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Ultrastructural Events of Human Reproduction as Revealed by High Resolution Electron Microscopy

Eventos Ultraestructurales sobre Reproducción Humana Demostrados por Microscopía Electrónica de Alta Resolución

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Abstract: In this paper will be reviewed, through an integrated analysis by light, scanning and transmission electron microscopy, recent results of our research group on some reproductive aspects including: a) Germ-somatic cell interactions during early ovarian development. b) Follicular development and oocyte–follicle cells associations through folliculogenesis. c) Microanatomy of the human egg and its vestments trough fertilization and early embryogenesis.

Key words: Reproduction, ultrastructure, electron microscopy, human

Resumen: En esta revisión se reporta un análisis integrado por microscopía de luz, electrónica de barrido y electrónica de transmisión de los recientes resultados del presente grupo de investigación en algunos aspectos incluyendo reproductor: a) Las interacciones de células germinales y somáticas durante el desarrollo temprano del ovario. b) El desarrollo folicular y asociaciones de oocyto-células foliculares a través de la foliculogénesis. c) Microanatomía del huevo humano y sus envolturas a través de la fertilización y embriogénesis temprana.

Palabras clave: Reproducción, ultrastructura, microscopía de luz y electrónica, humanos

Introduction

The ovary is a special gland that cyclically undergoes structural and functional changes in order to exert two principal secretory functions: the *endocrine* and *"exocrine"* or ovulatory function. These activities are possible thanks to the anatomical complexity of this organ, formed by two main cellular components, differing in origin and function: a germinal line and a somatic line. These cells reciprocally interact in a finely regulated manner, in both intrauterine and adult life.

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At present the morphodynamic events that lead to the formation, development, and postnatal activity of the female gonad are not completely understood. Thus, in order to contribute in clarifying these open questions we have studied, through an integrated light microscopy (LM), scanning and transmission electron microscopy (SEM and TEM) analysis, the main events of human reproduction from the primordial germ cells (PGCs) early appearance up to the embryo formation (6, 12).

Materials and Methods

The observations have been obtained from human specimens (ovarian biopsies, cumulus-enclosed oocytes and

fertilized eggs) and compared with other results from both humans and laboratory mammals. Samples were observed by LM, SEM and TEM. In some cases the intracellular structures have been exposed by applying the osmium-DMSO-osmium (ODO) method. This technique is based upon a maceration procedure that allows the removal of the excess of the cytoplasmic matrix from the cracked surface of the cells. Macerated samples have been then observed by a high-resolution SEM, in order to achieve a better comprehension of the spatial relationships among the cytoplasmic organelles (3). To better define the microstructure of *zona pellucida* (ZP) its amorphous material has been removed using a detergent (saponin), and its structural glycoproteins have been stabilized with a cationic dye (ruthenium red) followed by osmium-thiocarbohydrazide (OTO) treatment (4). The ZP of some of these samples has been fractured by means of a needle to expose the internal surface.

Results and Discussion

Primordial Germ Cells and Early Ovarian Development

Origin, migration and gonadal settlement of PGCs: The cells establishing the germ-cell line, both in the ovary and in the testis, are known as PGCs. The earliest morphological evidence of the of PGCs in mammals is in the endoderm of the dorsal wall of the yolk sac, near the developing allantois. In humans, this occurs during the third week post fertilization (p.f.) (Fig. 1). PGCs, actively proliferating, migrate by ameboid movements from the yolk sac epithelium to the gonadal anlage through the hind gut. This *PGCs migration* is very likely amplified by a passive translation mediated by the change of the growing embryo layout. PGCs are commonly seen later, during the fifth week of embryo development, in the dorsal mesentery, where they are capable of active migration through their own ameboid movements (Fig. 2). At the end of the fifth week, or during the sixth one, PGCs reach the gonadal anlage, *colonizing* the most superficial areas of the developing ovary (5, 6).

Morphodynamics of germ cell differentiation: At third week p.f., by both TEM and SEM, PGCs appeared rounded-shaped, with a diameter of about 15-20 μm. They are characterized by an eccentric nucleus and a cytoplasm relatively poor in organelles. During the active migration PGCs become spindle-shaped, with a long axis of about 30 μm. The plasma membrane elaborates protrusions and pseudopodia. A thin *"fuzzy coat" (glycocalix)* along with fibronectin completely covers the PGCs surface. This coat is believed to actively and dynamically establish an interaction between somatic cells and PGCs enabling these latter to correctly migrate towards the gonads. The nuclear envelope of PGCs appears somewhat irregular and their rough endoplasmic reticulum (RER) membranes, microtubules and microfilaments (many of which consist of actin) progressively increase in number. Since the ninth week p.f., the proliferating PGCs begin to differentiate in oogonia, that generally show ultrastructural features similar to gonadal PGCs. Oogonia tend to form clusters of dividing cells that exhibit identical chromosomal configurations, often joined by intercellular bridges. Since the 12th -13th week p.f., proliferating oogonia located in the inner cortex of the ovary begin to differentiate into oocytes. Nests of oocytes joined by intercellular bridges are not uncommon. Meiosis begins at this developmental stage and germ cell size increases. In the cytoplasm, mitochondria become numerous and disposed along the outer surface of the nuclear membrane and membrane-bound dense bodies can be found in the cytoplasm (Fig. 3). At 18-20 weeks until the term of ges-

