

Ultrastructure of human mature oocytes after slow cooling cryopreservation using different sucrose concentrations[†]

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BACKGROUND: We studied the ultrastructural characteristics of human mature oocytes frozen/thawed (F/T) using different concentrations of sucrose. Fresh human mature oocytes were used as controls. **METHODS:** The oocytes ($n = 48$) were fixed in 1.5% glutaraldehyde at sampling ($n = 16$) or after freeze/thawing performed using a slow cooling method with propane-1,2-diol 1.5 mol/l and sucrose at either 0.1 mol/l ($n = 16$) or 0.3 mol/l ($n = 16$) in the freezing solution. The oocytes were then processed for electron microscopy observations. **RESULTS:** Fresh and F/T oocytes belonging to both study groups were regularly rounded in sections, with a homogeneous cytoplasm and an intact zona pellucida (ZP). Organelles (mainly mitochondria–smooth endoplasmic reticulum aggregates and mitochondria–vesicle complexes) were abundant and uniformly dispersed in the ooplasm. The amount and density of cortical granules appeared to be abnormally reduced in some F/T samples, independently of the sucrose concentration in the freezing solution: this feature was frequently associated with an increased density of the inner ZP, possibly related to the occurrence of zona 'hardening'. Furthermore, slight to moderate microvacuolization was revealed in the ooplasm of some F/T oocytes, particularly in those treated with sucrose 0.3 mol/l. **CONCLUSIONS:** Freeze/thawing procedures are associated with ultrastructural alterations in specific oocyte microdomains, presumably linked to the reduced developmental potential of mature cryopreserved oocytes. Further work is needed to determine whether or not a high concentration of sucrose plays a role, at least in part, in producing the above alterations.

Key words: cryopreservation/human/oocyte/slow cooling/ultrastructure

Introduction

Oocyte cryopreservation may provide stored material for women requiring repeated *in vitro* fertilization (IVF) treatments, thus increasing the flexibility and clinical efficacy of fertility protocols, or in need to preserve their reproductive potential before undergoing potentially gonadotoxic treatments for neoplastic or other severe systemic diseases. Oocyte cryopreservation does not require the presence of a male partner, as embryo freezing does, and it is also recommended to couples who cannot accomplish embryo cryopreservation for moral or legal reasons (Paynter, 2000; Smith and Silva, 2004; Stachecki and Cohen, 2004; Gosden, 2005).

Cryopreservation of mature oocytes directly sampled from the pre-ovulatory follicles seems to be the best alternative approach to embryo cryopreservation, since the classic reports of Chen (1986) and Van Uem *et al.* (1987). However, in contrast to that reported for embryo freezing, the human mature oocyte may be negatively affected by freezing, due to its singular biological characteristics (Paynter, 2000; Coticchio *et al.*, 2004; Smith and Silva, 2004; Gosden, 2005). In fact, clinical and experimental observations report that the pre-ovulatory oocyte is highly susceptible to cryodamage, being exposed to thermal, mechanical or chemical forces during the freeze-thawing process. Part of these forces, such as generation of electric fields at the surface of the moving ice crystal front, intracellular ice crystal formation and ionic imbalance, may be directly related to the cooling/freezing process. However, other possible deleterious effects like the establishment of

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osmotic gradients, dehydration and chemical toxicity may be due to the cryoprotectant agents (CPAs) themselves (Al-Hasani *et al.*, 1987; Sathananthan and Trounson, 1989; Schalkoff *et al.*, 1989; Vincent *et al.*, 1990; Pickering *et al.*, 1991; Van Blerkom and Davis, 1994; Paynter *et al.*, 2005). Hence, in general, protocols in use for human oocyte cryopreservation are not yet optimized and clinical successes remain relatively low, although, during the last few years, a higher percentage of pregnancies has been obtained in a few IVF centres (Porcu *et al.*, 2000; Borini *et al.*, 2004, 2006; Chen *et al.*, 2005). For this reason, further experimentation on clinical trials is needed, in order to better understand the effects on human oocytes of all factors associated with cryopreservation and ultimately identify the cryopreservation procedure that points to best results.

Even cryopreserved human oocytes that survive cryopreservation and exhibit no apparent cellular damage by phase contrast microscopy (PCM) examination may display reduced fertilizability and arrested development during the early cleavage stage (Van Blerkom and Davis, 1994). The preservation of the structural integrity of the oocyte microdomains, with parallel maintenance of their physiological homeostatic and developmental functions, is essential for fertilization and early embryo growth. Compromising these structures, also in the absence of grossly detectable lesions, can deleteriously influence initial cryosurvival and further developmental competence of the oocyte.

The wide clinical and social impact of the procedures of cryopreservation and their current low performance have raised deep interest in clinicians, embryologists and reproductive biologists. Although the diagnostic–prognostic role of electron microscopy in assisted reproduction has been well established (Sathananthan *et al.*, 1993; El-Shafie *et al.*, 2000; Makabe *et al.*, 2006), nevertheless, detailed ultrastructural studies on the morphology of the subcellular microdomains in cryopreserved oocytes are objectively scarce. In particular, most of the studies performed on cryopreserved oocytes concern various mammals except humans (Sathananthan *et al.*, 1988a; Van Blerkom, 1989; Van Blerkom and Davis, 1994; Fuku *et al.*, 1995; Hochi *et al.*, 1996; Asada *et al.*, 2000; Strom Holst *et al.*, 2000; Hochi, 2003; Diez *et al.*, 2005). In addition, electron microscopy data related to the protocols currently in use are not reported in the earlier studies carried out in humans (Sathananthan *et al.*, 1987, 1988b; Schalkoff *et al.*, 1989; Van Blerkom and Davis, 1994).

The present study was undertaken to investigate by transmission electron microscopy (TEM) the possible effects that two alternative slow-cooling protocols using propane-1,2-diol (PrOH) 1.5 mol/l but differing in concentration of the non-penetrating CPA sucrose in the freezing solution (0.1 versus 0.3 mol/l) may have on the fine structure of human mature oocytes, in order to contribute to the identification of freeze-thawing procedures potentially able to ensure better results in terms of oocyte quality and competence for fertilization. In fact, although the combination of slow cooling and increased sucrose concentration (from 0.1 to 0.3 mol/l) improves oocyte survival after thawing from 40 to ~80% (Fabbri *et al.* 2001) and also increases metaphase II (MII)

spindle stability (Coticchio *et al.*, 2006), oocytes stored with this protocol may undergo very intense and rapid dehydration, associated with relevant osmotic stress (Paynter *et al.*, 2005), and implant with poor frequencies (Borini *et al.*, 2006).

