Ultrastructure of human mature oocytes after vitrification

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Abstract

Since the introduction of human assisted reproduction, oocyte cryopreservation has been regarded as an attractive option to capitalize the reproductive potential of surplus oocytes and preserve female fertility. However, for two decades the endeavor to store oocytes has been limited by the not yet optimized methodologies, with the consequence of poor clinical outcome or of uncertain reproducibility. Vitrification has been developed as the promising technology of cryopreservation even if slow freezing remains a suitable choice. Nevertheless, the insufficiency of clinical and correlated multidisciplinary data is still stirring controversy on the impact of this technique on oocyte integrity. Morphological studies may actually provide a great insight in this debate. Phase contrast microscopy and other light microscopy techniques, including cytochemistry, provided substantial morpho-functional data on cryopreserved oocyte, but are unable to unraveling fine structural changes. The ultrastructural damage is one of the most adverse events associated with cryopreservation, as an effect of cryo-protectant toxicity, ice crystal formation and osmotic stress. Surprisingly, transmission electron microscopy has attracted only limited attention in the field of cryopreservation. In this review, the subcellular structure of human mature oocytes following vitrification is discussed at the light of most relevant ultrastructural studies.

Introduction

Cryopreservation of the human oocytes is currently considered an attractive and useful technology to preserve fertility, although still not fully optimized. Cryostorage could be one of the few options for patients at risk of premature ovarian failure due to potentially gonadotoxic chemo/radio-therapies for malignancies, bone marrow transplantation protocols or a number of severe non-cancerous systemic diseases.[1-4] Oocyte cryopreservation might be used in women of reproductive age who choose to postpone childbearing. Cryopreservation of human oocytes also represents a valid alternative to legal, moral and religious problems concerning embryo freezing in some countries. Also, it does not require the presence of a male partner, thus having great impact on assisted reproduction technology.[5] However, in contrast to the preimplantation embryo cryostorage, it is quite difficult to adequately cryopreserve metaphase II (MII) human oocytes, mainly due to their large size, water content, high degree of cytoplasmic specialization and precise chromosomal arrangement.[6] Freezing of immature, germinal vesicle (GV) oocytes may elude some of the problems occurring during MII oocyte freezing, particularly those related to the cryo-damage of spindle and chromosomes. Nevertheless, the current inadequacy of *in vitro* maturation protocols represents an impediment for obtaining viable mature gametes after cryostorage of immature oocytes, thus significantly reducing the final performance of the whole procedure.[4,7]

Improvements in cryotechnology and modifications of conventional protocols of slow freezing have recently determined an increase of post-thaw oocyte survival, fertilization and embryo cleaving rates.[8] In spite of this, the pregnancy rates and clinical outcomes are still unsatisfactory or at least contradictory.[6] The cryopreservation technique of vitrification (an ultra-rapid freezing technique) offers new interesting perspectives in the field of oocyte cryopreservation, appearing less traumatic than slow freezing.[9-11] Vitrification technology is also described as an easy, quick, effective, low-cost and low-risk procedure.[9,12] However, due to the limited experience reported to date, further studies are needed to determine the actual efficiency and safety of this approach.[13] In particular, at the cellular level, little is known about the potentially detrimental effects of this procedure and, in particular, the high concentrations of the commonly used cryoprotectants.[4] In addition, some data are presented on the comparative efficiency of commercially available vitrification supports (named open and closed devices), but most concern non-human mammals.[4,14,15] A growing attention has been recently given to reduce cross-contaminations in the non-sterile environment of liquid nitrogen freezers, particularly in open devices, in which samples to be cryostored enter in direct contact with liquid nitrogen. On the other hand, even the closed carriers cannot theoretically avoid the transmission of microorganisms in the culture medium during the warming procedure due to a possible previous direct contact between the external surface of the carrier and accidentally contaminated liquid nitrogen.[16] Indeed, in the clinical practice both systems (open and closed) are allowed and routinely used, if aseptic procedures are carefully followed.

Oocyte morphology, as evaluated by phase-contrast microscopy, is an important and irreplaceable predictive indicator of oocyte quality, still in use to evaluate the success of a given assisted reproduction protocol. However, simple low-resolution morphological assessment is not fully adequate to measure oocyte fertility potential and developmental competence.[17] In fact, even human oocytes that survive cryopreservation and exhibit no apparent cellular damage by conventional microscopy examination may display reduced fertilizability and arrested development during the early cleavage stage.[18] The polarized light microscopy (PLM) technology allows to image meiotic spindles *in vivo* before and after cryopreservation and it is not harmful for the oocyte. A combined approach of PLM with immunocytochemistry has been successfully applied to detect alterations in the spindle configuration.

