EGYPTIAN JOURNAL OF AQUATIC RESEARCH 1687-4285
VOL. 31 NO. 2, 2005: 326-356.

OVARIAN FOLLICULAR ULTRASTRUCTURE OF THE OOCYTES OF *BOOPS BOOPS* WITH SPECIAL REFERENCE TO THE VITELLINE ENVELOPE DEVELOPMENT AND MICROPYLAR APPARATUS

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Keywords: thecal cells, granulosa cells, zona radiate, pore canals, micropyle

ABSTRACT

The study of the ovarian follicular epithelium of *Boops boops* revealed that it consists of two layers, an outer thecal layer and an inner granulosa layer. The epithelial follicle reaches its maximum growth during the primary yolk deposition stage, and then it begins to degenerate, as a preparation for ovulation. The zona radiata consists of 10 dense layers, alternating with 9 light layers. Zona radiate interna is less dense and much thinner than zona radiate externa. The pore canals in zona radiata of *Boops boops* has simple morphology, as it lacks any microfilaments, ornate surface structures or jelly coats. The micropyle of *Boops boops* is also simple as it lacks any microvilli in the accessory opening region, knobs, ridges, annular thickening or reinforcement as other species of family Sparidae. It is without a pit but has only one canal.

INTRODUCTION

Studies of fish reproduction usually require knowledge of the stage of gonad development in individual fish. Such knowledge is often based on visual inspection of the external appearance of the gonad. A more precise and detailed analysis requires the use of histological methods (West, 1990; Garcia-Diaz *et al*., 1997).

Identification of cellular changes however, may be difficult by light microscopy alone. Ultrastructural studies using scanning and transmission electron microscopy can resolve some of these difficulties, as well as provide additional valuable information to fish biologists (Selman and Wallace, 1986).

Several authors have studied the ultrastructure of oogenesis in fishes. Concerning the developmental stages of oocytes growth (Shackley and Kiny, 1977; Cruz-Landim & Cruz-Höfling, 1979; Bruslé, 1980), the cellular envelopes of the developing oocyte (Cruz-Landim *et al*., 1987; Cruz-Landim, 1990; Cruz-Höfling & Cruz-Landim, 1992), and the oocyte acellular covers during its growth and after egg maturation (Dumont & Brummet, 1980; Lopes *et al*., 1982; Hart *et al*., 1984; Cruz-Landim, 1990)

In fact, the structure of the follicular epithelium seem to be very much variable from one oocyte group to another. In the same way, the acellular covers that invest the oocyte during its development and after ovulation, do not have its origin and function well defined (Cruz-Landim & Cruz-Höfling, 2001).

These structures had a long-standing appear to the biologists since questions, as

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ovary steroid synthesis and adaptation of oocytes to certain environmental conditions have been attributed to the cellular and acellular coverings respectively.

It is well documented that vitelline envelope morphology vary between species and reflects adaptations to ecological conditions (Riehl, 1996). Accessory structures such as attaching filaments, fibrils and also jelly coats have been found in a great number of teleosts (Fausto *et al*., 1993). Their functional significance has been associated to the egg attachment among them or to the substrates (Riehl & Spartzner, 1998; Meijide & Guerrero, 2000).

The vitelline envelope of ovarian follicles in teleosts as in other vertebrates is a proteinaceous covering assembled outside the cell during oogenesis. Since the vitelline envelope acts as a mediator between the embryo and its environment. Therefore the present work aims to study of the developing process of the ovarian follicles and is relevant for understanding the origin and structure of the vitelline envelope.

MATERIAL AND METHODS

The fish samples used in the present work were obtained alive by commercial fishermen, from the Mediterranean coast near Kayet Bey castle at Anfoushy region, Alexandria. The fish were transported to the laboratory in aerated aquarium, dissected to determine sex and maturity stage. Then the gonads were cut into very minute pieces and immediately fixed in the universal E.M. fixative {glutraldhyde solution 4 %} and kept at 4^{o}C till processing.

The sample washing was done in 5% sucrose in 0.05M Cacodylate buffer overnight. Post fixation in 1% Osmium tetroxide in 0.2M cacodylate buffer. Rinsing and washing in buffer at pH 7.2-7.4. Dehydration, embedding, sectioning and staining with uranel acetate (saturated in 70% alcohol). The examination was done by using Jeol CX 100 electron microscope and Zeiss 109 transmission electron microscope.

For scanning electron microscope, the fixation of the sample was applied as previously said then, drying with critical point drier Samdri-PVT-3B and Coating with JEC-1100E ion Sputtering device.

RESULTS

Oogenesis phenomenon is known to initiate with the proliferation and differentiation of oogonias. These primordial germ cells, the smallest of the oogenic cells, are grouped in nests in the ovary and, after meiotic division and differentiation, they develop into oocytes. These are classified into groups, according to their stage of development, as determined by morphological changes in the nucleus, cytoplasm and follicular wall.

The primary growth phase in teleosts has traditionally been divided into two stages, the chromatin nucleolar phase and the perinucleolar phase (Yamamoto, 1956)

Ultrastructural analysis of chromatin nucleolar stage shows large, oval cells, located at the periphery of the ovarian lamellae either isolated or forming nests, with large oval nucleus. The nucleus holds a relatively large volume of the cytoplasm (Fig.2).

The nucleus is enclosed with a double membrane envelope showing a somewhat wavy contour. In some places, the outer membrane of the nuclear envelopes forms small out-pockets protruding into the cytoplasm.

The ground matrix of the nucleus is granular and of moderate density. The chromatin presents diffuse condensations. The nucleoli, several in number and found embedded in the matrix, with peripheral distribution (next to the nuclear membrane).

Within the cytoplasm, mitochondria are distributed near one pole of the cell or round nucleus. The oocyte cytoplasm is strongly basophilic during this period.

In this stage, the follicular epithelial cells are small in size and rather cuboidal in shape. They are situated nearly all around the oocyte except that part directly in contact with another oocyte, but not arranged in a single layer yet.

In the early phase of perinucleolar stage, the cellular volume of oocytes suddenly increases, a single layer of flat follicular epithelial cells enclosed the oocytes but they are not arranged regularly in a row. These follicular cells are fusiform with a narrow elongated or elliptical nucleus that includes clumps of condensed chromatin, irregularly distributed and sometimes associated with the nuclear envelope (Fig. 3). The cytoplasm of the follicular epithelial cells is filled with endoplasmic reticulum and small mitochondria.

The relation between the follicular epithelial cells and the oocyte is almost the same as in the younger oocytes. The surface of both the oocyte and follicular epithelial cells are smooth and run parallel to a narrow intercellular space. A basement membrane surrounds a group of both the oocyte and follicular epithelial cells closely. External to the basement membrane, several non germ cells may be found, but cannot yet be defined as theca cells (Fig. 4).

During this period, the oocyte cytoplasm is still strongly basophilic and homogeneous, it contains two types of electron-dense material: “nuage material”, which remains independent and is always located close to the nuclear membrane, and a material associated with mitochondria, known as the “intermitochondrial cement”.

The nucleus contains many easily distinguishable nucleoli that vary in number and diameter, next to the nuclear membrane.

The scanning electron microscope shows that the outer surface of the cytoplasmic growth eggs are completely covered by follicular epithelial cells (Fig. 5).

The secondary growth phase includes 3 vaculized stages as follows

The early vaculized stage in which a flat follicular cell layer is formed, that is composed of granulosa layer, a thick basal lamina and a squamous coat of thecal cells surround the oocyte. This thecal cell layer is composed of fibroblast-like cells, collagen fibrils and blood capillaries (Fig. 6).

Concomitant with the growth of the oocyte, and considered to be the first sign of specialization of the Oolemma, is the formation of the microvilli in the space between the growing oocyte and the follicular layer, called perivitelline space (extracellular space).

In this stage the surface of both the oocyte and follicular epithelial cells, no longer run in smooth and in parallel. A lot of ovular microvilli protrude into the perivitelline space as tubular micro-projections.

The granulosa cells develop microvillar processes, which are smaller in number and size to the ovular microvilli, which will penetrate the perivitelline space to a variable distance depending on the stage of the oocyte growth.

The ovular microvilli frequently are in close contact with the follicular microvilli, but no direct connection between the cytoplasm of the two cells has been observed. First, short microvilli appear on the oocyte surface. These cellular processes will interlace in an irregular path with those formed by the follicular layer.

The cytoplasm of the oocyte at this stage loses its basophilia and presents a dense granular matrix, with many mitochondria, vesicles with different electron densities, endoplasmic reticulum and ribosomes.

At the middle vaculized stage as the oocyte grows, the follicle differentiates and develops an external thecal layer and an internal granulosa layer, delimited by rather thick basal lamina (basement membrane) consisting of a serious of membranous layers.

The granulosa layer is still composed of cells with flattened morphology and shows cellular extensions (microvilli) that extend towards the oocyte surface through the perivitelline space, that become wider than previous stage. The ooplasm lost its basophilia (Fig. 7).

Outside the basement membrane, the theca cells of flat form are closely located. They generally stand in a row and have a flattened nucleus and the cytoplasm which contains a few small mitochondria, poorly developed endoplasmic reticulum, a small amount of vesicles and collagen fibers.

On the oocyte surface, the space between the oocyte and granulosa layer, appears to be progressively occupied by a material that appears to be synthesized by the oocyte.

The deposition of the dense and homogenous material goes on and forms the external layer of the vitelline envelope, the zona radiata externa. Later on, with in the same stage, oocytes show the presence of a second layer, the zona radiata interna, under the former one. This second layer is more coarsely aggregated and has less electron density than the zona radiata externa.

During this stage of development, zona radiata interna is relatively thicker than zona radiata externa. It consists of two to three rows of pore canals, while zona radiata externa consists of two rows only of pore canals.

At late vaculized stage the follicular epithelial layer, containing both the thecal cells and the granulosa cells that have increased in height but are still flat (Fig. 8).

The granulosa cells develop a rough endoplasmic reticulum that is filled with amorphous material, Golgi complex and considerable number of mitochondria and smooth endoplasmic reticulum.

