Review

Preservation of fertility in young cancer patients: contribution of transmission electron microscopy

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Abstract

During the last decade, new technologies in reproductive medicine have emerged to preserve the fertility of women whose gonadal function is threatened by premature menopause or gonadotoxic treatments. To offer an individualized approach to these patients, different experimental procedures are under investigation, including oocyte cryopreservation and cryopreservation and transplantation of ovarian tissue in the form of cortical fragments, whole ovary or isolated follicles. This review shows that transmission electron microscopy (TEM), combined with other in-vivo and in-vitro analysis techniques, is a valuable tool in the establishment of new experimental protocols to preserve female fertility. Ultrastructural studies allow in-depth evaluation of the oocyte's unique morpho-functional characteristics, which explain its low cryotolerance, and provide essential information on follicular, stromal and endothelial cell integrity, as well as cellular interactions crucial for normal folliculogenesis. In order to be able to offer appropriate and efficient options in every clinical situation, oocyte in-vitro maturation and ovarian tissue transplantation need to be optimized. Further development of new approaches, such as follicular isolation and whole ovary transplantation, should be encouraged. Fine ultrastructural details highlighted by TEM studies will be useful for the further optimization of these emerging technologies.

Keywords: *fertility preservation, oocyte, ovarian tissue, transmission electron microscopy, transplantation*

Introduction

For patients who need to undergo chemotherapy or radiotherapy, depletion of their follicular reserve will result in premature menopause and loss of fertility. Many cytotoxic agents have been shown to induce irreversible destruction of the follicular reserve, as reviewed by Donnez *et al.* (2006). Ultrastructural studies have allowed precise characterization of damage to ovarian tissue caused by gonadotoxic treatment (Familiari *et al.*, 1993; Raz *et al.*, 2002; Abir *et al.*, 2005). Familiari *et al.* (1993) evaluated, by TEM, the follicular density and morphology of ovarian follicles in patients treated by chemotherapy for Hodgkin's disease, and this demonstrated both a reduction in the number of ovarian follicles and impairment of the quality of those surviving. Abir *et al.* (2005) used TEM to compare ovarian tissue from patients treated or not by chemotherapy, and found deterioration in follicular quality after treatment, but no evidence of follicular cell or oocyte apoptosis. In particular, they found an increase in abnormal granulosa cell nuclei, oocyte vacuolization and basement membrane abnormalities in the ovarian tissue of patients subjected to chemotherapy. Another major concern is the possibility of generating chromosomal anomalies in growing oocytes after cytotoxic treatment (Candy *et al.*, 2000). A study in mice indicated that early fertilization post-chemotherapy may result in high pregnancy failure and malformation rates (Meirow *et al.*, 2001).

To preserve fertility in young patients undergoing cytotoxic treatment (radiotherapy or chemotherapy) for severe pathologies such as neoplastic or autoimmune diseases or for bone marrow transplantation, or those at risk of premature ovarian failure (hereditary premature menopause), three options may be considered: embryo, oocyte or ovarian tissue cryopreservation (Donnez and Bassil, 1998; Donnez *et al.*, 2000, 2005, 2006; Oktay, 2005; Kim, 2006).

The choice of the most suitable strategy for fertility preservation depends on different parameters: the type and dose of radio- or chemotherapy, the type of pathology, and the patient's age and partner status (for review, see Donnez *et al.*, 2006).

Embryo cryopreservation is currently the only established method of fertility preservation (Ethics Committee of the American Society for Reproductive Medicine, 1995). Unfortunately, this technique is subject to restricted indications, and it is not applicable to prepubertal girls, patients in whom ovarian stimulation is contraindicated, or women without a partner who do not wish to use donor sperm (Donnez *et al.*, 2006). An alternative involves cryopreserving female gametes prior to fertilization, either as single structures (cryopreservation of mature or immature oocytes) or embedded in the ovarian tissue (cryopreservation of ovarian tissue), but these procedures should only be performed in an experimental setting under IRB guidelines, according to the Practice Committee of the American Society for Reproductive Medicine (2006).