Materials and methods

Source of oocytes

This study was approved by the Public Health Agency of the Italian government, National Health Institute and the Institutional Review Boards of the participating clinics. Oocytes were obtained over a period between May 2003 and April 2005 from patients undergoing assisted reproduction treatment, with their informed consent and according to the current Italian laws. Only oocytes donated from women younger than 36 years (mean \pm SD: 32.6 \pm 2.9), whose infertility was due to a male factor, were used. More than one oocyte from the same patient was sometimes included in this study, but it did not systematically occur. Controlled ovarian stimulation was induced with long protocols using GnRH agonist and rFSH, according to the standard clinical protocols routinely utilized by the participating clinics. hCG (10 000 IU) was administered 36 hr prior to oocyte collection. Complete removal of cumulus mass and corona cells was performed enzymatically using hyaluronidase (20–40 IU/ml) and mechanically using fine bore glass pipettes. Only oocytes that were devoid of any sort of dysmorphisms at PCM examination, showing an extruded polar body I (PBI), thus presumably at the MII stage, were assigned to either the control or study groups. According to their assignment, oocytes were either fixed or frozen after a total period of time of 3–4 h following retrieval. During this period, oocytes were cultured in the medium used for standard IVF.

Cryopreservation solutions

Oocytes were cryopreserved using a slow-cooling method. The cryopreservation solutions were prepared using Dulbecco's phosphate-buffered solution (PBS) (Gibco, Life Technologies Ltd, Paisley, UK) and a Plasma Protein Supplement (PPS) (10 mg/ml) (BAXTER AG, Vienna, Austria). The freezing solutions were 1.5 mol/l PrOH + 20% PPS in PBS (equilibration solution) and 1.5 mol/l PrOH + either (a) 0.1 or (b) 0.3 mol/l sucrose + 20% PPS in PBS (loading solutions), as described by Fabbri *et al.* (2001). The thawing solutions were (i) 1.0 mol/l PrOH + sucrose + 20% PPS, (ii) 0.5 mol/l PrOH + sucrose + 20% PPS and (iii) sucrose + 20% PPS. Sucrose concentration in the thawing solutions was 0.2 or 0.3 mol/l depending of whether loading solution (a) or (b) had been used for freezing, respectively.

Freezing procedure

Oocytes were washed in PBS supplemented with 20% PPS and subsequently placed in the equilibration solution containing 1.5 mol/l PrOH + 20% PPS for 10 min. Afterwards, oocytes were transferred to the solution containing 1.5 mol/l PrOH + 0.1 or 0.3 mol/l sucrose + 20% PPS, at room temperature (RT) for 5 min, then loaded into plastic straws (Paillettes Crystal 133 mm; Cryo Bio System, France) and placed into an automated Kryo 10 series III biological freezer (Planer Kryo 10/1.7 GB). Temperature was gradually lowered from 20 to -8°C at a rate of $2^{\circ}\text{C}/\text{min}$. Manual seeding was induced during the 10 min holding ramp at -8°C . The temperature was decreased then to -30°C at a rate of $0.3^{\circ}\text{C}/\text{min}$ and finally rapidly to -150°C at a rate of $50^{\circ}\text{C}/\text{min}$. The straws were finally plunged into liquid nitrogen and stored for later use.

Thawing procedure

The straws were rapidly air-warmed for 30 s and then plunged into a 30°C water bath for 40 s. The CPA was removed at RT by step-wise dilution. Oocytes were expelled in the first thawing solution, 1.0 mol/l PrOH + sucrose + 20% PPS for 5 min, then equilibrated in 0.5 mol/l PrOH + sucrose + 20% PPS for additional 5 min. Finally, they were placed in sucrose + 20% PPS for 10 min before final dilution in PBS + 20% PPS for 20 min (10 min at RT and 10 min at 37°C). Before fixation, thawed oocytes were cultured for 3 h in 20 µl of drops of glucose-free medium normally used for embryo culture under warm mineral oil at 37°C in an atmosphere of 5% CO₂ in air.

Electron microscopy

A total of 48 mature oocytes were included in this study. Sixteen of them were fixed after ~4 h following retrieval and assigned to the control group. The other 32 oocytes were subjected to freeze-thawing procedures as detailed above and assigned at the sucrose 0.1 mol/l group ($n = 16$) or at the sucrose 0.3 mol/l group ($n = 16$), consistent with the concentration of sucrose in the cryopreservation solutions. Only oocytes that appeared of good quality when observed by PCM after freeze-thawing were selected for electron microscopy evaluation. As indicated by Veeck (1991), they should contain the morphological features of a clear, moderately granular cytoplasm, a narrow perivitelline space (PVS) with the PBI and an intact zona pellucida (ZP).

Oocytes were fixed and processed for TEM analysis as previously described (Nottola *et al.*, 1991; Motta *et al.*, 1995). Oocyte fixation was performed in 1.5% glutaraldehyde (SIC, Rome, Italy) in PBS solution. After fixation for 2–5 days at 4°C, the samples were rinsed in PBS, post-fixed with 1% osmium tetroxide (Agar Scientific, Stansted, UK) in PBS and rinsed again in PBS. Oocytes were then embedded in small blocks of 1% agar of about 5 × 5 × 1 mm in size, dehydrated in ascending series of ethanol (Carlo Erba Reagenti, Milan, Italy), immersed in propylene oxide (BDH Italia, Milan, Italy) for solvent substitution, embedded in Epon 812 (Agar Scientific, Stansted, UK) and sectioned by a Reichert-Jung Ultracut E ultramicrotome. Semithin sections (1 µm thick) were stained with Toluidine Blue, examined by light microscopy (LM) (Zeiss Axioskop) and photographed using a digital camera (Leica DFC230). Ultrathin sections (60–80 nm) were cut with a diamond knife, mounted on copper grids and contrasted with saturated uranyl acetate followed by lead citrate (SIC, Rome, Italy). They were examined and photographed using a Zeiss EM 10 and a Philips TEM CM100 Electron Microscopes operating at 80 kV.

According to Motta *et al.* (1988), the following parameters have been evaluated by LM and TEM and taken into consideration for the qualitative assessment of the ultrastructural preservation of oocytes: general features (including shape and dimensions),

microtopography, type and quality of the organelles, integrity of the oolemma, ZP texture, appearance of the PVS (width, presence of fragments, presence and characteristics of the PBI), arrangement of the MII spindle (in sections laying on appropriate planes), presence and extent of cytoplasmic vacuolization (Table I).

Results

The survival rates of cryopreserved oocytes with the 0.1 and 0.3 mol/l sucrose protocols were 41.2 and 71.4%, respectively, an outcome comparable to those published in previous articles (Fabbri *et al.*, 2001; Borini *et al.*, 2004, 2006; Chen *et al.*, 2005; Levi-Setti *et al.*, 2006). Among survived oocytes, good-quality gametes were ~90–95% of the total pool in both study groups.

