

## A STUDY ON ULTRASTRUCTURE OF ZONA RADIATA DURING OOCYTE DEVELOPMENT OF ZEBRAFISH (*Danio rerio*)

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**Abstract:** Fine structures of the zona radiata in zebrafish, *Danio rerio*, is examined and compared. The zona radiata (called as zona pellucid in mammals), is a porous structure located between oolemma and follicle cell epithelium in fish, has various functions. The zona radiata is an inner connection surface that regulates essential transition from follicle cells to oocytes and from oocytes to follicular layer. In this study, it is aimed to examine changeable and distinct structure of the zona radiata during oocyte development in zebrafish, which is a member of teleosts fish family. The structure of zona radiata were examined using light and transmission microscopy. The structure of zona radiata showed a morphological difference among the different species. The zona radiata structure could not be seen under light microscope in the pre-vitellogenesis phase, but it was seen as a thin line form at the edge side in the early vitellogenic phase. In the following phases, the tendency to become thicker zona radiata was observed. The existence of microvillus structures, which lie from the zona radiata to follicle cell layer and from this layer to the zona radiata, was observed by means of electron microscope. It was assumed that the oocyte ensures communication between two sides through microvillus structures.

**Keywords:** Zona radiata, Oocyte, Development, Ultrastructure, Zebrafish

**Özet:** Zebra balığında zona radiatanın ince yapısı incelendi ve karşılaştırıldı. Balıklarda oolemma ile folikül hücre epiteli arasında bulunan gözenekli bir yapı olan zona radiata (memelilerde zona pellusida), pek çok görevi yerine getirir. Zona radiata, granulosa hücrelerinden oositlere, oositlerden foliküler tabakaya gerekli hareketi düzenleyen bir iç alış-veriş yüzeyi gibi düşünülmektedir. Bu çalışmada amacımız, kemikli balık türü olan Zebra balığı (*Danio rerio*)'nda oosit gelişimi esnasında Zona radiatanın değişken, özgün yapısını incelemektir. Zona radiatanın yapısı ışık ve elektron mikroskobu kullanılarak incelendi. Farklı türler arasında zona radiata yapısı morfolojik farklılıklar gösterdi. Previtellojenik evrede zona radiata yapısı ışık mikroskopunda görülmedi, ancak erken vitellojenik evrede kenar bölgede ince çizgi halinde seçildi. İlerleyen evrelerde zona radiatanın kalınlığında artma gözlenmedi. Zona radiatadan folikül hücre tabakasına doğru uzanan, folikül hücre tabakasından da zona radiataya uzanan mikrovillus yapılarının varlığı elektron mikroskobu ile izlendi. Bu mikrovillus yapılarının aracılığı ile oositin iki yönde iletişimi sağladığı gösterildi.

**Anahtar Kelimeler:** Zona radiata, Oosit, Gelişim, İnce yapı, Zebra balığı

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## Introduction

Teleosts include more than half of the vertebrate species (Baldacci et al, 2001; Wekkawy and Osman, 2006). A key feature of teleost evolutionary success is their reproductive system, which must be functional in all aquatic environmental conditions. Much of the existing knowledge of the mechanisms involved in teleost fertilization is based on a few small model species such as zebrafish, medaka and bitterling, which have no real commercial value (Coward et al, 2002; Wekkawy and Osman, 2006). The Zona radiata in fish oocyte has three different sequential layers, including the oocyte plasma membrane or oolemma in the inner side, the Zona radiata in the middle and the follicle epithelium in the outer side. In developed follicle, the granulose layer and the theca layer form in the inner side and in the outer side, respectively. The Zona radiata has various functions. The nourishment of theca layer, of granulose layer, and of embryo and oocytes at their development phase takes place through the Zona radiata. It is also responsible for producing and secreting enzymes that hold a place in the re-ordering of certain layers of ovule membranes and/or in the digesting, the lyses, of the cell residues which are required to be cleaned at the end of the apoptosis, the programmed death. It also plays a significant role in the transition of small molecules to oocytes through connection routes and in the synthesis of membrane elements in some fish types (Selman and Wallace, 1989). The term of Zona radiata is used to mean vitellin membrane, chorion, chorionic vitellin membrane, and Zona pellucida in many books (Hosokowa, 1995). The Zona radiata embracing ovule is generally in an extra-cell matrix form with the permeable canals formed by inner cell layer of follicle epithelium and oocyte microvillus. This layer is composed of two sub-layers which have distinctive structural features in different fish. The inner layer is similar to the Zona pellucida in mammals and is composed of various carbohydrates and proteins. The task of this layer in embryonic development and fertilization is to protect the ovule. The outer side has a peculiar macro molecular structure that helps interaction between its own watery surrounding and ovule, and that rarely includes sulfate polysaccharide, carboxylate and glycoprotein