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Several studies analyzed spindle morphology, depolymerization and recovery in cryopreserved mammal and human oocytes but the correlation between the two methods is controversial.[19-22] Even if polscope can be considered an indicator of oocyte viability and could be used to help the selection of oocytes for insemination in intracytoplasmic sperm injection (ICSI) patients, immunocytochemistry associated with epifluorescence or confocal laser scanning microscopy (CLSM) provides great detail about cryoinjuries in individual fixed oocytes. Organelles highly sensitive to cryoinjuries such as meiotic spindles,[23,24] cortical granules (CG),[25] cytoskeleton components[26] can be studied using these combined approach. Moreover, the ability of CLSM to visualize in one focal plane the fluorescence associated with multiple markers renders this instrument extremely valuable for the study of co-localization of various markers, also allowing a 3-D reconstruction. Fluorescent *in situ* hybridization (FISH) is routinely used to detect aneuploidy using specific fluoroprobes for chromosome 13, 16, 18, 21 and X.[27] Recent advances in techniques of live cell imaging are becoming valuable tools to assess alterations in ooplasmic compartments. Intracellular calcium concentrations has been determined on fresh and thawed-warmed human oocytes, evidencing a better preservation of calcium dynamics in oocytes subjected to a close vitrification system than slow-frozen.[28] The developmental competences of vitrified oocytes can also be investigated analyzing the mitochondrial pattern or variations in the mitochondrial membrane potential. Variation in clustering and distribution of mitochondria has been found in mouse-vitrified oocytes, by staining the oocytes with specific mitochondrion selective probes as Mitotracker.[29]

However, to have the complete morphological view of cryopreserved oocytes, transmission electron microscopy (TEM) evaluation, alone or integrated with-immunocytochemical approach, is especially effective in estimating how cooling rates and cryoprotectants affect the oocyte structural integrity.[18,30] The impact of vitrification on the MII oocyte ultrastructural morphology has been investigated in several mammals such as bovines, porcine and rodents.[26,31-33] However, comprehensive studies on human vitrified-warmed oocytes are scarce and conclusive evidence concerning the identification of *top-quality* cryopreserved human oocytes in terms of ultrastructure has been generated just recently.[28,34-39] The present review was therefore aimed to identify the main subcellular targets of cryopreservation damage to assess the effectiveness of vitrification protocols on preserving the integrity of human mature oocytes.

