Qualitative and morphometric analysis of the ultrastructure of human oocytes cryopreserved by two alternative slow cooling protocols

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Abstract

Purpose To ascertain possible cell damage from cryopreservation, the ultrastructure of human oocytes cryopreserved by slow cooling was assessed.

Materials and methods Cryopreservation was performed through two protocols with one-step or two-step propanediol. Fresh control oocytes were examined for comparison. Samples were processed for transmission electron microscopy analysis. **Results** By light microscopy, both fresh and frozen-thawed oocytes appeared regularly rounded, with intact zona pellucida, and homogeneous cytoplasm. By electron microscopy

observation, organelles were abundant and uniformly dispersed. Mitochondria-smooth endoplasmic reticulum associations appeared regular. However, both the amount and density of cortical granules appeared abnormally reduced in frozen-thawed samples. Slight to moderate vacuolization was also found in the ooplasm of oocytes of both frozen groups. **Conclusions** Slow cooling ensures a good overall preservation of human oocytes. However, cytoplasmic vacuolization and cortical granule loss appears associated with cryopreservation, irrespective of the protocol used.

Capsule Slow freezing appears to ensure an overall good preservation of human oocytes. However, cytoplasmic vacuolization and cortical granules loss are recurrent forms of cell damage.

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Introduction

Since the introduction of controlled ovarian stimulation treatments in human in vitro fertilization (IVF), oocyte cryopreservation has been regarded as the ideal option to capitalize the reproductive potential of surplus oocytes and preserve female fertility. However, for almost two decades the endeavour to store oocytes has been frustrated by inadequate methodologies, with the consequence that the clinical outcome has been poor or of uncertain reproducibility [1, 2]. Concomitantly, embryo cryopreservation has developed as the preferred form of preservation. In the last several years, though, controlled rate slow cooling (CRSC) [3–5] and vitrification [6, 7] approaches have rapidly advanced, bringing oocyte cryopreservation into the range of treatments available for women suffering from, or at risk of, infertility. Nevertheless, the insufficiency of clinical data is still stirring controversy on the choice of the cryopres-

ervation method(s). Disagreement also exists on the entity and type of cell damage that the oocyte may suffer as a result of cryopreservation. For example, several lines of evidence have been reported for and against the hypothesis that the metaphase II (MII) spindle can be irreversibly disrupted by low temperature storage [8–13]. In effect, during the process of freezing-thawing (or vitrification-warming) the oocyte is exposed to a variety of physical and chemical conditions, such as low or suboptimal temperatures, formation of intracellular ice crystals (IIC), osmotic stress and replacement of intracellular water with cryoprotective agents (CPAs), that may endanger its mere survival [14]. The oocyte's ability to develop into a viable pregnancy may be jeopardized even in the absence of obvious injuries detectable by conventional microscopy [15]. It appears essential to define objective criteria by which to establish whether oocyte quality may be affected by cryopreservation, to the aim of supporting or ruling out the applicability of different protocols and appraise possible health risks for children born from cryopreserved oocytes. Oocyte quality may be investigated through a variety of biochemical [16], molecular [17] and microscopical methods [18, 19]. Each method has the potential to provide a specific and valuable insight into the constitution of the oocyte, although a definitive answer to the question as to whether oocyte cryopreservation can be safely performed clearly requires a multidisciplinary approach. Transmission electron microscopy (TEM) allows observation of fine details of cell microanatomy. Rather surprisingly, TEM has attracted only limited attention in the field of cryopreservation, especially in consideration of the fact that structural damage is one of the most likely adverse events associated with cryopreservation, as an effect of IIC formation and osmotic stress. More than 20 years ago, Sathananthan et al. [20] were the first to report damage to the zona pellucida (ZP), oolemma, and ooplasm in cryopreserved human oocytes. With the exception of an analysis of Van Blerkom and Davies published in the mid-nineties [21], further evidence on the ultrastructure of frozen-thawed oocytes has been gained only recently. Ultrastructural alterations involving the cortical granules (CG), ZP, organelle associations, and ooplasm were reported in coincidence with the application of particular dehydration and rehydration conditions [19, 22–24] or possibly specific CPAs [23], suggesting that TEM can represent a first line investigative tool.

