—Mini Review—
Ultrastructure of the Human Oocytes during In Vitro Maturation

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Abstract: *Following the maturation of follicular cells, the zona pellucida, the oocyte nucleus and cytoplasm, oocytes are ready for fertilization and further embryonic development. The mechanism of nuclear maturation has been investigated previously, however, cytoplasmic maturation has not been described clearly. From our ultrastructural observation, we defined cytoplasmic maturation as development of smooth endoplasmic reticulum, aggregation and development of mitochondria, and extension and ramification of microvilli on the surface of human oocytes. Furthermore, metaphase I oocytes, which show no specific features under light microscopy, have been found to undergo important changes in the whole maturation process. In metaphase I oocytes, organella such as mitochondria and smooth endoplasmic reticulum develop well and the communication between follicular cells is initiated. Thus, ultrastructural study may contribute to the elucidation of the complicated maturation process of human oocytes during in vitro maturation (IVM).*
Key words: Oocyte, Oogenesis, Maturation, Ultrastructure, In vitro maturation

Introduction

The oocyte is the largest cell in the human body, and is essential for the origination of a new life [1]. Although its function and morphology have been studied by many authors, its maturation mechanism has not been sufficiently clarified, especially in humans.

The IVM of oocytes in mammals was first reported by Pincus *et al.* [2]. Recently, the IVM of human oocytes has been clinically applied for patients with polycystic ovary syndrome [3]. The IVM procedures in humans have spread worldwide since the first report by Cha *et al.* [4]; however, their success rates of the procedure remain low. To improve the success rates, clarification of the oocyte maturation mechanism is required. For this purpose, in addition to physiological and endocrinological studies, a morphological approach, like those we used to elucidate the mechanism of exocytosis in decidual tissues [5, 6].

In this review, the ultrastructure of the oocyte during maturation in the IVM procedure from the germinal vesicle (GV) stage to the metaphase I and II stages is discussed to encourage greater understanding the maturation mechanism of human oocytes.

Oocyte Maturation

Primordial germ cells migrate to the gonadal ridge [7] and undergo mitosis to form oogonia, and the oocytes are pooled in the ovaries. Oogonia may initiate meiosis and then stop at the GV stage. On the other hand, developing follicular cells may form an unit with oocytes to be primordial follicles. The oocytes mature and have endocrine and autocrine relationships with the follicular cells [8].

In the initial stage of oocyte growth, neurotrophic factors such as neurotrophin 3 (NT3) and neurotrophin 4 (NT4), as well as brain-derived neurotrophic factors (BDNF) affect cells near the ovarian hilus which are invaded by nerve fibers [8]. Kit ligand, produced by follicular cells, functions with its receptor c-kit to initiate oocyte maturation by paracrine action. In addition, cyclic AMP plays a role in the expression of Kit ligand. Moreover growth differentiation factor 9 (GDF-9), a member of the TGF-β family, is only produced in oocytes and accelerates the maturation of follicular cells. Thus, the maturation process proceeds by mutual communication between follicular cells and oocytes. When follicular cells, as well as the zona pellucida,

Fig. 1. Light microscopic images of human oocytes. The germinal vesicle oocyte possesses a clearly defined vesicle (GV) in the cytoplasm (a). No characteristic components are observed in the metaphase I oocyte (b). A characteristic of metaphase II oocytes is the presence of the first polar body (PB) in the perivitelline space (c).

nucleus, and cytoplasm of the oocyte reach maturation, the oocytes become competent to undergo fertilization and further embryonic development. Nuclear maturation is known as GV breakdown and protrusion of the first polar body and cytoplasmic maturation is accomplished by growth of organella and structures in the cytoplasm. Among those maturing processes, cytoplasmic maturation is the most important for further development and acquirement of competence for fertilization. It may indicate the significant role of the cytoplasm abnormalities, such as vacuolar cytoplasm or central granulation, which result in low potential for development to good quality blastocysts [9].

