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Maturation of the Human Oocyte In Vitro: Nuclear Events During Meiosis (An Ultrastructural Study)

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Stages of meiotic maturation have been traced in human oocytes cultured in vitro from prophase I to telophase II, using electron microscopy.

Two hundred ten oocytes, aspirated at laparoscopy after appropriate hormonal stimulation, were cultured for 1–72 h and examined. Most oocytes were inseminated and the final phases of maturation were studied in fertilized ova.

The majority of oocytes had resumed meiosis and had matured to metaphase II. Some were arrested at metaphase I, and a few remained inactive at the germinal vesicle stage. Maturation of oocytes to metaphase II was achieved 3 h after laparoscopy; to telophase II, 3 h after insemination. Maturation of some oocytes to metaphase II may have occurred in the ovary.

Immature oocytes had eccentric nuclei containing dense fibrillar nucleoli. Corona cell processes formed complex junctions with the oolemma and a type of interaction analogous to neural transmission was observed. Maturing oocytes showed typical barrel-shaped spindles, but no centrioles or asters. Chromosomes were attached to microtubules via kinetochores. Midbodies were elaborated in the ooplasm and determined the plane of cleavage of polar bodies. Some aspects of oocyte degeneration, aberrations of oocyte maturation, and consequences of aging are also reported.

The significance of cytoplasmic maturation that occurs concurrently with nuclear maturation is discussed in relation to assessment of oocytes for in vitro fertilization.

Key words: oocyte maturation, meiosis, cumulus interaction, in vitro fertilization, laparoscopy, electron microscopy

INTRODUCTION

One of the salient prerequisites for successful in vitro fertilization (IVF) is obtaining a mature oocyte, just prior to ovulation. This is achieved by appropriate hormonal stimulation, monitoring of hormones in the blood and urine, ultrasound scanning of follicles, and correct timing of laparoscopy [Trounson, 1984; McBain and Trounson, 1984]. Maturation of oocytes with full developmental potential could be achieved by culturing aspirated oocytes 3–6 hr or more after laparoscopy, prior to

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insemination [Sathananthan and Trounson, 1982a; Trounson et al, 1982]. This is now a routine technique used in IVF.

The cortical changes that occur during oocyte maturation in vitro have been reported [Sathananthan and Trounson, 1982a; Sathananthan et al, 1984]. A preliminary report on the mature oocyte has also been published [Sathananthan and Lopata, 1980]. An earlier study of human oocyte maturation in vitro involved material collected from ovarian wedges removed after laparotomy [Zamboni et al, 1972]. This investigation traces the nuclear events that take place during maturation of preovulatory oocytes after laparoscopy, following appropriate stimulation, from the germinal vesicle stage up to fertilization. Morphological assessment of oocytes is routinely carried out in our IVF programme, and many aspirated oocytes have produced successful pregnancies, after fertilization and embryo transfer [Trounson and Wood, 1984].

MATERIALS AND METHODS

This study involved the ultrastructural examination of 210 oocytes for stages of maturation up to fertilization (Table 1).

The majority of women had tubal problems or infertility owing to unknown causes. They were stimulated with clomiphene citrate and/or human menopausal gonadotropin (HMG), followed by human chorionic gonadotropin (HCG) in the absence of an LH surge [Trounson et al, 1982; McBain and Trounson, 1984]. Daily plasma oestradiol and progesterone levels and plasma and urinary LH were monitored to predict time of ovulation and laparoscopy. Ultrasound was used to determine number, growth, and size of follicles; and oocytes were aspirated from mature follicles (16–25 mm in diameter) at laparoscopy, 32–36 h after the LH surge or HCG injection. Preovulatory oocytes were matured for 3–7 h before insemination in various media, including modified Ham's F_{10}, Earle's solution, Whitten's and Whittingham's T_6. Husband or donor semen was obtained by masturbation, washed, centrifuged, and layered to harvest motile sperm. Oocytes were inseminated with 10,000–100,000 motile sperm and incubated at 37°C under a gas phase of 5% O_2, 5% CO_2, and 90% nitrogen for 1–72 h. Most of the oocytes fixed for electron microscopy were unfertilized, although fertilized ova were examined for the final phases of maturation.

The eggs were fixed in 3% glutaraldehyde in 0.1 M cacodylate or phosphate buffer (pH 7.2–7.3) at 4°C. These were then postfixed in 1% aqueous osmium tetroxide (30–60 min), rapidly dehydrated in ethyl alcohol and acetone, and embedded in Araldite. Alternate series of thick sections (1 μm) and thin sections ($\sim$70 nm) were cut with glass and diamond knives using LKB and Reichert ultramicrotomes. Thick

TABLE 1. Maturation of Oocytes In Vitro—Electron Microscopy

Time in culture (h)[a]	No. of oocytes	GV stage	Metaphase I	Metaphase II	Telophase II[b]	Undetermined
1–10	18	2	2	6	8	—
11–72	192	10	36	114	12	20
Total	210	12	38	120	20	20

[a]Most oocytes were matured in culture for 3–7 h and then inseminated.

[b]All fertilized ova (telophase II) had a second polar body in the perivitelline space. Some of the GV and metaphase II oocytes were degenerating.

sections were stained with toluidine blue for light microscopy. Thin sections were mounted on uncoated copper grids, stained with alcoholic uranyl acetate and Reynold's lead citrate, and examined with a Philips 301 electron microscope.

RESULTS

Most preovulatory oocytes recovered by laparoscopy of large follicles (16–25 mm in diameter) had already resumed meiosis (Table 1). Occasionally, immature oocytes at the germinal-vesicle (GV) stage were also aspirated from smaller antral follicles (Fig. 1). Some metaphase I and II oocytes were recovered from large antral follicles (Figs. 2,3). Nuclear changes during maturation were traced from diplotene (Prophase I) to its completion (telophase II), after fertilization (Figs. 4–17).

General Organization of Oocytes

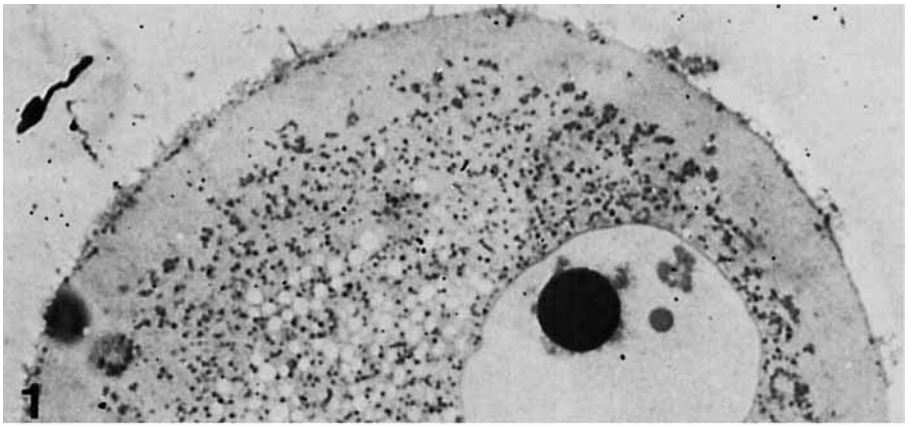
Preovulatory oocytes are initially surrounded by many layers of cumulus cells at the GV stage, which gradually disperse during maturation or when kept in culture. The cumulus, at first compact, expands and becomes gelatinous, and the cells progressively acquire lipid inclusions and vacuoles (luteinization). The corona cell processes that permeate the zona pellucida are retracted from the oocyte surface as the oocyte approaches maturity (Figs. 2,3), but their bulbous terminals may remain in the perivitelline space even after fertilization. Most cytoplasmic constituents are uniformly distributed in the ooplasm and consist of mitochondria, smooth endoplasmic reticulum (SER), Golgi membranes, annulate lamellae, lysosomes, and related inclusions, which have been described previously [Sathananthan, 1984; Sathananthan et al, 1984]. These organelles may be aggregated in the central ooplasm in GV oocytes. There is a marked increase in the number of cortical granules (CG) which form 1–3 discontinuous layers as the oocytes approach metaphase II and most of these granules release their contents at fertilization [Sathananthan and Trounson, 1982a,b].