To ascertain a possible cell damage and therefore obtain information useful for the choice of an appropriate cryopreservation method, we assessed the ultrastructure of human oocytes cryopreserved with two protocols involving either one- or two-step propanediol (PrOH) dehydration. The two protocols have been tested and are still being used clinically [3, 25], but nothing is known about their ability to preserve the cellular organization of the oocyte.

Materials and methods

Source of oocytes

This study was approved by the Institutional Review Board of the participating clinic. Oocytes were obtained over a period between June 2006 and January 2008 from patients undergoing ART treatment with their informed consent and according to the current Italian laws. Only oocytes donated from women younger than 30 years (mean $\pm$ SD: 25.7 $\pm$ 2.6), whose infertility was due to male or tubal factors, were used. More than one oocyte from the same patient was sometimes included in this study, but it did not systematically occur. Controlled ovarian hyperstimulation was induced with long protocols using GnRH agonist and rFSH, according to the standard clinical procedures routinely employed by the participating clinic [15]. Ten thousand IU of hCG were administered 36 h prior to oocyte collection. After retrieval, oocytes were cultured in IVF media (Cook IVF, Brisbane, Australia, or Sage IVF Inc, Trumbull, CT, USA). Complete removal of cumulus mass and corona cells was performed enzymatically using hyaluronidase (20–40 IU/ml), and mechanically by using fine bore glass pipettes. Only oocytes devoid of any sort of dysmorphisms at phase contrast microscopy (PCM) examination, showing an extruded first polar body (PBI), thus presumably at the MII stage, were assigned to either the control or study groups. According to their assignment, oocytes were either frozen or fixed after a period of 2 or 4 h, respectively, following retrieval. Frozen-thawed oocytes were cultured for further 2 h before fixation.

Two alternative sets of dehydration solutions were employed for this study:

One-step PrOH dehydration: 1.5 mol/l PrOH + 20% Plasma Protein Supplement (PPS, BAXTER AG, Vienna, Austria) in Dulbecco's phosphate-buffered solution (PBS, Gibco, Life Technologies Ltd, Paisley, Scotland) (equilibration solution) and 1.5 mol/l PrOH + 0.2 mol/l sucrose + 20% PPS in PBS (loading solution) previously described by Bianchi et al. [3].

Two-step PrOH dehydration: 0.75 mol/l PrOH and 1.5 mol/l PrOH (equilibration solutions) and 1.5 mol/l PrOH + 0.2 mol/l sucrose (loading solution) (Cook IVF, Brisbane, Australia).

Freezing procedure

One-step PrOH dehydration: oocytes were equilibrated in the equilibration solution (1.5 mol/l PrOH) for 10 min at room temperature (RT) and then transferred into the loading solution (1.5 mol/l PrOH + 0.2 mol/l sucrose) for 5 min.

Two-step PrOH dehydration: oocytes were equilibrated sequentially in solutions containing 0.75 and 1.5 mol/l PrOH

(7.5 min for each step) and then transferred for 5 min into the loading solution (1.5 mol/l PrOH+0.2 mol/l sucrose).

Oocytes were finally loaded in plastic straws (Paillettes Crystal 133 mm; Cryo Bio System, France), individually or in small groups (maximum three oocytes per straw). Straw temperature was lowered through an automated Kryo 10 series III biological freezer (Planer Kryo 10/1,7 GB) from 20°C to −8°C at a rate of −2°C/min. Manual seeding was performed at −8°C. This temperature was maintained in a hold interval of 10 min in order to allow uniform ice propagation. Temperature was then decreased to −30°C at a rate of −0.3°C/min and finally rapidly to −150°C at a rate of −50°C/min. Finally, straws were directly plunged into liquid nitrogen and stored for later use.