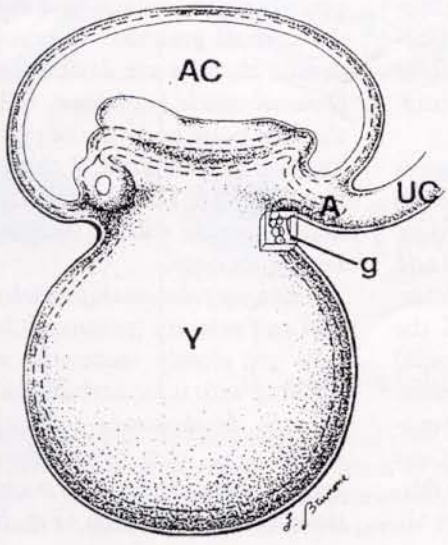
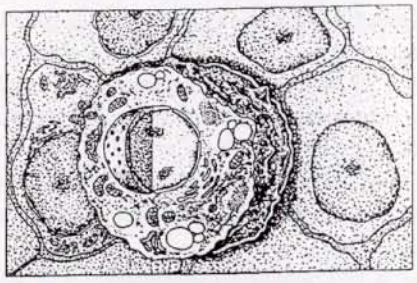
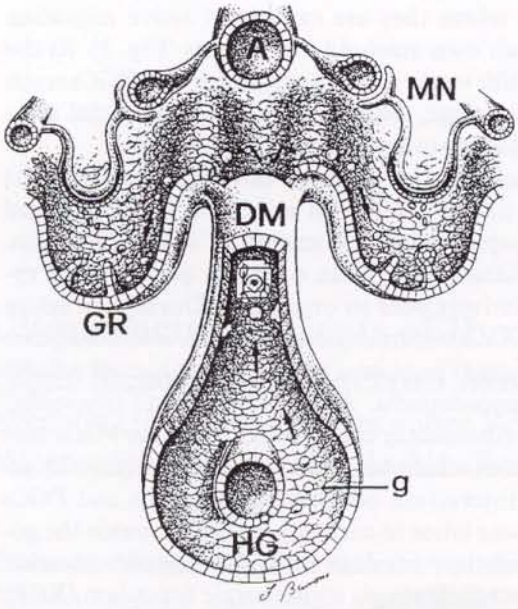


Figure 1. Origin of primordial germ cells (PGCs). Sagittal section of a 3-week-old human embryo. PGCs (g) are located in the wall of the yolk sac (Y) near the allantois (A). UC: future umbilical cord. AC: amniotic cavity. The main ultrastructural characteristics of a PGC starting migration are emphasized in the boxed area. (From Ref. 8, with permission)





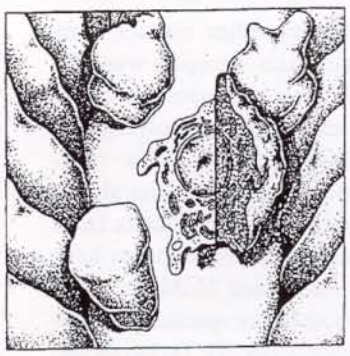


Figure 2. PGCs migration. Transversal section of an early human embryo. Migrating PGCs (g) can be found in the hind gut (HG) during the 4th week p.f., in the dorsal mesentery (DM) during the 5th week, in the genital ridge (GR) at the 5th-6th weeks. MN: mesonephric tubules and glomeruli; A: dorsal aorta. The main ultrastructural characteristics of a PGC actively moving the the dorsal mesentery are reported in the boxed area. (From Ref. 8, with permission)

tation a single layer of flattened somatic cells surrounds both oogonia and oocytes. These cells finally enclose them in primordial follicular structures. With these events, a true folliculogenesis begins (5) (Fig. 4).

Development of germ-somatic cell interactions: Somatic and germ cell lines reciprocally influence each other during the intrauterine life. In fact, the mechanisms controlling migration and settlement of PGCs in the developing ovary, and even their survival and differentiation during folliculogenesis, seem to be modulated by and dependent upon the adjacent somatic cells. At least in some vertebrates, PGCs migration seems to be also dependent on the production of chemotactic substances from the gonad-forming areas. Furthermore, some authors emphasize the possibility that, both in mouse and humans, the oriented migration of PGCs is partially guided by the substrate, and particularly, by the presence of specific components of the extracellular matrix such as fibronectin (see above). In addition, desmosomes, intermediate junctions, tight junctions and communicating gap junctions are as a rule often found between oogonia/oocytes and surrounding somatic cells at the time of folliculogenesis and during follicle maturation (5).