GV-Oocyte

Primary oocytes, arrested at the GV stage (diplotene of prophase I), had eccentric nuclei containing dense compact nucleoli associated with chromatin (Figs. 1,4). Less conspicuous inclusions such as "satellites" and smaller spherical bodies were also evident in the nucleoplasm. The nuclear envelope was perforated by numerous pore complexes (Figs. 5,15). Nucleoli were predominantly homogenous and fibrillar in appearance but may show darker spherical bodies (fibrillar centres) within their substance or located towards their periphery (Figs. 4,16). The GV oocyte represents the first arrest in meiotic activity.

First Meiotic Division

After a protracted period of inactivity, meiosis is resumed before ovulation. At the onset of resumption, the nucleus moves toward the surface, chromosomes condense, and an organelle-free zone appears at the region where the first polar body (PB_1) would be extruded (Fig. 5). Eventually, the GV is broken down [see Zamboni et al, 1972], a spindle composed of microtubules (MT) appears in the ooplasm, and homologous chromosomes align themselves on the equator of the spindle at metaphase I (Fig. 6). The meiotic spindle is more or less barrel shaped, and its MT end abruptly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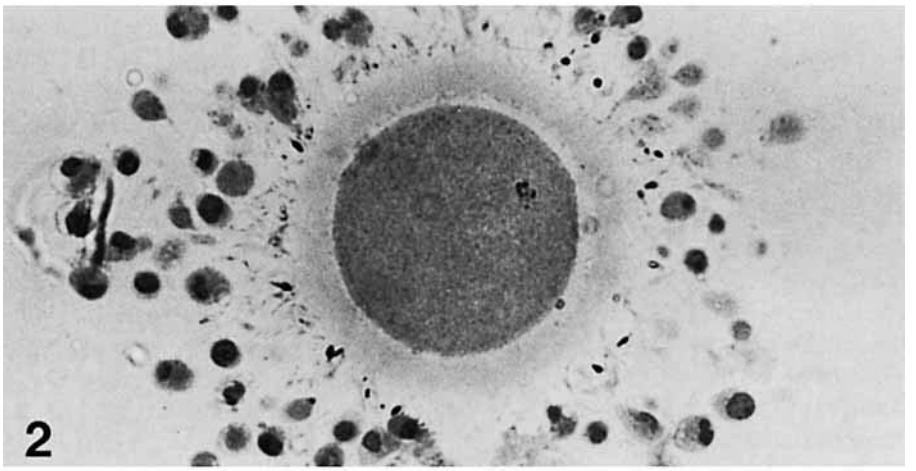
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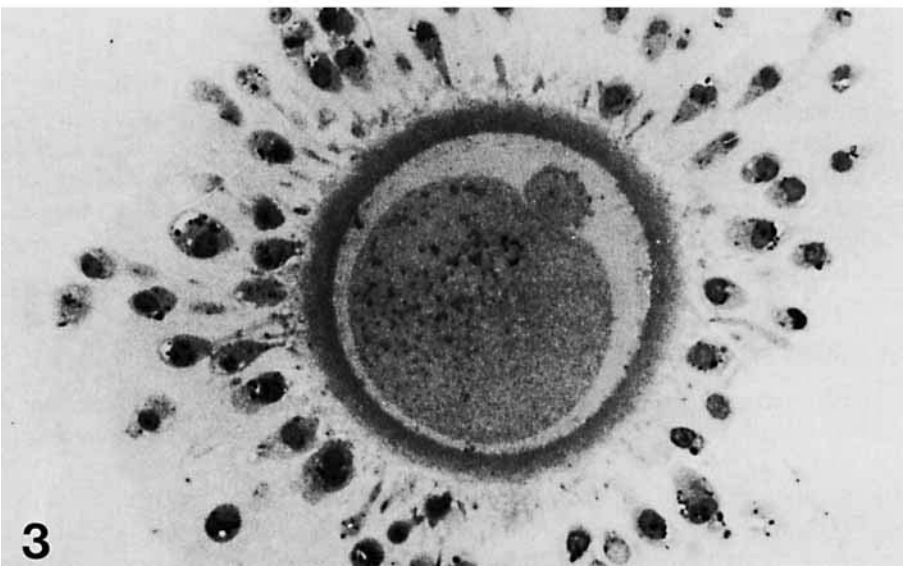
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at either pole, there being no centrioles or asters. Clusters of tiny vesicles may be associated with the poles of the spindle. The chromosomes present a fibrogranular texture and are attached to MT via crescent-shaped kinetochores (Fig. 7). Maturation proceeds through anaphase I to cytokinesis and PB_1 is finally formed. The spindle apparently rotates (Fig. 8) to its definitive position parallel to the oolemma in mature oocytes. The extruded chromosomes in PB_1 remain isolated and attached to residual MT via kinetochores (Fig. 17) and do not form a nucleus. PB_1 also contains CGs in addition to other organelles, and a peripheral band of microfilaments (MF) may appear soon after its abstriction [Sathananthan and Lopata, 1980].

Second Meiotic Division

The second maturation division begins almost immediately, as there is no interphase and as prophase II appears to be nonexistent. MT may diminish in number at this stage. Chromatids soon align themselves on the equator of the spindle (metaphase II), which is now oriented more or less parallel to the oolemma. Sections across the equator of the metaphase II spindle (metaphase plate) show chromosomes arranged almost in a circle, associated with MT (Figs. 3,9). The secondary oocyte enters the second meiotic arrest, at which stage they are ovulated and fertilized. Secondary oocytes, just prior to fertilization, show spindles that have again assumed a vertical position in the peripheral ooplasm, subjacent to PB_1 (Fig. 10). Chromatids are still organized near the equator and have already begun to separate (meta-anaphase II). This stage of maturity corresponds to that of tubal eggs in vivo.