Methodology

In this study, 10 immature and 12 mature oocytes donated for an IVM program were used. The IVM procedure had been conducted for patients with polycystic ovary syndrome. The oocytes retrieved were cultured for 24 h *in vitro* and matured oocytes were inseminated by intra cytoplasmic sperm injection. The fertilized and cleaved embryos were transferred at Day 3 or Day 5 stages. The cultured oocytes during IVM were pre-fixed at each stage immediately after culture. The study was approved by the IVF JAPAN Ethical Committee and the informed consent of all donors was obtained.

Donated oocytes were prefixed with 2% glutaraldehyde and 4% paraformaldehyde for 4 h at 4°C, and were washed and placed in PBS at 4°C overnight. They were then transferred at room temperature into serial dilutions of ethanol (30, 50, 70, 80, 90, and 100%), 10 min in each dilution, then transferred into a 1 : 1 mixture of 100% ethanol and n-butyl glycidyl ether for 10 min, and finally into the n-butyl glycidyl ether for 10 min, each step at room temperature. The prepared oocytes were then placed in a 1 : 1 mixture of n-butyl glycidyl ether and EPON 812 at room temperature, left overnight, and subsequently placed in EPON 812 for 10–12 h at room temperature. Following this, they were embedded in EPON 812 blocks and kept at 35°C for 10–12 h, 45°C for 10–12 h, and finally 60°C for 24 h.

The oocyte specimens in EPON 812 blocks were sliced by ultra microtome, and the ultrathin sections (~90 nm thickness) were stained with 0.6% tannic acid and 1.4% P-nitrophenol for 5 min, 5% phosphomolybdic acid for 60 min, and finally with Reynolds lead citrate for 5 min. These ultrathin sections were observed with a JEM-1011 transmission electron microscope (JEOL Datum Ltd., Tokyo, Japan).

Results and Discussion

Light microscopic appearance

Immature oocytes possessed a nucleus which was clearly defined by nuclear membrane and was characterized by a dense nucleolus (Fig. 1a). Metaphase I oocytes could not be characterized by light microscopic observation (Fig.1b). Extruded polar bodies were seen in the perivitelline space of metaphase II oocytes (Fig. 1c).

Ultrastructure of human oocytes

There are many studies of the morphology of oocytes of animals such as the mouse [10] and dog [11]. The ultrastructure of human oocytes has also been investigated and reported by several authors such as Zamboni *et al.*, 1972 [12]; Lopata *et al.*, 1980 [13], Suzuki *et al.*, 1981 [14]; Sathananthan, 1985 [15], 1994 [16]; Szöllösi *et al.*, 1986 [17]; Motta *et al.*, 1988 [18]; Sundström and Nilsson, 1989 [19]. In addition, human ovarian follicle kinetics have been elucidated by Gougeon [20]. Recently, the ultrastructural observation

method has been used to observe human oocytes after cryopreservation in order to assess methods of cryopreservation by authors such as Nottola, 2007 [21], 2008 [22]; Camboni, 2008 [23] and Gualtieri, 2008 [24].

The ultrastructural details derived from electron microscope (EM) images of the various components involved in oocyte maturation are presented below.

To acquire fine electron micrographs, an improved fixation technique is needed. Since, oocytes are large cells, and contain a lot of organella, complete permeation of fixation chemicals into the cells is difficult. Therefore, careful procedure for temperature is needed, because success of fixation depends on the temperature.