(Guraya, 1986; Selman and Wallace, 1989). With thin structure analyses, it was demonstrated that the Zona radiata forms in the pre-vitellogenic phase. In this study, the formation of Zona radiata during ooctid development in an osseous fish- the zebrafish- was examined, and the collected data were compared to the related literature. We describe here the fine structure of the envelope of the oocyte of the zebrafish.

## Materials and Methods

### Light Microscope

The ovary samples, which had been fixed in Bouin's solution and % 10 Neutral Formaldehyde with tampon, were later treated in routine histological processes and were sunk into paraffin. The sections in 6-7  $\mu\text{m}$  diameters were colored through PAS method so as to examine under light microscope. The sections were illustrated by using Olympus microscope and monitoring system.

### Transmission Electron Microscope (TEM)

Pieces of the eggs were immediately fixed by immersion in 2.5% glutaraldehyde in phosphate buffer for 24 h at 4°C. The specimens were washed in phosphate buffer for 1-3 h and then post-fixed in 1% osmium tetroxide for 2 h. The tissue pieces were placed in propylene oxide for 60 min, then in Epon 812.

Tissues were sectioned 1  $\mu\text{m}$  and stained to-luidine blue. Sections were examined under light microscope. Ultrathin sections were mounted in copper grids. Thin sections taken in about 0.5-0.7  $\mu\text{m}$  were contrasted by using Uranil Acetate – Lead Citrate. Later on, they were examined under transmission electron microscope (Jeol, 100) and their photographs were taken (Koç et al., 2008).

### Terminology of zona radiate

There is considerable variation in the nomenclature used to describe the external membrane of teleost eggs. Commonly used terms for this outer covering include chorion, zona pellucide, radiate membrane, egg membrane, vitelline membrane, vitellin envelope, egg shell (Yamagami et al., 1992; Wekkawy and Osman, 2006).

## Results and Discussion

The oocytes located in zebrafish ovary in different phases were examined at the same section (Figure 1). It was observed that primer oocytes are cells with oval nucleus having a few nucleoli (Figure 1. 2). In this phase, the nucleoli were circulated around the nucleus. In the first stage of growing up, the unchanged follicle layers were visible (Figure 2). The follicle diameters were small in this phase (Table 1, Figure 1). Flat follicular cell layer was delimited with a thick basal lamina. It was observed that theca layer is formed by fibroblast-like cells and many blood capillaries. Follicle layer was composed of flat cells with oval-shaped nucleuses. The nucleuses of oocytes in pre-vitellogenic phase were at centered position. In respect of light microscope data, the nucleoli seemed to be close to nuclear membrane (Figure 1). In vitellogenic phase, the size of oocytes escalated significantly. It was presumed that the first sign of the development of Zona radiata is the formation and taking shape of microvillus structures. It was observed that microvillus groups take shape in the area between follicle cells and oocyte (Figure 4, 5, 6, 7, 8). In the early vitellogenic phase, small microvillus groups were seen in oocyte surface (Figure 4). Microvillus groups had directed towards follicle cells in an irregular manner throughout all development phases (Figure 4, 5, 6). It was observed that microvillus groups have made connection with follicle cell surface by the progression of developmental phases (Figure 5, 6). The oocytes were much longer, bigger and more descent in the vitellogenic phase than they were at the beginning. This dimensional escalation was compatible with the nutrient accumulation in their cytoplasm. In this stage, it was observed that the thickness of the Zone radiata located between the ootid and follicle cells increased (Figure 1, 7). In the same stage, narrow and long spaces were observed on the Zona radiata (Figure 7, 8). These structures were comprised of microvillus groups lying reciprocally with each other between the oocyte surface and follicle cells. The follicle cells were observed with their cubic forms in this phase. Their nucleuses were big (Figure 5). Flat cells were seen in the theca layer. In this area, the shaped-blood cells were observed in flat fibroblasts and blood veins and lumens (Figure 6, 7).