Recent advances in mature oocyte cryopreservation procedures nevertheless herald a promising future for this approach (Porcu *et al.*, 2004; Borini *et al.*, 2007), and this is already included in clinical practice in several assisted reproduction centres. This technique has been successfully applied in humans. However, its effectiveness remains very low, with implantation rates ranging from 2.3 to 11% per thawed oocyte depending on the protocol (Levi Setti *et al.*, 2006; Gook and Edgar, 2007), underlining the importance of pursuing research in this field. Cryopreserving immature or mature oocytes is particularly challenging due to several unique morpho-dynamic and physiological features of oocytes (Familiari *et al.*, 2006; Gardner *et al.*, 2007; Gook and Edgar, 2007). As detailed by Smith *et al.* (2004), human and animal oocytes are especially sensitive to cryodamage at the level of the meiotic spindle, cytoskeletal components, cytoplasm and intracellular organelles, zona pellucida (ZP) and cortical granules (CG), although some of these alterations are reversible, as discussed in the review.

Oocyte cryopreservation cannot be applied, however, if the patient is prepubertal, the malignancy is oestrogen-sensitive (breast and ovarian cancer), or it is impossible to postpone therapy in order to induce superovulation. Cryopreservation and transplantation of ovarian tissue may be proposed in such cases as an experimental option to prevent fertility loss (Demeestere *et al.*, 2003; Oktay, 2005; Donnez *et al.*, 2006; Kim, 2006; Meirow *et al.*, 2007).

The ovarian cortex contains follicles mainly at primordial and primary stages, which are particularly resistant to cryopreservation procedures (Hovatta, 2005). At these stages, the follicles possess certain characteristics that make them less sensitive to cryoinjury, such as a low metabolic rate and number of granulosa cells, absence of a ZP and peripheral cortical granules, and a small immature oocyte (arrested in the prophase of the first meiotic division) with low amounts of intracytoplasmic lipids.

Ultrastructural studies have indeed demonstrated that ovarian tissue can be successfully cryopreserved with no significant alterations to follicular ultrastructure (Cortvrindt *et al.*, 1996; Oktay *et al.*, 1997; Gook *et al.*, 1999; Nisolle *et al.*, 2000; Abir *et al.*, 2001; Hreinsson *et al.*, 2003; Eyden *et al.*, 2004; Imhof *et al.*, 2004; Lucci *et al.*, 2004; Martinez-Madrid *et al.*, 2004a; Rodrigues *et al.*, 2004a,b; Camboni *et al.*, 2005; Fabbri *et al.*, 2006a,b; Santos *et al.*, 2006; Nottola *et al.*, 2007b; Fauque *et al.*, 2007). However, most investigations were conducted immediately after freezing, and further studies are clearly warranted to assess potential cryoinjury after incubation of the tissue (Gosden, 2000).

After thawing of ovarian tissue, different strategies have been explored to support follicular development and achieve oocyte maturation, including in-vitro culture and in-vivo transplantation approaches (Gosden *et al.*, 2002; Donnez *et al.*, 2004; Oktay *et al.*, 2004). Sustaining folliculogenesis *in vitro* is particularly challenging in humans due to the prolonged duration of follicular development (205 days) (Gougeon, 1996). Primordial follicles have not yet been grown beyond the preantral follicular stage when cultured as isolated or partially isolated follicles (Abir *et al.*, 2001), within adult tissue (Hovatta, 2004), or fetal tissue (Sadeu *et al.*, 2006), but complete in-vivo follicular development has been achieved after xenografting of human ovarian tissue (Gook *et al.*, 2003).

In humans, autotransplantation of cryopreserved ovarian cortical fragments is at present, the only procedure that has yielded live births (Donnez *et al.*, 2004; Meirow *et al.*, 2005), a clinical pregnancy (Demeestere *et al.*, 2006) and a biochemical pregnancy (Rosendahl *et al.*, 2006) after autologous transplantation.