1. Follicular cells and zona pellucida

Follicular cells, also known as granulosa or cumulus cells, play an important role in the process of oocyte maturation. In the early stage of oogenesis, one layer of follicular cells appears around the immature oocyte, and these follicular cells form primordial follicles. Based on cytoplasm enlargement, follicles possess two or three layers of follicular cells (secondary follicle) and several layers of follicular cells surround the oocyte in the GV stage. At this stage, the follicular cell nucleus occupies most of the cell, with few organella such as mitochondria and smooth endoplasmic reticulum (ER) (Fig. 4). These follicular cells possess features similar to fibroblasts. In the process of oocyte maturation, follicular cells also increase in number and change in morphology. Organelles increase in the follicular cells surrounding the oocytes as well. In a metaphase I oocyte, the size of the cell itself and the proportion of the nucleus increase remarkably. More mitochondria and smooth ER are seen in the cytoplasm and lipid droplets and microfilaments may appear. The communication between follicular cells and oocytes is initiated during this stage (described later). In the metaphase II oocyte, follicular cells separate, radiate, and disperse (i.e, the corona radiata). At this stage, the shape of the mitochondria appears different from those in GV and metaphase I oocytes; mitochondrial size increases and cristae are well identified in some cells. The smooth ER enlarges and becomes round, and lysosomes are sometimes observed in overmature cells. The follicular cells are metabolically coupled with oocytes via gap junctions, which play a role in communication similar to that reported in decidual cells [25]. Nutritional and regulatory elements responsible for oocytes functions are transmitted through the gap junctions [26].
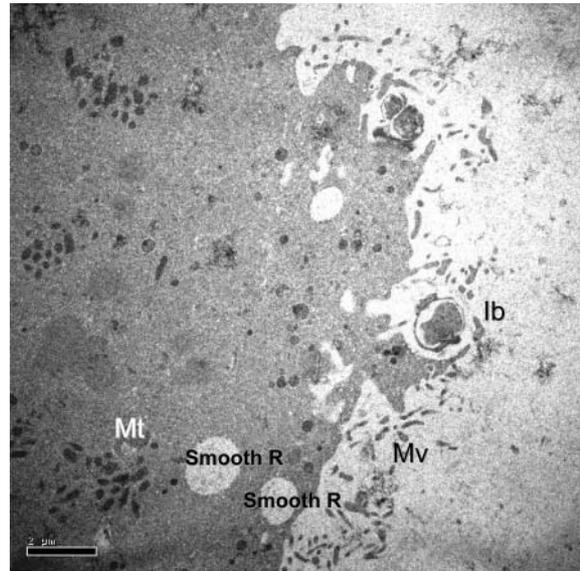
The zona pellucida undergoes maturation from

Fig. 2. An oocyte before *in vitro* culture. The germinal vesicle oocytes show few organella in the cytoplasm before culture. The microvilli (Mv) on the surface are short and have simple shapes. Oval or short tubular-shaped mitochondria (Mt) are observed in the cytoplasm. Few smooth endoplasmic reticula appear near the surface of the cell. Irregular shaped inclusion bodies (Ib) are present in the perivitelline space (8,000×, scale bar = 2 μm).

metaphase I to metaphase II. Spermatozoa never bind to the zona pellucida of the metaphase I oocyte, but will bind to the metaphase II oocyte with a mature zona pellucida [27].

2. Communication between the oocytes and follicular cells

In the metaphase I oocyte, the processuses extend from the follicular cells (Fig. 5), and penetrate the zona pellucida to reach the surface of the oolemma. This linkage is not observed in the mature oocytes. During the oocyte maturation process, maternally inherited genes are expressed in the earlier stages, and new proteins and m- RNA are synthesized in the later stages [28]. Part of the information required for these activities may be transmitted from follicular cells.