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. The membrane in mammals which corresponds to the zona radiata is the zona pellucida. The zona pellucida is a rich membrane in glycosaminoglycan and glycoprotein. The zona radiata is composed of mucopolysaccharide and glycoprotein. This membrane has characteristics of PAS-positive in fish and mammals. The vitellin envelope of teleosts is composed of 3 layers: Z1, Z2 and Z3 (Begovac and Wallace, 1989) also termed zona externa, interna and subinterna (Gillis et al. 1990). *S. dumerili*, during the maturation phase, the compacting of the vitelline envelope layers is apparent as the oocyte microvilli disappear, although remnants of vitelline envelope pores can still be observed (Grau et al. 1996). On the contrary, Kagawa (1981) reports the complete disappearance of the vitellin envelope pores in the yellowtail, *Seriola quinqueradiata* at this stage. As is the case in nearly all teleost fish, the zona radiata began to develop firstly after *primary phase* in zebrafish. At this stage, it was observed that there are follicle cells cumulated on the surface of oocyte. The structure of the zona radiata was homogeneous during the development phases. It was seen that the zona radiata that located between the oocyte and follicle cells had thickened at the *vitellogenic phase*. Light microscopy showed that the zona radiata was striated in early vitellogenesis. A similar finding has been reported for *Hemiodus* spp. (Garcia-Diaz et al. 1997, Brandao et al. 2003) found such striations during late vitellogenesis in *Serranus cabrilla* and *Serranus atricauda*. In the zona radiata of *Liza aurata*, each striated line represented a canal with pores opening at both ends. According to Shabanipour and Behroos (2004), zona radiata wasn't seen by light microscopy in the previtellogenic stage in *Liza aurata*. The zona radiata develop during the late stage of ovarian growth when vitellogenesis was about to end. During development of the oocyte in *Liza aurata*, the zona radiata is extended as primitive projections towards the inner surface which became more prominent as vitellogenesis progressed. Abdalla and Cruz-Landim (2003) also observed a perforated zona radiata with pore canals in *Piaractus mesopotamicus*. The microvillus groups located on the Zona radiata of zebrafish and lying from oocyte cell surface and follicle cells were observed in narrow and long spaces. These formations have been evaluated as

the evidence to structure-function correspondence. The thin structure of the ovary follicles of zebrafish was found to be in accord with the thin structure of the ovary follicles of *Lisa ramada* (İşisağ, 1996). In this study, the contributions of microvillus structures of follicle and theca cells to the formation of zona radiata were demonstrated accurately. As is observed in teleost fish in general and in zebrafish in particular, it was seen that as oocyte grows, the number of follicle cells has risen through mitosis and there has been a continuous follicle epithelium formation (Guraya, 1986). It was described that as is the case zebrafish, the zona radiata has formed at the further phases of development in *Fundulus heteroclitus*, and the zona radiata is composed of two layers, which are zona radiata internal and zona radiata external (Cerda et al. 1996). In teleost fish, a rapid thickening process is observed in the zona radiata during *maturation* and *ovulation stages* (Selman and Wallace, 1989). Accordingly, an obvious thickening process was seen in the zona radiata of zebrafish when the oocyte was growing. It was also supported by Guraya (1986) that the thickening tendency of the zona radiata in fish during oogenesis is caused by the rise in the ovule diameter and it lessens after ovulation. The main microscopic difference observed among the three species of *Hemiodus* was the thickness of the zona radiata layer of the oocyte. The grooves of the zona radiata observed in *Hemiodus ternetzi*, were pores or channels filled by filaments of the oocyte and of the follicular epithelium that could also be related to adhesiveness (Guraya, 1986). According to Li et al (2000) the ultrastructure of the zona radiata surface, the distribution density of pores and the size of eggs were also useful characters for distinguishing among the four species (*Mugil cephalus*, *Epinephelus malabaricus*, *Epinephelus coioides*, *Sciaenops ocellatus*), but the ultrastructural features of the micropyles were the most important of all for egg identification. A group of researchers found that the zona radiata is homogenous at the first phases of growing and the vitellin membrane has been composed of three different layers, which are Z1, Z2 and Z3, in the further phases (Begovac and Wallace, 1988; Selaman and Wallace, 1989). It was found that the growing process of vitellin membrane in *Novadan modestus* (black scraper) is very much similar to the case in teleost fish and this membrane is comprised of three different layers having a fibril structure internally (Hosokawa, 1985).