Although this technique has produced promising results, it is important to bear in mind that ovarian tissue grafts show only modest degrees of follicular survival, reducing the life span of the graft. Several studies conducted on hypogonadic severe combined immunodeficient (SCID) mice have demonstrated the ability of cryopreserved human ovarian follicles to initiate and maintain their growth and development up to the stage of ovulation in xenografts (Oktay *et al.*, 1998, 2000; Van den Broecke *et al.*, 2001; Kim *et al.*, 2002; Gook *et al.*, 2003, 2005). However, a high proportion of primordial follicles, ranging from 50 to 90% (Aubard *et al.*, 1999; Baird *et al.*, 1999; Nisolle *et al.*, 2000; Schubert *et al.*, 2007), appear to be lost after transplantation of cryopreserved ovarian tissue. It has been suggested that ischemic damage occurring before the establishment of neovascularization may be to blame, but the mechanisms underlying this follicular loss are largely unknown. Further structural and molecular studies on the early post-transplantation period and the regulation of follicular growth are clearly needed to progress in this field. TEM has proved to be useful in order to analyse the impact of grafting procedures on ovarian tissue integrity and provide further insights into any possible changes in follicular cells, stromal cells and oocytes. Indeed, interesting and intriguing TEM results on human follicular morphology after short-term xenografting have been obtained (Camboni *et al.*, 2005; Nottola *et al.*, 2007b); these will be discussed in this review.

Reducing the ischemic interval between transplantation and revascularization is essential to avoid loss of the follicular reserve and maintain function of the graft. In theory, the best way to achieve this is by transplantation of intact ovary with vascular anastomosis, allowing immediate revascularization of the transplant. The feasibility of autotransplantation of cryopreserved whole ovaries has been demonstrated in rodents (Wang *et al.*, 2002; Yin *et al.*, 2003; Chen *et al.*, 2005) and more recently in sheep (Bedaiwy *et al.*, 2003; Arav *et al.*, 2005; Imhof *et al.*, 2006). The authors are currently investigating the possibility of applying this approach to human ovaries (Martinez-Madrid *et al.*, 2004a, 2007; Jadoul *et al.*, 2007). Morphological assessment of ovarian integrity by TEM after whole human ovary cryopreservation is especially important in the context of this project, because of the absence of experimental xenotransplantation models to assess ovarian function after grafting. A crucial aspect of this procedure is the preservation of vessel integrity after cryopreservation, which is vital for reanastomosis of the ovarian vascular pedicle.

An alternative that may be considered is isolating ovarian follicles after thawing ovarian tissue, and growing them *in vitro* or *in vivo*. Theoretically, in-vitro culture is the best way to achieve follicular growth and maturation after cryopreservation and isolation. However, in-vitro culture of isolated (Abir *et al.*, 2001) or partially isolated (Hovatta *et al.*, 1999) human follicles has not yet shown satisfactory results. Another approach could therefore involve grafting isolated follicles. Cryopreservation and transplantation of isolated primordial follicles has been successfully achieved in mice (Carroll and Gosden, 1993) and preliminary studies indicate that isolated human follicles are viable and can grow after xenografting (Dolmans *et al.*, 2007). However, before this technique can be introduced into clinical practice, the first step is to optimize and standardize protocols for follicular isolation, and to evaluate the safety of the procedure. Isolation procedures may induce subtle alterations that irreversibly affect follicular developmental ability, which can be assessed by TEM and viability analysis. The value of these techniques has been clearly proved in the development of isolation protocols (Dolmans *et al.*, 2006).