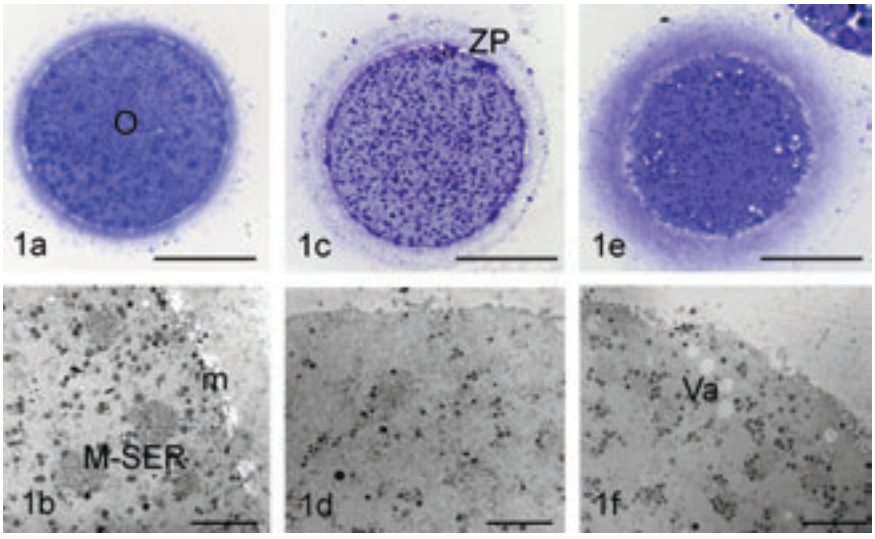

Figure 1. Fresh control (a, b), vitrified-warmed (c, d) and frozen-thawed by slow freezing (e, f) human oocytes. The general morphology and organelle microtopography are shown by LM (a, c, e) and TEM (b, d, f). No explicit differences in shape, dimensions and organelle distribution were found between fresh and cryopreserved oocytes. Apparent variations of ZP thickness (e) is an effect of the section plane (not equatorial). Vacuoles, detected only sporadically in both fresh (a, b) and vitrified-warmed (c, d) oocytes to the same extent, were numerous in the oocytes frozen-thawed by slow cooling (e, f). m, oocyte microvilli; O, oocyte; Va, vacuoles; ZP, zona pellucida; M-SER, mitochondria-smooth endoplasmic reticulum aggregates. Scale bars: (a) 45 µm; (c, e) 40 µm; (b) 4 µm; (d, f) 5 µm. Panels a,b,e,f: modified from Coticchio *et al.*[39]; panels c,d: modified from Nottola *et al.*[34]

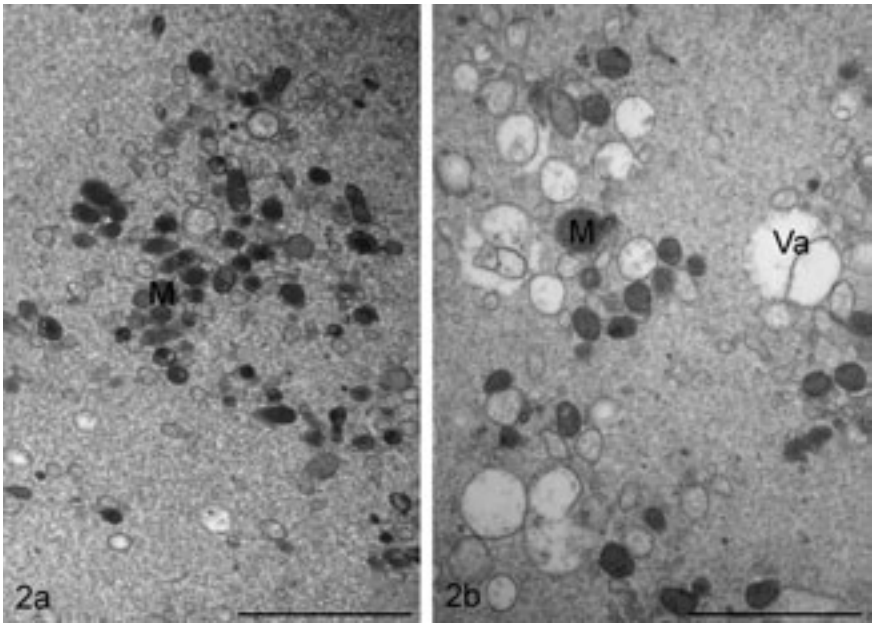

Figure 2. Vitrified-warmed (a) and frozen-thawed by slow freezing (b) human oocytes. TEM micrograph showing the paucity of vacuoles in (a) and the presence of an extensive vacuolization in (b). Va, vacuole; M, mitochondria. Scale bars: (a) 3 µm; (b) 2 µm. Panel b: modified from Coticchio *et al.*[39]

Ultrastructural studies

Electron microscopy techniques are the best tools to explore the cell ultrastructure, but involve important limitations. They rely on expensive technology and highly trained personnel. They can be hardly used for the analysis of large numbers of samples, being the process of specimen preparation and observation lengthy and almost entirely manual. Regardless, they can provide accurate and extensive information on the fine structure of the cell and its organelles.[18,34] Sathananthan *et al.* were the first to report damage to the zona pellucida (ZP), oolemma, and ooplasm in cryopreserved human oocytes.[40] With the exception of an analysis of Van Blerkom and Davies, in the mid-nineties,[41] further evidence on the ultrastructure of frozen-thawed oocytes has been gained only recently. Ultrastructural alterations involving the CG, ZP, organelle associations, and ooplasm were reported in coincidence with the application of particular dehydration and rehydration conditions,[25,38-41] suggesting that TEM can represent a first line investigative tool. Disagreement exists on the entity and type of cell damage that human oocyte may suffer as a result of cryopreservation. Several studies have reported pro and versus the hypothesis that the MII spindle can be irreversibly damaged by low temperature storage.[42] During the process of freezing-thawing the oocyte is exposed to a variety of physical and chemical conditions, such as suboptimal temperatures, formation of intracellular ice crystals, osmotic stress and replacement of intracellular water with cryoprotective agents, that may endanger its survival and competence and may cause subcellular changes undetectable by low resolution microscopy.[43] It appears essential to define objective criteria to establish whether oocyte quality may be affected by cryopreservation, in order to ruling out the applicability of different protocols and appraise possible health risks for children born from cryopreserved oocytes.