The thecal cells are characterized by the presence of numerous mitochondria, which has a rather complex internal structure, endoplasmic reticulum that is almost agranular, rough endoplasmic reticulum that is less well developed but nevertheless is often present. The moderately developed Golgi complex is close to the nucleus

Microvilli from the oocyte surface extend through the zona radiata and project deeply into the extracellular spaces of the overlying granulosa cells. The oocyte microvilli maintain close contact with the granulosa cells. They lie in close proximity to short microvillar extensions on the surface of the granulosa cells within the extracellular space, and in some places a single cytoplasmic process from the granulosa cells may come into contact with the microvillus of the oocyte within a pore canal.

At the end of the vaculized stage, the scanning electron microscope show that two main cell layers, an inner granulosa layer and an outer thecal layer, separated by rather thick basement membrane of connective tissue, represented the steroid-producing cells cover the surface of the oocyte. However, the granulosa cell layer is separated from the oocyte by vitelline envelope (Fig. 9).

The maturation phase includes different yolk deposition stages as follows

At primary yolk deposition stage, the steroid-producing thecal cell layer of these oocytes is very thick, consisting of two cell layers and had exhibited a considerable high nucleo-cytoplasmic ratio (Fig. 10).

The outer thecal cell layer are composed of fibroblast-like cells, containing lipid droplets together with a few mitochondria with lamellar cristae, and a small amount of tubular smooth endoplasmic reticulum.

Concerning the inner granulosa cell layer, until now, shows no marked change throughout the oocyte development. The most characteristic feature is the existence of a plentiful number of oval or spherical-shaped mitochondria with lamellar cristae and extensive smooth endoplasmic reticulum, which varied in size and shape, as well as the degree of opacity of their inclusions due to amorphous material. The rough endoplasmic reticulum appeared relatively ill developed but nevertheless was often present. Also lipid droplets, some of which with large sizes, were observed in those granulosa cells.

Concomitant with the oocyte growth, the zona radiata exhibit distinct striations with reticulate pattern, giving the zona radiata a more solid appearance. It increase in thickness specially zona radiata interna and was transversed perpendicularly by pore canals and certain process (microvilli) from both the granulosa cells and the oocyte. But

the microvillar process from the oocyte are seen no longer to penetrate deep into the perivitelline space of the overlying granulosa cells, that become narrower. The pore canals of zona radiata still contain microvilli from both the oocyte and granulosa cells.

Large irregular vacuoles and small oval vesicles may be observed in the peripheral of the oocyte cytoplasm. These vesicles, are called "cortical alveoli", have a size and consistency lower than and an appearance lighter than yolk vesicles.

At Tertiary yolk deposition stage, degeneration was observed to the thecal cells. Their nucleus disappears and they decrease very much in height (Fig.11).

Degeneration also occurs to the steroid-producing granulosa cells, which become compact, relatively small with elongated oval nuclei and a large amount of lipid droplets of different sizes are formed. Small sized mitochondria with tubular cristae and smooth endoplasmic reticulum with dilated cristernae filled with amorphous material can also be seen in the granulosa cell cytoplasm.

Zona radiata interna gets thicker and displays a multilaminar structure with alternating layers of similar densities. Up to ten different layers can be distinguished, while Zona radiata externa maintains a compact homogenous structure (Fig. 12).

The pores in the vitelline envelope increase in both their diameter and the distance between them. Microvilli crossing the vitelline envelope show an alternating shaped path and don't fill the whole pore canal.

Tangential sections of the vitelline envelope show the round pore canal section. Inside each pore, one and occasionally two microvilli can be seen. The thicker one belongs to the follicular cell and the thinner one to the oocyte (Figs. 12 and 13).

Microvilli extending from the ooplasm surface pass through the alternating pore canals and their tip project into the perivitelline space. Some of them come in direct contact with the microvilli of the overlying follicular cells, frequently at the base of the follicular microvilli (Fig. 11).

The most evident character for the preparation for ovulation is that, these microvillar processes either of follicular cells or from the oocyte are retracting and are no longer penetrating deeply into the perivitelline space, that become very much reduced (Fig. 11).

Prior to ovulation, complete degeneration occur to the thecal cells, leaving only granulosa cell layer with wide intercellular spaces between its cells and zona radiata covering the oocyte surface. Wide space will be seen also between the granulosa cells and zona radiata (Fig. 14).

The oocytes are ovulated to the ovarian cavity leaving the remains of the thecal and follicular layers in the ovarian lamella (empty follicles). Ovulated eggs are covered by the vitelline envelope, whose structure is similar to that observed in the late stages. The cytoplasm is now fully occupied with yolk platelets.

Pore canals in zona radiate and micropylar apparatus

Ripe eggs of *Boops boops* are spherical in shape about 2 mm in diameter. The surface of zona radiata is plain with a uniform distribution of pores. The pores had no lips throughout the course of development and increasing of thickness of zona radiate. The pores on the surface of eggs represent the space earlier occupied by microvilli from the developing oocytes and granulosa cells.

The eggs, which have a diameter, ranged from 250μ to 349μ (early vaculized stage), has a pore distribution density (distance between pores) equal to 0.24μ, with minimum and maximum values equal to 2d and 6d respectively (d=mm on magnification 15000). The pore-opening diameters are about 0.16μ with 2d and 4d as minimum and maximum values respectively (Table 1 and Fig. 1). Numerous agglutinates are observed scattered over the surface of zona radiata (Fig. 16).

The micropyle of the cytoplasmic growth eggs is cylindrical canal, which opened

outward like a hole in the epithelium follicle cells (Fig. 15). The canal has an average diameter of 1.4µ and 2µ along its short and long axis respectively. It lack any accessory openings at this stage of development at the animal pole, but numerous agglutinates are observed along the outer rim of the micropyle and on the surface of egg at the animal pole but not in the micropylar canal itself.

The micropyle of the early vaculized eggs is also cylindrical in shape and consists of micropylar canal and many accessory openings. The average diameter of the accessory openings is 0.2µ. The canal appeared to cross the 3 layers of cells covering the egg, follicular epithelium, granulosa, and zona radiata respectively (Fig. 18). The diameter of the micropylar canal at this stage of development is about 3.5µ.

Agglutinates are still observed on the outer rim of the micropyle and on the egg surface around the micropyle but decreased if compared with the previous stage.

The eggs with a diameter ranged from 350µ to 449µ (middle vaculized stage) have a pore distribution density, slightly higher than previous egg stage, equal to 0.3µ with 3d and 7d as minimum and maximum values respectively. The pore openings diameters increase to reach 0.19µ with minimum and maximum values equal to 2d and 4d respectively (Table 1 and Fig. 1).

The scattered agglutinations observed here over the surface of zona radiata are less than in the early vaculized stage (Fig. 17).

The eggs with a diameter ranged from 450µ to 549µ (late vaculized stage) have a pore distribution density equal to 0.56µ with 6.3d and 10.3d as minimum and maximum values respectively. The pore opening diameters decreased to measure about 0.16µ with 1.75d and 3.3d as minimum and maximum values respectively (Table 1 and Fig. 1).

The surface of zona radiata is smooth and the scattered agglutinations almost had disappeared (Fig. 19).

The micropyle of late vaculized stage is still cylindrical in shape, but the micropylar canal opened outward like a funnel (Fig. 20). The average diameter of the canal is about 5.5µ. It appeared as an opened depression in the follicular epithelium cells. May accessory openings were found in the surroundings of the micropyle, its average diameter was 0.45µ.

No further agglutinates are observed on the outer rim of the micropyle but some are scattered on the egg surface round micropyle.

The eggs with a diameter ranged from 550µ to 649µ (mature eggs) have increased pore distribution density equal to 0.75µ with 9d and 16d as minimum and maximum values respectively. The pore opening diameters is almost the same as in the late vaculized stage equal to 0.17µ with minimum and maximum values equals to 2d and 3d respectively.

The surface of zona radiata is plain and has only traces of agglutination (Fig. 21).

The eggs with a diameter ranged from 650µ to 749µ (ovulated eggs) have a pore distribution density that increased to reach 0.92µ with 10d and 18d minimum and maximum values respectively. The pore opening diameters increased sharply to become 0.2µ with minimum and maximum values equal to 2d and 4d. The surface of zona radiata is smooth and plain, almost devoid of any agglutination (Fig. 22).

The micropyle of the ovulated eggs consists of numerous accessory openings and oval canal of average diameter 3.5µ and 4.6µ on its short and long axis respectively (Fig. 23). The accessory openings are of different diameters at the animal pole round the micropylar apparatus ranging from 0.2µ and 0.55µ as minimum and maximum values respectively, with average pore distribution density equal 0.4µ.

DISCUSSION

The ultrastructure of developing follicle in *Boops boops*, seems to be similar to other oviparous teleosts but may differ in its thickness and component. The transition from

oogonium to oocyte occurs when the germinal cell enters meiosis.

Ravaglia and Maggese (2003) said that in the ovarian follicles of *Synbranchus marmoratus* many vesicular structures with heterogeneous material could be recognized. They are located mainly in the interfollicular space at the perinucleolar stage, and resemble multivesicular bodies. In more advanced stages these structures are seen not only at the perivitelline space, but also inside the pore canals of the vitelline envelope. Multivesicular bodies have been suggested to be lysosomes structures involved in the vitellogenin processing in the trout (Busson-Mabellot, 1984) and in the pipefish (Begovac and Wallace, 1988). In *Boops boops* these multivesicular bodies (lysosomes) are seen in the perivitelline space during the vaculized stages, intermingled with the microvilli from both the granulosa cells and the oocyte surface. Although we do not have direct evidence on the relation of multivesicular bodies with the vitellogenin translocation and processing in *Boops boops*, our observations are consistent with those made in other species.

As in many teleosts, the development of the vitelline envelope in *Boops boops* first becomes apparent with the appearance of the zona radiata externa, which is dense with homogenous structure, reduced thickness and structurally similar to that described in *Synbranchus marmoratus* (Ravaglia and Maggese, 2003). The zona radiata interna is less dense and generally has a fibrilar aspect. It has up to 10 dense layers, alternating with 9 light layers while the zona radiata interna of red sea bream, *Pagrus major* consists of seven reticular lamellae. Hart and Donovan (1983) and Ravaglia and Maggese (2003) reported that in *Brachydario rerio* and *Synbranchus marmoratus*, the zona radiata interna consists of 17 dense layers, alternating with 18 light layers.

