EMBJ.ORG FOR YOUNG DOCTORS

EUROMEDITERRANEAN BIOMEDICAL JOURNAL
2019,14 (34) 145–151
(*FORMERLY: CAPSULA EBURNEA*)

Review

CLINICAL ELECTRON MICROSCOPY IN THE STUDY OF HUMAN OVARIAN TISSUES

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ARTICLE INFO

Article history:
Received 19 October 2019
Revised 13 November 2019
Accepted 17 November 2019

Keywords:
Electron Microscopy, Oocytes, Ovary, Ovarian Neoplasms, Extracellular Vesicles.

ABSTRACT

Electron microscopy (EM) is a revolutionary imaging technique for obtaining high-resolution images of biological specimens in many fields, including the study of human ovarian tissues. EM, can be a powerful tool in many clinical fields, such as in therapeutic procedures for infertility. The success of Assisted Reproductive Technologies (ART), for example, is closely related to oocyte's quality, whose morphological features can be evaluated by EM to understand if the oocyte is mature, healthy and prone to fecundation. Moreover, EM studies are useful to demonstrate the responsiveness of the ovarian cells to environmental toxicants. EM can also be the starting point for the study of the release of extracellular vesicles from human ovarian cancer cells. These structures represent an attractive target of studies aimed at identifying clinically useful markers for liquid biopsies. This paper summarizes the potential applications of EM in ART and ovarian pathologies and its clinical implications.

1. Introduction

Electron microscopy (EM) has been a revolutionary imaging technique over the past 80 years, for obtaining high-resolution images of both biological and non-biological specimens.

The use of electrons as the source of "illuminating" radiation, given their very short wavelength, results in high-resolution images that provide outstanding information on the ultrastructural features of cell functions in health and disease. TEM (Transmission Electron Microscopy) for many aspects is "similar" to the light microscope and electrons pass through thin specimens generating a projection image. SEM (Scanning Electron Microscopy) relies on the emission of secondary electrons from the specimen's surface and provides detailed three-dimensional images of the cell surface.

The use of these two techniques, especially when combined, allows the study, for example, of the ultrastructural characteristics of the human oocyte *ex vivo*, as well as of the morphological features of healthy and ovarian tumour cells *in vitro*.

In the first case, these studies allow understanding if the oocyte is mature and healthy and, prone to fecundation. In the second one, they offer information that would enable to broaden the knowledge of healthy and cancer cells' biological processes paving the way for clinical applications, e. g — liquid biopsies.

2. Role of Electron Microscopy in clinical settings

EM is useful in several clinical fields as infectious diseases (1), oncology (2, 3), dentistry (4, 5), and in therapeutic procedures for infertility (3, 6, 7).

2.1. Assisted Reproductive Technologies

The success of Assisted Reproductive Technologies (ART) is closely related to oocyte's quality, which can be evaluated morphologically by EM (3).

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DOI: 10.3269/1970-5492.2019.14.34
 ISSN: 2279-7165 - Available on-line at www.embj.org

The purpose of this section is to describe the features of the fertilizable oocyte, and the main morphological alterations that may occur during procedures for infertility management by light (LM) and by TEM and SEM.

Observations by LM allow the morphological evaluation of the general characteristics of the oocyte (shape, size, general appearance of the ooplasm, integrity of the zona pellucida - ZP, perivitelline space - PVS, features of surrounding granulosa cells - GC). The analysis by TEM allows a more detailed examination and therefore, a better understanding of the subcellular characteristics of the oocyte itself (8).

On the other hand, to obtain more powerful, three-dimensional, fascinating and easily interpretable images of the surface of the oocyte and its coatings, the use of SEM is advisable (9).

Observed by LM, the healthy mature human oocyte has a round shape, and the ooplasm has a uniform texture with numerous uniformly dispersed organelles. The ZP, a glycoprotein matrix, forms a continuous layer, regular in thickness that surrounds the oocyte. A thin PVS space that embeds the 1st polar body, that is visible only in particularly favourable sections, separates the oocyte from the ZP.

It surrounds the oocyte both in the ovarian follicle and after ovulation. (Figure 1)

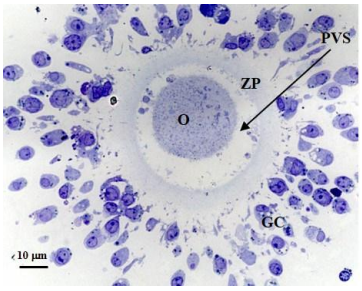

Figure 1. LM of a mature oocyte and its surroundings. ZP: zona pellucida, PVS: perivitelline space, GC granulosa cells, O: oocyte. Original magnification: 200x. Staining: toluidine blue. Scale bar is 10 µm.