In our experience, to this aim, the following structural and ultrastructural characteristics of the oocyte should be especially evaluated (methods are analytically reported by Nottola *et al.*[34]).

General features, *e.g.,* shape, dimensions, density of ooplasm, integrity of GV (in immature oocyte) and, in favorable sections, the arrangement of the MII spindle (spindle reassembly and chromosome alignment).

Vacuoles, lysosomes, multivesicular bodies (MVB)

Microtopography of the organelles:

- mitochondria
- mitochondria-smooth endoplasmic reticulum (M-SER) aggregates
- mitochondria-vesicle (M-V) complexes

CG pattern and ZP texture

Integrity of the oolemma

General features of vitrified oocytes

Cryopreservation does not significantly impair mammalian oocyte general microarchitecture.[34] The vitrified-warmed oocytes are regular in shape. Dimensions and density of the ooplasm in light microscopy (LM) examination of semithin sections may be compared to the fresh counterpart[34,35] (Figure 1). Indeed, human mature oocytes subjected to the vitrification procedures may show general ultrastructural features compatible with criteria of maturity and viability similar to those found in slow-frozen human oocytes

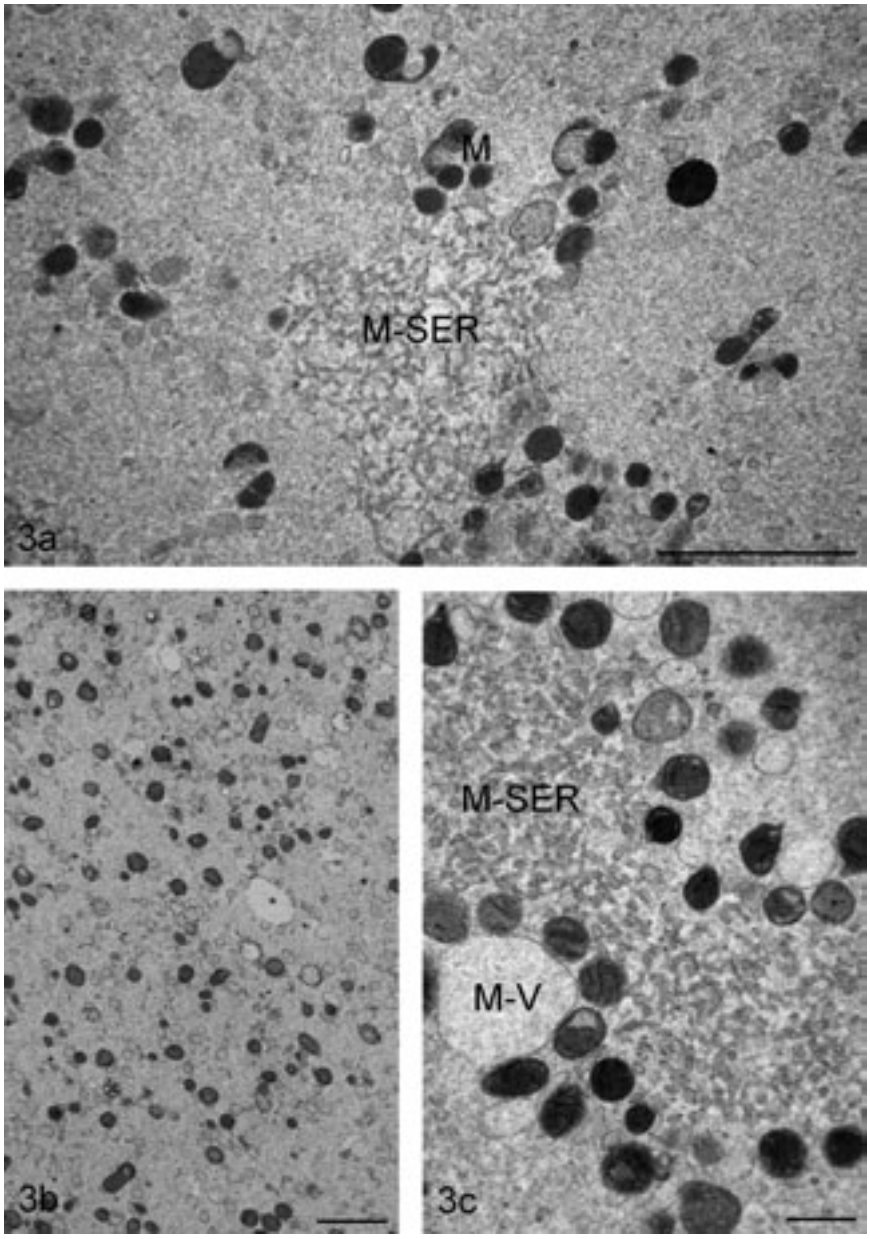

Figure 3. Fresh control (a) and vitrified-warmed (b, c) human oocytes. Mitochondria are generally rounded and provided with a few peripheral or transverse cristae. Dumb-bell shaped mitochondria (likely dividing) can be occasionally found in the ooplasm (a). Voluminous aggregates between mitochondria and smooth endoplasmic reticulum are seen (a, c). Note in (b) the absence of regularly shaped M-SER aggregates in the ooplasm. M, mitochondria; M-SER, mitochondria-smooth endoplasmic reticulum aggregates; M-V, mitochondria-vesicle complexes. Scale bars: (a) 2.5 µm; (b) 2 µm; (c) 0.5 µm. Modified from Nottola *et al.*[34]

and in fresh oocytes as well.[36,37,44,45]