Completion of Maturation

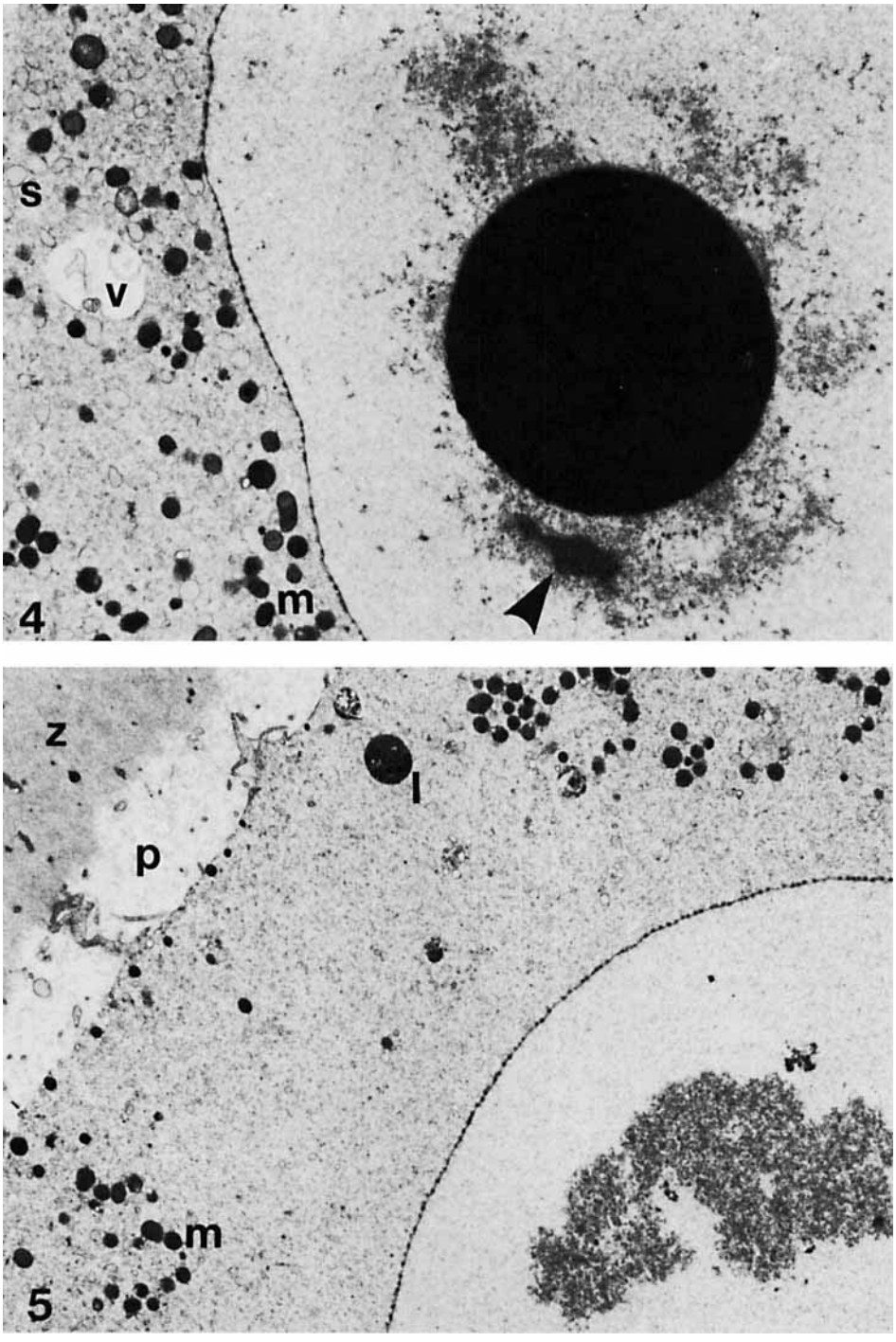
The second meiotic division is completed only at fertilization. The daughter chromatids separate (anaphase II), after sperm penetration and oocyte activation (Fig. 11). Dense material of the midbody appears at the equator of a broad spindle. Eventually, at telophase II, the second polar body (PB_2) is constricted at the level of the midbody, and chromatids separate to either pole of the spindle, which has now contracted and assumed a characteristic barrel shape (Fig. 12). The chromatids fuse with one another, condense, and form nuclei at either pole, whilst MT are still attached to them (Figs. 13,14). Nuclear envelopes are assembled by flattening of vesicular elements of the SER on the periphery of the condensed chromatin masses, resulting in the formation of a nucleus in PB_2 and a female pronucleus in the ooplasm. During late telophase II, PB_2 is abstricted by further contraction of the spindle in the midbody region, aided by the activity of peripheral MF [see Lopata et al, 1980]. PB_2 is usually devoid of CGs, as it is formed soon after CG exocytosis.

Figs. 1–3. Oocyte maturation—light micrographs.

Fig. 1. Immature (primary) oocyte arrested at the germinal-vesicle stage. The nucleus is eccentric and contains a dense, compact nucleolus. The cortex is devoid of organelles except for a discontinuous layer of cortical granules. The dark granules in the ooplasm are predominantly mitochondria. Note centralized vacuolation of ooplasm and a sperm attached to the zona. ×950.

Fig. 2. Maturing oocyte at metaphase I showing chromosomes in peripheral ooplasm. The cumulus cells have begun to disperse and retract from the oocyte surface. Many sperm are attached to the zona. × 243.

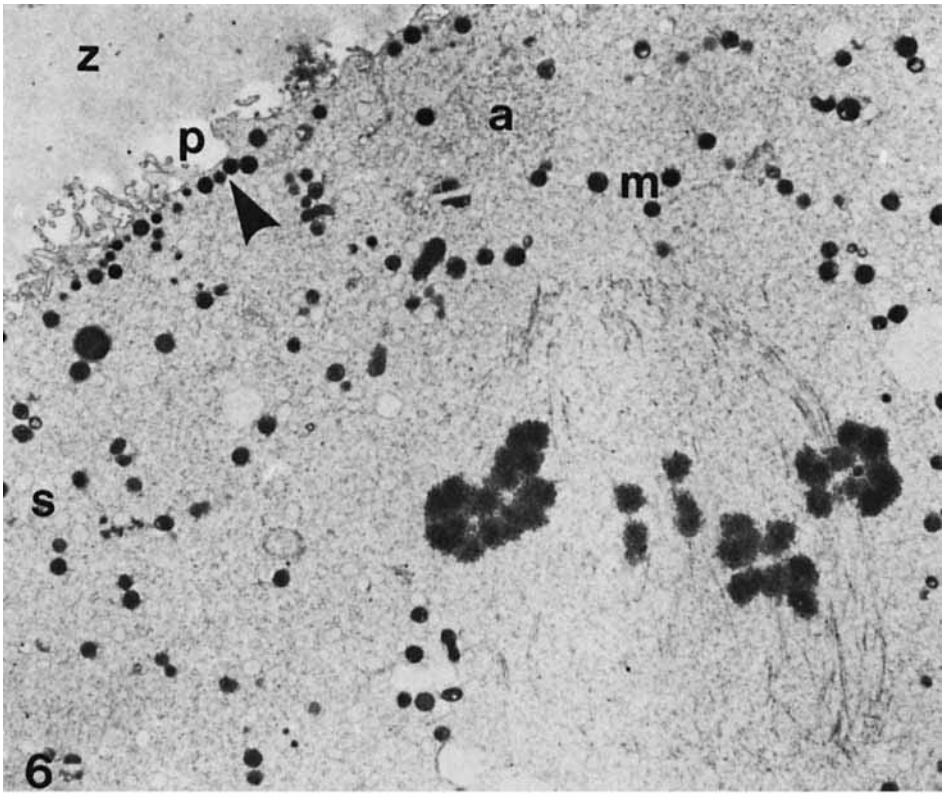
Fig. 3. Mature (secondary) oocyte at metaphase II, sectioned through chromosomes (metaphase plate) and first polar body. Most follicle cells have retracted their processes from the oocyte-surface and contain dark lipid globules and clear vacuoles. Note clumping of organelles caused by accidental injury to the oocyte. ×243.



Figs. 4–14. Electron micrographs of oocyte maturation.

Fig. 4. Nucleus of a primary oocyte arrested at the germinal-vesicle stage. It has an envelope perforated by pores and contains a dense, compact nucleolus associated with chromatin and a "satellite" body (arrow). m = mitochondria; s = vesicular SER; v = vacuole. ×8,190.

Fig. 5. Primary oocyte at resumption of meiosis. The germinal vesicle has approached the surface and contains condensed chromatin. An organelle-free zone is visible where the first polar body would be eventually extruded. A dense, compact nucleolus associated with this chromatin was also revealed in serial sections. z = zona; p = perivitelline space; m = mitochondria; l = secondary lysosome. ×6,300.