By LM on semithin sections, fresh control and frozen/thawed (F/T) oocytes belonging to both study groups were of a regular round shape, 90–100 µm in the maximum diameter, with a clear, homogeneous cytoplasm and an intact ZP (Figure 1a–c, insets). A certain degree of cytoplasmic vacuolization was detected in the cytoplasm of a part of F/T oocytes (Figure 1c, inset).

By TEM analysis at low magnification, the organelles were abundant and uniformly dispersed in the ooplasm of fresh and F/T oocytes belonging to both protocols of cryopreservation (Figure 1a–c). At higher magnification, mitochondria were the most prominent organelles. Mitochondria were found closely intermingled with tubular membranes of smooth endoplasmic reticulum (SER) anastomosed with each other or, less frequently, associated with small vesicles filled of a slightly electron-dense material, forming, respectively, mitochondria–SER (M–SER) aggregates (Figure 1d–f) and mitochondria–vesicle (MV) complexes (Figures 1e and 2a). Larger M–SER aggregates were scattered in the cortical areas of the ooplasm (Figure 1a–c). Mitochondria were round or oval, with a diameter varying from 0.5–0.8 µm, provided with few peripheral arch-like or transversal cristae and with a matrix showing a moderate electron density (Figures 1d and 2a–d). Elongated forms with a central constriction ('dumb-bell'-shaped mitochondria), commonly interpreted as dividing mitochondria, were sometimes observed in the ooplasm (Figure 2a). Small tubular elements of SER, apparently isolated, were also occasionally observed.

Presence of spherical cortical granules (CGs) just beneath the oolemma, varying in diameter from 300 to 400 nm, was a

Table I. Ultrastructural changes in human mature oocytes cryopreserved using different sucrose concentrations

| Treatment | Number (%) of oocytes | | | | | | | | | | |
|-------------------|-----------------------|------------|---------------------------|--|-------------------|------------|-----------|----------|-----------|----------|------------|
| | Examined | | Damaged in | | | | | Oolemma | ZP | PVS | Vacuoles |
| | Shape | Dimensions | General features | | | Organelles | | | | | |
| | | | Organelle microtopography | Mitochondria M–SER aggregates (MV complexes) | Cortical granules | | | | | | |
| Untreated | 16 | 1 (6.2%) | 0 (0%) | 0 (0%) | 0 (0%) | 1 (6.2%) | 1 (6.2%) | 1 (6.2%) | 0 (0%) | 1 (6.2%) | 1 (6.2%) |
| Sucrose 0.1 mol/l | 16 | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) | 7 (43.7%) | 1 (6.2%) | 6 (37.5%) | 1 (6.2%) | 4 (25%) |
| Sucrose 0.3 mol/l | 16 | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) | 1 (6.2%) | 6 (37.5%) | 1 (6.2%) | 5 (31.2%) | 0 (0%) | 11 (68.7%) |