3. Oolemma and microvilli

The development of microvilli is distinctive. At the GV stage, microvilli appear as short processuses from the surface of oolemma (Fig. 2). At the metaphase I stage, microvilli grow in size and increase in number. At the metaphase II stage, microvilli mature and show
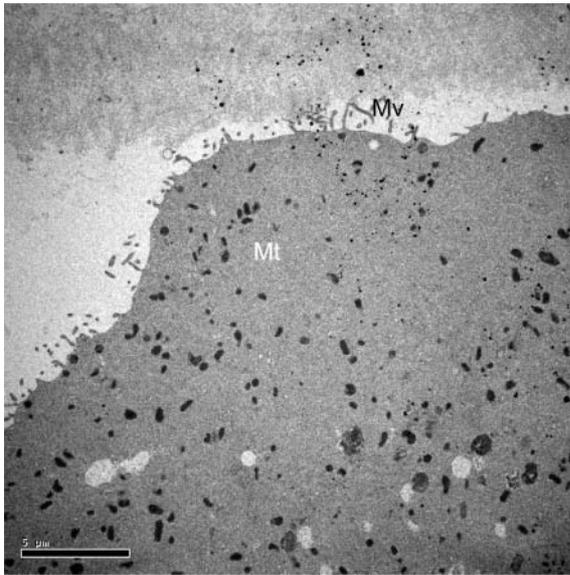
Fig. 3. A germinal vesicle oocyte cultured *in vitro* for 6 h. The oocyte still has low and undeveloped microvilli (Mv) on the surface. Round-shaped mitochondria (Mt) are scattered in the cytoplasm (5,000×, scale bar = 5 μm).
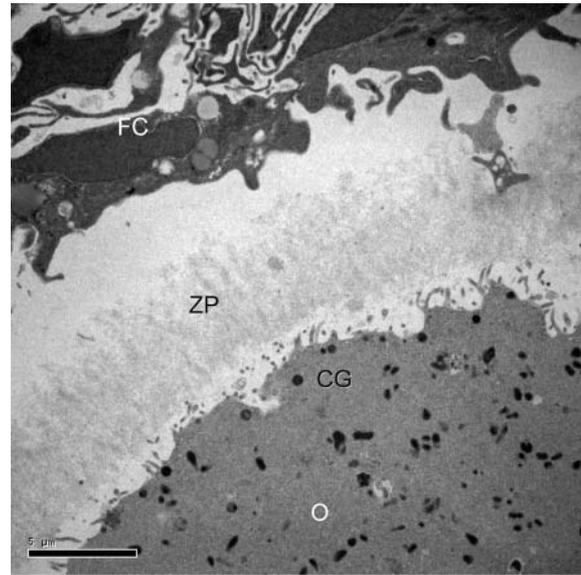

Fig. 4. Follicular cells (FC) and, zona pellucida (ZP) of an oocyte (O) cultured *in vitro* for 6 h. A large, condensed nucleus occupies the follicular cell and appears similar to fibroblasts. At this stage, communication between the oocyte and follicular cells has not been initiated. There are a few cortical granules (CG) in the periphery of the oocyte (5,000×, scale bar = 5 μm).

complicated ramifications (Fig. 8). They appear to absorb substances secreted from the ooplasm, such as enzymes and proteins. Microvilli activity is most prominent during polar body extrusion.

4. Cortical granules

Cortical granules are characteristic components that appear beneath the surface of the oolemma, and are composed of a distinct membrane with differentially stained small particles. They have a homogenous core surrounded by a thin halo next to the membrane [29]. The function of cortical granules is uncertain; however, it is related to fertilization and the prevention of polyspermia [30]. At the GV stage, cortical granules are dispersed throughout the whole cytoplasm, but are not prominent (Fig. 4). At the metaphase I stage, their numbers increase and they appear denser. At the metaphase II stage, these align beneath the surface of the oolemma. Such alignment is an indication of oocyte maturation [31].

5. Mitochondria

Mitochondria play an essential role in oocyte development, and are composed of a double membrane, the internal part of which forms cristae that extend into a matrix. The matrix contains mitochondrial

DNA which is different from that in the nucleus. Mitochondria produce energy by oxidative phosphorylation through the electron transport system.