2.1.1. Ooplasm

The ooplasm is rich in organelles, mainly represented by aggregates formed by mitochondria and membranes and by cortical granules (CGs) (Figure 2).

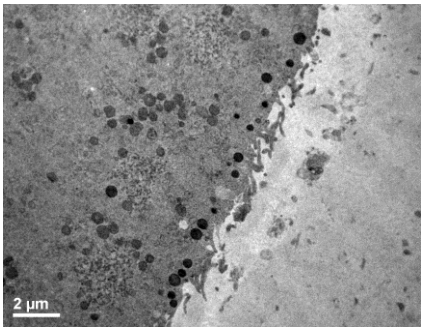

Figure 2. TEM: cortical cytoplasm and inner ZP of a normal human mature oocyte. Scale bar is 2µm.

2.1.1.A. Cytoplasmic aggregates

Mitochondria are the most abundant organelles in the ooplasm of mature human oocytes. They have a spherical or oval shape, a dense matrix and a few arciform or transversal peripheral crests. Mitochondria are characteristically associated with membranes of the smooth endoplasmic reticulum (SER), resulting in the formation of peculiar aggregates located mainly in the cortical area of the ooplasm. These structures are composed of large aggregates of tubules anastomosed to each other and closely associated with mitochondria (M-SER aggregates), or they appear as small vesicles containing a moderately electron-dense material, surrounded by mitochondria (MV complexes).

These aggregates are likely involved in the energy production and storage at fertilization and during first embryo cleavages. Structural dysmorphism of SER, detectable by phase-contrast microscopy, has been associated with compromised embryo development and implantation, thus confirming the crucial role of M-SER during fertilization and the first embryo cleavages.

The ratio of M-SER aggregates/MV complexes seems to be influenced by in vitro culture.

The ultrastructural analysis revealed a general decrease in M-SER aggregates amount and a proportional increase in atypical MV complexes in in vitro matured oocytes and after prolonged culture (Figure 3).

These complexes, presumably derived from a morphodynamic remodelling of the aggregates mentioned above. They can be considered ultrastructural markers of an oocyte aging both in vitro and in vivo (10,11).

2.1.1.B. Cortical granules

The CGs, in mature oocytes, are placed immediately below the oolemma, in a linear, continuous and regular fashion, they are round and electron-dense.

CGs are responsible for the so-called cortical reaction, a rapid and massive release of CG content in the PVS that occurs only at fertilization. The cortical reaction and the consequent hardening of the inner layer of the ZP (zona reaction) are necessary to block polyspermy.

They originate from the Golgi apparatus when the oocyte is still immature and progressively increase in number during oocyte maturation, migrating towards the oolemma.

Several alterations in number, localization and electron density of CGs appeared in aged oocytes (10,11) (Figure 3), during procedures of cryopreservation (6,8) and during in vitro culture (9, 11).

Oocytes retrieved from women of advanced age display an abnormal reduction in the amount and inner density of suboolemmal CGs. In in vitro matured oocytes isolated CGs, still migrating toward the cell periphery, have been sometimes detected in the inner ooplasm demonstrating an impaired cytoplasmic maturation (10).

Cryopreservation of mature oocytes influences the distribution of CGs. They generally appear sparse, forming a discontinuous layer irrespective of the protocol applied while the cryopreservation of oocytes at the germinal vesicle stage does not seem to influence the morphology of CGs (6, 8, 12, 13).

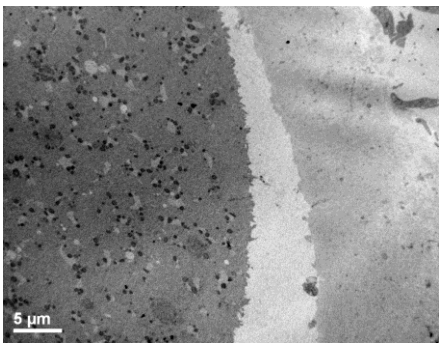

Figure 3. TEM: cortical cytoplasm and inner ZP of a dysmorphic human oocyte. Scale bar is 5µm.

2.1.1.C. Other organelles

The presence of multiple vacuoles in the ooplasm is a sign of oocyte degeneration, and they appear during procedures of slow freezing (12, 13). Vacuoles appear as empty structures, irregularly rounded and bordered by a membrane, often interrupted. Vacuolization after cryopreservation may represent one of the most accurate markers of cryodamage sensitivity.