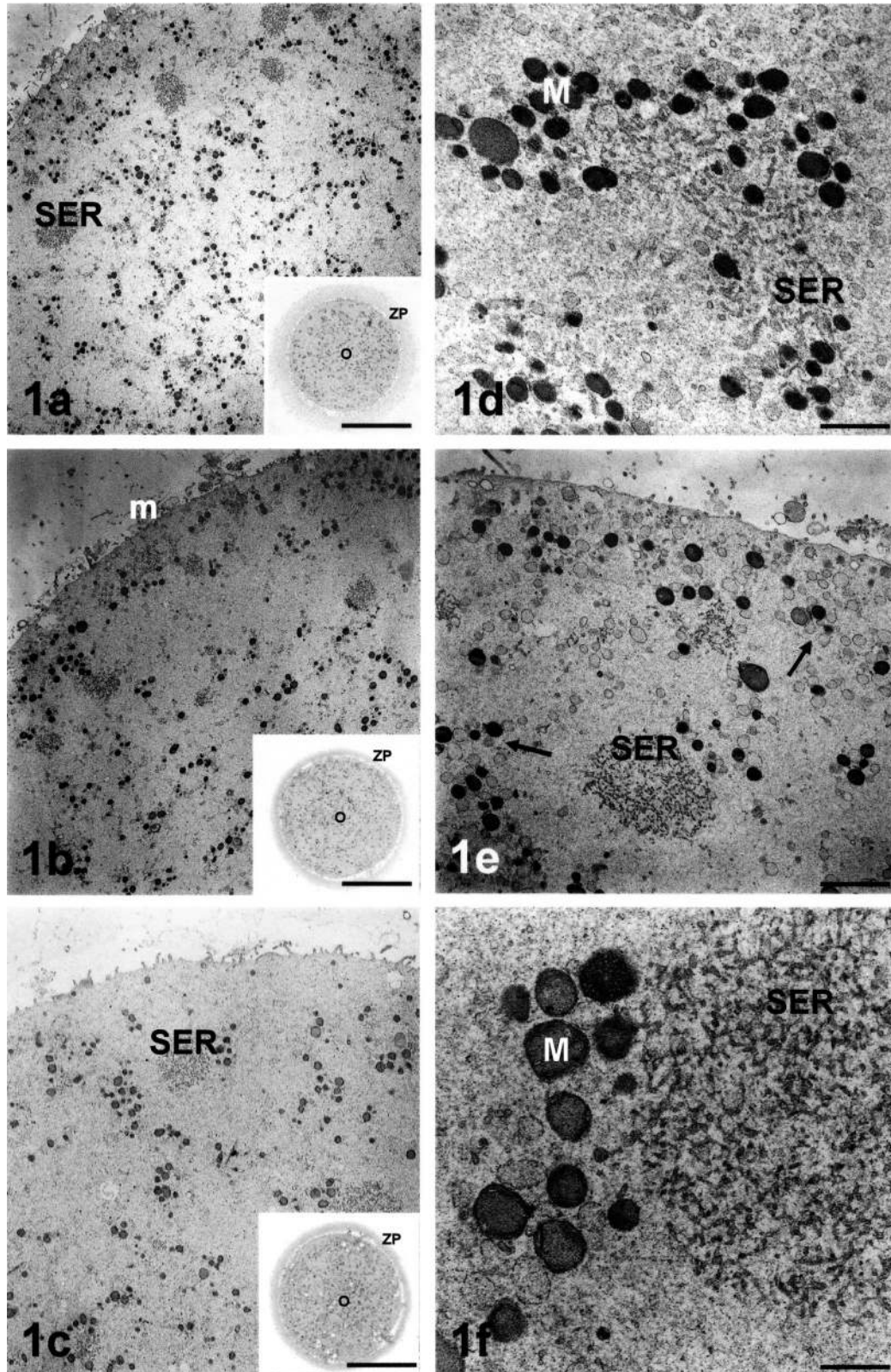


Figure 1. Human mature oocytes. The general morphology and organelle microtopography are shown by transmission electron microscopy and light microscopy (insets) in (a) (control group), (b) (sucrose 0.1 mol/l group) and (c) (sucrose 0.3 mol/l group). No overt difference in shape, dimensions and organelle distribution was found among groups. The apparent increased thickness of the zona pellucida (ZP) of the oocyte shown in (a), inset, is an effect of the section plane (not equatorial). A slight degree of vacuolization is seen in the oocyte belonging to the sucrose 0.3 mol/l group (c, inset). (a–c) (insets): O, oocyte. (a–c): SER, smooth endoplasmic reticulum; m, oocyte microvilli. Note also the presence of voluminous aggregates between mitochondria (M) and elements of SER in (d) (control group), (e) (sucrose 0.1 mol/l group) and (f) (sucrose 0.3 mol/l group). (e): arrows, small mitochondria–vesicle complexes. Bar: 4.5 μm (a–c); 45 μm (a–c, insets); 0.9 μm (d); 1.8 μm (e); 0.6 μm (f).

constant feature in all the oocytes observed. However, both amount and density of CGs appeared abnormally reduced in some F/T samples with respect to fresh oocytes, independently of the sucrose concentration in the cryoprotectant solution (Figure 3a–c). In F/T samples, CGs were also occasionally observed to be discharging their content into the PVS (Figure 3d). These abnormal features in F/T samples were frequently associated with an increased density of the filamentous texture of the inner aspect of the ZP (Figure 3e).

In fresh and F/T oocytes, the oolemmal surface was continuous and folded into numerous short microvilli projecting into a narrow, light PVS (Figures 1a–c and 3a–e). In sections laying on appropriate planes, the PBI was detected in the PVS (that was wider in this region) and the MII spindle was found in the ooplasm. The PBI typically contained condensed, aggregated chromatin, not delimited by a nuclear envelope, and scattered CGs (Figure 4a). The spindle (observed in 9 fresh and in 15 F/T oocytes, 7 of which were treated with sucrose 0.1 mol/l

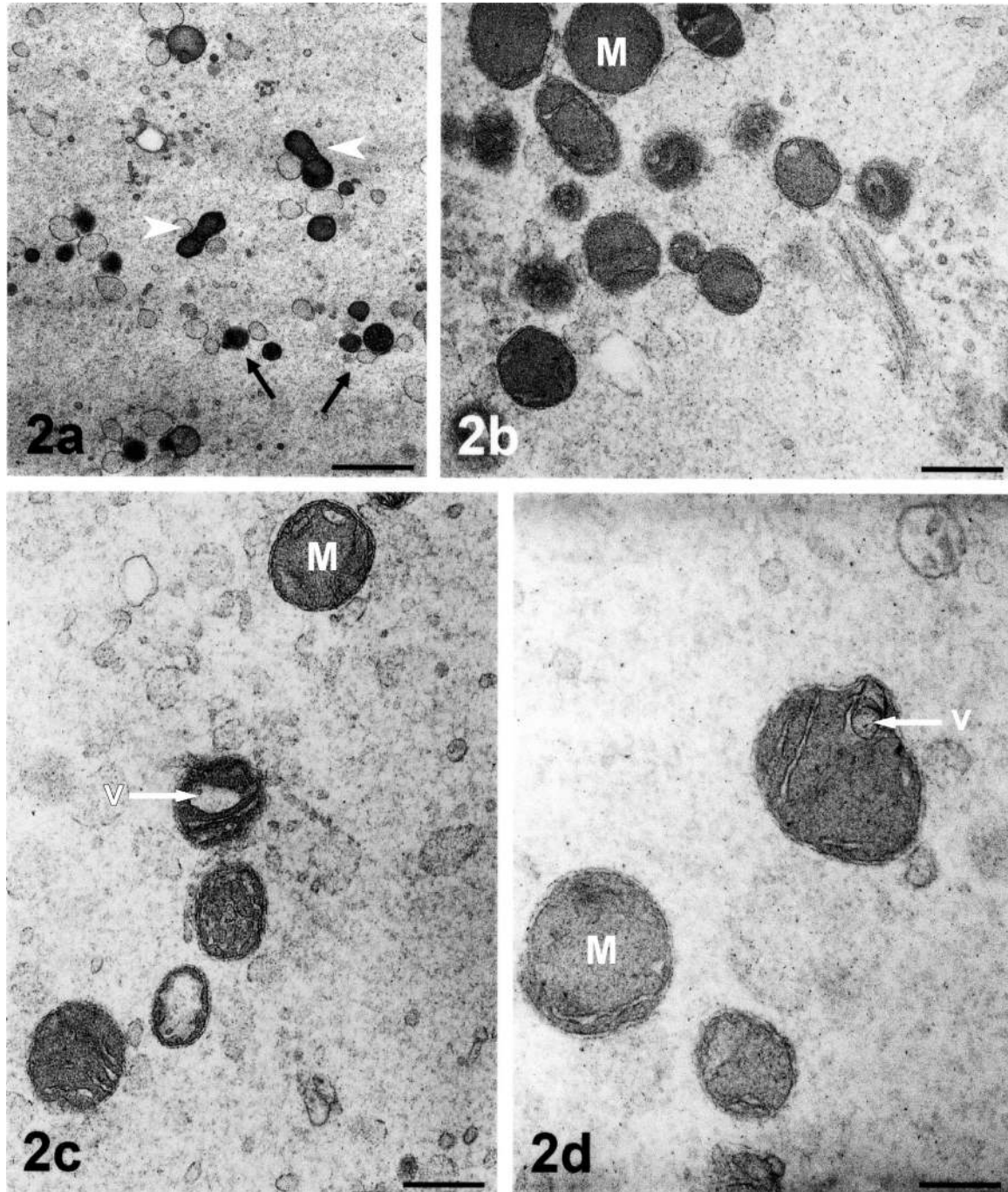


Figure 2. Human mature oocytes. Rounded mitochondria (M) with few peripheral arch-like or transverse cristae and a moderately electron-dense matrix are seen in (a) and (c) (sucrose 0.1 mol/l group) and also in (b) and (d) (sucrose 0.3 mol/l group). For comparison, see Figure 1d (control group). (a): arrows, small mitochondria–vesicle complexes. ‘Dumb-bell’ shaped mitochondria are also seen (arrow heads). (c) and (d): note the presence of clear vacuoles (v plus arrow) inside the mitochondrial matrix. Bar: 1.0 μm (a); 0.5 μm (b and c); 0.25 μm (d).

and 8 with sucrose 0.3 mol/l) assumed a peripheral position in the ooplasm and consisted of chromosomes and associated microtubules converging at each pole. Two chromatids were seen in each chromosome, presenting usual granulo-fibrillar microstructure. No asters (centrioles) were found at the two poles of the spindle, which appeared surrounded by M-SER aggregates and small MV complexes (Figure 4b).