According to Schmehl and Graham (1987), the zona radiata in *Oncorhynchus tshawytscha*, chum salmon (*O. kisutch*), pink salmon (*O. gorbuscha*), brown trout (*Salmo trutta*), rainbow trout (*Salmo gairdneri*) and lake trout (*Salvelinus namaycush*) examined consisted of an outer adhesive coating, a thin densely staining zona radiata externa with pore canal plugs and a thick, fibrous zona radiata interna with a fibrous network on the inner surface. There was a two layer adhesive coating over the zona radiata externa in all species except pink salmon in which only one layer was observed. There were structural differences among species in the adhesive layer, zona radiata externa and plugs in the pore-canal openings. It was explained that the Zona radiata internal gets a fibril and complex structure in teleost fish, when the oocyte is growing (Kobayashi, 1985; Koya et al., 1995; Selman and Wallace, 1989).

### Conclusions

In zebrafish, during further growth of the oocyte and vitellogenesis, the zona radiata played an increasing role in the transport of material required for yolk synthesis. Ultrastructural analysis has shown that the zona radiata is formed during the early vitellogenic stage. The zona radiata is extended as primitive projections towards the inner surface. These structural changes in the ZR were correlated with the appearance of primitive projections that later extended as finger-like structures. This structural change resulted in an increase in surface area and agreed with the importance of the ZR in transporting the material necessary for production yolk during vitellogenesis.

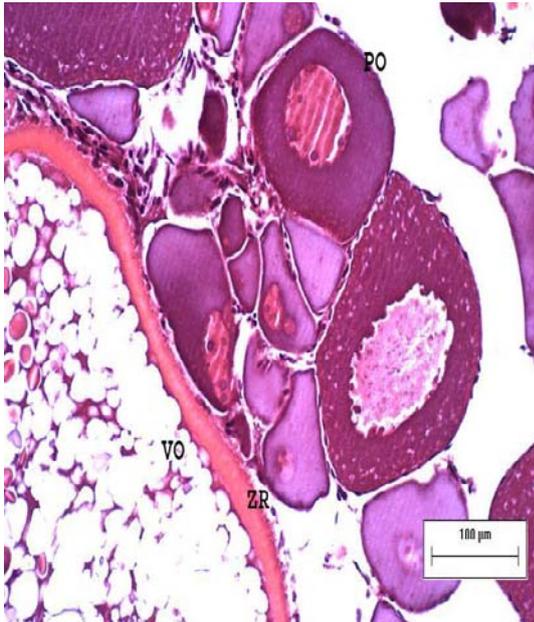


Figure 1

**Figure 1.** General overview of oocytes during the phases of growing..Vitellogenic oocyte (VO),Primary oocyte (PO), PAS staining X10

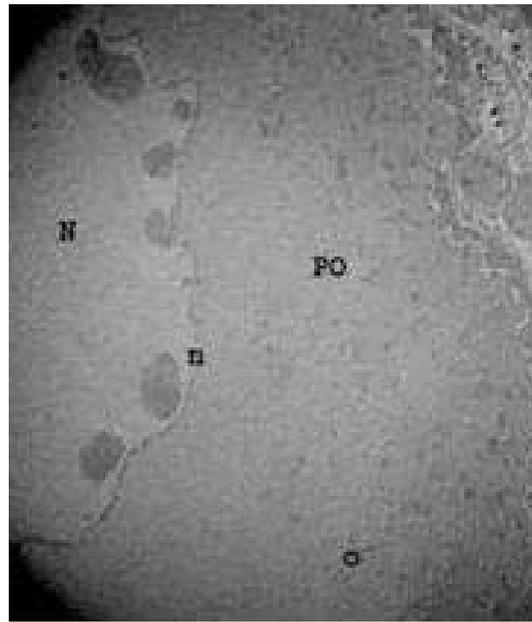


Figure 2

**Figure 2.** The electron micrograph of primer oocyte. Primary oocyte (PO), ,Oolemma (o) Zona radiata (ZR).Nukleus (N),nukleoli (n).x3000