The aim of this review is to summarize all the available scientific literature relating to existing structural and ultrastructural studies of the female gamete after different laboratory procedures for the preservation of female fertility, emphasizing the importance of TEM in the evaluation and comparison of different protocols. With a view to their future routine application in clinical practice, it is important to first establish whether oocyte and ovarian tissue cryopreservation, follicle isolation and ovarian tissue transplantation may compromise oocyte and ovarian tissue morpho-physiology. In this regard, TEM may provide essential information on cellular and tissue integrity, which cannot easily be obtained by other approaches. This review shows that TEM morpho-functional studies have made a great contribution to the development of new options for the preservation of female gametes. The review focuses on human oocytes and ovarian tissue but, since most protocols were initially set up in animal models, results from animal studies are also summarized. Although there are many differences between species (Gandolfi *et al.*, 2006), important lessons can be learned from these studies.

Cryopreservation of mature oocytes

Mature oocytes often fail to survive the freeze-thawing process, showing lower rates of survival and development than embryos (Gook and Edgar, 2007). Their high susceptibility to cryodamage may be explained by their unique morpho-dynamic features: large size, water- and cold-sensitive intracytoplasmic lipid content and chromosomal arrangement. Ultrastructural damage to the meiotic spindle (Pickering and Johnson, 1987; Sathananthan *et al.*, 1987, 1988a,b, 1992; Wu *et al.*, 2006), cytoskeletal components (Vincent *et al.*, 1990a), cytoplasm and intracellular organelles (Sathananthan *et al.*, 1987; Hochi *et al.*, 1996), ZP (Sathananthan *et al.*, 1987; Strom Holst, 2000; Wu *et al.*, 2006) and CG (Vincent *et al.*, 1990b; Al Hasani and Diedrich, 1995; Fuku *et al.*, 1995a,b; Hyttel *et al.*, 2000; Valojerdi and Salehnia, 2005; Ghetler *et al.*, 2006; Nottola *et al.*, 2007a) are commonly reported in mature animal and human oocytes after cooling and freezing, as detailed below.

During the final maturation process, oocytes undergo cytoplasmic modifications, as well as redistribution and neosynthesis of organelles, showing a complex and fragile organization of nuclear and cytoplasmic compartments at the metaphase-II stage (MII) oocytes. Critical steps during cryopreservation that may alter oocyte ultrastructure include exposure to high concentrations of cytotoxic cryoprotectant and the freezing step itself (including chilling and induction of crystallization). Vitrification of mature oocytes may offer advantages over conventional slow-programmed freezing protocols by improving oocyte ultrastructure and physiology preservation and post-thaw survival rates (Gardner *et al.*, 2007), but needs to be more fully investigated before its clinical application can be considered (Sathananthan and Trounson, 1989; Schalkoff *et al.*, 1989; Gook and Edgar, 2007).

Premature exocytosis of cortical granules

During the early stage of oocyte growth, membrane-bound spherical or slightly ovoid organelles derived from the Golgi apparatus comprise structures called cortical granules (CG) (Cran and Esper, 1990; Ghetler *et al.*, 2006), which are typically observed by TEM. In mature human oocytes, CG are aligned in subplasmalemmal areas (Motta *et al.*, 1988; Sathananthan *et al.*, 1993, 2006) and physiologically release their contents into the perivitelline space during fertilization by means of a process called the cortical reaction, which produces modifications to the inner ZP area (zona reaction), causing ZP hardening. This mechanism is responsible for the block to polyspermic, preventing the penetration of supernumerary spermatozoa into the oocyte (Vincent *et al.*, 1990a; Sathananthan *et al.*, 2006).

Several authors have used TEM to identify an abnormal reduction in the number of CG in the cytoplasm of mature oocytes in humans and animals after different cryopreservation protocols (Vincent *et al.*, 1990a; Al Hasani and Diedrich, 1995; Fuku *et al.*, 1995a,b; Hyttel *et al.*, 2000; Valojerdi and Salehnia, 2005; Ghetler *et al.*, 2006; Nottola *et al.*, 2007a).