Thawing procedure

Thawing was carried out at RT. Straws were removed from liquid nitrogen, warmed in air for 30 s and then plunged in a water bath at 30°C for 40 s. Cryoprotectant step-wise dilution was performed as follows:

One-step PrOH dehydration: Thawed oocytes were released in 1.0 mol/l PrOH, 0.3 mol/l sucrose and 20% PPS and incubated for 5 min. Afterwards they were transferred in 0.5 mol/l PrOH, 0.3 mol/l sucrose and 20% PPS for additional 5 min. Finally oocytes were placed in 0.3 mol/l sucrose and 20% PPS for 10 min before final dilution in buffer including 20% PPS for 20 min (10 min at RT and 10 min at 37°C). All these solution were prepared in PBS buffer.

Two-step PrOH dehydration: Thawing was performed as described for the one-step PrOH protocol employing manufactured solutions containing the same proportions of cryoprotectants (Cook IVF, Brisbane, Australia).

Electron microscopy

A total of 45 mature oocytes were included in this study. Fifteen of them were fixed after 4 h following retrieval and assigned to the control group. The other 30 oocytes were assigned to the study groups and subjected to freeze-thawing procedures as described above. Fifteen survived oocytes for each group were selected for electron microscopy evaluation. Oocytes were considered survived if they had 1. rounded, regular shape; 2. clear, moderately granular cytoplasm; 3. narrow perivitelline space (PVS) with the PBI and, 4. intact, colourless ZP. Oocytes were fixed and processed for TEM analysis as previously described [19]. 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 80KV.

Statistical analysis

The presence of vacuoles larger than 1 µm was evaluated at the light microscopy level on at least three equatorial sections per oocyte (distance between the sections: 3–4 µm), and values were expressed in number of vacuoles per 100 µm² of the oocyte area. The evaluation of cortical granule density was performed through collection of TEM microphotographs of whole surface profiles at ×6300 magnification on three equatorial sections per oocyte. Images were further enlarged on the PC screen, in order to easily recognize and count CG. Values were expressed in number of CG per 10 µm of the oocyte linear surface profile [24]. Statistical data were shown as mean±SD. *P*-value and statistical significance were evaluated by Student's *t* test. Significance was defined as *P*<0.05.

Results

In total, 45 mature oocytes, 15 for each of the three groups (one fresh and two frozen-thawed), were analyzed. According to Motta et al. [26], the following parameters were evaluated by LM and TEM and taken into consideration for the qualitative morphological 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) and presence and extent of ooplasmic vacuolization.

General appearance of the ooplasm

A preliminary evaluation was performed with semi-thin sections observed through LM. In fresh controls, the ZP resulted regular and uninterrupted. The cell profile appeared

spherical and occupying almost entirely the intrazonal space. The ooplasm texture was regular and without discontinuities (Fig. 1a). Regularity, sphericity, and size of the ZP and ooplasm were well preserved also in cryopreserved samples. However, in these specimens, and particularly in those frozen with the two-step protocol, the ooplasm presented with small, interspersed, circular areas in which staining and matter consistency were reduced (Fig. 1b,c).

TEM analysis revealed an overall similarity between fresh and frozen-thawed oocytes. In general, appearance, distribution and mutual association of organelles did not seem to be affected by cryopreservation (Fig. 1d–f). In appropriate sections, MII chromosomes were found eccentrically located in the ooplasm, and the PBI was detected in the perivitelline space. Some distinct differences emerged, however, from a more thorough observation, as described below.

Cortex and ZP

In fresh material, the cortical district showed a regular oolemma with numerous microvillar formations (Figs. 1d, 2a). The area was rich in CG aligned peripherally in a continuous sub-olemmal array (Fig. 2a). In cryopreserved

oocytes, CG were also located abutting the oolemma. In some sub-olemmal domains CG appeared less abundant and/or electron-dense (Fig. 2b–e). The mean number $\pm$ SD of CG per 10 μ m was 9.07 ± 0.45 , 3.78 ± 1.75 , and 2.89 ± 1.08 in the control, one- and two-step groups, respectively (Table 1). These differences between fresh and cryopreserved groups were highly significant ($P<0.0001$). In coincidence with the areas poorer in GC, the inner ZP surface showed some degree of compaction perhaps secondary to modifications caused by enzymatic activities presumably released in the peri-vitelline space following CG exocytosis (Fig. 2f).