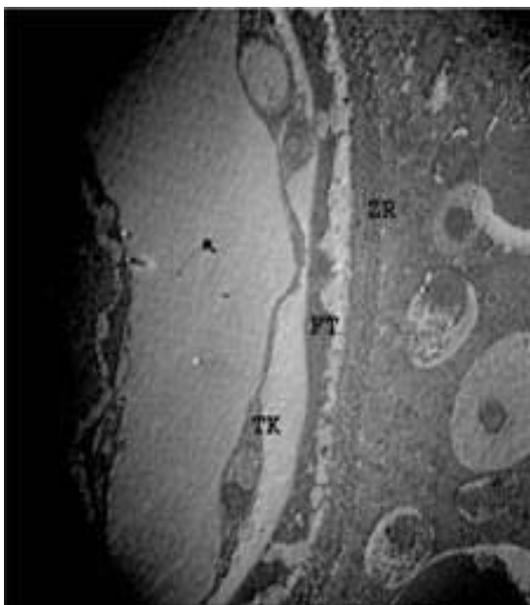


Figure 3

**Figure 3.** The electron micrograph of pre-vitellogenic oocyte. Zona radiata (ZR), Follicular layer (FT), Teca layer (TK), Cortical alveolus (Ca).x3000

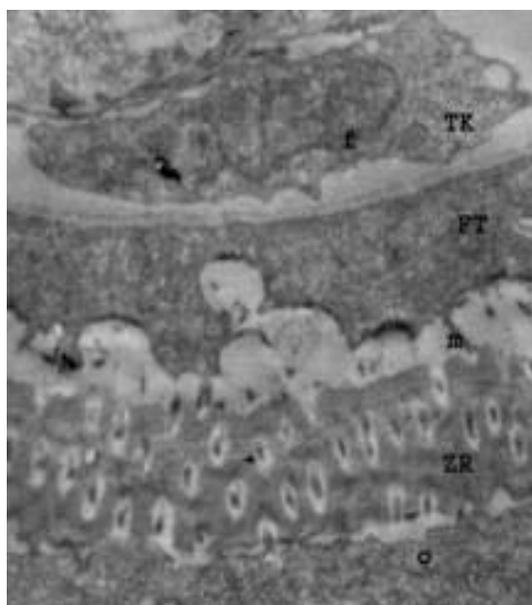


Figure 4

**Figure 4.** The electron micrograph of pre-vitellogenic oocyte. Zona radiata (ZR), Follicular layer (FT), Teca layer (TK), oolemma (o), microvillus (m). x12000

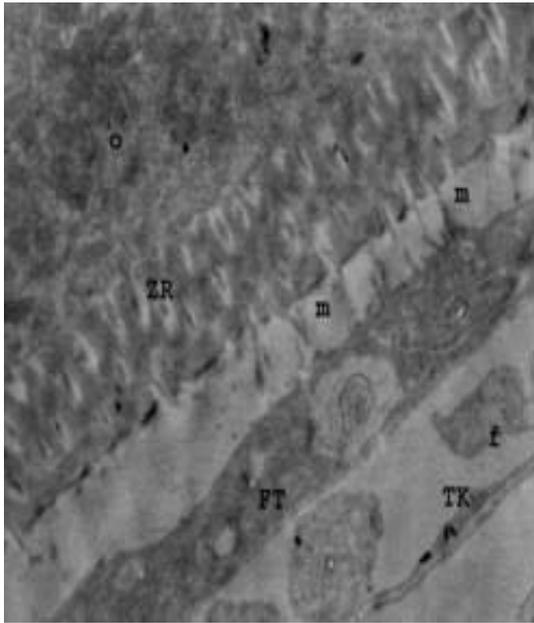


Figure 5

**Figure 5.** The electron micrograph of zone radiate and follicular layer in vitellogenic oocyte. Zona radiate (ZR), Follicular layer (FT), Teca layer (TK), fibroblast (f), oolemma (o), microvillus (m) .x12000