Germ cell reduction in number: The number of germ cells, that left the yolk sac consisting of a few hundred cells, rapidly increases by mitosis during their migration. Later, after their settlement in the developing ovary and further differentiation in oogonia this proliferative activity highly increases. Thus, at the fifth month p.f. the number of germ cells (now at the stage of oogonia) reaches a peak of about 6-7 millions. A very impressive germ cell reduction in number occurs during the prenatal development, leading the germ cells to reach just one million before birth. A degeneration process begins during the fifth month p.f. affecting oogonia and, above all, primary oocytes in zygotene and pachytene stages. A less conventional, alternative process, occurring in mouse and humans, that seems to promote a reduction in number of germ cells before birth, is the extrusion of these cells on the surface of the gonad and their subsequent elimination into the coelomic cavity, Fig. 4.

Follicular Development and Oocyte-Follicle Cells Associations

Oocyte morphology: By TEM, oocytes in primordial and primary follicles show a large, eccentrically placed vesicular nucleus with a conspicuous nucleolus. Mitochondria are round or irregular, with arched cristae, arranged in numerous layers around the nucleus. A Golgi complex, membranes of endoplasmic reticulum, vacuoles, lipid droplets and annulate lamellae can be frequently seen close to the nucleus, corresponding to the so-called *vitelline Balbiani body*. During further stages of development the organelles become much more dispersed in the ooplasm and the Golgi starts to assembly the cortical granules. In preovulatory (mature) oocytes drastic changes are detectable by the occurrence of the *germinal vesicle breakdown*, followed by the extrusion of the first polar body in the perivitelline space and by the location of metaphase II chromosomes near the cell surface (Fig. 3). The ultrastructural characteristics of the mature oocyte will be thoroughly examined in the following sections.

Oocyte relationships with follicular cells: In primordial and primary (preantral) follicles flattened follicular cells are closely associated with the growing oocyte, forming with it numerous focal cell contacts, zonulae adherents, desmosomes and gap junctions. We also observed long, thin cytoplasmic extensions of follicular cells penetrating into the oocyte through deep invaginations of the oolemma (Fig. 5). By ODO method and