Lysosomes and multivesicular bodies, associated with vacuoles, are present in the ooplasm of damaged oocytes. The presence of these organelles correlates with autolysis/autodigestion processes.

2.1.2. Oolemma and perivitelline space

The oolemma of mature oocytes presents digitiform protrusions (microvilli) projecting into the PVS. They are involved in sperm binding and fusion. Typical microvilli are multiple, regularly distributed, long and thin. Dysmorphism of microvillar pattern occurs during aging (10, 11), during in vitro culture (6, 9) and during cryopreservation (12, 13) (Figure 3).

2.1.3. Zona pellucida

Examined at the SEM, the external surface of the ZP that surrounds mature and healthy oocytes presents a typical spongy appearance, and, by TEM, it consists of granular filaments and agglomerates (14, 15). It allows species-specific fertilization, prevents polyspermy, and permits the acrosome reaction. An altered appearance of ZP, especially a compact internal layer, may suggest a premature release of CGs and may compromise the fertilization (10).

2.2.Ovarian environmental toxicology

The ovary is the target of various environmental toxicants. Pesticides seem to be particularly important, in this context. They are chemicals used extensively in agriculture and, in recent years, exposure to pesticides has attracted much attention in the scientific community, especially concerning possible adverse reproductive effects. Pesticides produce adverse effects on female fertility mainly through the mechanism of endocrine interference (e.g. interaction with hormone receptors, steroidogenesis and hormone metabolism) (16).

The high interest in this problem stimulated programs of pesticide residue monitoring, providing useful data for the assessment of indirect exposure to endocrine disruptors in the general population.

These substances often have physical-chemical properties that make them very stable and slow to degrade over time (lipophilicity), remaining in the environment (soil, food and water) for many years. The exposure, therefore, takes place both in the agricultural professions and in the general population, through the food residues/air and water contamination (16). Unfortunately, there are no ultrastructural studies in humans. Indeed, specific animal models largely contributed to the knowledge of pathophysiological bases of follicular depletion due to exposure to pesticides. It seems that the main target of pesticides is the somatic compartment of the ovarian follicle.

The cells surrounding the oocyte contribute to oocyte maturation and fertilization through gap junction communication (9).

Several studies investigated the role of the follicular microenvironment in the maturation of the oocyte. Follicular structures sustain the development of fertilizable oocyte and early embryo, primarily through hormone synthesis (17, 18).

Besides, the follicular cells are quite sensitive to environmental toxicants, such as endocrine disruptors agents. Indeed, EM studies demonstrated a dose-related degeneration of the granulosa cells (GCs), after exposure to endocrine disruptors (19-21).

The GCs in the antral follicle consists of two populations: the parietal cells and the cumulus oophorus cells (whose deeper cells are called corona radiata cells). The parietal GCs have a cuboidal or polyhedral shape and are joined together by a dense network of cytoplasmic protrusions. The corona radiata cells have an elongated shape and are separated by large intercellular spaces (Figure 1) due to the phenomenon of cumulus expansion. However, both populations present typical ultrastructural characteristics of steroidogenic cells. The nuclei are oval and have one or often two prominent nucleoli. In the cytoplasm, numerous mitochondria, electron-dense lipid droplets, and cisternae belonging to the rough endoplasmic reticulum and Golgi complex are present (22-24).

Exposure of pesticides at low doses causes moderate reduction of cell contacts, occasional membrane blebbing (Figure 4) and sporadic chromatin margination and condensation. Highest doses lead to extensive cytoplasmic vacuolization, severe membrane blebbing, rupture of membrane, up to, in the most damaged samples, cell lysis, with the presence of abundant cellular debris. All these features are considered ultrastructural signs of apoptosis.

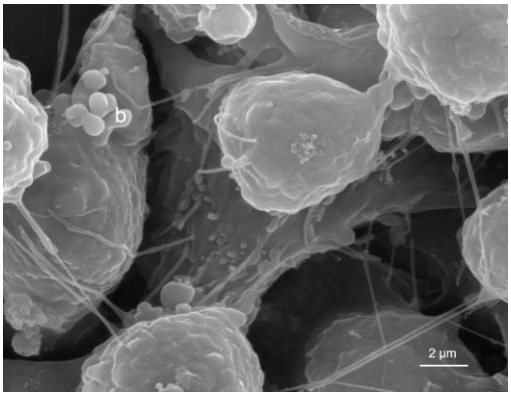

Figure 4. SEM: Mouse granulosa cells cultured with low dose of pesticide Mancozeb. b: blebs. Scale bar is 2µm.