Mitochondria and smooth endoplasmic reticulum associations

Close topographic association between mitochondria and smooth endoplasmic reticulum elements is a common feature in mature oocytes, confirming the intimate cooperation of these two types of organelles. In fresh oocytes, several mitochondria-smooth endoplasmic reticulum (mitochondria-SER) aggregates were found diffusely distributed throughout the cytoplasm, with a prevalent localization in the innermost regions. Cross-section of SER elements were different in size, presumably the larger corresponding to

Fig. 1 Fresh control oocytes (a,d); cryopreserved, one-step PrOH dehydrated oocytes (b,e); cryopreserved, two-step PrOH dehydrated oocytes (c,f). The general morphology and organelle microtopography are shown by light microscopy (LM) (a–c) and transmission electron microscopy (TEM) (d–f). No overt difference in shape, dimensions and organelle distribution was observed among fresh and cryopreserved oocytes. The apparent reduced dimensions and increased zona pellucida thickness of the oocyte shown in c is an effect of the section

plane (not equatorial). Clear vacuoles were detected by LM in cryopreserved oocytes, located in both inner (b) and outer, peripheral (c) areas. These vacuoles (V), when observed by TEM, appear as empty, scarcely electrondense structures (f). a, O: oocyte; ZP: zona pellucida. c, C: residual corona cells. d, O: oocyte; ZP: zona pellucida; m: oocyte microvilli. Bar is: 45 μ m (a); 40 μ m (b–c); 4 μ m (d); 3 μ m (e); 5 μ m (f)

Fig. 2 Fresh control oocyte (**a**); cryopreserved, one-step PrOH dehydrated oocytes (**b,c**); cryopreserved, two-step PrOH dehydrated oocytes (**d–f**). A rim of electrondense cortical granules (CG plus arrows) is seen just beneath the oolemma in **a** (fresh oocyte). Cortical granules form a discontinuous layer in cryopreserved oocytes, being either grouped in some areas (CG plus arrows, **c,e**) or almost totally absent in other (**b,d**). Areas of increased density are present in the inner aspect of the zona pellucida (ZP) in **f**. The dense ZP corresponds to an area of the cortical ooplasm virtually devoid of cortical granules. **a,d,e**, m: oocyte microvilli. Bar is: 1.4 μm (**a**); 1.8 μm (**b,d**); 1 μm (**c**); 0.8 μm (**e**); 2 μm (**f**)

cisternae and the smaller to tubuli. Both types of elements are believed to form an intricate and uninterrupted intracellular compartment. Mitochondria were found often adjacent or intimately juxtaposed to SER elements. In all samples, size varied between 0.5 and 1.0 μm and appeared

either rounded or, less frequently, oval, presumably in dependence of section plane orientation. The mitochondrial lumen showed a relatively electron dense matrix and, occasionally, was crossed by small transverse cristae (Fig. 3a). No obvious differences were found in specimens

Table 1 Morphometric evaluation of the presence of cortical granules and extent of vacuolization in human mature oocytes after cryopreservation by two alternative slow cooling protocols including either

one- or two-step PrOH. Values are expressed as mean $\pm$ SD. Statistical analysis is calculated between columns

	Fresh	Cryopreserved	
		One-step	Two-step
No. of cortical granules/10 μm	9.07 $\pm$ 0.45 ^a	3.78 $\pm$ 1.75 ^b	2.89 $\pm$ 1.08 ^c
No. of vacuoles (1.0–2.5 μm in diameter)/100 μm^2	0.45 $\pm$ 0.48 ^e	1.06 $\pm$ 0.94 ^f	1.64 $\pm$ 0.95 ^g

^{a, b} $P < 0.0001$; ^{a, c} $P < 0.0001$; ^{b, c} $P = 0.1$

^{e, f} $P < 0.03$; ^{e, g} $P = 0.0002$; ^{f, g} $P < 0.1$

of both cryopreserved groups, the topography and numerosity of mitochondria and SER associations being apparently undisturbed (Fig. 3b,c).