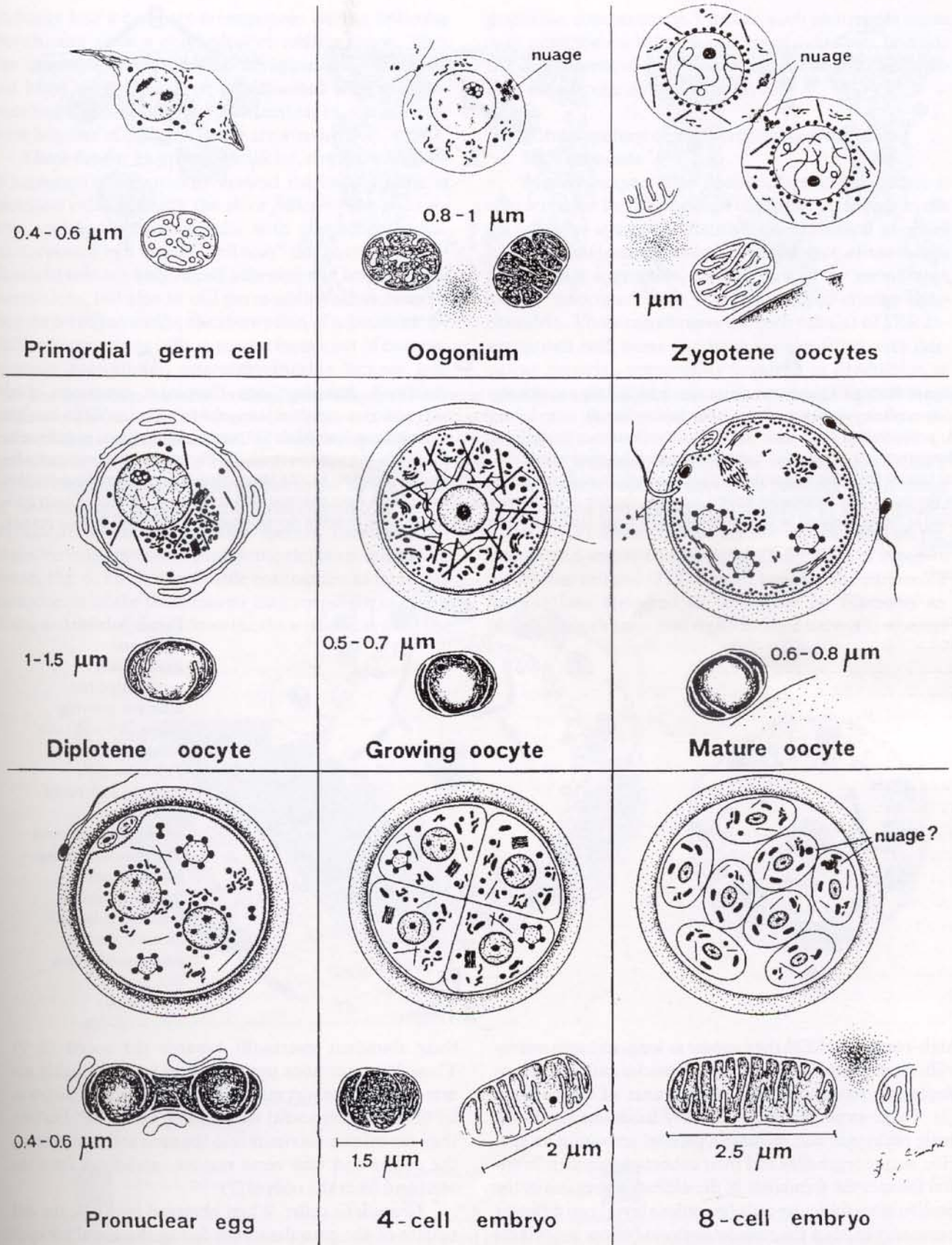


Figure 3. This figure summarizes, from an ultrastructural point of view, the morphodynamic changes occurring in the human female gamete from primordial germ cell stage, oogenesis, maturation up until fertilization and early embryo development. Changes in microtopography, size, shape, cristae configuration and matrix density of the mitochondria have been also detailed in each section of the figure. (From Motta et al., 2000. Human Reprod., in press).

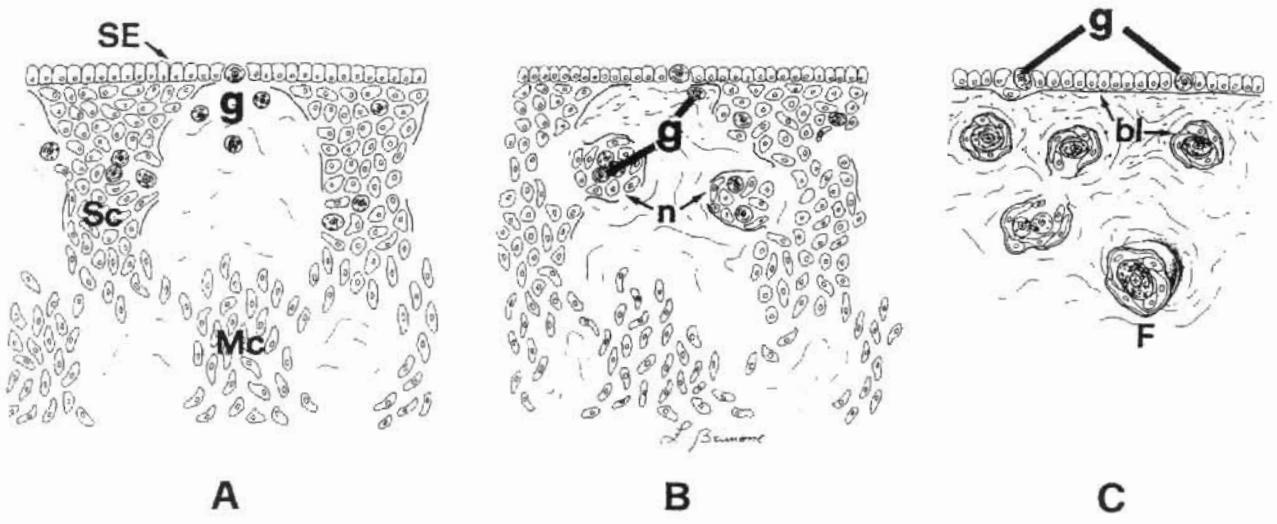


Figure 4. This figure illustrates the relationships occurring between the proliferating surface coelomic epithelium and the mesonephric components of the developing human ovary as well as their respective contribution to the formation of follicular cells. **A**: proliferation of somatic cords, from both surface epithelium (Sc) and mesoneprhos (Mc); SE: surface epithelium; g: germ cells. **B**: segregation of nests (n), containing more than one germ cells (g), included in the somatic cords. **C**: Formation of a continuous basal lamina (bl) both under the surface epithelium and around the primordial follicles (F), the latter deriving from the fragmentation of the nests. The figure also underlines that the fate of germ cells (g) (elimination from the ovarian surface or formation of early follicles) is closely related to the formation of a basal lamina during human prenatal ovarian development. (From Ref. 8, with permission)