Exposure of fresh MII mouse (Schalkoff *et al.*, 1989; Vincent *et al.*, 1990a) and human (Schalkoff *et al.*, 1989) oocytes to cryoprotectant at room temperature causes a release of CG, associated with ZP damage. A reduction in CG number has also

been reported in mature bovine oocytes, both after simple exposure to vitrification solution (Fuku *et al.*, 1995a) and after vitrification itself (Fuku *et al.*, 1995b; Hyttel *et al.*, 2000). Similarly, in a recent ultrastructural study, a significant decrease in CG was reported in pre-ovulatory human oocytes after freeze-thawing with propane-1,2-diol (PROH) plus sucrose at different concentrations (Nottola *et al.*, 2007a). Concomitant with this decrease in CG number, compaction of the inner area of the ZP with loss of its physiological texture was also observed (Fuku *et al.*, 1995b; Nottola *et al.*, 2007a). These ultrastructural modifications have been interpreted as premature exocytosis of CG, as a consequence of impairment of the microfilament network responsible for organelle movements, including CG migration (Smith *et al.*, 2004).

However, other ultrastructural studies on oocyte cryopreservation suggest that cryopreservation and premature CG exocytosis are not always connected. Gook *et al.* (1993) found an abundance of CG in human oocytes cryopreserved with PrOH, and Sathananthan *et al.* (1987, 1992) reported well-preserved CG in pre-ovulatory human oocytes cooled to 0°C with or without dimethylsulfoxide (DMSO) exposure, and in mouse oocytes after cooling. Moreover, in two morphological studies comparing human oocytes frozen–thawed with PrOH and sucrose and fresh human oocytes (Van Blerkom and Davis, 1994; Jones *et al.*, 2004), no reduction in CG number was reported.

Zona pellucida hardening

It has been reported that premature zona hardening associated with premature release of CG may be responsible for reduced fertilization rates in cryopreserved oocytes (Johnson *et al.*, 1988; Schalkoff *et al.*, 1989; Van Blerkom and Davis, 1994; Smith *et al.*, 2004). Alterations in ZP ultrastructure associated with decreased sperm binding capacity were identified by scanning electron microscopy (SEM) in canine oocytes frozen to –20°C (Strom *et al.*, 2000). However, Sathananthan *et al.* (1987) found no difference in sperm binding or zona penetration, despite marked damage to the ZP in frozen–thawed mature human oocytes, and Kazem *et al.* (1995) suggested that a compromised zona reaction, due to a reduction in CG number, may allow the penetration of supernumerary spermatozoa.

Intracytoplasmic sperm injection (ICSI) could circumvent the problem of sperm penetration block (Gook *et al.*, 1995; Kazem *et al.*, 1995), but as ICSI does not appear to re-establish normal fertilization rates, other fundamental cryodamage must occur after cryopreservation of mature oocytes (Borini *et al.*, 2004).

Mitochondrial damage

Changes to cytoplasmic membranes and organelles, particularly mitochondria, are other possible consequences of oocyte cryopreservation. Alterations in mitochondrial morphology, as well as in the association between mitochondria and smooth endoplasmic reticulum (SER) elements (membranes and vesicles), may influence the developmental competence of human oocytes (Van Blerkom, 2004). In particular, it has been suggested that alterations in mitochondrial polarity and the mitochondria-SER association may be among the factors determining embryo development failure, as they are involved in free intracellular calcium regulation (Jones *et al.*, 2004; Van Blerkom, 2004).

Exposure to cryoprotectant alone does not seem to affect cytoplasmic ultrastructure of oocytes, since normal cytoplasmic organelles, including mitochondria, were observed in fresh oocytes exposed to PrOH and DMSO plus sucrose (Schalkoff *et al.*, 1989). It is the decrease in temperature that appears to have an influence on oocytes. Chilling has been implicated in the modification of membranes (Ghetler *et al.*, 2005), which may affect their integrity, while freezing has been shown to cause mitochondrial enlargement and alterations in the relationship of SER elements with mitochondria in bovine oocytes (Schmidt *et al.*, 1995). Sathananthan *et al.* (1988a) also observed mitochondrial swelling and SER element damage after cooling human oocytes to 0°C.