3. Ovarian cancer: contribution of basic research to biomarkers' identification

Ovarian cancer is the deadliest gynaecological malignancy. It has a poor prognosis, the overall 5-year survival rate being of approximately 50%. The 5-year survival rate could rise to 90% if the cancer is within the ovary at the diagnosis, but this occurs only in 20% of patients (25). The disease consists of a heterogeneous group of neoplasia, of which about the 90% are epithelial (mucinous, serous, endometrioid, and clear cell subtypes) (26); most of the women are diagnosed at the stage of and die for a high-grade serous ovarian cancer subtype (HGSOC). HGSOC is way more aggressive than the low-grade serous ovarian cancer, and it presents a poorer prognosis (27).

There is a great debate regarding the origin of HGSOC (27, 28, 29). Cancer cells may arise from fallopian tubes, whose inner surface is lined by an epithelium consisting of ciliated cells (30). On the other hand, cancer cells may originate from the ovarian surface epithelium (OSE), which is composed of a single layer of flat/cuboidal epithelial cells (31).

Anyhow, an early diagnosis is desirable for offering patients a better chance of being cured using available therapies, consisting of a combination of surgery and chemotherapy (32). Standard methods for ovarian cancer diagnosis are copious and include instrumental or serological investigations (for a review see 33). In this regard, in the last years, the liquid biopsy, a valid alternative to tissue biopsy, has been watched as a promising tool for ovarian cancer detection, diagnosis and therapy response monitoring (34).

Liquid biopsy refers to the analysis, into the blood, of circulating tumor cells (CTCs), circulating tumor DNA (ctDNA) and exosomes (EXOs) released from primary and/or metastatic tumors. The possibility to screen blood samples for markers (DNA, RNA, proteins) of tumor origin (in the form of CTCs or ctDNA or EXOs) has the benefit of being less invasive, painful and risky for patients and the potential to reflect the biological status of parental cancer cells (35).

Primary tumor standard tissue biopsies are unlikely able to reflect the broad heterogeneity that is typical of most tumors. There being a plausible possibility to miss the most aggressive subclones and, consequently, relevant information to correctly direct clinical treatments (36).

The further advantage of the possibility to proceed with repeated liquid biopsies (usually not feasible with standard tissue biopsy) to monitor the pathology after treatments is worthy of serious consideration.

Liquid biopsy from blood samples, in oncology, can rely, as mentioned, on intact circulating tumor cells, circulating cell-free nucleic acids (cfDNA, cfRNA or cfmiRNA) and other structures, such as exosomes released into the bloodstream by primary or metastatic cells (36).

Circulating tumor cells are cells that have been able to detach from tumor and to extravasate into the bloodstream; they are rich in DNA, RNA and proteins useful as cancer markers.

Cell free nucleic acids derive from apoptotic and necrotic tumor cells and are discharged into the bloodstream as well.

Exosomes are lipid bilayer-enclosed extracellular vesicles (EVs). They are secreted by tumor cells into the tumor microenvironment; from there, they may reach almost all biological fluids. EXOs, whose composition reflects that of parental cells, contain proteins, lipids and nucleic acids; the latter are way more stable inside EXOs, in contrast with their pronounced instability into the blood (36).

Normal and tumor cells release EXOs along with microvesicles (MVs), both functioning as intercellular messengers.

EXOs and MVs mainly differ for their size and, most important, cell origin. EXOs range in size from 30 to 100 nm. They develop within the multivesicular bodies by inward budding, and ultimately, are released into the extracellular space. MVs have a diameter from 50 to 1000 nm (but can go up to 10 μm) and are formed directly from an outward budding of the plasma membrane. EVs cargo includes proteins, such as enzymes, growth factors, growth factor receptors, cytokines and chemokines, lipids, and nucleic acids, including mRNA, miRNA, ncRNA, and genomic DNA (37). Molecular studies revealed both similarities and differences in the composition of EVs, as compared to the progenitor cells.

Several studies evidenced the potential use of EVs in liquid biopsy, as a biomarker in non-tumoral (mostly cardiovascular and autoimmune diseases) and tumoral diseases (breast, prostate, colorectal, lung, pancreatic, ovarian cancers and many others) (33, 37-39).

Some molecules (Del-1, Fibronectin, Survivin-2B), for example, may be overexpressed in EVs from breast cancer patients. EVs-associated PTEN could be a useful marker to detect prostate cancer patients (40); glypican-1 in cancer EVs allows to discriminate between patients with pancreatic ductal adenocarcinoma and benign pancreatic patients (41).