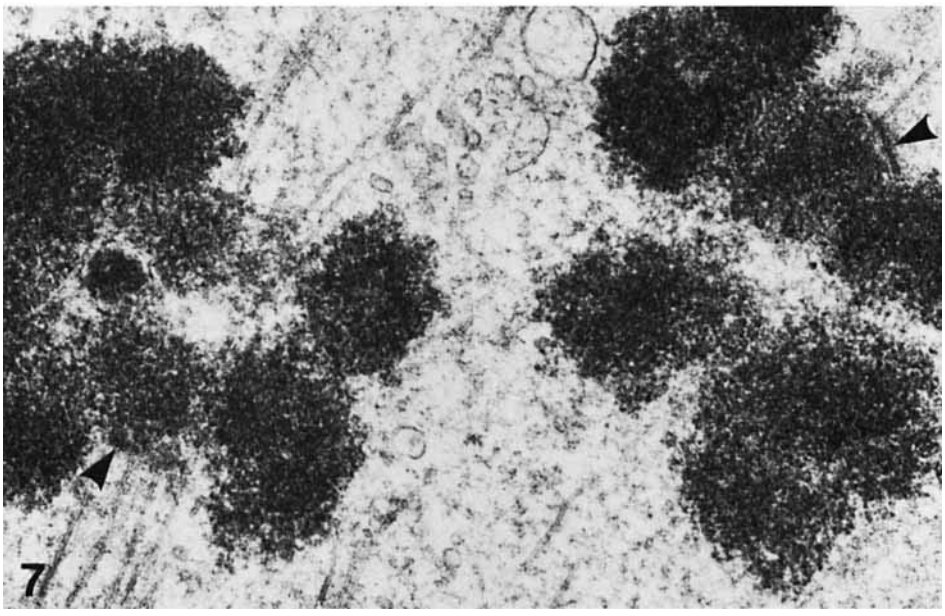
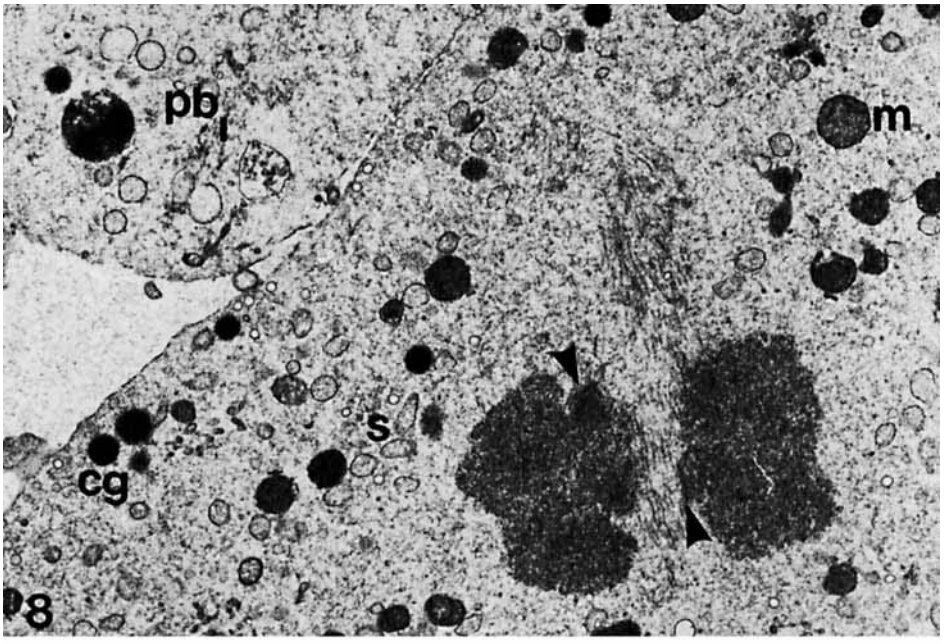


Fig. 6. Maturing oocyte at meta-anaphase I. Paired chromosomes are aligned in the equatorial region of the meiotic spindle, which consists of microtubules that end abruptly at either pole, there being no centrioles or asters. Microvilli and cortical granules (arrow) are seen at the surface of the oocyte. z = zona; p = perivitelline space; m = mitochondria; s = vesicular SER; a = aggregate of small SER. × 6,300.

Fig. 7. Two pairs of homologous chromosomes of the oocyte in Figure 6 showing insertion of spindle microtubules into kinetochores of each chromosome (arrows). The chromosomes have a fibrogranular texture. ×32,130.



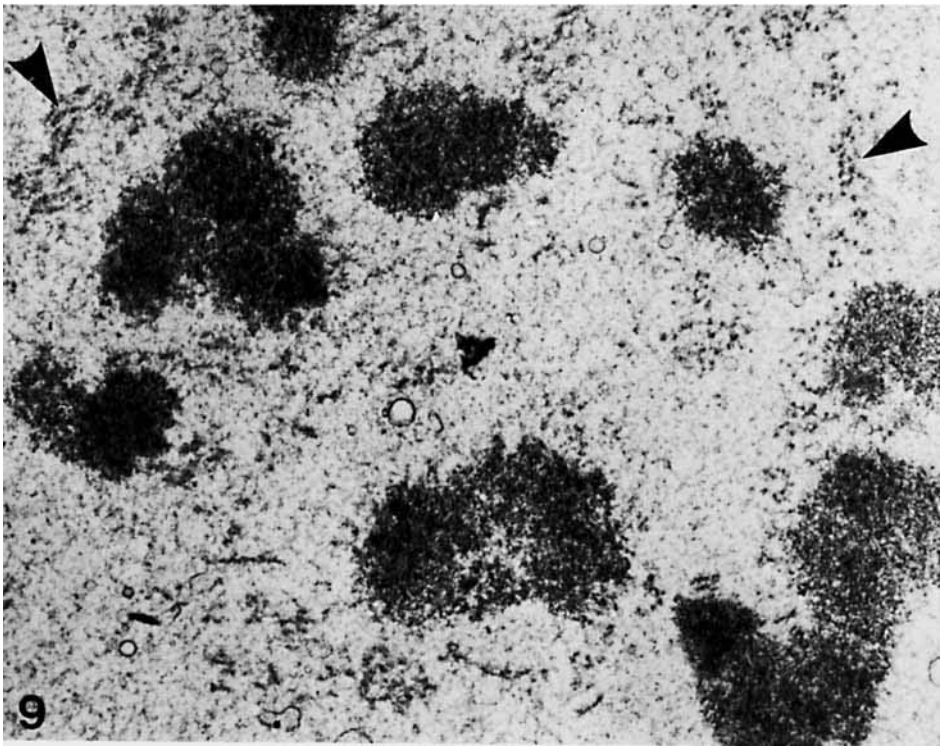


Fig. 8. Mature secondary oocyte at late telophase I, soon after extrusion of the first polar body (pb_1). A typical dyad consisting of two chromosomes separated by microtubules inserting into kinetochores (arrows) is visible. The spindle is oriented obliquely and seems to be rotating to its usual position parallel to the oolemma. cg = cortical granules; m = mitochondria; s = vesicular SER. ×13,860.

Fig. 9. Cross section through the metaphase II spindle (metaphase plate) of a mature secondary oocyte (see Fig. 2). The microtubules are cut transversely or obliquely (arrows), and the chromosomes are more or less arranged in a circle. ×17,640.

