Nova Biologica Reperta 3 (2): 157-162 (2016)

ریز ساختار زونا ردیاتا در طول مراحل رشد تخمک لقاح نیافته گوپی زنده زا (*Poecilia reticulata*)

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دریافت: ۱۳۹۴/۱۰/۰۱ / پذیرش: ۱۳۹۵/۰۴/۱۴

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چکیده. زنده زایی فرایند پیشرفته‌ای است که در برخی ماهیان استخوانی دیده می‌شود. تخمک ماهیان با پوششهای متفاوتی حفاظت می‌شود که اولین آنها پس از غشاء تخمک (اولما) زونا ردیاتا (ZR) با ساختاری فاقد سلول است. زونا ردیاتا تنوع زیادی در ضخامت، ساختار و احتمالا عملکرد در تخمک ماهیان متفاوت و همچنین مراحل مختلف رشد تخمک‌ها دارد. در تحقیق حاضر ریز ساختار زونا ردیاتا در اطراف تخمک ماهی گوپی (*Poecilia reticulata*) توسط میکروسکوپ‌های نوری و الکترونی روبشی در مراحل مختلف رشد مطالعه شده است. زونا ردیاتا در مراحل ۱ و ۲ رشد تخمک دیده نشد. در مرحله ۳ زونا ردیاتا بصورت نواری نازک بدور تخمک یافته شد. ضخامت و پیچیدگی ساختاری در زونا ردیاتا تخمک مرحله ۴ پیشرفت کرده ولی در مرحله ۵ واجد ظاهری متفاوت و کاستی در ضخامت شد. مشخصه‌های سطح خارجی، ویژگیهای منافذ و عملکرد احتمالی زونا ردیاتا در طول نمو تخمک بررسی شد.

واژه‌های کلیدی. تخمک، زونا ردیاتا، ساختار، عملکرد، ماهی گوپی

Fine structure of zona radiata during growth stages of unfertilized oocyte in viviparous guppy (*Poecilia reticulata*)

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Received: 22.12.2015 / Accepted: 04.07.2016

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Abstract. Viviparity is an advanced reproducing process observed in certain bony fishes. Fish oocyte is protected by different coverings, the immediate one over oolemma being a non-cellular membrane known as Zona Radiata (ZR). ZR has shown variations in thickness, configuration and probably function at different fish oocyte and oocyte growth stages. In the present research work the ultrastructure of zona radiata around oocytes of guppy (*Poecilia reticulata*) has been studied by light and scanning electron microscopy methods concerning different oocyte growth stages. ZR was not observed at stages I and II. At stage III ZR was observed as a thin layer around the oocyte. It increased in thickness and complexity at stage IV (vitellogenesis) but showed different appearance and declined in thickness during the following stage. External surface characteristics, features of pore canals and probable function of ZR during oocyte development were also investigated.

Keywords. function, guppy fish, oocyte, structure, zona radiate

INTRODUCTION

Viviparity is a process in which eggs are fertilized internally and undergo development within the maternal reproductive system (Kunz-Ramsay, 2004) and has proved to be a highly successful mode of reproduction that has evolved independently many times and with many variations in a wi-

[DOR: 20.1001.1.24236330.1395.3.2.8.4]
[DOI: 10.21859/acadpub.nbr.3.2.157]

[DOR: 20.1001.1.24236330.1395.3.2.8.4]

[DOI: 10.21859/acadpub.nbr.3.2.157]

de range of taxonomic groups (Hoar & Randel, 1988). Embryos of viviparous teleosts develop either in the lumen of the ovary (intralumenal gestation) or in the ovarian follicle (intrafollicular gestation). Intrafollicular gestation is common in four families of viviparous teleosts: the Poeciliidae, the Anablepidae, the Clinidae, and the Labriosomidae, where the follicle wall is the principal maternal tissue involved in the maternal-embryonic relationship (Wourms, 1981; Wourms *et al*., 1988).

The Oocytes of viviparous teleost fishes are also covered by a non-cellular envelope known as Zona Radiata (ZR). The ZR of viviparous teleosts has rarely been described in the literature (Wourms *et al*., 1988). Earlier thin ZR layer was reported around developing eggs of *Mollienisia sphenops* and *Xiphophorus helleri* (Zahnd & Porte, 1962), as well as *Platypoecilus maculates* (Erhardt & Götting, 1970).

There are structural differences in the ZR from fishes of different systematic and ecological positions which help scientists identify ichthyopl - anktons (Colmenero *et al*., 2015). ZR also prese - nts certain adaptations during pre and post-spawning and egg development (McMillan, 2007) making contribution in various functions during oocyte growth (Lönning & Hagstriim, 1975; Grove & Wourms, 1983; Riehl & Greven, 1990, 1993).