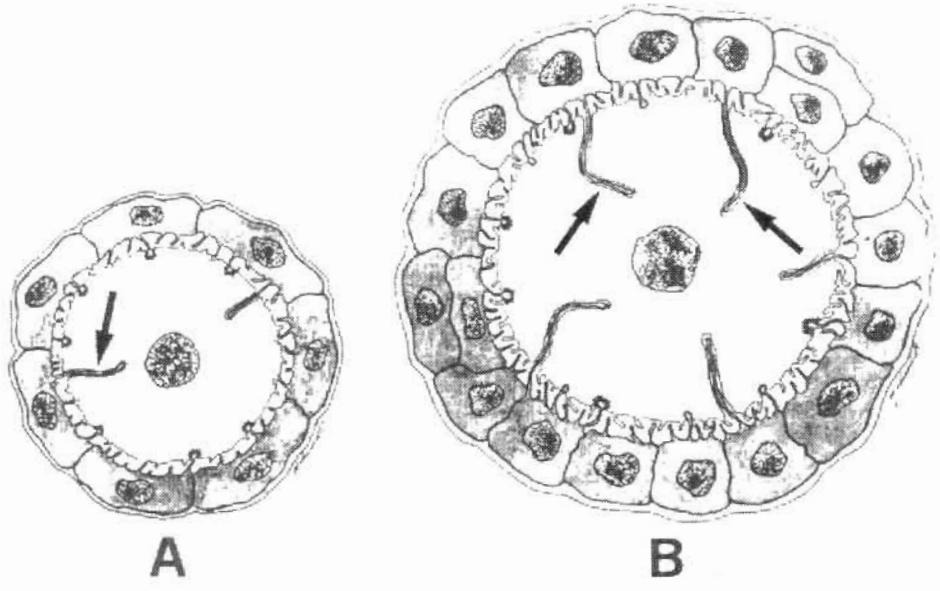


Figure 5. In this figure are dynamically represented two different growing stages (A and B) of a primary follicle. Follicular cells project a number of short microvilli towards the oolemma. The presence of long intraooplasmic microvilli arising from follicular cells (arrows) are also emphasized (From Ref. 5, with permission)

high-resolution SEM they appear as long, tortuous microvilli, closely associated with Golgi vesicles and smooth endoplasmic reticulum (SER) membranes of the ooplasm (3). These structures may probably modulate the metabolic exchanges and mediate a parallel activation on specific oocyte organelles and their cohort of enzymes. In antral follicles the formation of the antrum segregates in the proliferating follicular cells (granulosa layer) two different compartments: *i.e.* the *cumulus oophorus* (whose inner layer is called *corona radiata*) and the *parietal granulosa cells*. By high resolution SEM after the ODO maceration technique corona cells show a characteristic apical polarization of their abundant microvilli towards the oocyte (3, 7). These long, tortuous projections as well the highly numerous oocytic short microvilli much facilitate the transfer of substances useful for building up the ZP. Further, they can release nutrients into the zona and from there to the oocyte and vice versa remove catabolytes from the zona and from the oocyte (7).

Granulosa cells: When observed by SEM, the cell surface of the granulosa cells facing the antral cavity is smooth and often covered by a thin layer of granular/filamentous substance, which most likely corresponds to *follicular fluid*. On the contrary, peripheral granulosa cells

gradually lose a compact arrangement during follicular growth, and show a polyhedral or stellate shape. They also develop short processes, invaginations, microvilli and blebs. Granulosa layer is delimited from the surrounding theca cells by a basal lamina, to which many thick bundles of collagen fibers are attached.

Theca layers: In growing follicles, stromal cells start to aggregate concentrically around the basal lamina of granulosa cells, forming the *theca folliculi*. The *theca interna* contains numerous cells with steroidogenic features, covered by a typical "cell coat" that may play a fundamental role not only in cell adhesion and in cell-to-cell interactions, but also in cell permeability, thus controlling the secretion and/or the absorption of substances. By SEM, adjacent theca cells appear to form a net of communicating intercellular, microlabyrinthine lacunae into which numerous microvilli are projected. Secretory products of theca cells (androgens) may be stored in this extracellular compartment and/or directed towards the granulosa layer. The *theca externa* layer is composed by cellular elements similar to those that in the earlier stages of development populate the undifferentiated interstitium. In several mammalian species, including humans, varying amount of contractile elements can also be found, Fig. 6. Tonic or pulsatile contraction of muscular components of the *theca externa* may cooperate in mobilizing and driving fluids from the *theca interna* toward the granulosa compartment. Further, such contractile tissue may nevertheless help the process of ovulation, favoring the detachment of the cumulus oophorus and the expulsion of follicular components (1, 7).

Ultrastructure of the Fertilizable Oocyte and Its Vestments

Mature oocyte: The healthy, fertilizable oocyte is characterized by the presence of the *first polar body* in the perivitelline space, the distribution of cortical granules in subplasmalemmal areas, the occurrence of numerous cytoplasmic aggregates, mainly formed by membranes closely associated with rounded, arched-cristae mitochondria. These membranes are both tubules of SER anastomosed each other and large vesicles filled with flocculent material, presumably involved in production of substances and/or in generating reservoir of membranes useful for fertilization and early embryogenesis (8) (Fig. 3).