Vitrification of mature oocytes may prevent severe damage to cytoplasmic organelles and membranes, according to Valojerdi and Salehnia (2005). They analysed the ultrastructure of MII mouse oocytes after either cryopreservation by slow freezing or by vitrification, followed by fertilization. In the slow-frozen oocytes, some mitochondria were found to be swollen. After fertilization, slow-frozen oocytes showed extensive vesiculation in their cytosol, which may correspond to mitochondria or SER, while vitrified oocytes did not show any mitochondrial alterations. However, the ultrastructural observations of Valojerdi and Salehnia (2005) contradicted the findings of other studies. Hochi *et al.* (1996) reported that vitrification or exposure to vitrification solution induced swelling of mitochondria, coupled with a reduction in matrix density. Alterations to the mitochondria and SER association and an increase in the volume of vacuoles were also reported after vitrification of bovine oocytes (Fuku *et al.*, 1995b).

Membranous organelle damage

Changes in temperature were also shown to affect membranous organelles. Studies on mouse MII oocytes revealed that the endoplasmic reticulum, Golgi apparatus and nuclear envelope were all sensitive to cooling (Sathananthan *et al.*, 1992). Deformation and disruption of cellular membranes, as well as degeneration and disappearance of microvilli, were detected in slow-frozen (Sathananthan, 1987; Valojerdi and Salehnia, 2005) and vitrified (Sathananthan and Trounson, 1989) human oocytes. In in-vitro matured bovine oocytes frozen with glycerol, the plasma membrane showed multiple ruptures, with numerous cytoplasmic vesicles dislocated in the perivitelline space (Schmidt *et al.*, 1995).

On the other hand, Valojerdi and Salehnia (2005) showed that the ultrastructure of vitrified mouse MII oocytes was similar to fresh oocytes, showing no alterations in cellular membranes. The same results were recently achieved in pre-ovulatory human oocytes frozen–thawed with PrOH plus sucrose at different concentrations (Nottola *et al.*, 2007a). As illustrated in **Figure 1**, the general morphology and organelle microtopography of mature human oocytes was well preserved after freezing and thawing.

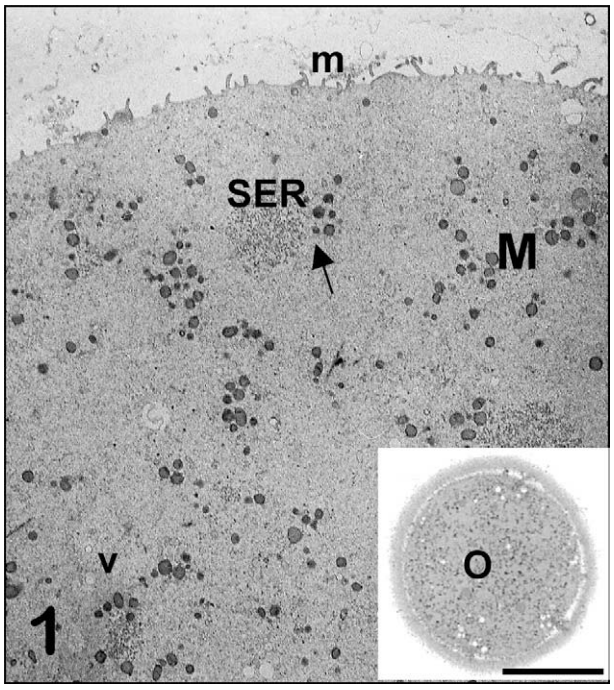

Figure 1. Mature human oocytes frozen–thawed with propane-1,2-diol plus sucrose 0.3 mol/l. No alterations were found in shape, dimensions or organelle distribution, but a slight degree of vacuolization (v) was seen. Also note the presence of voluminous aggregates between mitochondria (M) and SER elements (arrow). O, oocyte; SER, smooth endoplasmic reticulum; m, oocyte microvilli. Bar: light microscopy (inset): 45 µm; transmission electron microscopy: 4.5 µm (from Nottola *et al.*, 2007a, with permission).