These results indicated that there are differences between *Boops boops* and other species, yet they all have one common feature. As the oocyte development goes on, the zona radiata interna is the layer that gets a complex multilaminar and fibrilar pattern (Genta and Barbieri, 1993; Koya *et al*., 1995 and Ravaglia and Maggese, 2003). These variations are often used as taxonomical tools to identify species (Riehl and Greven, 1992 and Hirai, 1993).

The zona radiata plays a very important role in protecting the fertilized egg and the embryo from the surrounding environment. In demersal and bathypelagic eggs, this membrane has a greater thickness than in pelagic eggs developing in the upper water layers (Makeyeva and Mikodina, 1977).

One important and till now not satisfactorily answered question in which cells, the oocyte and/or the follicular cells, supply the vitelline envelope components (Shackley and Kiny, 1977; Tesoriero, 1977 and Dumont and Brummet, 1980).

Recently it has been shown that some specific glycoproteins of the vitelline envelope are synthesized in the liver under estrogenic induction before the beginning of the synthesis of vitellogenin (Yamagami *et al*., 1992 and Seapigliati *et al*., 1999). These data are accordance with the proposal of Hyllner *et al*. (1991 & 1995), suggesting a tertiary origin of the vitelline envelope, because most of the biggest glycoproteins are synthesized out of the ovary and transported to it by the blood stream. On the other hand, Begovac and Wallace (1988) demonstrated that in *Synbranchus scovelly*, the principal biochemical components of the inner layer of the vitelline envelope are proteins originated inside the follicle layer rather than in the liver.

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 formation of the vitelline envelope (Hamlett *et al*., 1999).

In *Boops boops*, both the zona radiata externa and zona radiata interna seem to be formed by the oocyte itself. The vesicles with

different electron dense material found in the peripheral ooplasm releasing its content into the perivitelline space support this idea.

Koya *et al*. (1995) and Ravaglia and Maggese (2003) reported that the vitelline envelope appears first in *Hexagrammus octogrammus* and *Synbranchus marmoratus* at the perinucleolar stage. These data agree with the description of Hyllner *et al*. (1991) for *Oryzias mikyss* were they shown, using histochemical methods, that the deposition of vitelline envelope proteins was previous to the active intake of vitellogenins by the oocyte. However, in other species, the initial point of development of this structure is variable. In *Chelon labrosus* and *Liza aurata* the depostion of homogenous and dense material in the perivitelline space occurs during the onset of vitellogenesis (Brusle, 1985).

The present results shows that, in *Boops boops*, the formation of the zona radiata begins during the vaculized stages, but it increase in thickness in the yolk deposition stages. So, it is quite clear that the appearance of the zona radiata vary between species, being reported at at the end of the yolk vesicle stage by Baglin (1982) and Fahmy (1997).

During oocyte development the vitelline envelope is regularly crossed by thousand of microvilli processes from the oocyte and the follicular cells. These microvilli get in contact in different ways and allow the oocyte to grow up by transferring nutrients and growing factors (Kobayashi, 1985).

In the chum salmon, Kobayashi (1985) and in the *Synbranchus marmoratus*, Ravaglia and Maggese (2003) reported that 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 perivitelline 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 junctions, gap, tight or intermediate as reported by selman and wallace, (1989), however Ravaglia and Maggese (2003), could not detect which kind of junction is present in the *Synbranchus marmoratus*. They reported that they could not find any microvilli processes that cross the vitelline envelope and enter in contact with cortical alveoli. These microvilli seem to finish inside the pore canals, as was reviewed in many other species by Guraya (1986). Kobayashi (1985) reports that this is probably due to the fact that the follicular microvilli are formed and enter the radial canals during late phases of development of the egg envelope.

Photomicrographs of vitelline envelope tangential sections, containing mainly a unique microvillar process from the oocyte within each pore canal, support this theory. In this case, and according to Hamlett *et al*. (1999), exogenous nutrients needed for the oocyte growth should arrive to the ooplasmic surface after passing through the intercellular spaces between follicular cells and should be incorporated into the oocyte by diffusion and active transport or by pinocytosis.

In *Boops boops*, our results show that, in the tangential sections of the vitelline envelope, each round pore canal has two microvilli, the thicker one belongs to the follicular cells and the thinner one to the oocyte. Microvilli extend from the ooplasm surface and pass through the alternating pore canals and project their tip into the perivitelline space. Some of them come in direct contact with the microvilli of the overlying follicular cells, frequently at the base of the follicular microvilli.

In the vaculized stages, the three layers of wall were formed, zona radiata, zona granulosa and follicular epithelium (thecal cells), as their arrangement from inside to outside the oocyte with homogeneous distribution of the walls. Harris (1986) used the term “primary envelope” to replace the commonly confused names zona radiata, zona pellucida, and vitelline membrane.

Chorion and "secondary membrane" to refer to the outer layer of thecal and granulosa cells.

The present study has also indicated that both follicular cell layers (thecal and granulosa) have exhibited both quantitative and qualitative variations concomitant with the successive maturity stages of oogenesis.

It is now well established that steroid producing cells are characterized by ultrastructural features such as mitochondria with typical tubular cristae, smooth endoplasmic reticulum and lipid droplets (Chrisiensen and Gillim, 1969; Kurosumi and Fujita, 1974). In the present study the thecal cells were found to have most of these features, closely resembling thecal steroid-producing cells, previously described in *Oncorhynchus kistch* and *Oncorhynchus gorbuscha*, *Salmo gairdneri*, *Oncorhynchus rhodurus*, *Oreochromis mossambicus* by (Nagahama *et al*., 1978; Van den Hurk and Peute, 1979; Kagawa, 1985 and Smith and Haley. 1987) respectively.

The above-mentioned fish with thecal cells are salmonid or fresh water species and the present study demonstrated ultrastructurally the presence of the thecal cells in marine teleosts. Histochemical data for steroid producing cells in the thecal layer of marine teleosts have been reported in *Scomber scomber* (Bara, 1965) and *Trachurus mediterraneus* (Bara, 1974).

In teleosts, as in other non-mammalian vertebrates, it has been demonstrated that a female-specific protein (vitellogenin), which is synthesized by the liver in response to estradiol-17β, is released into the blood and then transported to the ovary (Ng and Idler, 1983; Wallace and Selman, 1985), and the microvilli are thought to be the sites of substance exchange between the follicle cells and the oocyte.

In the present study, a close contact between microvillar processes of granulosa cells and the oocyte surface maintained during only vitellogenesis. Although Hurley and Fisher (1966) suggested the possibility of protoplasmic continuity between follicular cell and oocyte via some microvilli, as evidenced by the absence of intervening membranes between them, yet no cytoplasmic continuity at the points of contact between the oocyte microvilli and follicle cells processes was seen in the present study. Therefore, the transport of materials across their membranes in the *Boops boops* ovary is probably mediated by diffusion and active transport at the molecular level and by endocytosis (Selman and Wallace, 1986 and Matsuyama *et al*., 1991). However, details of the mechanism and the sites of conversion of vitellogenin into the yolk proteins are unknown. The regulation and mechanism of substances through the granules cells and oocyte surface need to be determined at the molecular level.

From maturation to ovulation, the vitelline envelope suffers many remarkable changes in its morphology. Ravaglia and Maggese (2003) noticed in *Synbranchus marmoratus*, that the inner layer of the egg envelope in its late maturational stage becomes more compact and the multilaminar structure seems to be less evident, getting a more homogenous appearance, as was described in other teleosts (Selman and wallace 1989; Matsuyama *et al*.1991).

Another important observed change is the retraction of microvilli, where the pore canals become narrower as the microvilli processes from both the oocyte and the follicular cells begin its retraction. This retraction is generally followed by the occlusion of the pore canals and it is plugging by dense material .The zona radiata externa acquires a continuous aspect and the zona radiata interna striation is less apparent. However, high magnification by scanning electron microscope photomicrographs of the ovulated eggs of *Synbranchus marmoratus* (Ravaglia and Maggese, 2003) show the vitelline envelope surface with regular depressions, suggesting only a superficial plugging of the pore canals.

During the early stage of oocyte maturation in *Boops boops*, the most prominent changes in the granulosa cells

include stretching of the granulosa cells, forming wide intercellular spaces, and dilation of the rough endoplasmic reticulum, which is filled with amorphous material. Although the detailed chemical nature and function of this phenomenon are unknown, yet these findings are in good agreement with previous observation on the corresponding cells during oocyte maturation in the ovary of *Oncorhynchus kisutch* (Nagahama *et al*., 1978), *Salvelinus leucomaenis* (Kagawa *et al*., 1981) and *Oncorhynchus rhodurus* (Kagawa, 1985).

Teleostean ovaries –in general- are known to secrete steroid hormones (Fostier *et al*., 1983) and according to Nagahama (1983), at least five different cellular sources have been considered to be implicated in the process of steroid production in the ovaries of such fishes. These sites included the granulosa cells, certain thecal cells, the corpus luteum, corpora atretica and interstitial gland tissues.

In accordance with the above postulation, it was presently noticed that during the process of oogenesis, suspected steroidogenic cells were indicated in the thecal and granulosa layers forming the follicular oocytes envelopes. These cells had manifested marked alternations, being reflected in the form of an obvious increase in both number and size, as well as a high content of lipid droplets. These features were taken as an indication of the obvious involvement of those cells in the process of steroidogenesis of ripe oocytes. These observations conform those presented by Shackley and kiny (1977) in *Blennius pholis*, Rosenblum *et al*. (1987) in *Ictalurus nebulosus* and Matsuyama *et al*. (1991) in *Pagrus major*. In the teleost ovary, Fostier *et al.* (1983) reported that the steroidogenic activities in the teleost ovary have been reported to be localized in the interstitial cells in addition to the thecal and granulosa cells, according to the process and stage of oogenesis.

In vitro experiments on amago salmon, *Oncorhynchus rhodurus*, using isolated thecal and granulosa cell layers (Kagawa *et al*., 1982 and Young *et al*., 1982) have indicated that both follicle layers are necessary for the course of estrogen production in response to salmon gonadotropin. The thecal layer is thought to be involved in the production of estrogen precursors (mainly testosterone), which are converted to estradiol-17β in the granulosa cell layer. Thus taking biochemical data on salmonid fish into consideration, it seem likely that the thecal cells are the major cellular sites of steroid synthesis in *Boops boops*. Mousa (1994) studying *Mugil cephalus*, proved a positive correlation between oocytes dimensions and their contents of estradiol-17β hormone.