In immature GV-stage oocytes, there is no evidence on the presence of ultrastructural damages to the nuclear envelope, nucleoplasm, nucleolar bodies, euchromatin and heterochromatin (*unpublished results*). In matured oocyte, occasional detection of meiotic spindles in both fresh and cryopreserved oocytes is obtained by LM and TEM only in particularly favorable sections. In these sections, the spindle of cryopreserved oocyte appears usually well structured. As above stated, since it is not possible to obtain repetitive and systematic information on spindle morphology by means of electron microscopy alone, other morphological approaches mainly based on immunocytochemistry are needed.

Vacuoles, lysosomes, multivesicular bodies

A slight vacuolization of the cytoplasm is rather normal in GV oocyte and pronuclear eggs, but is very rare in MII oocytes.[34,46] Different degrees of vacuolization have been detected in MII oocytes after cryopreservation. Following vitrification it is becoming evident a cryodevice dependency to vacuolization. In fact, only rare vacuoles were occasionally detected in human MII vitrified by the open carrier devices Cryoloop and Cryoleaf[34] (Figures 1c,d and 2a). On the contrary, a slight degree of vacuolization was found in human MII oocytes vitrified with CryoTip (closed device).[28,35] Abundant vacuolization was instead constantly observed in human mature oocytes after slow-freezing, irrespective of the procedure applied[25,36-39,46] (Figures 1e,f and 2b). Vacuolization may be a non-specific response of the oocyte to cryoinjury and/or osmotic stress. Multiple vacuoles in the ooplasm of human oocytes may be caused by swelling and coalescence of isolated SER. They are considered the morphological expression of a degenerative process,[30,38] as shown by the recurrent finding of associations of vacuoles with MVB and lysosomes.[34,36] MVB are considered a type of digestive organelle related to exchange processes occurring at the oocyte surface, whilst secondary lysosomes are involved in degradation of oocytic materials. However, both organelles, lysosomes in particular, may be considered as typical regressive markers when associated with vacuoles in the oocyte.[47,4]. Thus, as the appearance of vacuoles in human mature oocytes might ultimately lead to a reduced oocyte competence to fertilization and/or impaired embryo development,[46] the quasi absence of vacuolization in the vitrified-warmed oocytes could be interpreted as a marker of a good quality of the oocyte.[34]