Oxidative phosphorylation by the respiratory chain produces ATP, an important energy source for cells. This activity is encoded by both nuclear and mitochondrial DNA. At the GV stage, there are fewer mitochondria compared with at other stages and they are small in size, mostly round, but sometimes tubular in shape (Fig. 2). At the metaphase I stage, their number increases and elongated mitochondria appear (Figs. 6 and 7). In mature oocytes, the appearance of mitochondria is characteristic and the number of mitochondria increase and aggregate in the center of the cells (Figs. 9 and 10). Immature oocytes have a scant population of cytoplasmic organelles, which mainly cluster around the nucleus. Accompanying this mitochondrial maturation, other organella migrate closer to the cell periphery [32]. Sundstörm [31] reported on the cytoplasmic changes related to oocyte maturation and noted a clustering of mitochondria and an aggregation of tubuli in the smooth ER surrounded by mitochondria.
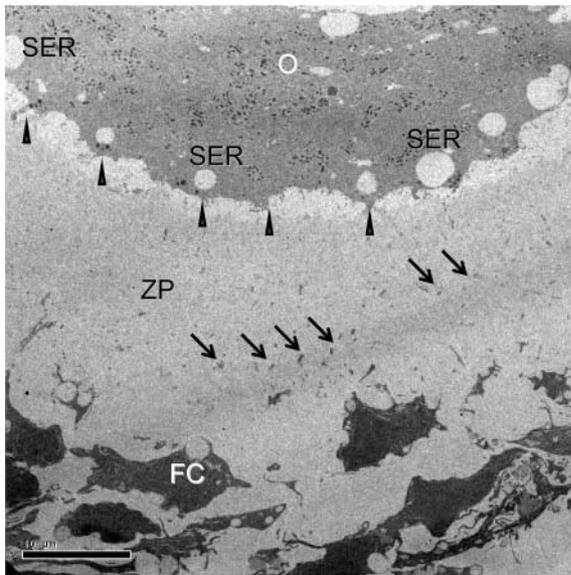
Fig. 5. Follicular cells (FC) and zona pellucida (ZP) of an oocyte (O), and cultured *in vitro* for 12 h. Processuses extend through the thickened zona pellucida to the oocyte (arrow head). At the part of the surface of the oolemma from which the processuses start, oolemma projections are present. Many penetrations are observed in the zona pellucida (arrow). Prominent smooth endoplasmic reticulum (SER) is present adjacent to the oolemma (2,500×, scale bar = 10 μm).
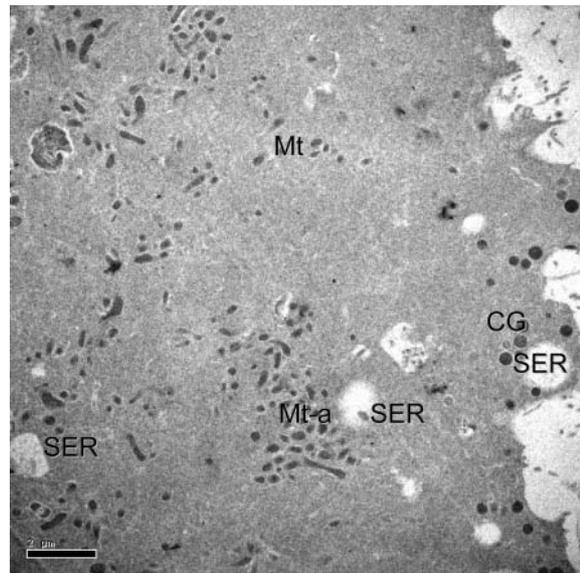

Fig. 6. An oocyte cultured *in vitro* for 12 h. The number of mitochondria (Mt) has increased, and some Mt exist separately, while others are aggregated (Mt-a) at this stage, and partially seen in the cytoplasm. Smooth endoplasmic reticulum (SER) is oval-shaped. Cortical granules line part of the oolemma surface (8,000×, scale bar = 2 μm).

6. ER

The ER has many functions in the oocyte. Proteins are synthesized, cut, and folded in the ER. In addition, proteins are transported by the budding ER to other organella such as the Golgi apparatus [33]. Moreover, the ER is associated with the synthesis of mRNA and lipids and is a place for storing calcium; it also adjusts the volume of calcium in the cells.

Smooth ER is seen in all stages of oocyte maturation. There are two smooth ER shapes; vesicular and tubular. Smooth ER is well known to be associated with the Golgi apparatus and is associated with protein and mRNA synthesis. At the GV stage, vesicular smooth ER is scattered and the vesicles are small (Fig. 2). At the metaphase I stage, they grow in size and increase in abundance (Fig. 6). While only small vesicular smooth ER are present at the initial stage of oocyte maturation, the tubular type smooth ER can be observed in metaphase I oocytes. A ballooned form of smooth ER appears at the oocyte periphery (Fig. 5) in the late stage of metaphase I, and its appearance indicates active protein synthesis. This suggests that the ballooned
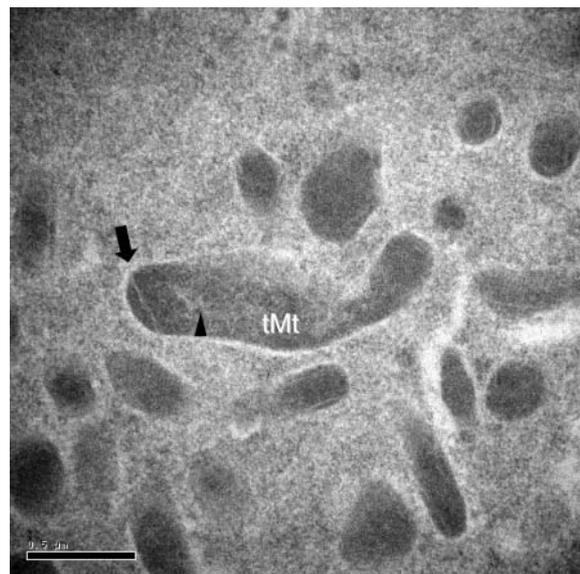