The ZR plays a significant role in controlling interactions between the external and internal egg environments. Therefore, knowledge of its fine structure can assist in obtaining a better understanding of environmental effects on egg development (Riehl & Kock, 1989).

As viviparity is an advanced strategy in reproduction and since viviparous species are fewer compared with oviparous fishes, fine architecture, probable functions and developmental transition of ZR might differently feature other biological and evolutionary importance. Guppy fish (*Poecilia reticulata*) being viviparous, easily maintained and widely accessible was selected to study the ultrastructure of ZR with restricted functions compared with oviparous fishes.

MATERIAL AND METHODS

Female guppy fish were obtained from a local ornamental fish dealer. The ovary of tiny guppy was rather small, unpaired and light yellow in color. Ovaries were fixed in Bouin's solution for 24 h, transferred to %70 ethanol, dehydrated in a series of graded ethanol solutions, and made transparent by xylene. Samples were embedded in paraffin, wax and sectioned at 5 µm thick by Leica rotary microtome. Sectioning was carried out in an order of 25 serial sections as a batch. The number of batches depended on the thickness of the tissue being processed. 5 intervening sections of each batch were sampled for staining by Hematoxylin and Eosin and the rest were kept safely for further investigation and SEM preparation. H&E stained sections were mounted permanently and studied by light microscopy method.

Preparation for SEM

Preliminary investigation of H&E stained sections revealed various histological aspects of ZR but to exploit the details of events happening in stained slides, a selected single unstained preidentified paraffin section of a serially sectioned ribbon, partly processed for staining, was mounted on a 1×1 cm cover slip after being spread in warm water bath.

It was left in room temperature for 24 hours, carefully deparaffinized in situ by drops of xylene and finally cleared by drops of 90% alcohols for several times. It was, then, placed in desiccator to be preserved from humidity and dust particles. Prepared slide was, then, glued on a stub, gold covered and scanned by Hitachi S-4160 SEM.

RESULTS

Elliptical shaped immature oocytes were abundant in the mid-dorsal region of the ovary while the ventral region contained spherical mature oocytes. Based on Light microscopy and SEM images, oocyte growth stages I and II possessed typical features observed in oocytes of bony fishes and ZR appear in neither of the two stages.

Stage III (Cortical alveoli stage)

The average diameter of oocytes at this stage was 138.14 µm. Stage III was identified by presence of cortical alveoli found at inner cell margin, slowly emerging into larger vesicles toward the cell center. Follicular epithelium (FE) gradually thickened and zona radiata (ZR) appeared as a thin membrane (of about 2.15 µm in thickness) between oolemma and follicular epithelium (Fig.1a). Electron microscopic observations showed that at the beginning of stage III, ZR did not show a proper configuration though the ZR outer surface had highly porous appearance and primary pore canals were formed with no projections passing through.

ZR external surface appeared to be quite rippled

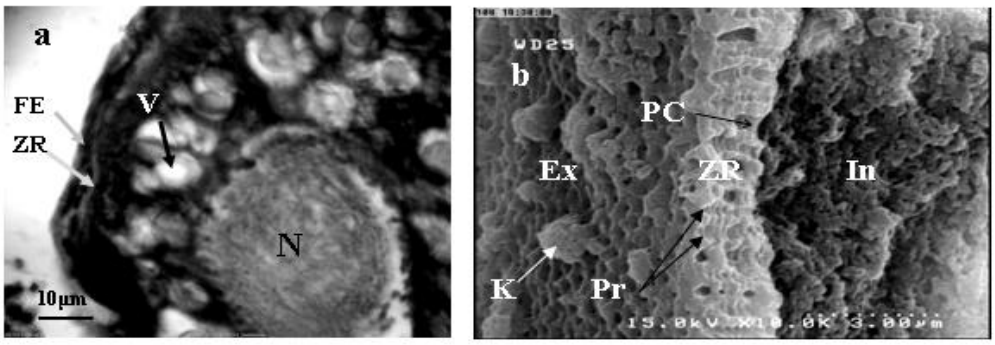

Fig. 1. *P. reticulate*. **a**: Oocyte at stage III (cortical alveoli stage). Note the thin ZR and vesicles that are increasing in number. **b**: SEM micrograph of oocyte at stage III. Cross section of stratified ZR, rippled external surface and pores leading into canals, microvilli or any type of projections through pore canals are absent. ZR: Zona Radiata, FE: Follicular epithelium, V: Vesicle, N: Nucleus, Ex: External surface of oocyte, Pr: Pores leading to canals, PC: Pore canal, In: Internal or inner surface, K: Knobs.

carrying many pores and knob-like structures which were dispersed irregularly (Fig.1b). A cross section of ZR reveal ed its stratified structure and that almost every pore had communication with the interior of oocyte directly through a passage or canal (pore canal) crossing the ZR (Fig.1b).