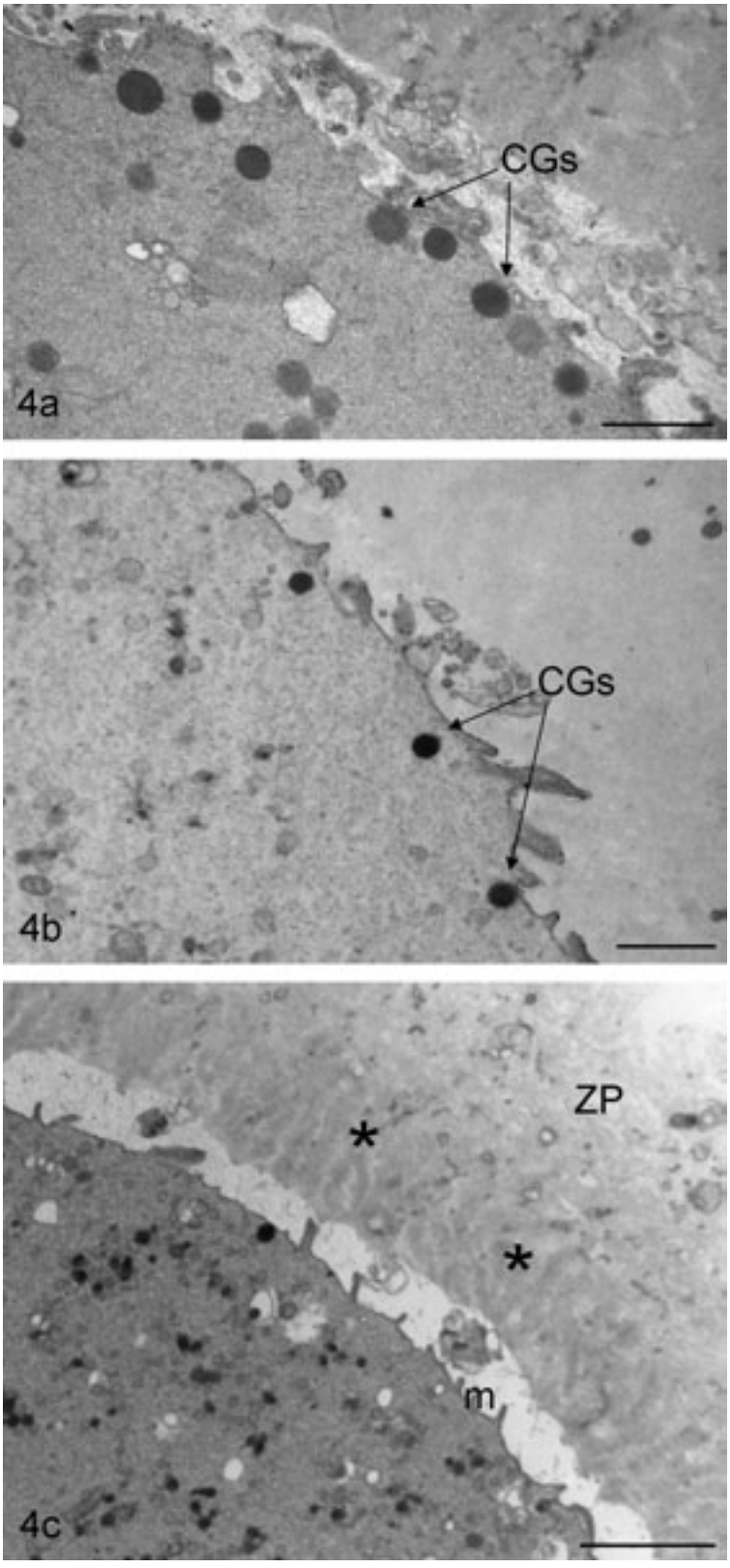

Figure 4. Fresh control (a) and vitrified-warmed (b, c) human oocytes. A rim of electron-dense CG (arrows) is seen just beneath the oolemma of the fresh oocyte (a). Instead, CG are sparse and form a discontinuous layer in vitrified-warmed oocytes (b, c). Note in (c) the increased compaction of the inner aspect of the ZP (asterisks) and the reduced number of microvilli. The dense ZP in c corresponds to an area of cortical cytoplasm virtually devoid of CG. CGs, cortical granules; m, microvilli; ZP, zona pellucida. Scale bars: (a) 1.1 µm; (b) 1.3 µm; (c) 2.5 µm. Panels a,b: modified from Nottola *et al.*[34]