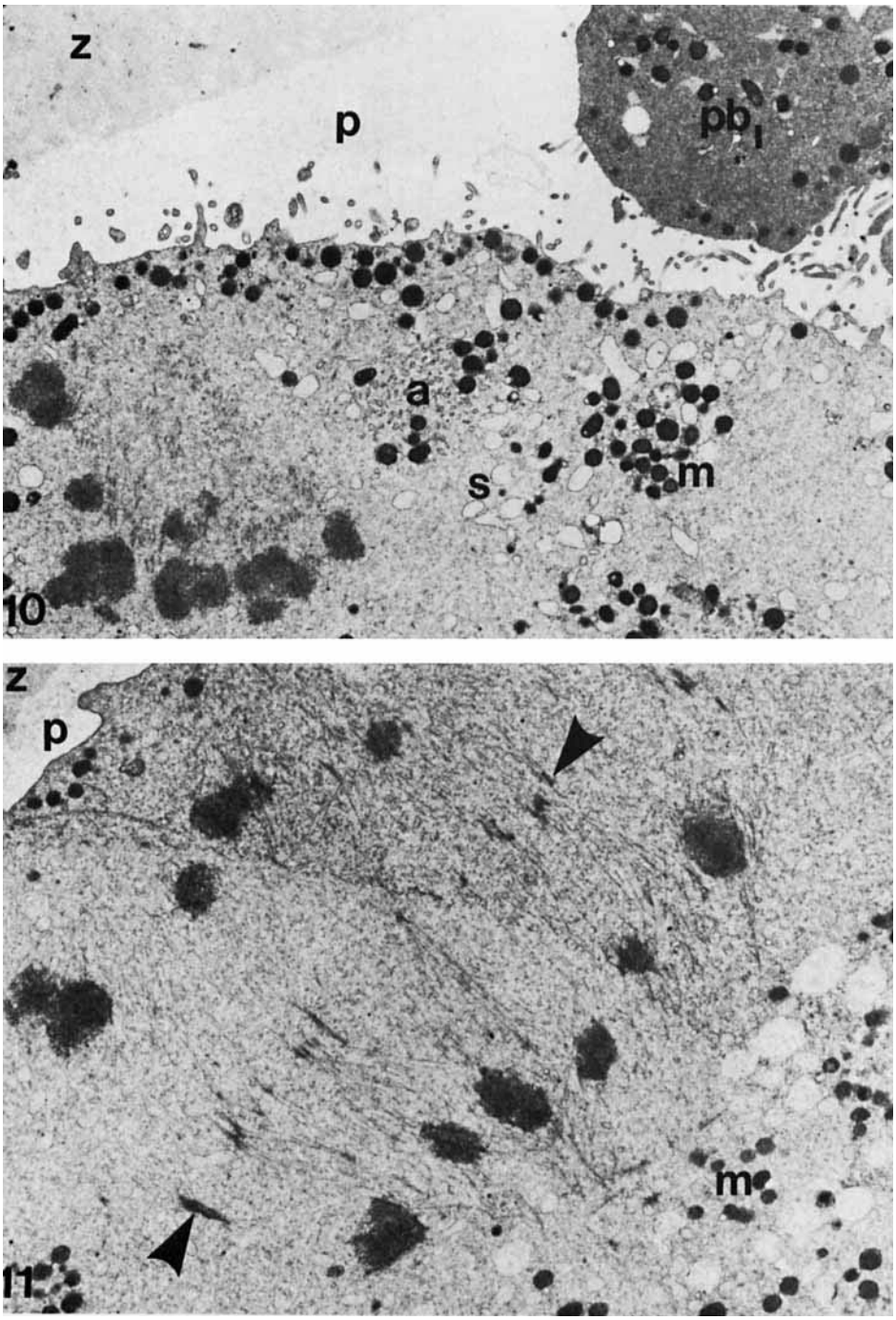


Fig. 10. Secondary oocyte at meta-anaphase II, just prior to fertilization. The spindle has moved toward the surface and has rotated vertically. The chromosomes are about to separate and many cortical granules are seen beneath the oolemma. One chromosome is subjacent to the oolemma. z = zona; p = perivitelline space; pb_1 = first polar body; m = mitochondria; s = vesicular SER; a = aggregate of small SER. ×6,300 (2 h postinsemination).

Fig. 11. Anaphase II spindle of a fertilized ovum soon after sperm penetration. The spindle is rather wide and consists of microtubules, ending abruptly at either pole. The chromatids have separated, and dense material of the interbody is beginning to appear at the spindle equator (arrows). z = zona; p = perivitelline space; m = mitochondria. ×10,710 (2 h postinsemination).

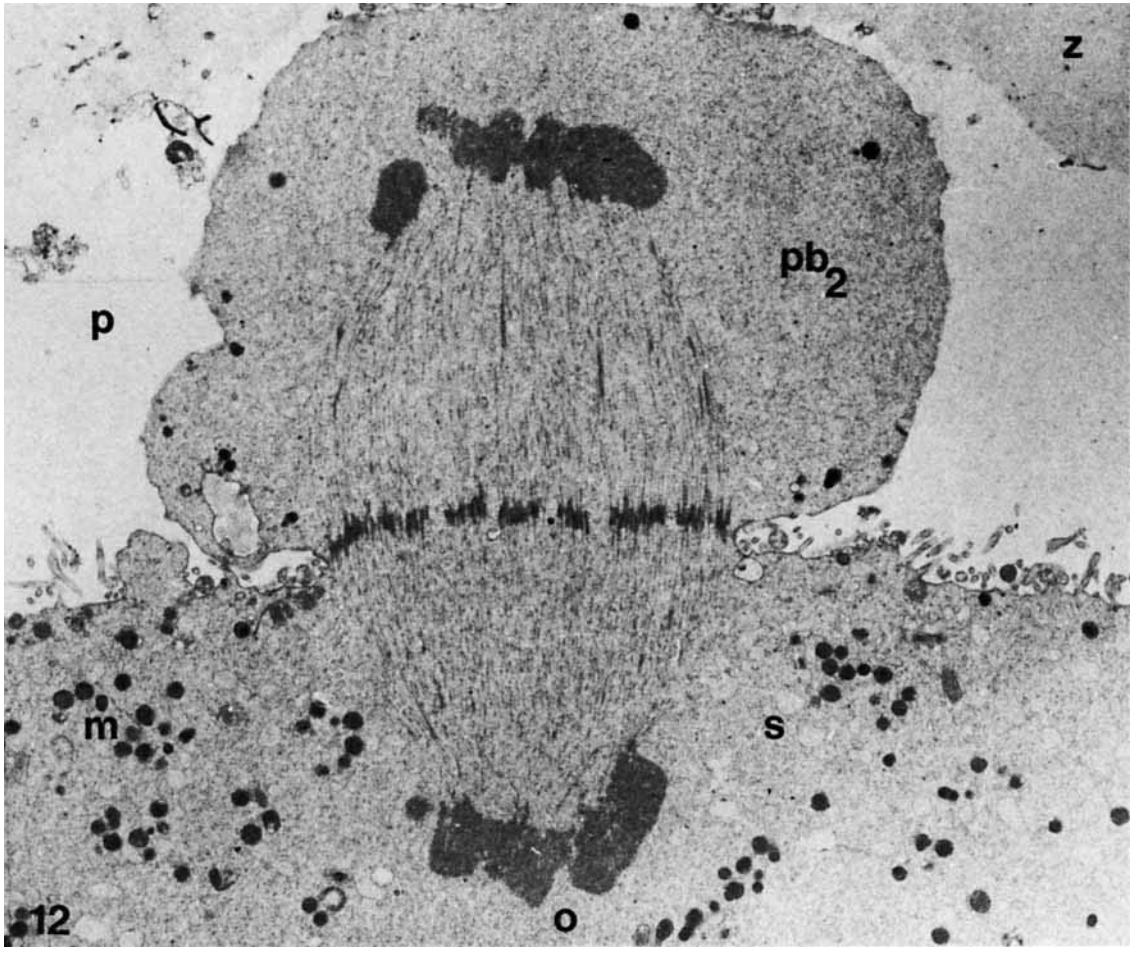
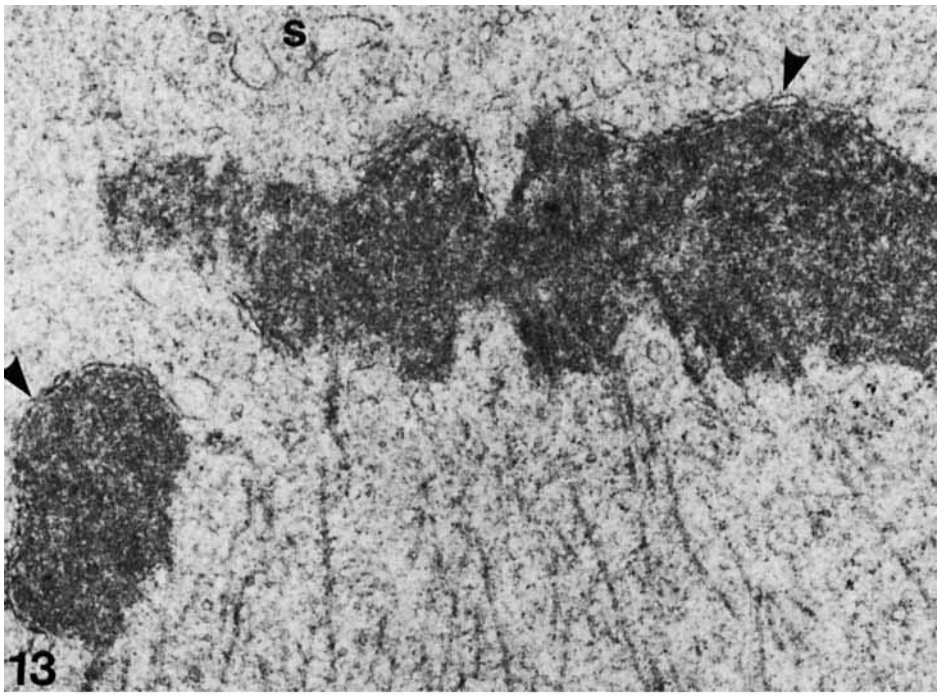