Stage IV (Vitellogenesis stage)

The average diameter of oocytes at this stage was 272.08 µm. The oocyte was occupied by yolk globules. External wall of nucleus was crenated, follicular layer thickened further and a well-developed 3.92 µ thick zona radiata was clearly visible (Fig. 2 a). At this stage, the noncellular ZR proper possessed well-grown projections with a free end towards the inner side of the oocyte (Fig.2 b, d). The projections were quite variable in appearance and their stout bodies were crossconnected (Fig. 2 b, d). ZR was still stratified but certain pore canals were not continued to connect the exterior of the oocyte to its interior. The canals found their way in by either direct communications initiating from exterior pores or indirectly through a half way secondary rout (Fig. 2 d). A cross-section of projections revealed (Fig. 2 c and d) that the architecture behind striated appearance observed in histological preparation was formed by the inter spaces alternated by projections. Late at this stage, the thickness of zona radiata was reduced to as much as 2.5 µm.

Stage V (Matured stage)

The average diameter of oocyte at this stage was 874.65 µm. Nucleus was disappeared. Yolk globules turned to be homogeneous, mass occupying whole oocyte (Fig.3a) leaving only a few vacuolees. Follicular epithelium was turned to be thin and loose over zona radiata being also reduced in thickness (1.5 µm) (Fig. 3b). The ZR of unfertilized oocyte showed smooth surface, bore ornamental folds and reduced number of pores. Cross section of ZR revealed loss of complexity in overall configuration and nature of pore canals.

DISCUSSION

Five stages of oocyte growth were identified in guppy (*Poecilia reticulata*). Zona Radiata of oocytes in stages I and II was not observed by either light or electron microscopy as in many teleosts until appearance of cortical alveoli (Anderson, 1967; Iwamatsu & Kobayashi, 2002). In *P. reticulata*, during the third stage of oocyte growth a narrow ZR, which was barely detectable under light microscope, started to form between follicular cells and oolemma. It seems that thin ZR has been accepted as a characteristic of viviparous fishes like *Heterandria formosa* (Gravemeier & Greven, 2006).

The rough external surface of ZR was greatly porous and pores were communicated directly to the interior surface through a short passage or canal (Fig.1 a,b).

Unlike *Heterandria formosa*, which lacked lamellae, the pore canals of *P. reticulata* in stage III revealed a stratified (lamellar) appearance which had been reported earlier for *Dermogenys pusillus* (Flegler, 1977) and *Pimephales promelas* (Manner *et al.*, 1977). Pore canals have been investigated to be the result of microvilli penetration through ZR raised from oolemma which finally reached follicular cells (Droller & Roth, 1966; Grove & Wourms, 1994; Giulianini & Ferrero, 2000; Iwamatsu & Kobayashi, 2002; Kagawa, 2013) but they were not prominent enough in *P. reticulata* to be identified as striation on histological observations.

[DOR: 20.1001.1.24236330.1395.3.2.8.4]
[DOI: 10.21859/acadpub.nbr.3.2.157]