Slight to moderate microvacuolization was revealed also by TEM in the ooplasm of some F/T oocytes, particularly in those treated with sucrose 0.3 mol/l (Figure 5a–c). Vacuoles, varying in diameter from 2.5 to 4 μm , were surrounded by a limiting membrane often interrupted, thus appearing as light spaces opening in the ooplasm. They were empty or, more rarely, irregularly filled with a flocculent or fine fibrillar substance (Figure 5c).

The quality and incidence of ultrastructural changes occurring in mature oocytes during the freeze-thawing procedures object of our study are summarized in Table I. These

changes, although related to cryopreservation and, in some cases, to the specific protocol in use, did not appear associated to the provenience of the oocytes (from the same patient or from different patients).

Discussion

The positive outcome of IVF is strictly dependent on the morpho-functional viability of the oocyte. In fact, in the pre-ovulatory period, the female gamete undergoes a series of maturative changes which, in the absence of degenerative alterations or dysmorphisms, render it competent for fertilization and even capable of generating a viable embryo (Familiari *et al.*, 2006).

As reported in Materials and methods, we decided to limit our ultrastructural analysis to a population of apparently good-quality oocytes by PCM because their evaluation could have a major clinical relevance. In fact, only cryopreserved oocytes

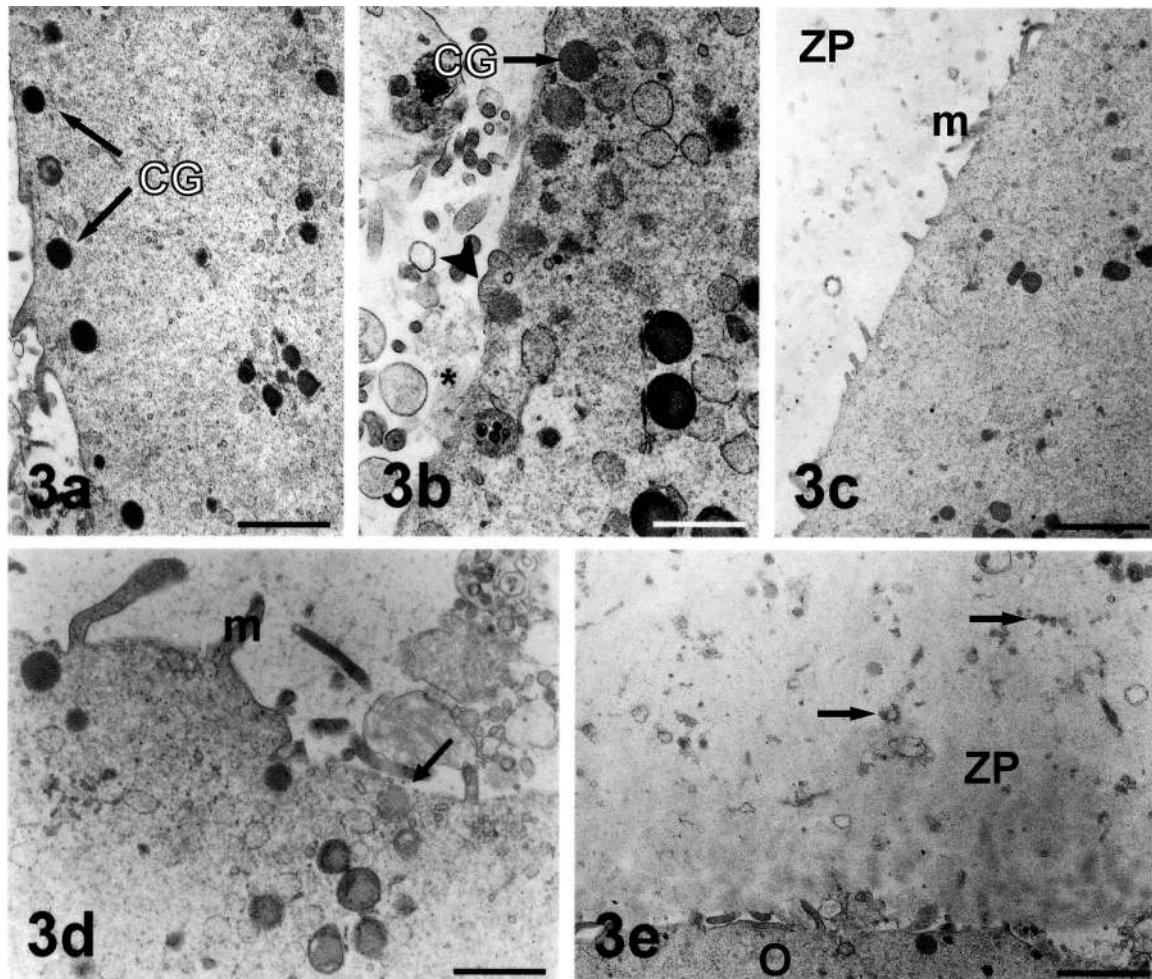


Figure 3. Human mature oocytes. Presence, amount and density of cortical granules are shown. A single, regular row of electron-dense cortical granules (CG plus arrows) is seen just beneath the oolemma in (a) (control group). Cortical granules (CG plus arrow) appear instead reduced in density in (b) (sucrose 0.1 mol/l group) and frankly absent in (c) (sucrose 0.3 mol/l group). In (b), a slightly electron-dense material is seen in the perivitelline space (asterisk), possibly representing the exocytosed content of cortical granule(s). The oolemma at the site of release is denser in appearance (arrowhead) as a consequence of the incorporation of the cortical granule membranous envelope. (d and e) (sucrose 0.3 mol/l group): note the presence of a cortical granule discharging its content in the perivitelline space (PVS) in (d) (arrow); note also the abnormally dense appearance of the inner aspect of the ZP in (e), associated with an oocyte (O) cortex almost devoid of cortical granules. Numerous remnants of follicular cell processes and other debris (arrows) are seen in the zona texture. Microvilli (m) are seen projecting from the oolemma surface in the PVS of all samples (a–e). Bar: 1.5 μm (a); 0.7 μm (b); 2.3 μm (c and e); 0.9 μm (d).