Vacuolization

Vacuolization is an important ultrastructural dysmorphism frequently observed in oocytes subjected to cryopreservation procedures. Oocyte vacuolization seems to be associated with fertilization failure and alterations in embryo cleavage and preimplantation development after fertilization (El Shafie *et al.*, 2000; Ebner *et al.*, 2005).

Vacuolization has been detected in mature bovine oocytes (Fuku *et al.*, 1995a,b; Hyttel *et al.*, 2000; Schmidt *et al.*, 1995) and immature horse oocytes after exposure to vitrification solution alone (Al Hasani and Diedrich, 1995; Hochi *et al.*, 1996), following vitrification (Fuku *et al.*, 1995b; Hochi *et al.*, 1996; Hyttel *et al.*, 2000), and after freezing with glycerol (Schmidt *et al.*, 1995). In humans, Schalkoff *et al.* (1989) detected cytoplasmic vacuolization in fresh MII oocytes exposed to cryoprotectant. Moreover, Nottola *et al.* (2007a) observed moderate microvacuolization in frozen–thawed human oocytes, particularly those frozen with PrOH and high concentrations of sucrose (0.3M).

Depolymerization of the meiotic spindle

The meiotic spindle is the major microtubular structure in mature oocytes, responsible for spatial organization and subsequent migration of chromosomes during meiotic division (Smith *et al.*, 2004). It is known to be sensitive to low temperatures (Zenzes *et al.*, 2001; De Santis *et al.*, 2007) and cryoprotectants (Johnson and Pickering, 1987; Van der Elst *et al.*, 1988). Changes to the structure and organization of microtubules suggest that cryopreservation of oocytes at the MII stage may frequently be accompanied by irreversible damage to the spindle, which can affect chromosomal organization and alignment and even cause fertilization failure. Data are nevertheless still conflicting: while some studies show that certain freezing protocols preserve meiotic spindle organization (Gook *et al.*, 1993, 1994; Van Blerkom and Davis, 1994; Baka *et al.*, 1995; Stachecki *et al.*, 2004; Coticchio *et al.*, 2006), others report alterations (Zenzes *et al.*, 2001; Boiso *et al.*, 2002), such as spindle microtubule depolymerization and disorganization. Furthermore, it has been postulated that, due to the dynamic nature of microtubules, normal spindle configuration may be recovered after rewarming, thanks to a process of spindle repolymerization (Smith *et al.*, 2004).

To assess the effect of cryopreservation protocols on meiotic spindle organization, a number of techniques have been applied, such as immunofluorescence (Baka *et al.*, 1995; Zenzes *et al.*, 2001; Stachecki *et al.*, 2004), polarization (Bianchi *et al.*, 2005; Rienzi *et al.*, 2005) and confocal microscopy (Boiso *et al.*, 2002; Coticchio *et al.*, 2006) and TEM (Sathananthan *et al.*, 1987, 1988a,b, 1992; Wu *et al.*, 2006). Using TEM, Sathananthan *et al.* (1992) found that temperatures below 15°C affected the meiotic spindle of porcine oocytes: at 4°C with the microtubules suffering total or partial depolymerization, while at 0 and -7°C, they completely disappeared. These authors showed that, at lower temperatures, the chromosomes tended to clump or rotate and centrosomal material was fragmented with no evidence of the spindle poles. However, spindle structure was restored after rewarming at 37°C. In another study with human oocytes, the same team (1988a) obtained similar results at lower temperatures. Spindles from oocytes cooled to 0°C were disassembled due to the extensive depolymerization of

the microtubules. Only a few microtubules could be identified, and these were at the poles, bundled together, or associated with chromosomes. Some oocytes cooled with DMSO at 0 and 8°C showed evidence of microtubules, but the spindles were still disorganized and abnormal in structure. This study demonstrates that meiotic spindles are very sensitive to cooling and that DMSO was not able to provide satisfactory stabilization of the spindle.