Vacuoles

The small circular areas, displaying a reduced staining in semi-thin sections, were the object of a more thorough observation at the ultrastructural level. These formations were delimited by membranes that were at times interrupted and characterized, in some parts, by densely organized indentations or niches. The inside of these compartments appeared scarcely electron-dense in comparison to the surrounding cytoplasm and devoid of any sort of particulate matter or membranous system. They were classified as vacuoles, being their appearance reminiscent of vacuolar structure found sometimes in immature germinal vesicle (GV) stage oocytes. These vacuoles, found primarily—but not exclusively—in the peripheral or even sub-olemmal regions, appeared more numerous in cryopreserved samples (Fig. 4a–d). Slight to moderate vacuolization was, respectively, revealed in the ooplasm of frozen oocytes of both one-step and two-step groups but with different frequency (30 and 20% vs. 50% and 25%, respectively). Morphometric analysis revealed that differences between fresh and frozen-thawed samples were statistically significant. In particular, the mean number $\pm$ SD of vacuoles of 1.0–2.5 μm in diameter per 100 μm^2 was 0.45 ± 0.48 , $1.06\pm$

0.94 , and 1.64 ± 0.95 in the control, one-step, and two-step groups, respectively (Table 1).

Discussion

Recent achievements have made oocyte cryopreservation the methodology that perhaps is currently attracting most interest in human IVF, having shown the potential to alleviate the ethical and legal problems associated with embryo storage, make oocyte donation safer, and represent an alternative for fertility preservation in women. In fact, after approximately 20 years of efforts in basic and clinical research [1, 2, 4, 14, 15, 27–31], oocyte cryopreservation has been described to achieve high standards of clinical efficiency [3, 6, 7, 32]. Objective clinical evidence is emerging at a relatively slow pace, because IVF centres that routinely cryopreserve oocytes are still few and, consequently, current data are too small or dishomogeneous to draw definitive conclusions. Research endeavours may contribute to the expedient identification of optimal cryopreservation conditions that would assist the embryologist's choice of a more appropriate cryopreservation procedure. In this work, we describe the ultrastructure of human mature oocytes cryopreserved by two CRSC protocols that have been introduced in the clinical practice [3, 25] but of which no information concerning possible cryodamage is available. In the two protocols, dehydration

Fig. 3 Fresh control oocyte (**a**); cryopreserved, one-step PrOH dehydrated oocyte (**b**); cryopreserved, two-step PrOH dehydrated oocyte (**c**). Mitochondria (M, **a**) are generally rounded and with few peripheral or transverse cristae. Voluminous aggregates between mitochondria and elements of smooth endoplasmic reticulum (SER, **a**) are seen in fresh and cryopreserved samples. Small cisternae presumably deriving from dilated smooth endoplasmic reticulum (arrows, **a**) are also commonly observed. Bar is: 0.8 μm (**a**); 1 μm (**b**); 1.2 μm (**c**)

Fig. 4 Cryopreserved, one-step PrOH dehydrated oocytes (a,b); cryopreserved, two-step PrOH dehydrated oocytes (c,d). Numerous clear vacuoles (V) varying in dimensions are seen in the ooplasm. The vacuoles appear empty, with a limited, and often interrupted,

membrane. A secondary lysosome (Ly) is observed in association with vacuoles in d. b, m: microvilli. Bar is: 1 μm (a); 1.4 μm (b); 2 μm (c,d)