The granulosa cells of vitellogenic oocytes of *Boops boops* contained features suggestive of protein synthesis as abundant rough endoplasmic reticulum and well developed Golgi complex. As described earlier, biochemical data have suggested that the granulosa cells of amago salmon also play an important role in the production of estrogen. These cells have important enzymes as aromatase and 17β-hydroxysteriod dehydrogenase, which convert estrogen precursors produced in the thecal layers to estrogen (Young *et al*., 1982). Although, no biochemical data are present in our study, these many ultrastructural resemblanes in the thecal and granulosa cells between *Boops boops* and amago salmon, suggest that this two-cell model for the synthesis of follicular estrogen in the amago salmon may be applicable to that in *Boops boops*.

Pore canal and micropylar apparatus of external egg membranes had been used as a taxonomic character for species identification of fish eggs (Riehl & Schulte, 1978) and micropylar microstructures are considered to be species specific (Riehl, 1980). The outer surface of the chorion and the microstructure of the micropyle are the noteworthy features for egg identification and phylogenetic study. However, the outer surface of the chorion generally doesn't show remarkable differences in microstructure among species in a genus or family, while the micropyle is the initial isolating mechanism for preventing

interspecific hybridization (Chen *et al*., 1999).

Gopalakrishnan *et al*. (2002) discovered by using scanning electron microscope, the fine structure of the egg envelope and micropyle of unfertilized spawned eggs of rohu (*Labeo rohita*).

Micropyles of pelagic eggs are only small depression of the surface envelope, while in demersal eggs are funnel-shaped with a wide outer pit and a canal (Mikodina, 1987).

The micropyle apparatus of *Boops boops* is without a pit but only has one canal, which opened outward, like a funnel in the ovulated eggs. This finding agrees with Chen et al., 1999 who worked by using scanning electron microscope on species identification and phylogenetic interference among four species of family Sparidae by studying micropylar ultrastructure. He reported that, the micropyles of the four species of Sparidae are without a pit but only have one canal.

The micropylar apparatus of *Boops boops* is considered to be simple one, if it is compared with other species of family Sparidae (*Sparus sarba*, *Acanthopagrus latus*, *Acanthopagrus schlegeli* and *Pagrus major*) studied by Chen *et al*., 1999. *Sparus sarba* has lots of little knobs on the thickened annuli of the canal, and the annular thickenings showed a clockwise spiral arrangement from the bottom to the outer opening in the channel; *Acanthopagrus latus* has annular reinforcement on the sides of the canal in two clockwise-spiral ridges and the reinforcement had no knobs and were smooth; *Acanthopagrus schlegeli* has microvilli in the region of accessory openings, the micropylar canal was reinforced by two clockwise-spiral thickened annuli in the canal wall and the annular reinforcement had many knobs but with no microvilli; *Pagrus major* has long microvilli in the accessory opening region, the micropyle canals were reinforced by 10 thickened annuli in the canal sides and many knobs were found on the thickened annuli.

The micropylar apparatus of *Boops boops* in the present study lack any microvilli in the accessory opening region, knobs, ridges, annular thickening or reinforcement, so accordingly it is simple if compared with other species of family Sparidae.

The arrangement of accessory openings in *Acanthopagrus schlegeli* is randomly while that of *Pagrus major* is of radial type (Chen *et al*., 1999). The arrangement of accessory openings in *Boops boops* is randomly like that of *Acanthopagrus schlegeli*. The average diameters of the accessory openings are 0.38µm, 0.45µm, 0.38µm and 0.19µm for *Sparus sarba*, *Acanthopagrus latus*, *Acanthopagrus schlegeli* and *Pagrus major* respectively (Chen *et al*., 1999), while that of *Boops boops* is 0.37µm and the average diameter of the outer openings of the micropylar canal is 5.58µm, 5.36µm, 4.67µm and 7.2µm for *Sparus sarba*, *Acanthopagrus latus*, *Acanthopagrus schlegeli* and *Pagrus major* respectively (Chen *et al*., 1999), and that of *Boops boops* is 4.6µm.

The pore canals in zona radiata of *Boops boops* have a simple morphology, in comparison to the outer covering of certain adhesive or pelagic eggs like *Clupea harengus* and *Cyprinus carpio*. The eggs envelopes of the latter are studded with microfilaments, ornate surface structures or jelly coats (Guraya, 1986; Gopalakrishnan *et al*., 2002).

The surface of zona radiata in *Boops boops* is plain with uniform distribution of pores. The pores had no lips. According to Riehl & Spartzner (1998) a smooth zona radiata constitutes a less complex egg attachment apparatus that would not assure strong adhesiveness.

Numerous agglutinates were observed scattered over the surface of zona radiata especially at eggs of smaller size (cytoplasmic growth cells) and decrease till it disappear in the ovulated egg. Similar agglutinates were observed on the outer rim of the micropyle in young developed eggs. According to Mikodina (1987), the agglutinates seen along the rim, the micropylar canal and on the surface of zona

radiata can be the residues of the cytoplasmic processes of granulosa cells.

Notice that the diameter and the density of the pore distribution varied according to the egg region. At the vegetal pole, they had a similar diameter and were uniformly distributed. Whereas at the animal pole, they varied in diameter and their density increased towards the micropyle (Rizzo *et al*., 2002).

The average pore diameter in the zona radiata in the eggs of *Boops boops* increased towards the completion of maturation of eggs, it ranged in diameter from 0.16µm in eggs of diameter ranged from 250µm to 349µm (early vaculized eggs) to be 0.2µm in the ovulated eggs (650µm-749µm). The average distance between pores also increased towards ripening of eggs, it ranged from 0.24µm in early vaculized stage to 0.92µm in ovulated eggs.

Amanze & Iyenager (1990) described eggs of the cyprinid *Barbus conchonius* with a micropyle region that consists of seven to ten grooves and ridges, which are directed into the micropylar canal. Riehl & Putzner (1991) showed that the eggs of catfish *Sturisoma anreum* have a zona radiata, which exhibits 22 furrows that run from the vegetal to the animal pole.

All this previously described ridges and furrows are radial, this spiralling pattern in Luciocephalus species with partial termination in the micropyle pit remains absolutely unique among fish.

Amanze & Iyenager (1990) were able to demonstrate with time-lapse video microscopy and computer-aided analysis of sperm movement that the grooves guided sperm cells into the micropyle. They calculated that the guidance role of the micropylar region would enhance sperm penetration and/or fertilization by as much as 99.7%. The surface structures observed in Luciocephalus species supports the opinion of Amanze & Iyenager (1990) that such a pattern could function as a sperm guidance system.

Li *et al*., (2000) study the zona radiata of marine fish eggs in four perciforms fishes, from three different families (Serranidae, Sciaenidae and Mugilidae), he concluded that pore diameter did not differ significantly for the fishes in the same genus, but was significantly different for different genera, even when the genera were in the same family while the egg surface ultrastructure, pore distribution density and egg size are all useful characters for distinguishing among the fish species, and that the ultrastructure features of the micropyles are the most important for egg identification.

This finding agree with (Riehl and schulte, 1978; Riehl 1980,1993) who reported that zona radiata may be useful for distinguishing among fishes from different families or orders but the ultrastructural features of the micropyle can serve as taxonomic characters that are species-specific.

Mikodina and Makeeva (1980) demonstrated that inspite of great similarity between the eggs of silver carp (*Hypophthalmichthys molitrix*), bighead (*Aristichthys nobilis*), grass carp (*ctenopharyngodon idella*) and black amur (*Mylopharyngodon piceus*), the structure of their micropyle and egg membranes differed greatly and were species specific.

Table (1): Relation between distance between pores and pores diameters with ova of Boops boops at different diameters

Egg diameter in µm	Distances between pores in divisions			Average distance between pores in µm	Pore diameter in divisions			Average Pore diameter in µm
	Min	Max	Average		Min	Max	Average	
250 - 349	2	6	3.7	0.24	2	4	2.5	0.16
350 - 449	3	7	5	0.3	2	4	2.85	0.19
450 - 549	6.3	10.3	8.4	0.56	1.75	3.3	2.5	0.16
550 - 649	9	16	11.3	0.75	2	3	2.6	0.17
650 - 749	10	18	13.9	0.92	2	4	3.1	0.2

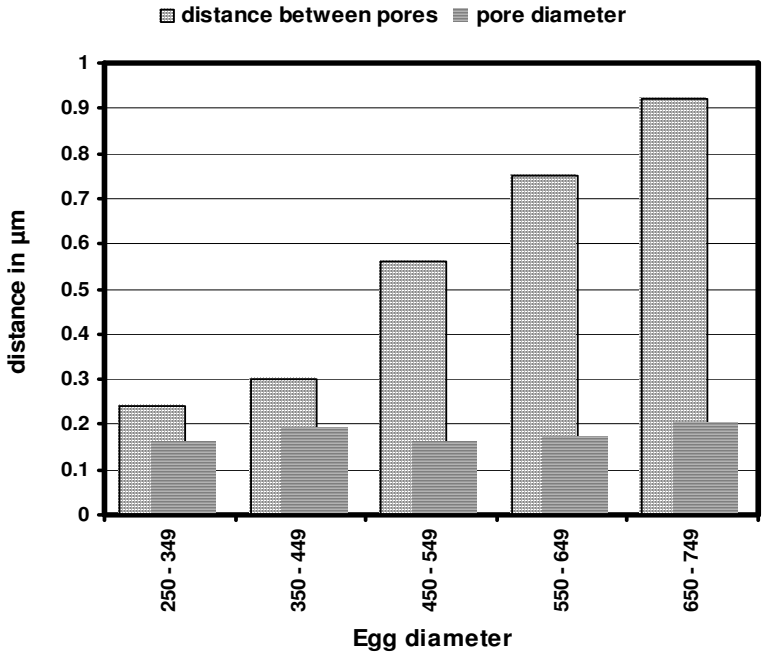
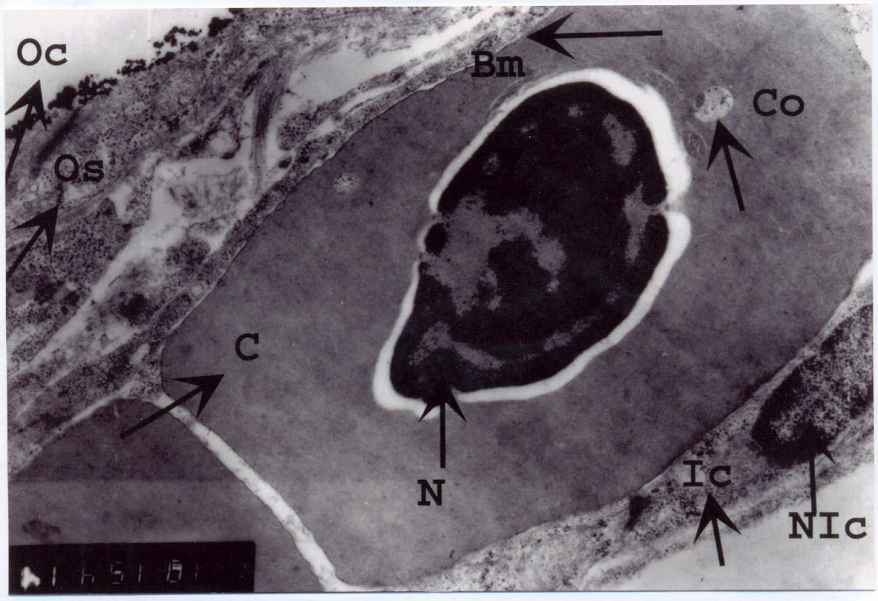