Microtopography of organelles

Human oocytes after vitrification frequently (50%) show atypical, immature M-SER aggregates.[34] The arrangement of M-V complexes and the fine structure of mitochondria are similar in both fresh and vitrified-warmed samples[34] (Figure 3). Mitochondria show good morphological preservation after vitrification, both in mouse and human oocytes.[33,34] Oocyte mitochondrial damage (*e.g.,* modifications in shape and surface, swelling, reduced density and alteration of the cristae) has been frequently detected in some other mammals subjected to different vitrification protocols.[26,31,32]

M-SER disorganization with maintenance of mitochondrial integrity has been also found in human MII oocytes subjected to a slow freezing protocol using ethylene glycol (EG) as cryprotectant.[37] Thus, the alterations in shape and/or size of M-SER aggregates observed in the vitrified-warmed oocytes, even in the absence of clear mitochondrial alterations, were related more to the use of EG in the vitrification solutions than to the cryodamage itself. EG is widely used in vitrification procedures because of its lower toxicity if compared with other cryoprotectants.[49] However, when two solutions containing different concentrations of EG were tested, a higher proportion of human oocytes showing normal morphological features was associated with the use of the vitrification solution containing a lower EG concentratio.[9]

M-SER aggregates are precursor of M-V complexes.[50] Mitochondria and associated cytoplasmic membranes may play a role in the neoformation of membranes during early embryogenesis.[51] M-SER aggregates may also regulate local concentrations of free calcium and ATP production, thus acting on different cellular activities including mediation of calcium-dependent signal transduction pathways at fertilization.[52,53] The ultrastructural changes in the arrangement of M-SER aggregates could be, among other factors, potential determinants of a reduced oocyte competence for fertilization, possibly due to disturbances in calcium homeostasis. It should be also considered that variations in shape and size of M-SER aggregates, in the absence of detectable damage in both SER tubules and mitochondria, could be related to different morphodynamic stages of these structures.

Cortical granules pattern and texture of zona pellucida

The number of CG may be abnormally reduced in vitrified-warmed oocytes[34] (Figure 4). A compaction of the inner aspect of the ZP, with the loss of its typical filamentous texture[54] (Figure 4c), due to the presence of large areas of filaments packed together, was sometimes found in the same samples. These are ultrastructural markers of the occurrence of a premature exocytosis of the CG content into the perivitelline space (PVS), with the resulting hardening of the inner aspect of ZP. Such hardening may halt sperm penetration at fertilization and this problem can be overcome only by ICSI.[4] CG pattern changes were found not only in vitrified oocytes, in human and other mammals, but also in oocytes subjected to different protocols of slow freezing.[25,31-34,36-39] Thus, CG exocytosis, a phenomenon that indicates a possibility of illegitimate oocyte activation, does not seem to be dependent upon the procedure applied or to the cryoprotectant used, but it is a quite common phenomenon occurring during oocyte cryopreservation, although some studies reported contrasting results.[3,55]

Integrity of the oolemma

Vitrified-warmed oocytes are surrounded by an intact, continuous oolemma and mostly provided with microvilli projecting into a PVS with regular shape, width and content[30,48,50]) (Figure 4c). A fraction (about 30%) of the warmed oocytes, however, showed focal surface areas with rare and/or short microvilli.[34] Similar ultrastructural features have been found in human oocytes subjected to a slow freezing protocol based on the use of EG.[37] Conversely, a normal pattern of microvilli was detected in human slow-frozen oocytes cryopreserved with propane-1,2-diol.[36] Structural damage of microvilli in cryopreserved oocytes could be related to a primary alteration of the sub-oolemmal cytoskeletal web occurring during cryopreservation.[33] The improper microvilli distribution may ultimately contribute to fertilization failure, due to ineffective spermatozoon-oocyte fusion.[56]

Conclusions

Human mature oocytes subjected to the vitrification show good overall ultrastructural preservation. In particular, the quasi absence of cytoplasmic vacuolization seems to be the most relevant marker of quality in vitrified oocytes using open carriers. However, the finding in the same oocytes of immature M-SER aggregates as well as the sparse CG with altered ZP and focal microvilli reduction, emphasize the need for further ultrastructural studies, associated with an immunocytochemical approach. It should be also pointed out that the reduction in the number of CG, associated to a zona hardening, implies the use of ICSI as the insemination technique of choice after cryopreservation. This review clearly validates the use of electron microscopy and correlated morphofunctional techniques in the assessment of oocyte cryodamage, also in precise evaluation and refinement of cryopreservation protocols.

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