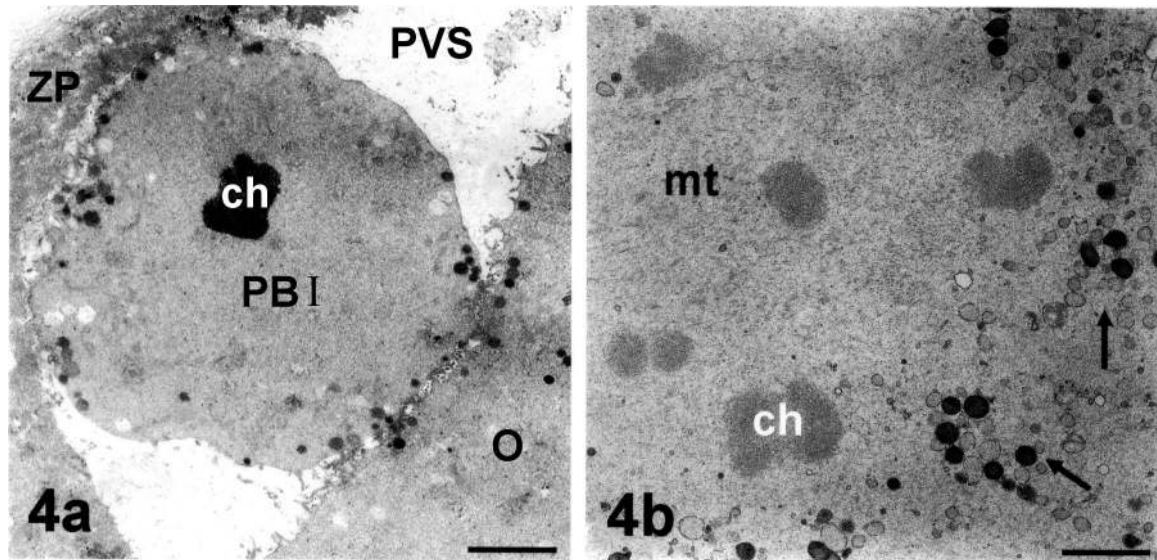


Figure 4. Human mature oocytes (sucrose 0.1 mol/l group). Note the presence of the polar body I (PBI) in the PVS (a) and of the meiotic spindle, composed of microtubules (mt) and associated chromosomes (ch) in the ooplasm (b). (a): O, oocyte; ch, condensed chromatin in the PBI. (b): arrows, small mitochondria-vesicle complexes. Bar: 3.3 μm (a); 1.5 μm (b).

which appear normal and viable after thawing are routinely selected and inseminated during IVF cycles.

In this study, the oocytes (fresh and F/T belonging to both study groups) appeared regular in shape, dimensions and overall arrangement of the ooplasm by both LM and TEM. Reasonably good preservation of the general organization of the cytoplasm has been also found in oocytes cryopreserved by slow cooling to low (-50°C to -80°C) or high (-35°C to -36°C) subzero temperatures in 1.5 M dimethylsulphoxide (DMSO) or 1.5 M PrOH (Sathananthan and Trounson, 1989). In contrast, distortion and shrinkage of the oocyte, accompanied by some disorganization of the cytosol, evident as organelle-free patches, have been found in pre-ovulatory human oocytes cooled to 0°C with or without DMSO (Sathananthan *et al.*, 1988b). Ultrarapid freezing and vitrification also determined an extensive disorganization of the ooplasm (Sathananthan and Trounson, 1989).

The mature oocyte shows a complex cytoplasmic organization as a consequence of the remodelling occurred during the final stages of maturation, when neogenesis, modification and redistribution of organelles in specific ooplasmic areas did occur. The most common ultrastructural feature observed in the ooplasm of all the cryopreserved oocytes was the presence of numerous well-preserved, typical mitochondria, often arranged with SER elements to form voluminous M-SER aggregates and, less frequently, small MV complexes. The same features were also found in the fresh controls and are superimposable to those described for fresh, healthy oocytes (Sundstrom *et al.*, 1985a,b; Szollosi *et al.*, 1986; Motta *et al.*, 1988; El.Shafie *et al.*, 2000). All SER elements, although showing different profiles (tubules and vesicles) and often appearing as independent structures by TEM, actually belong to the same interconnecting system of linked membranes, which are continuous with each other (El.Shafie *et al.*, 2000; Familiari *et al.*, 2006; Makabe *et al.*, 2006). Such a distribution

of SER throughout the oocyte cytoplasm has been confirmed by scanning laser confocal microscopy analysis (Van Blerkom, 2004). SER membranes appear also capable of transforming into each other, as revealed by the detection of intermediate features between tubules and vesicles by TEM (Motta *et al.*, 1988). In this regard, M-SER aggregates presumably represent the precursor of MV complexes, which develop later during the maturational phase (Sundstrom *et al.*, 1985b; Motta *et al.*, 2000).

Different roles are hypothesized for these particular membrane systems and associated mitochondria. They may work in conjunction to obtain: (i) rich pool of energy reserve; (ii) production/secretion of substances (nutrients, growth factors) useful for fertilization and (iii) rapid neogenesis of plasma and nuclear membranes during the first embryo cleavages (Motta *et al.*, 1988, 2000, 2003). In addition, it has been recently pointed out that regulation of intracellular calcium sequestration and release is depending upon coordinated activity of mitochondria and associated SER (Van Blerkom, 2004; Makabe *et al.*, 2006). In this regard, as shown by fluorescence microscopy, mitochondrial respiration and ability to participate in the regulation of calcium homeostasis are positively related to mitochondrial polarity, and putative highly polarized mitochondria with associated SER elements are distributed in pericortical areas (Van Blerkom *et al.*, 2002; Jones *et al.*, 2004; Van Blerkom, 2004).

Mitochondrial dysfunctions and/or abnormalities may influence the competence for fertilization of human oocytes and could be a critical determinant of human embryo developmental failure (Motta *et al.*, 2000; Van Blerkom, 2004). Mitochondria and associated membranes may be sensitive to cooling. In fact, when observed by TEM in some pre-ovulatory human oocytes cooled to 0°C with or without DMSO, mitochondria underwent swelling and the elements of SER were also damaged by the cooling procedure