Zona pellucida: By SEM, the outer surface of the ZP of preovulatory oocytes shows the presence of numerous fenestrations that render it comparable to a sponge-like structure (9). Fenestrations are not recognizable on immature and atretic oocytes (Fig. 7). By SEM of saponin, ruthenium red and OTO treated samples, the surface ZP fenestrations appeared to be formed by filaments arranged in both large and tight-meshed network, whereas

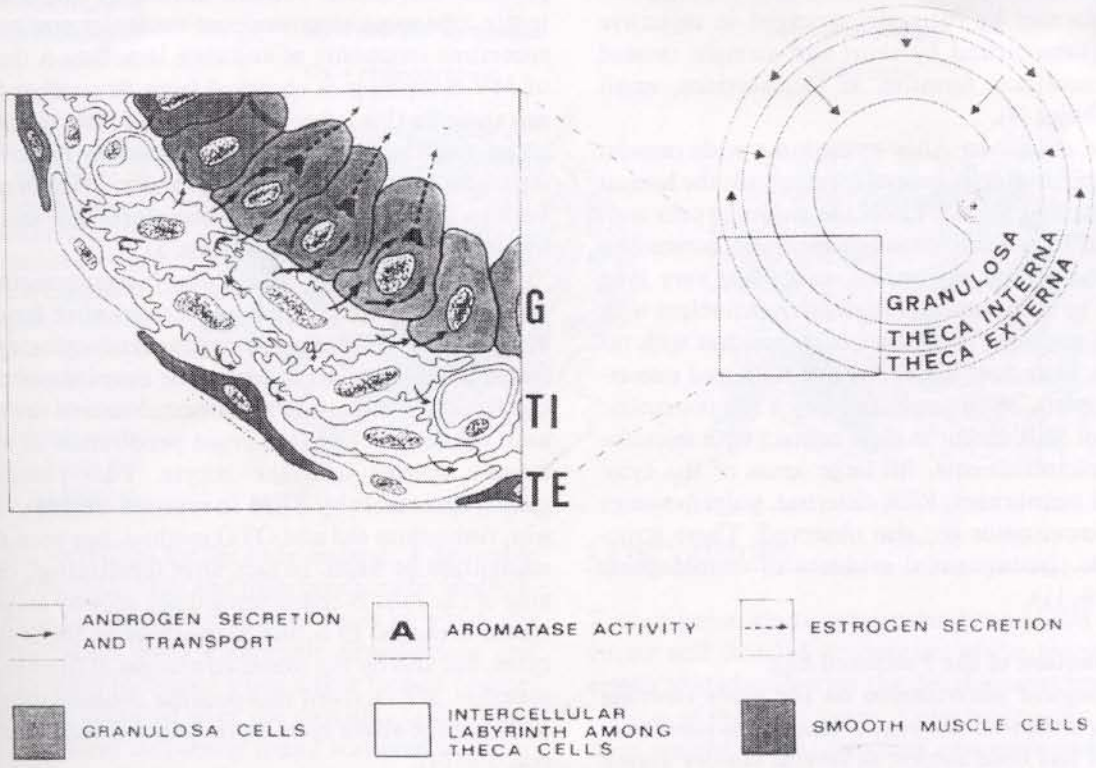


Figure 6. The main characteristics of the theca interna are reported in this figure. In the boxed area on the left is depicted the net of labirynthine intercellular lacunae and their possible role in the storage of fluids as well as in addressing the androgens toward (arrows) the granulosa layer, where they undergo aromatization. In addition, the presence of smooth muscle cells (SMC) in the theca externa is emphasized (s). The arrows in the schema on the right indicate the dynamics of the SMC contraction in the follicle (From Ref. 6, with permission)