Several studies also suggest the emerging role of EVs as diagnostic biomarkers in ovarian cancer. The levels of blood circulating EXOs increase from benign ovarian disease to stage I-IV of ovarian cancer (42) and some miRNAs levels rise in women with invasive ovarian cancer compared to healthy patients or women with benign ovarian cancer (41). More recently, in a multiplex analysis, levels of CA-125, EpCAM and CD24 associated with EVs from ovarian carcinoma were found elevated in 15 women with ovarian cancer compared to 5 healthy controls (35). Mucins MUC1 and MUC16 associated with serum or ascites EVs in ovarian cancer patients are also papabile biomarkers for improved diagnosis of the disease (44).

As for many cancer biomarkers, the in-depth knowledge of the pathophysiological processes is mandatory for clinical applications.

The role of EVs in all ovarian cancer processes is relevant. For example, EVs from ovarian ascites are rich in proteolytic enzymes, such as MMPs, cathepsin B and uPA, whose activation increases extracellular matrix degradation, thus contributing to the pro-invasive characteristics of tumor cells and facilitating their invasion and metastatic ability (45, 46). Ovarian cancer EVs influence angiogenesis, drug resistance, immune escape and tumor microenvironment modulation (44, 47-49).

The evidence that tumor cells release a higher number of EVs than healthy cells undoubtedly suggests the role of such EVs in tumor progression. Indeed, observing the cell surface, by SEM technique, it is possible to notice profoundly different behaviour between healthy cells and tumor cells. Normal Human Ovarian Surface Epithelium (OSE) cells show that they are very spread and completely flat, they are round-shaped, very regular, and the plasma membrane surface appears very smooth (Figure 5).

In contrast, the shape of ovarian tumor cells appears very irregular, and the plasma membrane reveals a lot of dynamic movements that lead to abundant EV release (MVs, in particular). These extracellular vesicles are of extremely heterogeneous size, ranging between 200 and 800 nm (Figure 6).

The identification and the subsequent extensive studies of these phenomena have paved the way for the many experimental studies that have clarified most of the pathways in which EVs are involved, suggesting from time to time which molecules could represent a right candidate as diagnostic and prognostic biomarkers.

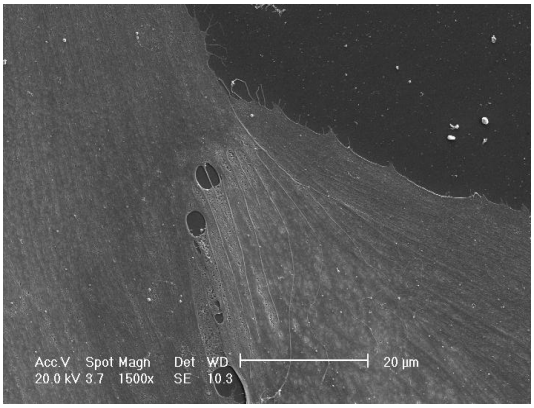

Figure 5. Normal Human Ovarian Surface Epithelium cells (OSE) observed by SEM. Scale bar is 20 µm.

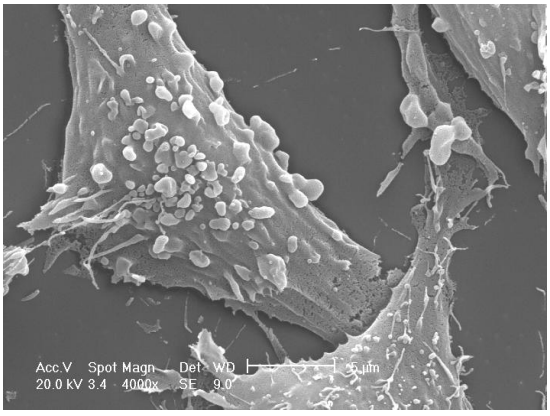

Figure 6. Human ovarian cancer cells (Ovarian endometrioid adenocarcinoma 1A9 cells) observed by SEM. Scale bar is 5 µm.

4. Conclusions

EM could be useful in many aspects of ovarian tissue studies. The explanation of physiological structural and sub-structural parameters of the mature oocyte is useful for a comparison with the dysmorphisms that may occur during several clinical settings, especially in infertility management. In this perspective, it is also essential to understand the mechanisms of action of pesticides on female fertility to understand better the pathologies induced by environmental factors. Undoubtedly the exposure to pesticides compromises the ovarian follicular health in animal models; it would be necessary to obtain ultrastructural data also in humans.

On the other hand, the dissection of biological processes in basic research leads the way to the identification of biomarkers useful for diagnostic and prognostic purposes in ovarian cancer.

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