vitellogenic molecule (Wallace, Selman, 1990). Our findings support the hypothesis of Wallace and Selman (1990) about the pathway of vitellogenin into the follicle. We assume that it moves through the basal lamina, strongly marked by the antibodies, via the intercellular spaces between neighboring follicle cells. Vitellogenin is not found in the follicle cells but in the immediate proximity of them. In zona radiata canal pores, vitellogenic molecules contact the vitellogenic receptors in the plasmalemma of the microvilli and through receptor-mediated endocytosis they enter the oocyte (Opresko, Wiley, 1987, Tyler et al., 1991, Lancaster, Tyler, 1994, Manannos et al., 1997).

The results from our electrophoretical studies on vitellogenic follicles and blood serum of tench agree with the studies performed on other teleosts-*Carassius auratus* and *Archosargus probatocephalus* (Greely et al., 1986), *Fundulus heteroclitus* (Selman, Wallace, 1983), *Oryzias latipes* (Marakami et al., 1990), *Brachidanio rerio* (Selman et al., 1993), *Verasper moseri* (Matsubara, Sawano, 1995). Applying SDS-gels, a protein band is found in blood serum with molecular weight about 200 kD, and in the vitellogenic follicles typical bands are found with similar molecular weights of 122-197 kD, and three more in the lower molecular weight range. We have shown that in the tench, vitellogenesis occurs only in spring. Our antibody did not detect vitellogenin in the blood serum or the follicles in the autumn-winter period. Tench passes the winter with praevitellogenic follicles, in contrast to closely related species *C. carpio, C. carassius, H.molitri*).

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