is achieved by one step or two step. Differences in the protocols could be associated with changes in osmotic stress. Electron microscopy techniques are powerful tools to explore the cell ultrastructure, but involve important limitations. They rely on expensive technology and highly trained personnel. Furthermore, they cannot be used for the analysis of large numbers of samples, being the process of specimen preparation and observation lengthy and entirely manual. Regardless, they can provide extensive and detailed information on the fine structure of the cell and its organelles. Ultrastructural analysis is particularly suited to study possible effects of cryopreservation, a process that exposes the cell to mechanical stress irrespective of the methodological approach. Sathananthan [20] was the first to introduce electron microscopy for the study of cryopreserved human oocytes. He observed fractures in the zona, oolemma anomalies, and extensive disarrangement of the ooplasm. By simply cooling fresh oocytes to 0°C in the presence of dimethylsulfoxide (DMSO) for less than an hour, he also noticed that elements of the endoplasmic reticulum, Golgi, mitochondria and the cytosol were affected to some extent [33]. Limits of ultrastructural analysis as a tool to assess the normalcy of cryopreserved oocytes emerged also from a study of Van Blerkom and Davies [21]. These authors reported cytokinesis anomalies and developmental arrest in embryos developed from cryopreserved human oocytes, despite electron microscopy analysis carried out after thawing had failed to demonstrate gross cytoplasmic alterations in frozen-thawed oocytes.

Thereafter, electron microscopy studies on cryopreserved human oocytes eclipsed for over a decade, as a effect of the inability of the cryopreservation methodology available in the nineties to ensure high survival rates. With the advent of improved protocols [4, 34, 35], novel ultrastructural evidence was generated. Comparing fresh oocytes with others frozen with a CRSC involving 0.1 mol/l sucrose as an extracellular CPA, Ghetler et al. [36] found massive reduction in the number of CG as a effect of cryopreservation, concluding that stored oocytes should be micro-injected rather than inseminated by standard IVF to prevent possible fertilization failure secondary to zona hardening. In effect, ICSI (intracytoplasmic sperm injection) had been suggested to be the elective route to achieve fertilization in cryopreserved oocytes [27] and has now become a standard of treatment [6, 7, 14, 29]. The question of a non-physiological discharge of CG in cryopreserved oocytes remains controversial. By using epifluorescence microscopy, Gook et al. [37] showed that the staining specific for CG was not decreased in cryopreserved material in comparison to fresh controls. They also demonstrated that that fertilization could be achieved for frozen-thawed oocytes through standard IVF [28]. More recently, the successful use of standard IVF has been reported by other authors [38]. Conversely, ultrastructural analysis has confirmed that a partial loss of CG occurs after cryopreservation. We observed this circumstance qualitatively in oocytes cryopreserved with CRSC protocols involving the use of 0.1 or 0.3 mol/l sucrose as a constituent of the freezing

solution [19]. Similar findings were described by Gualtieri et al. [24]. Consistent with this, we found that oocytes cryopreserved by a CRSC protocol including ethylene glycol (EG) as an intracellular CPA [23] or by vitrification [39] were also affected by a loss of CG. Here, we extend and confirm our previous experience and provide quantitative data suggesting that about two thirds of the original population of CG is lost, and presumably their contents are released into the perivitelline space, as a result of cryopreservation. This phenomenon appears rather ubiquitous in cryopreserved oocytes, irrespective of the cryopreservation approach (CRSC or vitrification) or specific protocol. It may be worth noting that the one-step protocol described in this work appears to induce a smaller CG loss and could be preferential to the two-step procedure for clinical use. Whether cortical granule release is sufficient to cause zona hardening in frozen-thawed oocytes is still matter of debate, but the fact that we observed thickening and change in texture of the inner surface of the ZP is in line with this hypothesis.

In our study, another prominent characteristic found in cryopreserved oocytes was the increased presence of vacuolar formations. The nature of these formations has not been well characterized. They may be found in fully-grown immature oocytes, but in metaphase II oocytes they are rare and have been interpreted as a sign of cytoplasmic immaturity or, *vice versa*, ageing. Because vacuoles could be a general response to injury [40], they may represent a manifestation of oocyte stress after cryopreservation. The difference in the mean number of vacuoles per area unit was not statistically significant between the one- and two-step protocol. Regardless, again we suggest that the one-step protocol, that is less affected by the formation of vacuoles, may be preferred for clinical application. The one-step protocol seems to be associated to a higher clinical outcome [3], although this has not been confirmed through a prospective randomized control trial. Rather consistently, the presence of vacuoles was described by our group as well as by others in association with a variety of CRSC protocols [19, 23, 36]. It is tempting to hypothesize that vacuoles located peripherally may evolve from crypt-like invaginations and clusters of endocytic vesicles which form in the oocyte cortex following simple exposure to CPA, as shown by Schalkoff et al. [41]. However if this assumption is correct, it remains to be explained why oocytes vitrified through the cryoleaf method, which are exposed to high concentrations of CPAs, do not exhibit an increase in the number of vacuoles, as we recently reported [39]. Regardless their origin, vacuoles may be adopted as a specific indicator of cryodamage and reduced developmental potential in cryopreserved oocytes, considering that an increased number of vacuoles [19] and a reduced developmental ability [14, 15] is associated to certain CRSC protocols. Preliminary evidence suggest that vitrification protocols