Fig. 12. Completion of second maturation (telophase II) at fertilization. The spindle is barrel-shaped and composed of numerous microtubules. Chromatids have separated, and the second polar body (pb_2) is being constricted at the level of the dense interbody, which has now contracted. z = zona; p = peritvitelline space; o = ooplasm; m = mitochondria; s = vesicular SER. ×6,370 (3 h postinsemination).



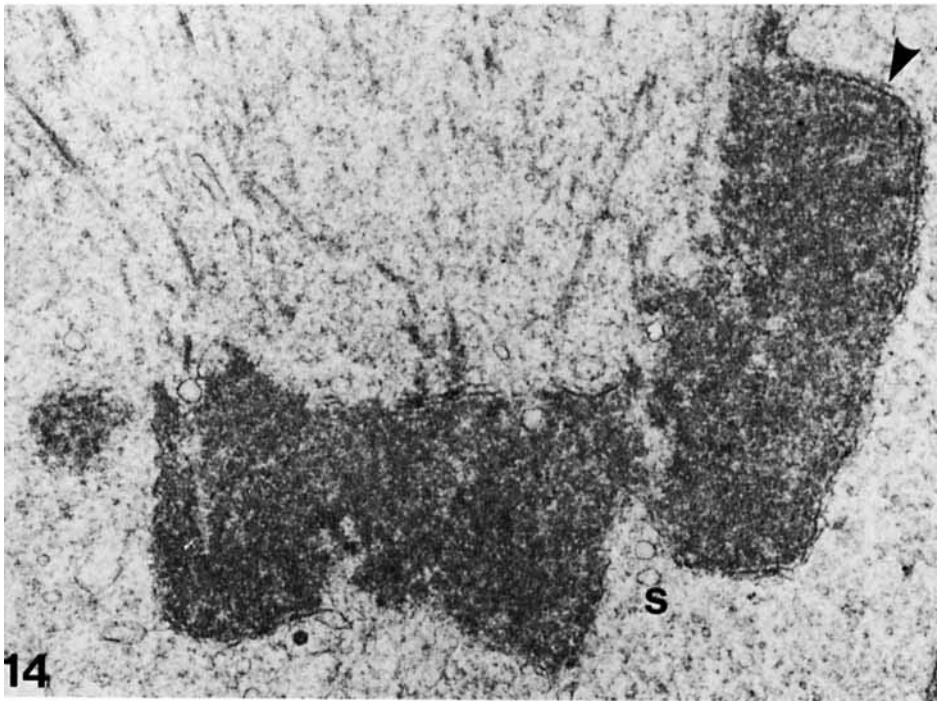
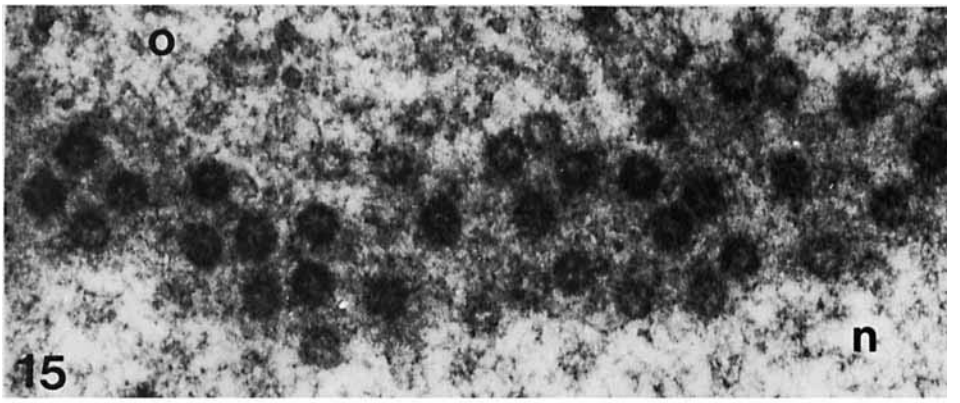


Fig. 13. Extruded chromosomes in the second polar body (Fig. 12) fusing with one another and forming a nucleus. Parts of the nuclear envelope (arrows) are being assembled by small and large vesicular elements of the SER (S). Microtubules are inserting into the chromatin mass. ×24,570.

Fig. 14. Chromatids condensing at the ooplasmic pole of the second meiotic spindle (Fig. 12) to form a female pronucleus. Its envelope is formed in the same way as shown in Figure 13. Microtubules are still associated with the chromatin. s = vesicular elements of SER. ×24,570.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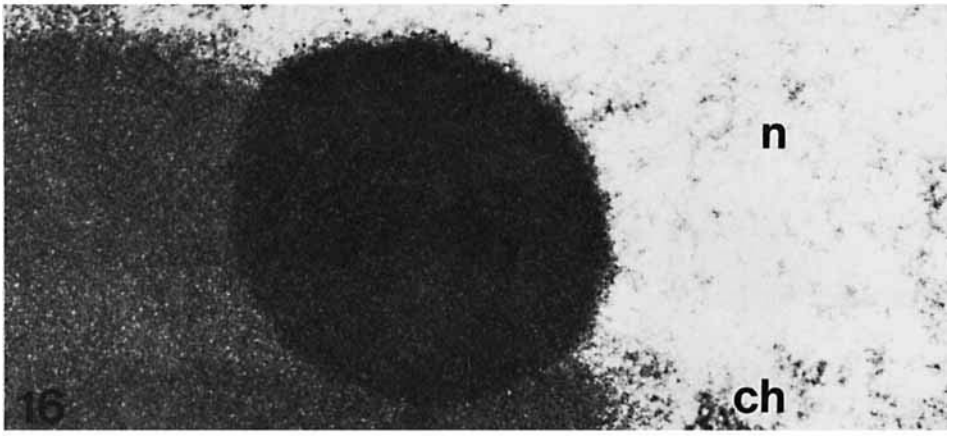
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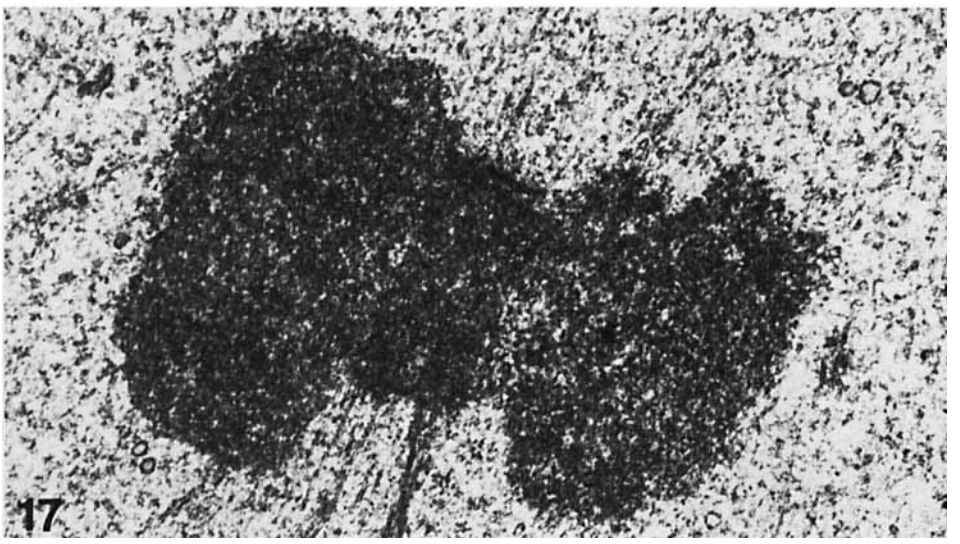
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Cumulus-Oocyte Interaction