Fig. 7. High magnification image of oocyte cytoplasm of an oocyte cultured for 12 h. The development of mitochondria is dominant and many long tubular shaped mitochondria (tMt) are observed. Note the cristae (arrow) and small particles (arrow head) in the Mt (50,000×, scale bar = 0.5 μm).
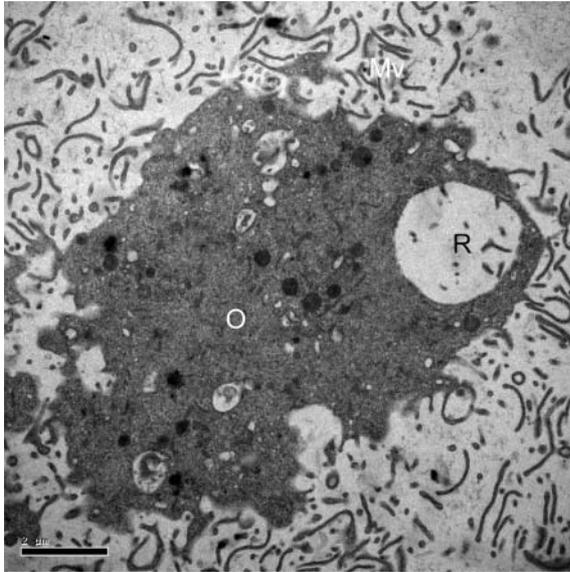
Fig. 8. An oocyte (O) cultured for 24 h. The micrograph shows the edge of the oocyte, where the development of microvilli (Mv) is prominent. The Mv extend into the perivitelline space, branching intricately. Retraction of oolemma can be observed at the periphery (R) (10,000×, scale bar = 2 μm).
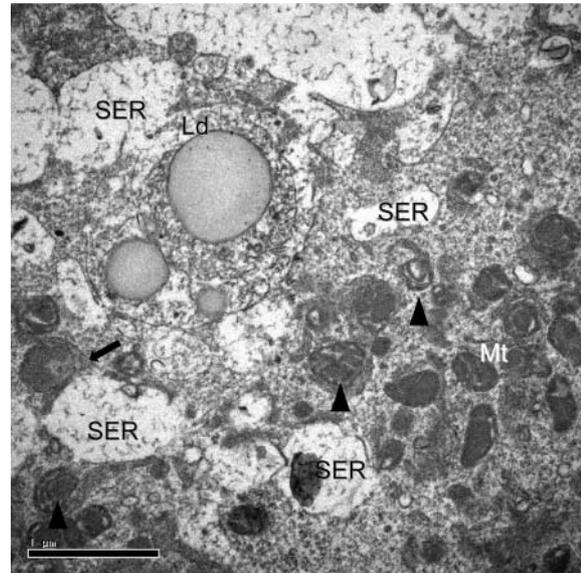