Fig (1): Relation between pores diameter and distance between pores with the ova of *Boops boops* at different



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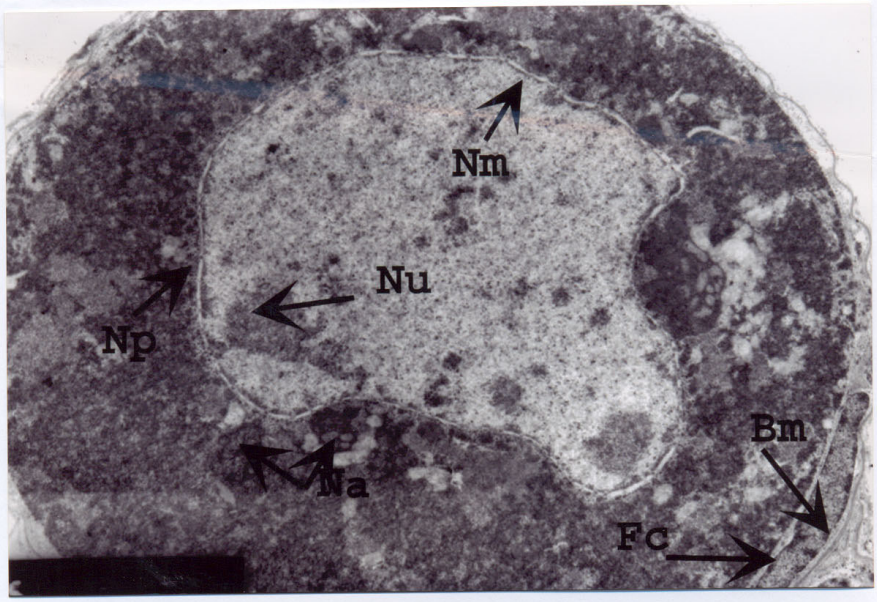

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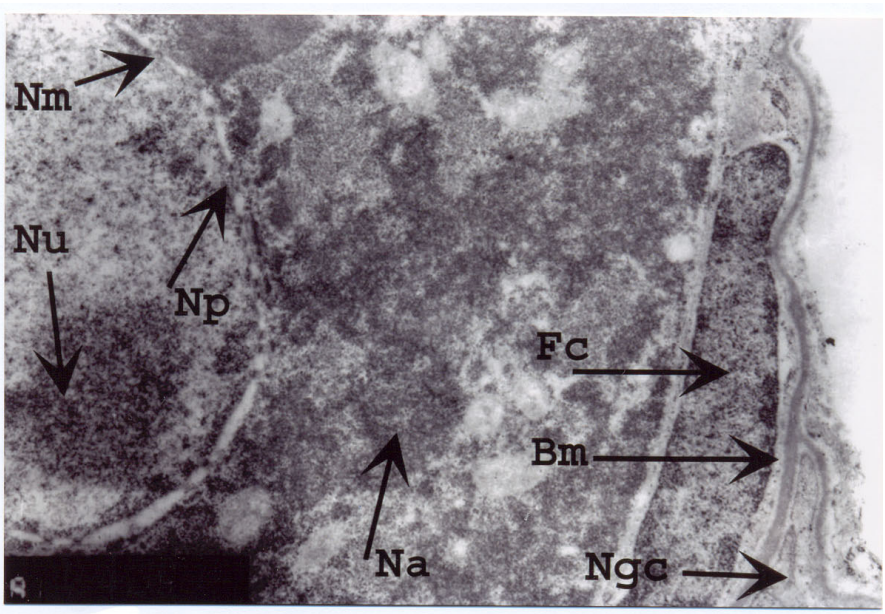

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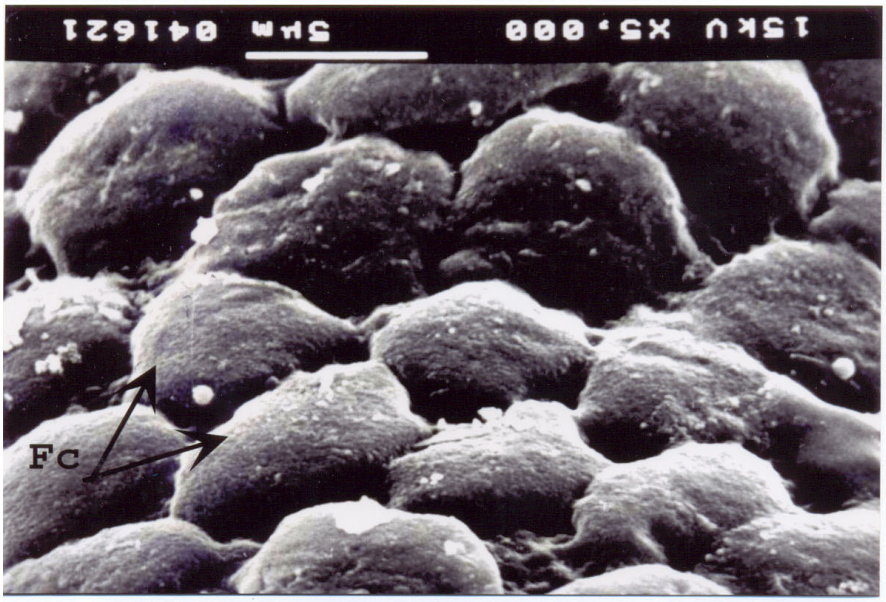

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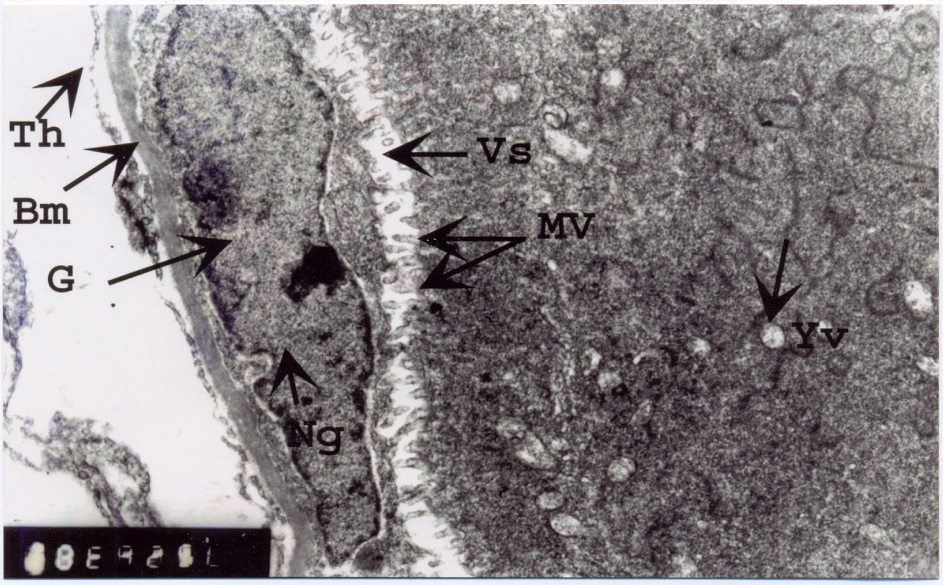

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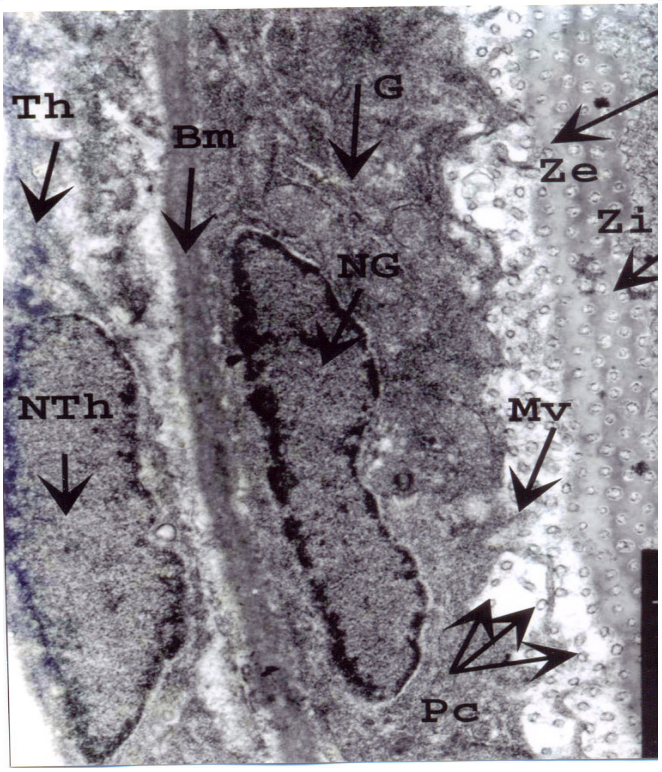
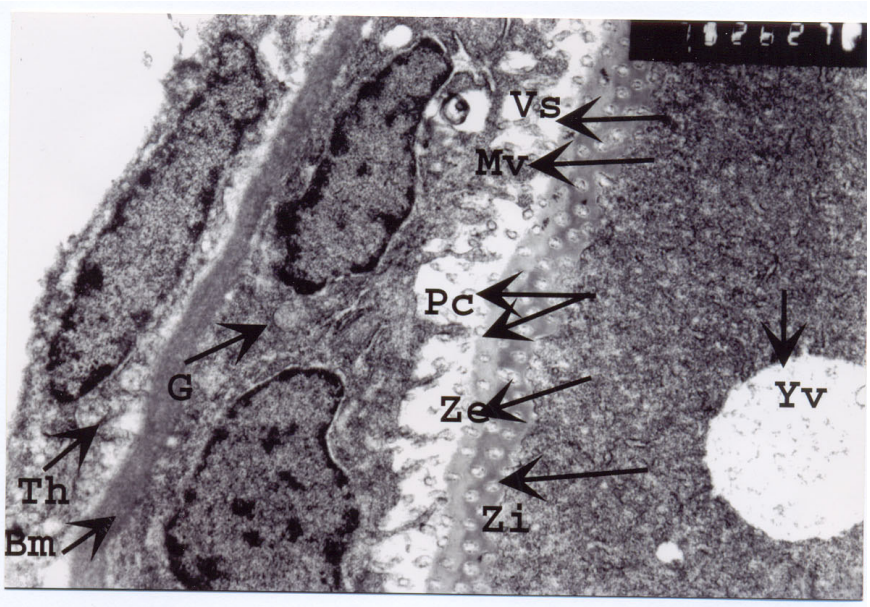