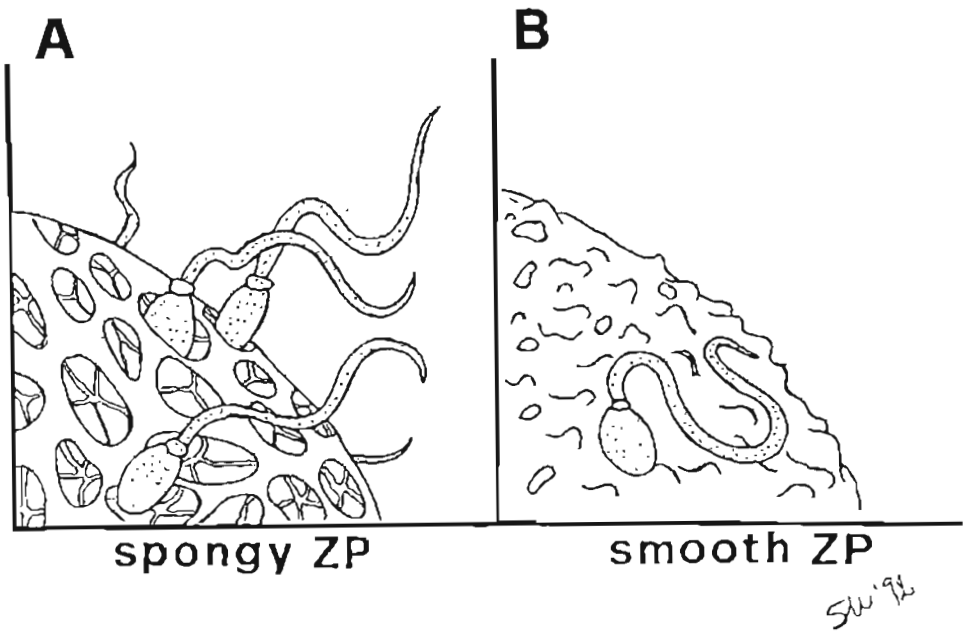


Figure 7. A spongy, fenestrated zona pellucida (ZP) surrounded by numerous penetrating spermatozoa is seen in figure A; Note in figure B the presence of a compact, smooth ZP that does not show areas of sperm attachment and penetration. (From Notrola et al., 1993. Ass Reprod Techn/Androl, Kiawah, SC, 4: 309-317, with permission)

only a tight-meshed network characterizes the outer zona surface of unfertilizable oocytes. The sperm binding seems enhanced by the spongy structure of the ZP. By SEM, the inner zona surface of oocytes at any stage generally shows a granular appearance. However, by correlated TEM and SEM analysis of saponin, ruthenium red and OTO treated samples, the ZP reveals a fine organization, being formed by filaments arranged in repetitive structures, characterized by short and straight twisted filaments sometimes forming, at intersections, small rounded globules (4).

Cumulus oophorus: After ovulation a wide *cumulus mass* of about 20,000 cells generally surrounds the human oocyte. By both SEM and TEM, the cumulus cells usually appeared irregularly round-shaped and covered by numerous membrane expansions and often very long microvilli. The cumulus cells show an oval nucleus with one to three nucleoli. Elongated mitochondria with tubular cristae, abundant membranes of SER and numerous lipid droplets, often surrounded by a few concentric membranes of SER and/or in close contact with microtubules and microfilaments, fill large areas of the cytoplasm. Golgi membranes, RER cisternae, polyribosomes and microperoxisomes are also observed. These structures provide circumstantial evidence of steroidogenic activity (1, 10, 11).

Ultrastructure of the Fertilized Egg

Morphological observations on the early cleaving embryo: The ultrastructure of conventional *in vitro* fertilized material has been subject of several studies during the last decades, showing peculiarities similar to *in vivo* fertilized eggs. By TEM, *pronuclear eggs* appear large, spherical- or oval-shaped, with regular pronuclei containing dense nucleoli. A number of mitochondria-vesicles (MV) complexes are scattered throughout the cytoplasm. Mitochondria appear spherical and provided with arched cristae. Numerous microvilli projected into a narrow perivitelline space, where remnants of polar bodies are found. *Two-to-four cell eggs* show similarly sized nucleated blastomeres. Small cell fragments in the perivitelline space and vacuolized blastomers may be also present. The nuclei contain uniformly dispersed chromatin, one to six electrondense nucleolar precursors and sometime fragments of annulate lamellae. A decrement of MV complexes is observed from two-cell to four-cell egg stage. In this latter stage, mitochondria become elongated and their matrix appears scarcely electrondense, occasionally showing clear vacuoles. Membranes and vesicles belonging to Golgi complexes are seen in the blastomere cytoplasm (1, 8) (Fig. 3).

Zona Pellucida: The spongy arrangement of the outer surface of the ZP is not altered after fertilization and during early cleavage up to the blastocyst stage. After fertilization the inner aspect of the zona instead dramatically changes according to the occurrence of the so called *zona reaction*, in order to avoid penetration of supernumerary sperms into the oocyte. This phenomenon, clearly detectable by TEM in samples treated with saponin, ruthenium red and OTO method, has been also well recognized by SEM. In fact, after fertilization, the inner side of the zona is characterized not only by areas of filaments arranged in a fine network as in the mature oocytes, but also by the presence of areas of filaments fused together. We ascribed this peculiar condensation of filaments to the above zona hardening that leads to the zona reaction (4).

Cumulus oophorus: Cumulus cells after ovulation and particularly after fertilization seem to undergo a process of luteinization, both *in vivo* and *in vitro* (Fig. 8) (see also above). The general enhancement of the steroid-synthetic properties suggests a precise paracrine role of