Specialized junctional complexes were observed between corona cell processes and the oolemma, particularly in GV oocytes. These processes traverse the thickness of the zona (Fig. 18) and have MT, MF, and membrane-bound vesicles with granular contents that were associated with Golgi complexes within the cell body. More MF and vesicles are found toward the swollen terminals of these processes at the oocyte surface (Fig. 19). At the junctional complex, the cell membranes are separated by a space that appears to be obliterated at points of increased electron density. These may represent gap junctions or nexi. Buttonlike terminals containing numerous MF and vesicules were occasionally observed apposed to oolemma in the perivitelline space of fertilized ova, as well.

Abnormal Oocyte Maturation

The common aberrations of oocyte maturation include its arrest at metaphase I, disorganization of spindle MT, and consequent scattering of chromosomes and formation of subnuclei, resembling pronuclei of fertilized ova. Nucleation of PB_1 was observed in a few oocytes, which could be mistaken for PB_2. Oocytes aged in culture may show centripetal migration or peripheral accumulation of CGs and rounded stumpy microvilli.

Oocyte Degeneration

Few oocytes arrested at the GV stage or metaphase II, recovered at laparoscopy, had already degenerated in the follicle (atresia). They appeared dense and heterogenous with the light microscope and showed characteristic centralized vacuolation or extensive lysosomal degeneration with pycknotic nuclei. Others prolonged in culture showed subtle changes in fine structure characterized by swelling of SER and clumping of mitochondria.

DISCUSSION

The sequence of events leading to oocyte maturation in the human follows the usual mammalian pattern and agrees in most details with that of the mouse [Zamboni, 1970, 1971, 1972; Thompson et al, 1974]. The fine structure of the meiotic spindle, chromosomes, and kinetochores also closely resembles that described for this animal [Calarco, 1972]. The barrel-shaped configuration of the spindle, including organization of microtubules and the midbody, is especially noteworthy, as it conforms to the

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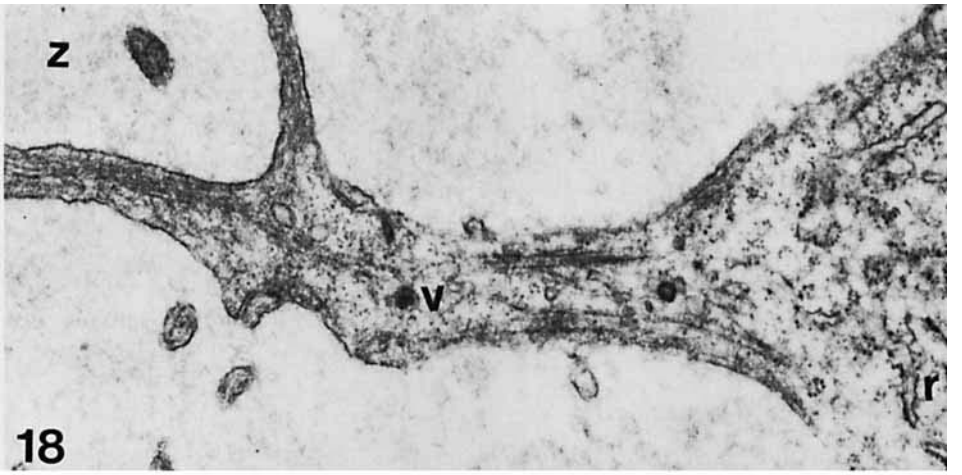
Figs. 15–17. Electron micrographs of nuclear components.

Fig. 15. Tangential section of the nuclear envelope of a germinal-vesicle oocyte. The nuclear pore complexes consist of annuli organized of granular or fibrillar subunits. n = nucleoplasm; o = ooplasm. ×63,000.

Fig. 16. Part of a dense compact nucleolus of a germinal-vesicle oocyte showing a predominantly fibrillar structure. A denser spherical body (fibrillar centre) is seen at its periphery and may be found deep within the nucleolus. ch = chromatin; n = nucleoplasm. ×49,140.

Fig. 17. Isolated chromosome within the first polar body of a fertilized ovum. Residual microtubules are seen inserting into the kinetochore on either side of the chromosome. The kinetochore appears to form a band about the constriction of the chromosome. ×39,690.



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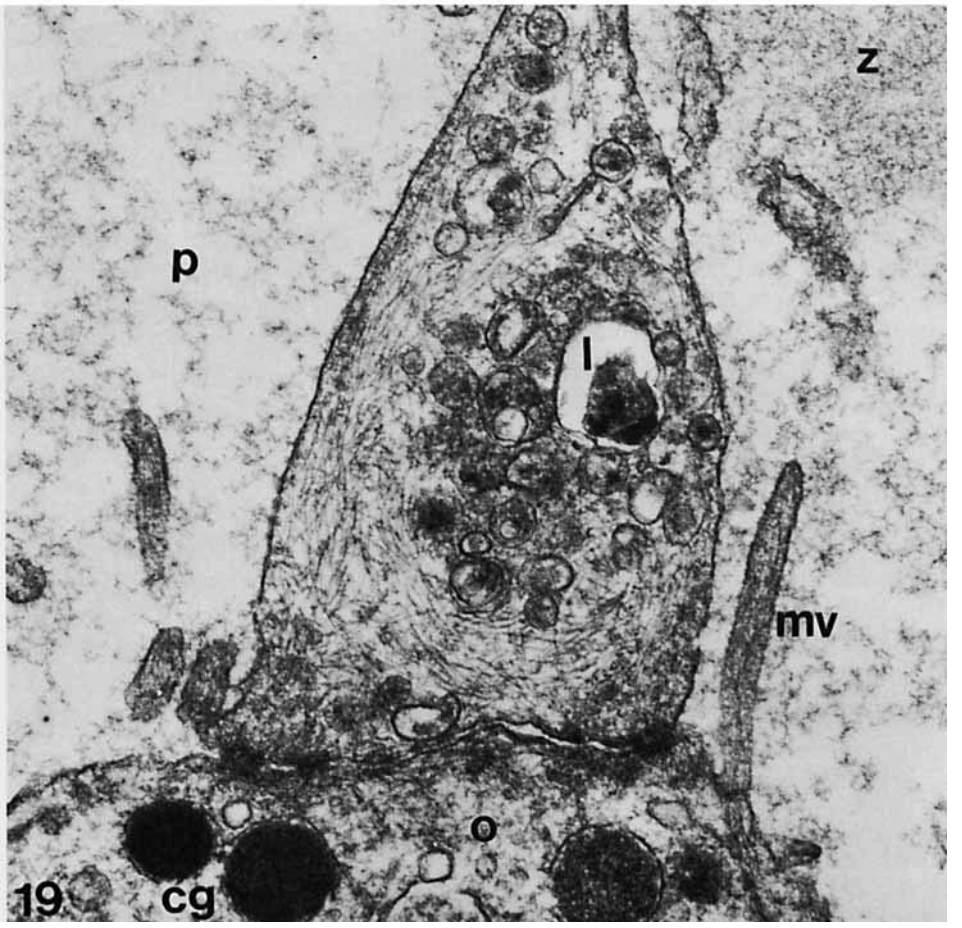