Figure (7) **X7.500**



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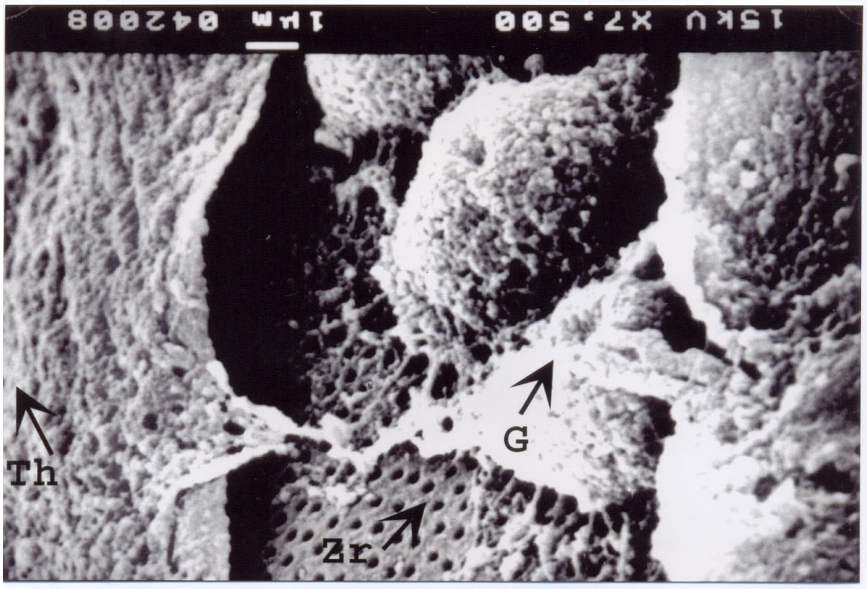

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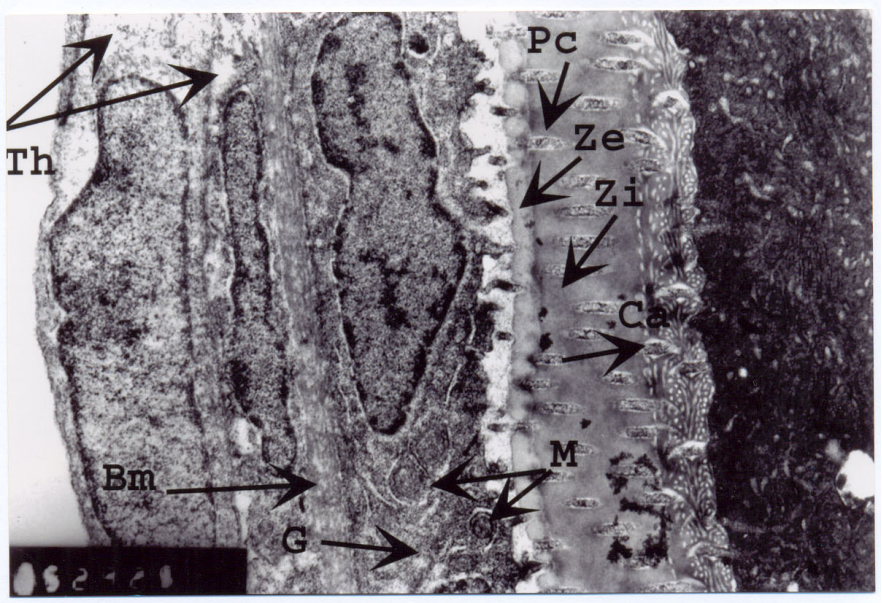

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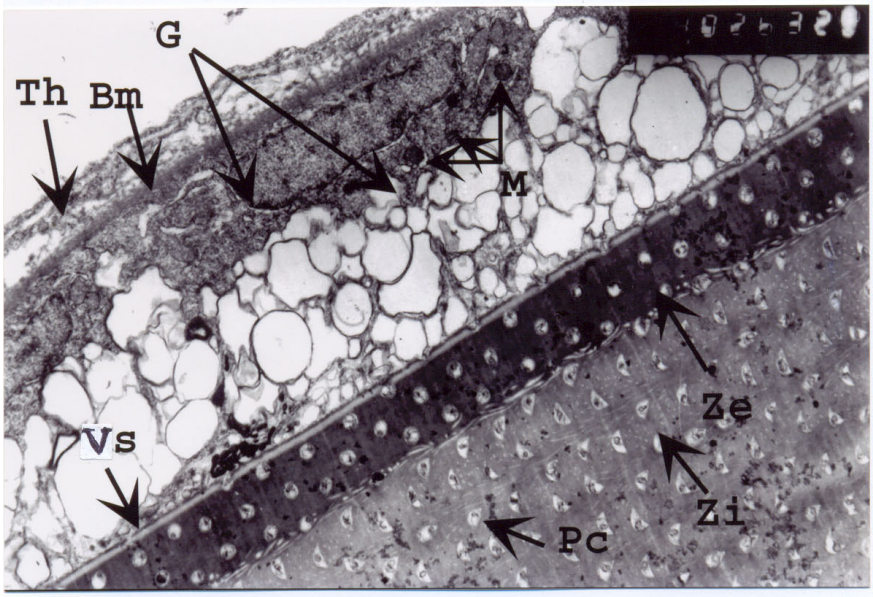

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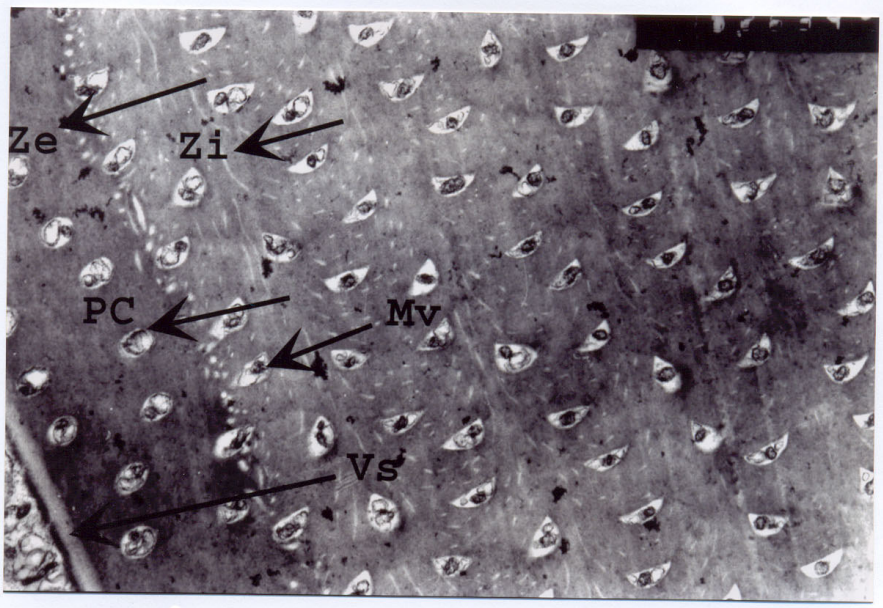
Figure (11)



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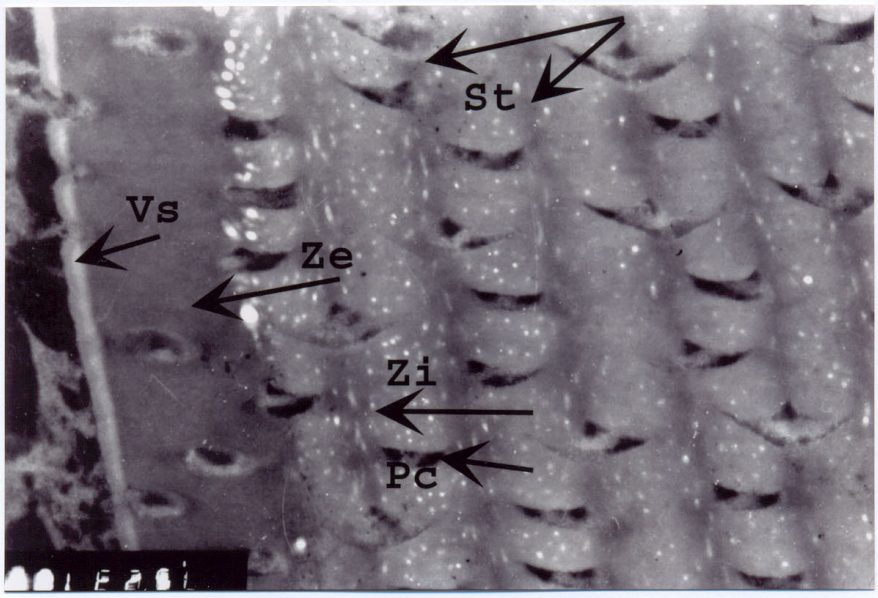