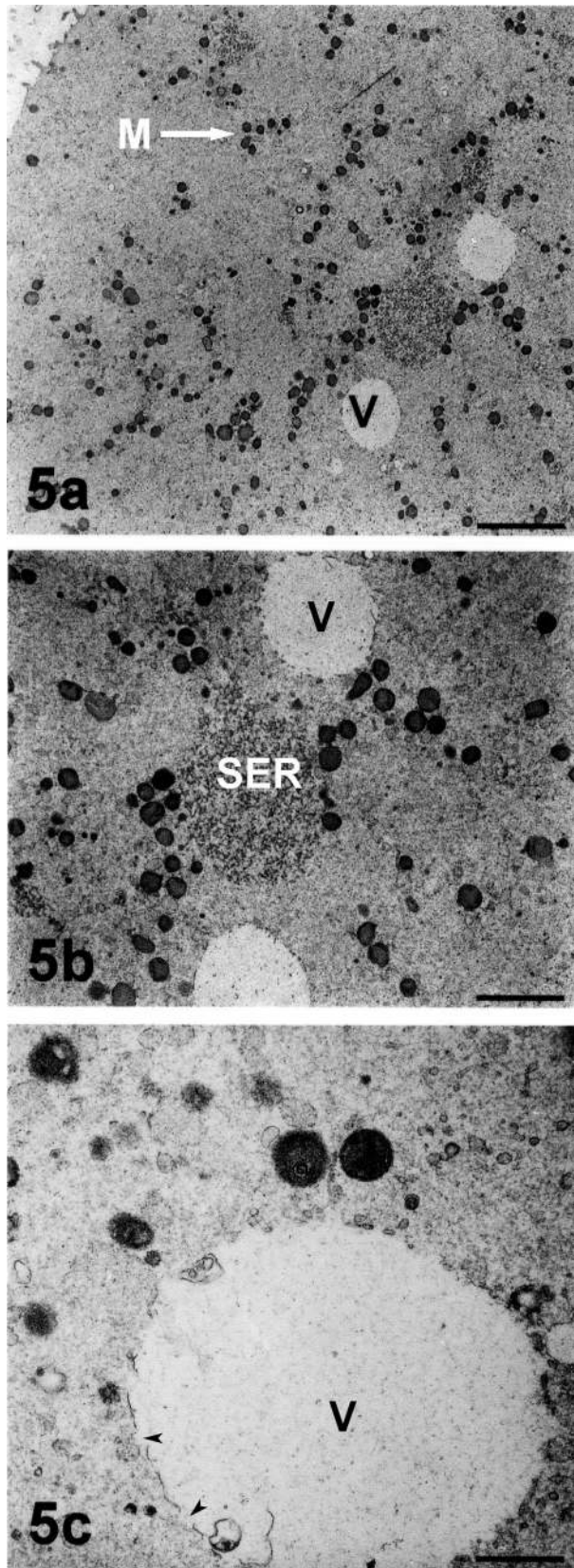


Figure 5. Human mature oocytes (sucrose 0.3 mol/l group). Clear vacuoles (V) are seen in the ooplasm which, in spite of this, appears populated by numerous typical organelles (a and b). (a): M plus arrow, mitochondria. Vacuoles (V) generally appear empty and delimited by a membrane often interrupted (arrowheads) (c). Bar: 4.5 μm (a); 1.8 μm (b); 0.7 μm (c).

(Sathananthan *et al.*, 1988b). A normal complement of organelles, including mitochondria, was instead present elsewhere in the cytoplasm of fresh oocytes treated with CPAs alone (PrOH and DMSO plus sucrose 0.2 M) at RT (Schalkoff *et al.*, 1989).

Interestingly, by fluorescence microscopy, cryopreservation of human MII oocytes (performed using 1.5 M PROH with sucrose 0.2 M) was accompanied by loss of high mitochondrial polarity in some oocytes, associated with a significantly reduced capacity to up-regulate the levels of intracellular free calcium after thawing, but not with apoptotic changes (Jones *et al.*, 2004). The possibility that differences in the state of mitochondrial polarization between human MII oocytes is a factor in outcome after embryo transfer is intriguing, especially in view of current pregnancy results with thawed oocytes that demonstrate high frequencies of post-implantation failure. Thus, although we found by TEM a morphologically normal complement of M-SER aggregates in the cryopreserved oocytes belonging to both study groups, our results cannot be predictive of possible alterations in mitochondrial polarity and calcium metabolism.

In human mature oocytes, CGs are stratified in one/three rows in subplasmalemmal areas (Baca and Zamboni, 1967; Sathananthan and Trounson, 1982; Sundstrom *et al.*, 1985a,b; Motta *et al.*, 1988; Sathananthan *et al.*, 1993, 2006). CGs contain mucopolysaccharides, proteases, tissue type plasminogen activator, acid phosphatase and peroxidase (Strömstedt and Byskov, 1999). Although some of these granules may occasionally discharge their content independently of fertilization (Lopata *et al.*, 1980; Szollosi *et al.*, 1986), nevertheless, a sudden and massive physiological release of CG content in the PVS occurs only at fertilization ('cortical reaction'). The cortical reaction and the consequent hardening of the inner aspect of the ZP ('zona reaction') are finalized to prevent the penetration of supernumerary spermatozoa into the oocyte (polyspermy) (Sathananthan and Trounson, 1982; Sathananthan *et al.*, 1993, 2006; Familiari *et al.*, 2006).

In our study, we observed that, in a certain percentage of F/T oocytes belonging to both study groups, the amount and density of CGs appeared abnormally reduced when compared with those of fresh controls. A compaction of the inner aspect of the ZP, with the loss of its typical regular filamentous texture (Familiari *et al.*, 1992) due to the presence of large areas of filaments packed together, was also frequently found in the same samples. These data could be interpreted as the ultrastructural correlates of a premature, non-finalistic exocytosis of the CG content into the PVS with the consequent hardening of the inner side of the ZP. In addition, in our study, these features seem independent from the sucrose concentration in the cryoprotectant solutions. However, the incidence and amount and even the actual cause of CG exocytosis after cryostorage or simple cooling are still a matter of debate. In fact, other morphological studies indicated a comparable distribution and density of CGs in fresh and F/T (with PrOH and sucrose as cryoprotectants) MII-stage human oocytes, suggesting that a significant CG exocytosis may not be always associated with cryopreservation, although these observations do not preclude the possibility that a partial CG exocytosis in some other

areas would not be detected (Van Blerkom and Davis, 1994; Jones *et al.*, 2004). Similarly, abundant CGs were observed in all oocytes analysed, cryopreserved by a slow freeze-rapid thawing method using PrOH as cryoprotectant (Gook *et al.*, 1993). CGs appeared also well preserved in pre-ovulatory human oocytes cooled to 0°C with or without DMSO (Sathananthan *et al.*, 1988b). Conversely, a significant reduction in granule complements by TEM has been described in fresh human oocytes treated with CPAs at RT (Schalkoff *et al.*, 1989). Such a premature release of CG after cryostorage could be a consequence of an impairment of the actin microfilamentous texture that, in human oocytes, normally forms a cortical layer (Pickering *et al.*, 1988) and controls organelle movements, including CG migration and even exocytosis (Smith and Silva, 2004). The hardening of the ZP that follows CG exocytosis may be in part responsible for a reduction in oocyte fertilizability after cryopreservation (Schalkoff *et al.*, 1989; Pickering *et al.*, 1991; Smith and Silva, 2004), although the application of intracytoplasmic sperm injection (ICSI) can bypass some of the problems due to ZP alterations (Gook *et al.*, 1995; Kazem *et al.*, 1995).