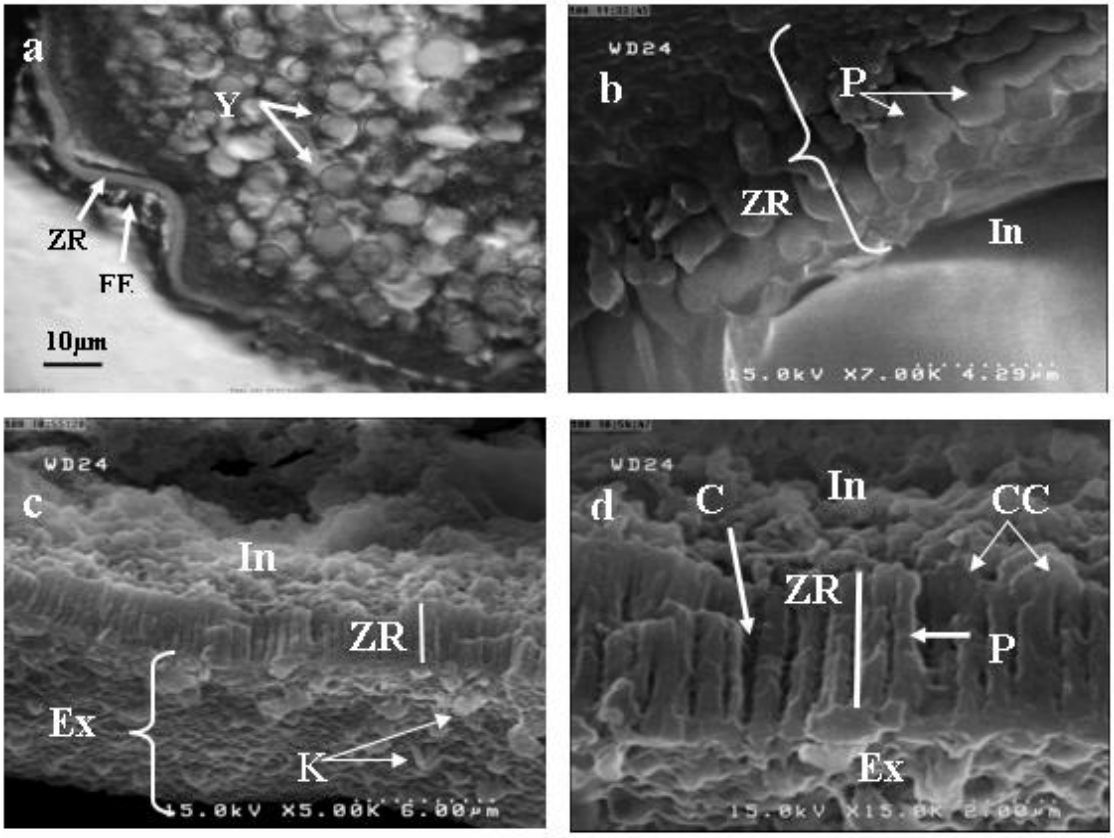

Fig. 2. a: *Poecilia reticulata*, histological micrograph of oocyte in stage IV (vitellogenesis) occupied by yolk globules and showing broader ZR. **b:** SEM micrograph of guppy fish oocyte in late stage IV. Intact ZR partitioning structures in the form of projections heading to the interior of oocyte. Cross connections of projections presents a complicated architecture. Oolemma and content of oocyte are not shown. **c**: SEM micrograph of oocyte at vitellogenic stage (IV). Cross section of an oocyte at stage IV. Please note the exterior of oocyte. Rippled surface of exterior accompanied by knob – like structures (K). **d:** Magnified micrograph C. Stratification of ZR shown along the partitions. Pore canals in between partitions connecting exterior to interior of oocyte. Cross section of ZR going through partitions and cross connections manifesting non tubular nature of partitions and the routs from exterior to interior are not always straight forward. ZR: Zona radiata, In: Internal or inner surface, Ex: External or outer surface, C: Canal, FE: Follicular epithelium, P: projections, CC: Cross connections, Y: Yolk globules, K: Knobs.