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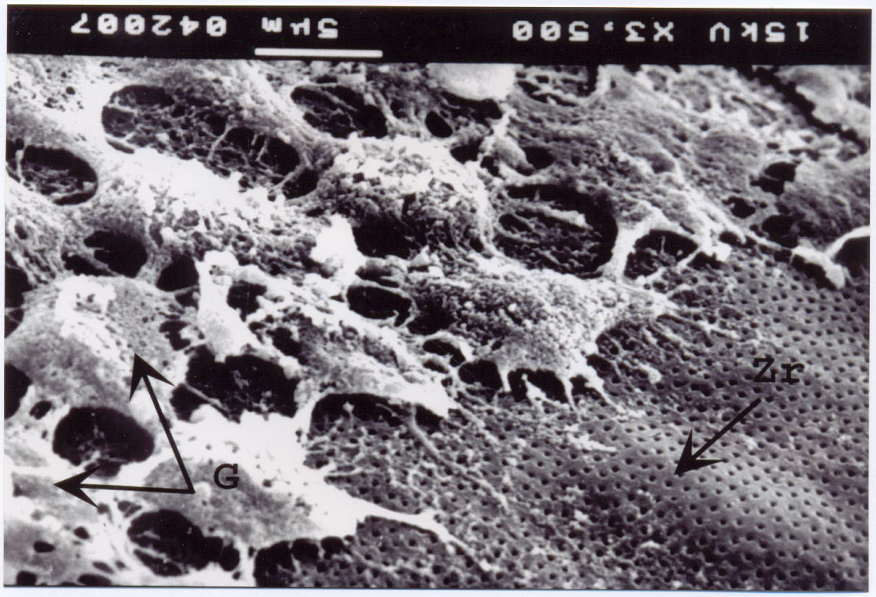

Figure (14)

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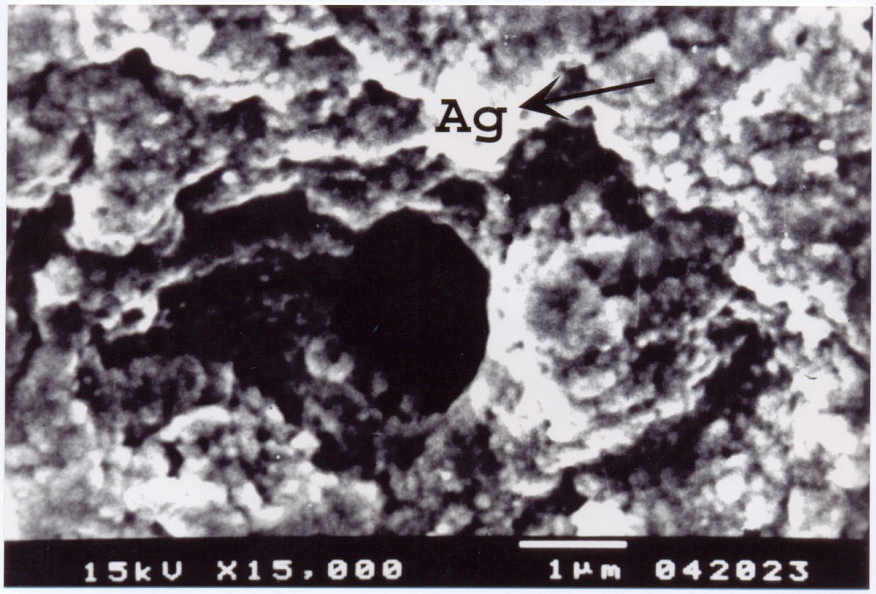

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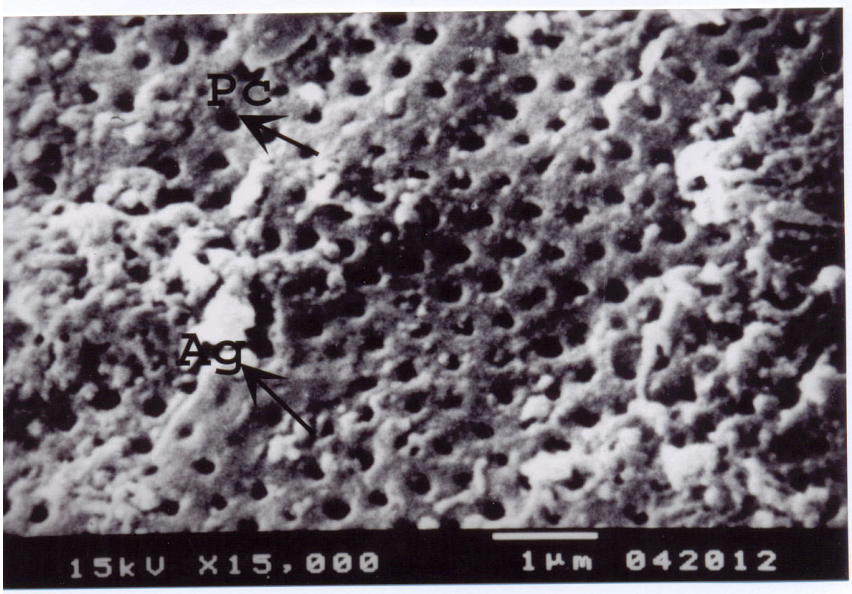
X (15.000)



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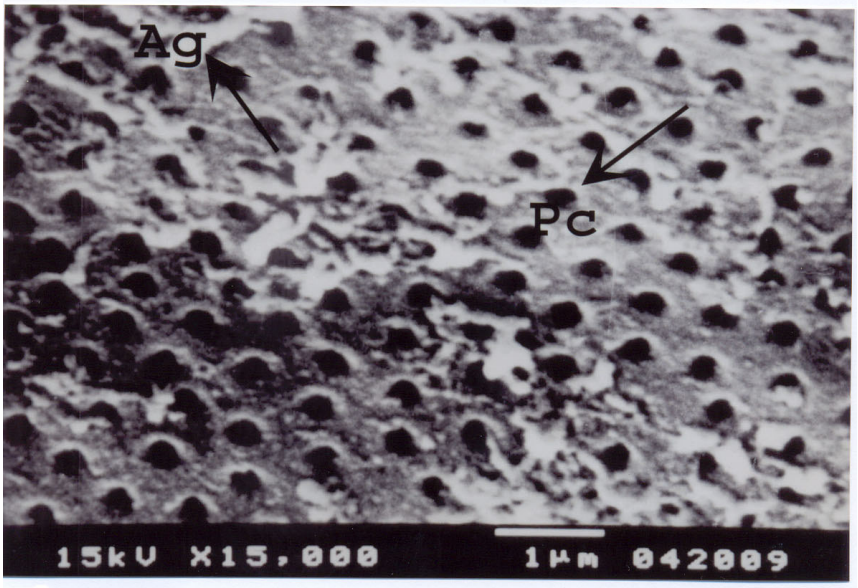

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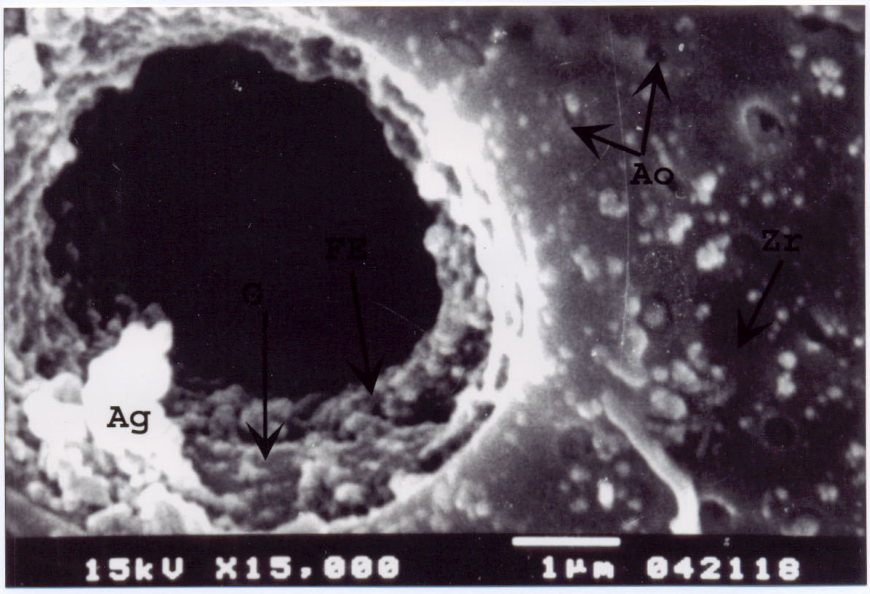

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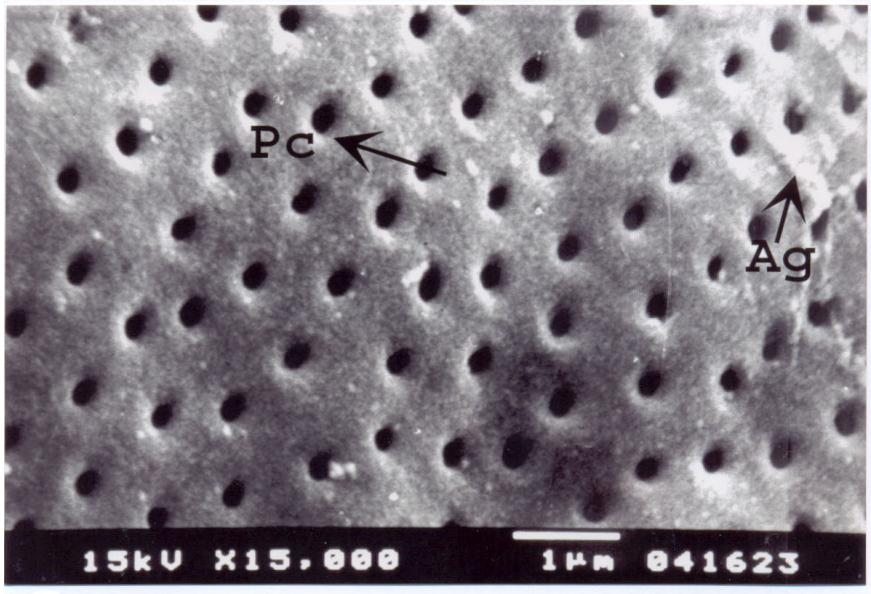