may be compatible with high implantation ability [7] and a normal occurrence of vacuoles [39]. This inference, however, is rather speculative and requires accurate verification, having the above mentioned vitrification data generated by two different containment devices, the cryotop [7] and cryoleaf [39].

Finally, we compared the condition of mitochondria and SER elements between fresh and cryopreserved oocytes. Individually, these two types of organelles play well characterized roles in all cells. In particular in oocytes, not only are mitochondria central to the energy generation process, but also can produce intermediates of the tricarboxylic acid cycle and reducing equivalents that may be employed in antioxidant defense. The SER complex is instead well characterized as an organelle system competent to the translation, modification, packaging and delivery of proteins destined to the plasmalemma or exocytotic compartments. Together in the oocyte, the association between mitochondria and SER elements contribute to the generation of the typical oscillations in cytosolic free calcium which are triggered by the fertilizing spermatozoon and are interpreted by the oocyte biochemical machinery as a trigger for the fertilization [42]. Magnitude and frequency of calcium oscillation have also been found to influence much later events in development, such as organogenesis and fetal growth [43]. For such reasons mitochondria-SER aggregates are particularly important for the physiology of the oocyte. After cryopreservation with the two CRSC protocols adopted in this study, we did not detect signs of perturbations in mitochondria or SER elements, separately or in close association with each other. Distribution, amount, shape and electron-density of these organelles were virtually indistinguishable between fresh and cryopreserved groups. Previously, we reported that mitochondria-SER associations were undisturbed in oocytes cryopreserved with CRSC protocols relying on ProH as an intracellular CPA [19] and used in the clinical practice [14, 15]. Conversely, other cryopreservation conditions may be in fact deleterious to mitochondria-SER aggregates. In particular, in oocytes cryopreserved by a CRSC protocol including EG as an intracellular CPA, we noticed pronounced disorganization of these aggregates [23]. This lead us to exclude this protocol as a candidate method applicable for clinical purposes. Why some cryopreservation conditions can generate damage to the mitochondria-SER aggregates is still to be understood. It might be worth discriminating between the effects of individual intracellular CPAs, because mitochondria-SER aggregates disarrangements so far have been reported only in association with the use of EG, but not ProH. Concerning this, it is interesting to note that we found noticeable, although moderate, underdeveloped mitochondria-SER aggregates in oocytes vitrified with a solution containing EG [39]. However, such findings are

largely insufficient to conclude that EG can specifically and directly affect the constitution of mitochondria-SER aggregates.

Conclusions

In conclusion, this study confirms that CRSC ensures a good overall preservation of the oocyte. In frozen-thawed material, ZP, oolemma, mitochondria and mitochondria-SER associations were found similar to fresh controls. Our data also support the notion that CG are partially lost in cryopreserved oocytes, suggesting the appropriateness of ICSI as the preferred method to achieve fertilization. Moreover, vacuolization appears as a recurrent form of cell damage caused by cryopreservation. Because our observations suggest that one-step PrOH dehydration is less affected by post-thaw vacuolization, such a cryopreservation condition might be preferable in order to better preserve oocyte quality after cryopreservation and possibly achieve a higher clinical outcome. This supports the opinion that TEM analysis provides valuable and objective criteria to assess cell injury from cryopreservation.

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