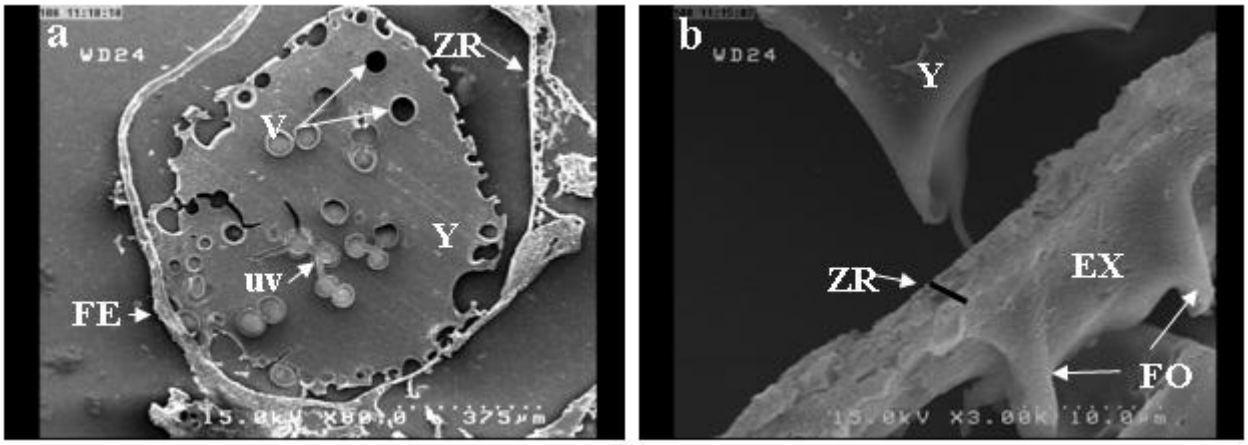

Fig. 3. a: SEM micrograph of matured oocyte (stage V) of guppy (*Poecilia reticulata*). Vacuoles are uniting in homogenous yolk. Loose follicular epithelium and thin zona radiata are visible a distance from yolk (perivitellin space). **b:** SEM micrograph of zona radiata (ZR) of the same oocyte. Zona radiata thickness is clearly decreased, structure simplified, striations disappeared and number of pores greatly reduced. Folds are present on exterior. FE: Follicular epithelium, V: Vacuoles, uv: Uniting vacuoles, Y: Yolk, ZR: Zona radiata, EX, External surface of zona radiata, FO: Folds.

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Successive stage IV was obviously important for the process of vitellogenesis. ZR was characterized by thickening, more complex appearance (cross connections) and larger pore canals being distinguished as striation in histological preparations (Fig. 2a,d). The configuration and architectture of ZR gradually attaining complication, entering and during the stage IV, has been described as a common feature of oocyte growth of oviparous and viviparous teleosts (Anderson, 1967; McMillan, 2007).

Azevedo (1974) explained that in viviparous Xiphophorus helleri ZR was composed of a single layer and reached its maximum thickness by the end of vetillogenesis and TEM micrographs of ZR in oocytes of viviparous Lebistes reticulatus showed penetrations of microvilli of oolemma caused complication in structure (Droller & Roth, 1966).

Changes in complexity of ZR have been concerned in relation to different transferring materials crossing ZR and, therefore, functioning as a mediator (Flegler, 1977; McMillan, 2007). In many teleosts the complexity of ZR was conserved at the maturity stage and during the post-ovulation period, which suggested other functions for ZR (Laale, 1980). ZR in maturing stage (V) of oocytes in *P. reticulata* bore extensive reverse modification leading to simplicity in appearance, reduction in thickness and also number of pore canals. Similarly Gravemeier & Greven (2006) reported mature oocytes of *Heterandria formosa* possess a more homogenous zona. At maturity the oocytes of *P. reticulata*, the rough external surface of ZR turned smooth with diffused fewer pores and low striation.

Such reverse mode of changes could be expected because the major function of ZR was already performed as nutrient mediator during oocyte growth stage. In a similar manner, disappearances of ZR features related to vitellogenesis in matured oocyte of viviparous *Heterandria formosa* and *Xenotoca eiseni*, and microvilli withdraw of oocyte in oviparous species have been stated by Gravemeier & Greven (2006). Apart from mediating nutrients, some other functions are also attributed to ZR, such as protecting ovulated oocyte against polyspermy (Ginsburg, 1961; Laal, 1980; Hart, 1990), mechanical and chemical hazards (Pommeranz, 1974; Yamagami *et al*., 1992; Kagawa, 2013), bacterial action (Bell *et al*., 1969; Hagenmaier & Wilhelm, 1972) and serving as anchor or attachment mode (Riehl & Patzner, 1998; Breining & Britz, 2000).

In viviparous fish though ZR might have a protecting function to prevent polyspermy but it could not have any other role found in oviparous species. Therefore, it was probable that ZR might continue the mediatory function even after fertilization and during gestation. Droller & Roth (1966) has extensively des cribed the occurrence of proliferating pinocytosis during vitellogenic stage in Lebistes reticulatus. On the smooth surface of ZR of matured oocyte (stage V) in *Poecilia reticulata*, it is most likely that extensive folding (Fig. 4b) provided lager surface area to permit the entrance of micro molecules by endocytosis (McMillan, 2007).

The scattered knob like structures on external surface of ZR of growing (stage III) and vitellogenic (stage IV) oocytes of *P. reticulata* were disappeared in matured ones. Riehl & Greven (1990) and Riehl (1991) mentioned the one-layered electron dense zona radiata of *Ameca splendens* bore short processes that were thought to be remnants of attaching filaments. They argued the filaments might be related to fish phylogeny and evolution and reduced envelope could facilitate a more effective exchange of gases .

ACKNOWLEDGEMENT

Authors are thankful to the Deputy of Research and Technology, Guilan University for financial support.

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Mirnategh, S.B., Shabnipour, N. and Kaviani, E.F. 2016. Fine structure of zona radiata during growth stages of unfertilized oocyte in viviparous guppy (*Poecilia reticulata*). – Nova Biol. Reperta 3 (2): 157-162.

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