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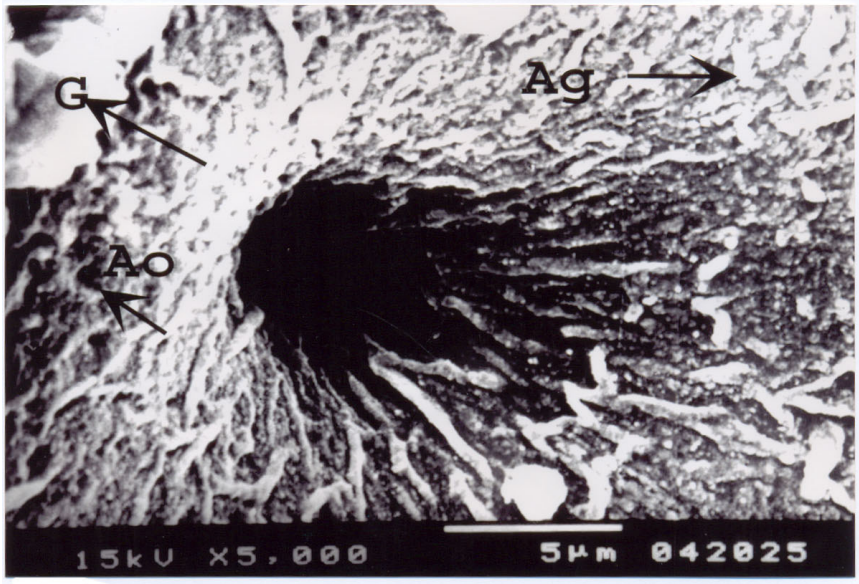

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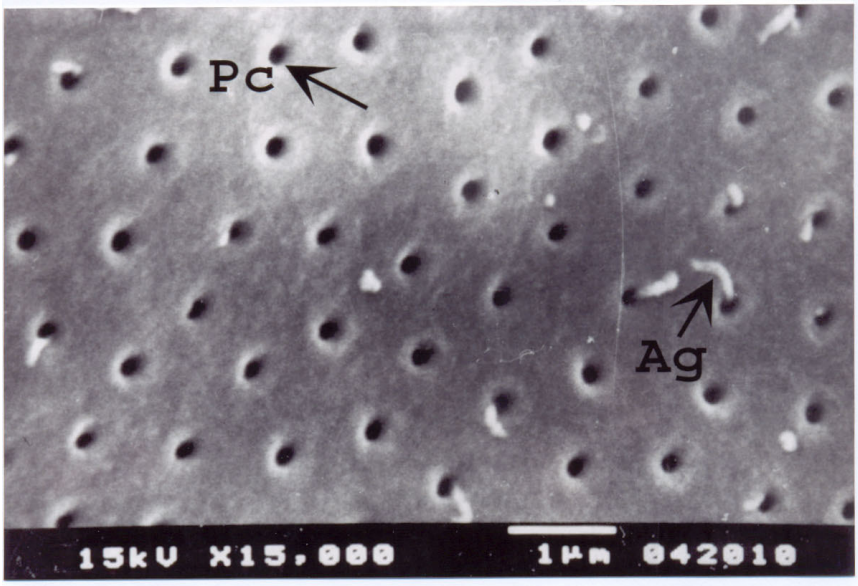

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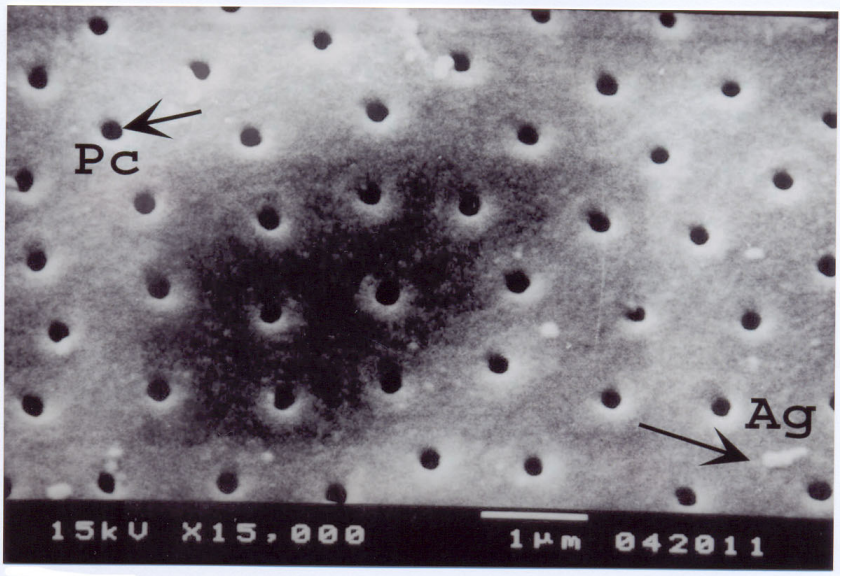

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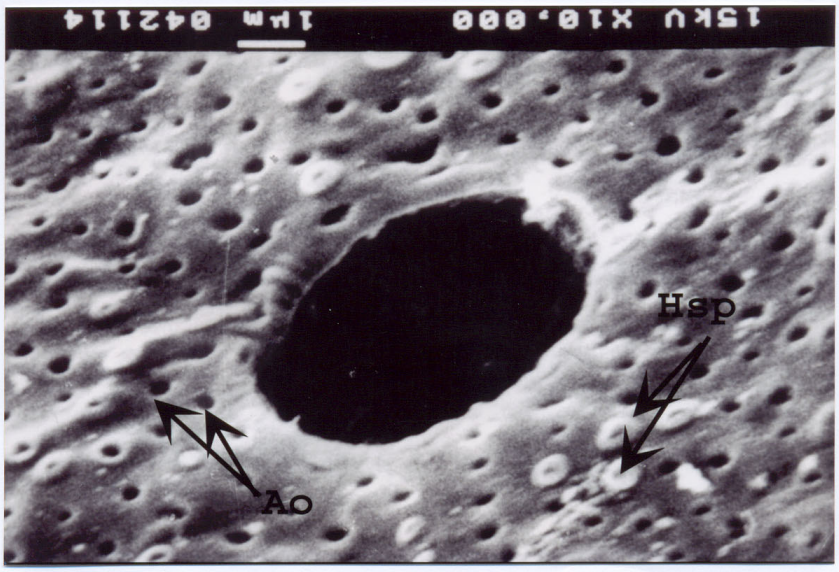

Figure (23) X (10.000)

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Figure legends

Figure 1. Graph showing the relation between pores diameter and distance between pores with the ova of *Boops boops* at different diameters.

Figure 2. Electromicrograph of T.S. in ovaries of *Boops boops* showing oogonia embedded in the ovarian stroma.

Figure 3. Electromicrograph of T.S. in ovaries of *Boops boops* showing an early cytoplasmic growth cell (pre-nucleolus stage).

Figure 4. Electromicrograph of T.S. in ovaries of *Boops boops* showing magnification to the wall of early cytoplasmic growth cell.

Figure 5. Scanning electron microscope showing the outer surface of the late cytoplasmic growth eggs. Notice that it is completely covered with follicular epithelial cells.

Figure 6. Electromicrograph of T.S. in ovaries of *Boops boops* showing the wall of early vaculized oocyte.

Figure 7. Electromicrograph of T.S. in ovaries of *Boops boops* showing the wall of middle vaculized oocyte.

Figure 8. Electromicrograph of T.S. in ovaries of *Boops boops* showing the wall of late vaculized oocyte.

Figure 9. Scanning electron microscope showing the outer surface of the late vaculized oocytes.

Figure 10. Electromicrograph of T.S. in ovaries of *Boops boops* showing the wall of primary yolk deposition oocytes.

Figure 11. Electromicrograph of T.S. in ovaries of *Boops boops* showing the wall of tertiary yolk deposition oocyte.

Figure 12. Electromicrograph of zona radiata interna and externa of tertiary yolk deposition oocytes.

Figure 13. Electromicrograph of zona radiata interna and externa of tertiary yolk deposition oocytes showing the striation between the multilaminar alternating pore canals.

Figure 14. Scanning electron microscope showing the outer surface of the mature oocytes. Notice the complete degeneration of the thecal cell layer and the wide intercellular spaces between the granulosa cells.

Figure 15. Electromicrograph showing the micropyle of the cytoplasmic growth eggs by scanning electron microscope.

Figure 16. Electromicrograph showing the pore canals in zona radiata on the eggs ranged in diameter from 250µ to 349µ by scanning electron microscope.

Figure 17. Electromicrograph showing the pore canals in zona radiata on the egg ranged in diameter from 350µ to 449µ by scanning electron microscope.

Figure 18. Electromicrograph showing the micropyle in zona radiata on the surface of the early vaculized oocyte by scanning electron microscope.

Figure 19. Electromicrograph showing the pore canals in zona radiata on the egg ranged in diameter from 450µ to 549µ by scanning electron microscope.

Figure 20. Electromicrograph showing the micropyle in zona radiata on the surface of the late vaculized oocyte by scanning electron microscope.

Figure 21. Electromicrograph showing the pore canals in zona radiata on the egg ranged in diameter from 550µ to 649µ by scanning electron microscope.

Figure 22. Electromicrograph showing the pore canals in zona radiata on the egg ranged in diameter from 650µ to 749µ by scanning electron microscope.

Figure 23. Electromicrograph showing the micropyle in zona radiata on the surface of the ovulated eggs by scanning electron microscope.

Abbreviations

1. Ao = accessory openings.
2. Ag = agglutinations.
3. Bm = basement membrane.
4. C = cytoplasm.
5. Ca = cortical alveoli.
6. Co = cytoplasmic organelles.
7. Fc = follicular cells.
8. G = granulosa layer.
9. Hsp = head of the sperm.
10. Ic = interstitial cells.
11. M = mitochondria.
12. Mv = microvilli.
13. Na = nuage.
14. Ng = nucleus of granulosa cell.
15. Nm = nuclear membrane.
16. Np = nuclear pores.
17. Nu = nucleolus.
18. NIc = nucleus of interstitial cells.
19. Ngc = non germ cells.
20. Nth = nucleus of thecal cell.
21. Oc = ovarian cavity.
22. Os = ovarian stroma.
23. Pc = pore canal.
24. St = striations.
25. Th = thecal layer.
26. Vs = perivitelline space.
27. Yv = yolk vesicle.
28. Ze = zona radiata externa.
29. Zi = zona radiata interna.
30. Zr = zona radiata.