Fig. 10. High magnification image of cytoplasm of an oocyte cultured for 24 h (a metaphase II oocyte). Mitochondria (Mt) are aggregated in the center of the cytoplasm and cristae are clearly observed in some Mt (arrow head). Ballooned smooth endoplasmic reticulum (SER) can be observed. Some SER is associated with mitochondria (arrow). Lipid droplets (Ld) appear round in shape (50,000×, scale bar = 1 μm).
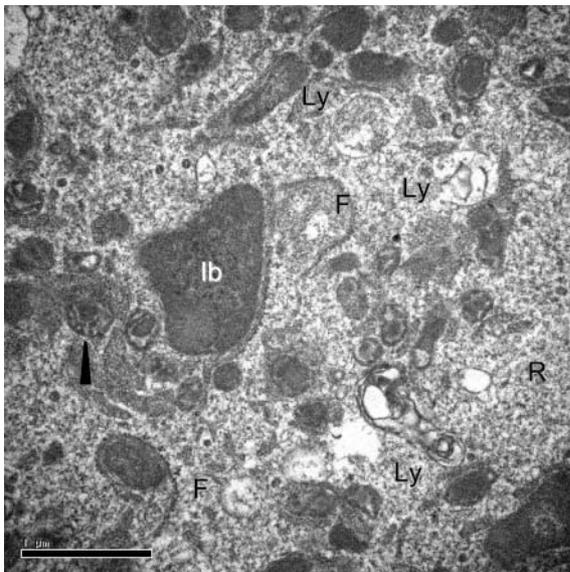

Fig. 9. The cytoplasm contains numerous different organella in metaphase II oocytes. Fibrillar components are frequently observed and some surround the mitochondria. Free ribosomes (R) are clearly observed. The existence of large inclusion bodies (Ib) and lysosomes (Ly) are also characteristic at this stage. Mitochondria cristae are clearly defined (arrow head) (50,000×, scale bar = 1 μm).

form may be related to the synthesis of cortical granules and their exocytosis. At the metaphase II stage, many smooth ER are ballooned, and are associated with mitochondria (Fig. 10). Rough ER are also the most commonly observed organelle in the cells; however, they are rarely seen in oocytes.

7. Golgi apparatus

The Golgi apparatus is composed of multilayered cisternae and small granules, and is a common organelle for the synthesis of proteins. They are rarely observed in growing oocytes, and are reported to be related to the synthesis of cortical granules [34].

8. Lysosomes

The lysosome is an organelle that functions to recycle unnecessary and digested substances from cells. Waste products that are taken into the cells by autophagy and endocytosis are hydrolyzed in the lysosome. Useful substances after hydrolysis are absorbed into the cytoplasm and unnecessary ones are left as residual bodies.

There are two kinds of lysosomes in the cytoplasm:

primary lysosomes contain enzymes for hydrolysis; and secondary lysosomes that contain enzymes and waste products. Both types are seen in metaphase II oocytes (Fig. 9), but not in GV oocytes (Fig. 2).

9. Annulate lamellae

The annulate lamellae is a multicisternal structure in the cytoplasm, and is rarely observed in an oocyte. However, they may be observed during any stage of maturation and their appearance is known as an indicator of cell degeneration.

Pores, similar to those in the nuclear membrane, are observed in the lamellae, and it has been reported that annulate lamellae will transform itself into ER cisternae [35].

Conclusion

Recently, endocrinological, immunological, and genetic methods have been applied to the elucidation of cell functions in oocytes. However, the investigation of morphology, especially at the microstructural level, gives us fundamental knowledge of the various aspects of oocyte maturation. Modern EM capabilities have progressed remarkably along with associated computer improvements. The EMs are no longer expensive complicated instruments available only to select researchers. Currently available portable scanning EMs are placed on a table and used for study by children in Japanese primary schools.

Previously, many researchers have described the ultrastructure of human oocytes; however, much remains to be investigated. Thus far, many of these ultrastructural studies have been performed using immature oocytes obtained from *in vitro* fertilization programs. These oocytes had failed to mature, and thus, their data may not be sufficient for elucidating the maturation mechanism of human oocytes for the improvement of IVM procedures. Hence, this study is significant in terms of the materials acquired from the patients undergoing IVM.

The IVM procedures have been widely applied, clinically, by many centers. However, the IVM use has generally been limited to polycystic ovary syndrome patients. If the morphological aspects of oocyte maturation are clarified and better clinical results are achieved, the IVM might become an acceptable alternative to conventional IVF.

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