In our study, F/T oocytes were surrounded by an intact, regularly structured plasma membrane provided with numerous microvilli projecting into a normal-looking PVS in terms of shape, width and content. These ultrastructural characteristics were all superimposable to those shown by fresh, healthy oocytes (Motta *et al.*, 1988; Sathananthan *et al.*, 1993, 2006; El-Shafie *et al.*, 2000; Familiari *et al.*, 2006). Deformation and disruption of plasma membranes as well as disappearance of microvilli were instead found in some vitrified oocytes (Sathananthan and Trounson, 1989).

Proper organization of the oocyte cytoskeleton, particularly of its microtubular fraction, is essential for normal spindle formation and chromosome segregation. Several studies have demonstrated that exposure of oocytes to cooling, CPAs or freeze-thawing can induce alterations (depolymerization and disorganization) in the spindle microtubules. In contrast, due to the dynamic nature of the microtubule polymerization/depolymerization process, it is possible that spindle re-polymerizes in a normal fashion upon rewarming (Smith and Silva, 2004).

In our study, when observable in favourable sections, the MII spindle was regularly barrel-shaped, anastral, with slightly pointed poles (Sathananthan *et al.*, 1993; Familiari *et al.*, 2006) and properly positioned in the ooplasm of both fresh and F/T oocytes. These features suggest the occurrence of spindle restoring/reassembling during the short period after thawing.

Actually, the meiotic spindle appears ultrastructurally well preserved also in fresh, CPA-treated oocytes (Schalkoff *et al.*, 1989). In contrast, in pre-ovulatory human oocytes cooled to 0°C with or without DMSO, MII spindles were disassembled, due to extensive depolymerization of microtubules, and chromosomes clumped together or were scattered in the cortical ooplasm, particularly in the absence of DMSO (Sathananthan *et al.*, 1988b). However, in this latter study, oocytes were not cultured after cooling, thus only short-term effects of cooling have been determined, whereas possible

spindle recovery with microtubule re-polymerization could not be evaluated. Scattering of chromosomes in MII oocytes may lead to the impairment of pronuclear assembly after fertilization, with the consequent formation of micronuclei (Sathananthan *et al.*, 1988a, 1993; Sathananthan and Trounson, 1989) and development of the condition of aneuploidy, that may severely impair subsequent embryonic development (Smith and Silva, 2004). Spindle damage was also detected in oocytes that failed to fertilize after slow-freezing to -196°C with DMSO and after vitrification (Sathananthan *et al.*, 1987).

However, although Sathananthan *et al.* (1988b) reported good ultrastructural preservation of spindle with sample fixation at both 22 and 37°C, nevertheless other authors emphasize that the absence or disorganization (depolymerization) of the spindle should be considered technical artefacts if samples are routinely and not specifically treated during electron microscopy procedures. According to these authors, in order to allow best visualization of spindle microtubules, oocytes should be fixed with warmed fixative and subsequently handled at 37°C (El-Shafie *et al.*, 2000). These technical requirements, together with the actual difficulty to constantly and completely visualize by TEM the microtubular scaffolding of the spindle and associated chromosomes, lead us to take into consideration other approaches to the study of the spindle morphodynamics during cryopreservation, such as confocal microscopy examination.

By confocal microscopy, spindle and chromosomal configurations appear severely compromised in cryopreserved MII oocytes (Boiso *et al.*, 2002). Sucrose concentrations seem also to differently affect MII spindles in cryopreserved oocytes: in particular, as recently reported by our group, protocols adopting higher sucrose concentrations in the freezing solution seem to promote the retention of an intact chromosome segregation apparatus comparable in incidence to fresh oocytes (Coticchio *et al.*, 2006). However, according to Mullen *et al.* (2004), increasing sucrose concentration will impose greater osmotic stress and will increase the possibility of causing damage to the spindle, although these authors did not include in their treatments penetrating CPAs, which may influence microtubule dynamics (Johnson and Pickering, 1987).

In our study, slight to moderate microvacuolization was revealed in the ooplasm of some F/T oocytes, particularly in those treated with 0.3 mol/l sucrose. Large, cavernous crypts immediately beneath the oolemma have been observed by other authors in fresh, CPA-treated human oocytes (Schalkoff *et al.*, 1989). The presence of vacuoles has also been reported in bovine oocytes after vitrification (Fuku *et al.*, 1995) and in horse oocytes, both after vitrification and after exposure to vitrification solution alone (Hochi, 2003; Hochi *et al.*, 1996). Vacuolization can be considered a non-specific feature commonly found in cells that are responding to an injury (Ghadially, 1982) and is probably the most apparent and dynamic cytoplasmic dysmorphism in human oocytes when routinely examined by PCM (Ebner *et al.*, 2005). In detail, a slight degree of vacuolization is commonly found in immature and aged oocytes, but it is usually absent in fresh, healthy MII oocytes (Sundstrom *et al.*, 1985b; Sathananthan *et al.*, 1993;

El.Shafie *et al.*, 2000; Makabe *et al.*, 2006). When present in mature oocytes, vacuoles may arise either spontaneously or by fusion of pre-existing vesicles derived from SER and are usually filled with a fluid that is virtually identical to the perivitelline fluid (Van Blerkom, 1990). Oocyte vacuolization may be associated with other ultrastructural cytoplasmic alterations such as the presence of secondary lysosomes and myelinic figures, damaged mitochondria, ruptures in the oolemma and reduced amount of microvilli, all aspects of frank degeneration in the oocyte (Motta *et al.*, 1988; El.Shafie *et al.*, 2000). However, oocyte vacuolization deserves further investigation even in the absence of other alterations, because it is considered to be the most important ooplasmic feature that is associated with fertilization failure in both IVF and ICSI (Ebner *et al.*, 2005). In addition, when vacuolated MII oocytes—as seen by PCM—undergo fertilization, they rarely cleave (El.Shafie *et al.*, 2000). A detrimental effect of oocyte vacuolization on preimplantation development up to the blastocyst stage has also been hypothesized (Ebner *et al.*, 2005).

Thus, in our opinion, the presence of cytoplasmic vacuolization might lead to an impairment of the developmental potential of the F/T oocytes, not only exerting a short-time detrimental influence on oocyte cryosurvival and fertilization but also inducing a long-term negative effect on embryo implantation rate, possibly more pronounced when oocytes are cryopreserved using high concentration of sucrose, in agreement with previous clinical studies (Borini *et al.*, 2006).

In conclusion, our ultrastructural data suggest that human mature F/T oocytes may show general ultrastructural markers of maturity and viability similar to those found in fresh oocytes. However, freeze-thawing procedures may generate ultrastructural alterations in specific oocyte microdomains, presumably responsible for the reduced developmental potential of cryopreserved oocytes. In this regard, in further studies, it should be taken into account that high concentrations of sucrose may exert a role, at least in part, in producing the above alterations.

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