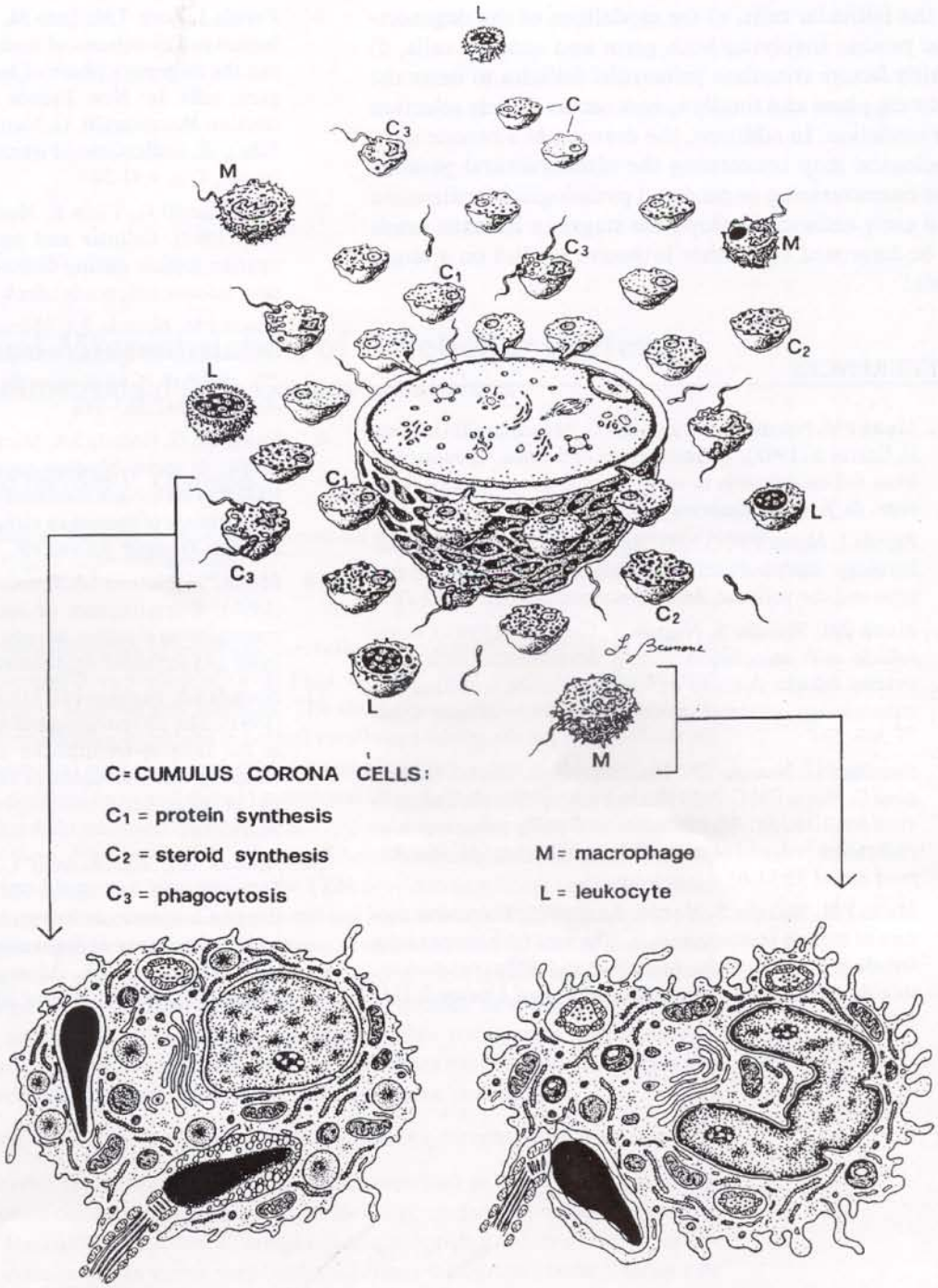


Figure 8. The presence of different cell subpopulations in the cumulus mass is portrayed in this figure. C: cumulus-corona cells; C_1: protein synthetic cells; C_2: steroid synthetic cells; C_3: phagocytic cells. M: macrophages. L: lymphocytes. The cells forming the subsystem of spermiophagic elements, *i.e.* C_3 (cumulus-corona cells capable of phagocytosis) and M (monocytes activated to macrophages) have been enlarged at the bottom of the figure. (From Ref. 10, with permission)

the cumulus secretions for the early embryo. A favorable effect of the cumulus cells and their products on fertilization has been confirmed by clinical trials in which the rate of fertilization, early embryonic development and even implantation increased when either human oocytes or embryos are cultured with the cumulus cells. Blood elements have been frequently found scattered inside in the cumulus mass. Among them, leukocytes and macrophages may probably act not only as "scavenger" -like cells but also as modulators of the steroid secretion of the neighboring cumulus cells by production of cytokines (1, 10-12) (Fig. 8).

Conclusions

Transmission electron microscopy and SEM offer an accurate and detailed description of the morphodynamic events that characterize the development and the cyclic activity of the adult ovary. These techniques also allow a deep insight of the intriguing processes underlying fertilization and early embryo development. Unfortunately, some aspects of these processes are still unclear and –in our opinion– need to be further investigated. In particular a) when and where germ cells separate from the somatic cell line, b) the origin of the ovarian blastema and

of the follicular cells, c) the modalities of the degenerative process involving both germ and somatic cells, d) which factors stimulate primordial follicles to enter the growing phase and finally e) how occurs follicle selection for ovulation. In addition, the drawing of a precise morphological map concerning the ultrastructural parameters characterizing normal and pathological fertilization and early embryo development stages in humans needs to be improved by further intensive studies on a larger scale.

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