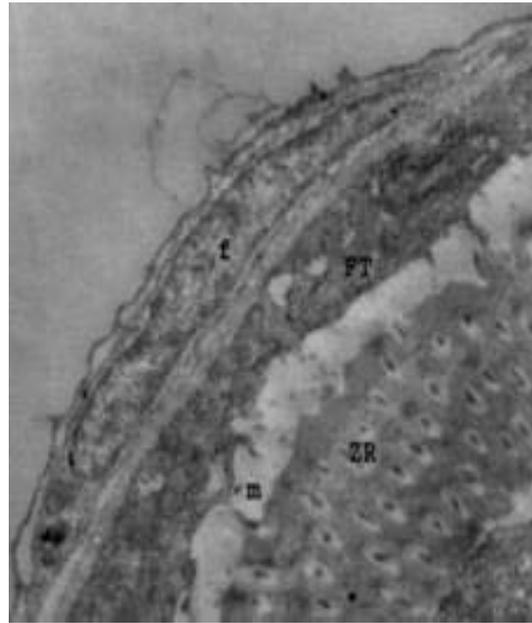


Figure 6

**Figure 6.** The electron micrograph of vitellogenic oocyte Zona radiate (ZR), Follicular layer (FT), fibroblast (f), microvillus (m).x12000

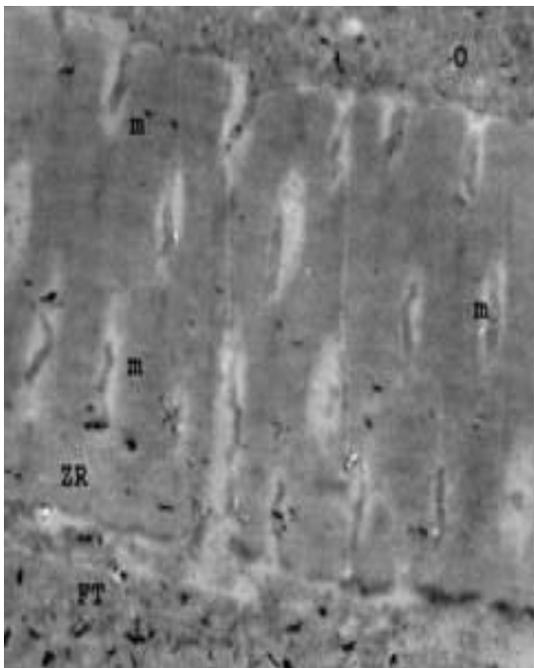


Figure 7

**Figure 7.** The electron micrograph of the zona radiate of oocyte in vitellogenic phase. Oolemma (o),Follicular layer (FT), Zona radiate (ZR) Mikrovillus (m).x25000

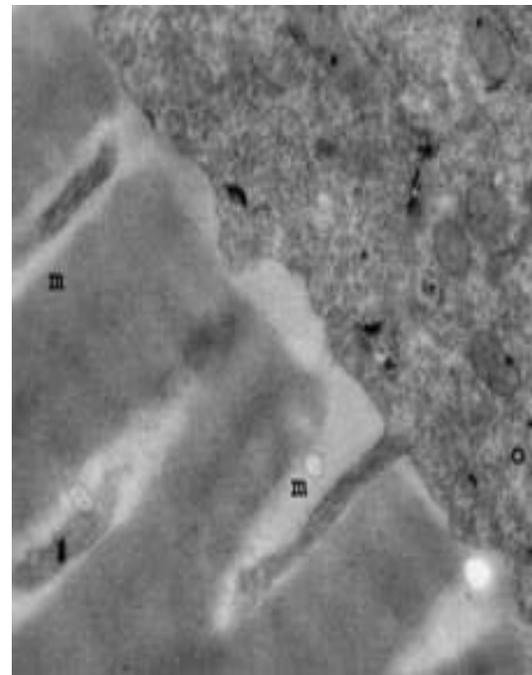


Figure 8

**Figure 8.** The electron micrograph of zona radiate in vitellogenic phase. Mikrovillus (m), oolemma (o). x25000

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