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classical description of these structures in various mammals, visualized with the light microscope. Midbodies seem to determine the plane of cleavage of polar bodies.

The process of maturation of human oocytes in vitro, after clomid and/or HMG stimulation followed by laparoscopy, is more or less identical to that observed without stimulation, after laparostomy [Zamboni et al, 1972]. However, the time taken for completion of oocyte maturation after laparoscopic recovery is much quicker than that recorded at laparotomy, where most of the oocytes required 32–40 h or more to reach metaphase II. This was often achieved only after 3 h in culture and the process was completed to telophase II 3 h after insemination. The state of maturation of the oocyte recovered, of course, depends on the timing of laparoscopy after the preovulatory LH surge of HCG injection in the stimulated cycle. HCG is thought to stimulate the final phase of maturation and induce ovulation and should be carefully administered after maximum steroid production [McBain and Trounson, 1984]. Most oocytes have invariably resumed meiosis in the stimulation regimes used in our clinics and were at stages varying from metaphase I to metaphase II of maturation. Oocytes recovered closer to the time of ovulation approaching metaphase II are more likely to yield embryos with full developmental potential after fertilization [Trounson, 1984]. Hence laparoscopy is usually scheduled about 32–36 h after the spontaneous plasma LH surge of HCG injection [Trounson, 1984; Mohr and Trounson, 1984]. Fertilizable oocytes are usually recovered from mature follicles (16–25mm diameter) with large antra. These preovulatory oocytes, once aspirated, are then cultured for a further 3–6-h period to allow them to complete maturation to metaphase II before they are inseminated [Sathananthan and Trounson, 1982a; Trounson et al, 1982]. This procedure has produced higher pregnancy rates and is now routinely used in most clinics. Occasionally mature oocytes at metaphase II have been aspired at laparoscopy, but their assessment with the light microscope is not easy with the cumulus intact. These should have one polar body and no evidence of a nucleus.

Immature GV oocytes may sometimes be aspirated from nonovulatory follicles, usually smaller than 16 mm in diameter. It is generally known that GV oocytes can spontaneously resume meiosis when introduced into culture [Zamboni, 1972; Suzuki et al, 1981]. Many of these oocytes matured in culture, were successfully fertilized, and produced a few pregnancies after embryo transfer (Veeck, 1984]. Oocytes normally require their follicular environment to complete maturation [Crosby and Moor, 1984], and this meiotic competence is influenced by a variety of factors [Tsafriri et al, 1983]. The incidence of GV oocyte recovery in our clinics has been very low, perhaps owing to better timing of laparoscopy.

Apart from nuclear events of maturation, cytoplasmic factors and cumulus-oocyte interaction play important roles in oocyte maturation [Thibault, 1977; Soupart, 1980; Szollosi and Gerrard, 1983; Crosby and Moor, 1984]. The cytoplasmic events

Figs. 18–19. Electron micrographs of cumulus cell processes.

Fig. 18. Corona cell process permeating the thickness of the zona of a germinal vesicle oocyte. The cell body (right) contains ribosomes and endoplasmic reticulum (r). Microtubules and microfilaments funnel into the process which is branched. Numerous microfilaments are seen in the distal region of the process. z = zona; v = vesicle. ×32,130.

Fig. 19. Swollen terminal of a corona cell process forming a junctional complex with a germinal-vesicle oocyte. The cell membranes show dense areas that may represent gap junctions or desmosomes. The terminal contains microfilaments, vesicles with granular contents, and a body resembling a lysosome (l). Microvilli (mv) and cortical granules (cg) are seen at the oocyte surface. z = zona; p = perivitelline space; o = ooplasm. ×49,140.

include cortical maturation of the oocyte expressed by proliferation of CGs [Sathananthan and Trounson, 1982a] and the possible elaboration of a maturation-promoting factor [see Crosby and Moor, 1984] and a sperm-decondensing factor in the ooplasm [Yanagimachi, 1978]. Redistribution of cytoplasmic organelles occurs [Szollosi and Gerrard, 1983] and changes relating to the organization of microfilaments and SER have also been observed [Sathananthan et al, 1984]. CGs are formed from peripheral hypertrophic Golgi complexes, bands of microfilaments disappear, whilst aggregates of small SER become more prominent. There are evidently changes that also take place in the zona, composed of glycoprotein, that increase its receptivity to sperm attachment [Suzuki et al, 1981; Sathananthan et al, 1982]. More sperm attach to the zonae of maturing oocytes than to those of GV oocytes. Changes in the cumulus involve dispersion of the cells, retraction of their processes from the oocyte surface, uncoupling existing cell-junctions, and their progressive luteinization. However, nuclear maturation might be independent of cumulus cell dispersion and uncoupling of cell junctions. This has been observed in a few cultured oocytes.

The complex interrelationship demonstrated between corona cell processes and the oolemma of GV oocytes is noteworthy. It is tempting to postulate a type of cellular interaction analogous to neural transmission, where secretory vesicles may be transported down the corona cell process aided by microtubules, which perhaps act as guide rails. The microfilaments seem to have a contractile function and probably play a role in the initial extension and later retraction of the processes from the oocyte surface. These processes are known to form gap junctions with the oolemma through which small molecules could be transported to the oocyte [Szollosi and Gerrard, 1983; Crosby and Moor, 1984]. The mode of transport of larger molecules is not clear, and this aspect of cumulus-oocyte interaction needs further investigation.

The aberrations of oocyte maturation also conform to those reported previously [Zamboni et al, 1972; Thompson and Zamboni, 1975]. The ageing of oocytes, in particular, is of considerable importance in IVF, as there is evidence that they become unfertilizable or, if fertilized, result in polyspermy. Explosions of CG, observed in some polyspermic ova [Sathananthan and Trounson, 1982b], could be traced to ageing oocytes showing peripheral aggregations of CGs [Sathananthan et al, 1984]. Scattering of chromosomes during maturation may cause chromosome nondisjunction or lagging and also lead to the formation of subnuclei. These nuclei may be confused with pronuclei in the assessment of fertilization in vitro. It must, however, be noted that since meiosis is a long-drawn-out process, being initiated in the fetal ovary, chromosome abberations occur in vivo, as well, and may be expressed in the final stages of maturation in vitro.

In conclusion, oocyte maturation is a complex process and both compartments of the cumulus-oocyte complex undergo significant morphological as well as physiological and biochemical changes during maturation. All of these events are triggered directly or indirectly by gonadotropic hormones (FSH and LH), which stimulate synthesis of steroids by follicle cells, particularly oestrogen and progesterone, that may also play an important role in the maturation process (see Crosby and Moor [1984] for review).

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