Cryopreservation of immature oocytes

An alternative approach is to freeze immature oocytes at the germinal vesicle stage (GV oocytes) and mature them *in vitro*. At the GV stage, oocyte chromatin is diffused and the cell is still at the diplotene stage of prophase I, when no polymerized tubules are present. This strategy offers some practical advantages, because hormonal stimulation is not needed (Gosden, 2005), and freezing GV oocytes avoids the risk of meiotic spindle damage and cytogenetic abnormalities during subsequent cellular divisions.

Some reports have shown favourable results after immature oocyte cryopreservation (Boiso *et al.*, 2002). However, according to other studies, immature oocytes do not freeze better than those at the MII stage (Al Hasani and Diedrich, 1995; Fuku *et al.*, 1995b; Ghetler *et al.*, 2006). In fact, after freeze-thawing immature oocytes, the risk of structural damage to the cytoskeletal components, cytoplasm organelles, ZP and CG is high. Ghetler *et al.* (2006) demonstrated an increase in CG exocytosis by confocal microscopy and a drastic reduction in CG number and vesicle formation in the ooplasm by TEM in GV and MII human oocytes frozen with PROH as the cryoprotectant.

Van Blerkom (1989) reported that, after vitrification, >90% of GV mouse oocytes were capable of resuming meiosis and undergoing normal nuclear and cytoplasmic maturation to the MII stage after in-vitro maturation (IVM), in spite of disruption and profound alterations to the structure and organization of the nucleolus, nucleus and cytoplasm suffered during dehydration. Indeed, in this study most cytoplasmic and nuclear perturbations appeared to return to normal after post-thaw culture. Nevertheless, irreversible chromosomal and cellular changes were observed after vitrification of GV mouse oocytes, such as premature chromosomal condensation, mixing of nucleoplasmic and cytoplasmic components prior to GV breakdown, and externalization of chromatin fragments into the cytoplasm after reformation of the oocyte nucleus. These results demonstrate that vitrification is associated with disorders that could adversely affect embryo development after fertilization and illustrate the feasibility of generating fertilizable oocytes containing deleted segments of DNA.

Swelling of mitochondria has also been reported, with reduced matrix density, vacuolization in subplasmalemmal areas and destruction of cell–cell interactions in vitrified immature horse oocytes (Hochi *et al.*, 1996). Another study showed that cryoprotectant exposure or vitrification induced pronounced ultrastructural modifications in microvilli, mitochondria, vesicle formation and the ooplasm of GV bovine oocytes (Al Hasani and Diedrich, 1995; Fuku *et al.*, 1995b).

After thawing, GV oocytes need to undergo nuclear and cytoplasmic maturation to become fertilizable, but techniques to achieve this *in vitro* are still suboptimal. Indeed, although recent studies have reported a marked improvement in pregnancy outcomes after IVM of fresh GV oocytes (Jurema and Nogueira, 2006), only one live birth has resulted from a cryopreserved GV oocyte subsequently matured *in vitro* (Tucker *et al.*, 2004). In summary, the future of GV-stage oocyte cryobanking depends on the optimization of cryopreservation protocols and the development of reliable IVM procedures (Hovatta, 2005; Kim, 2006).

Cryopreservation of ovarian cortical fragments

A number of studies have proved that human ovarian tissue can be successfully cryopreserved, showing good survival, morphology and function after thawing, leading several assisted reproduction centres to develop cryobanking of ovarian tissue as an experimental option for cancer patients (Hovatta, 2005). Morphological and functional recovery of ovarian tissue after thawing was evidenced by live/dead assays, organ culture, xenotransplantation to immunodeficent mice and light microscopy (LM) and TEM analyses (Donnez *et al.*, 2006).

The majority of follicles in the ovarian cortex are at primordial (70–90%) and primary (20–30%) stages, the most resistant stages to cryoinjury and ischemic damage (Hovatta, 2005). However, designing a cryopreservation protocol for ovarian tissue is challenging owing to its cellular heterogeneity. Cryoprotectant permeation capacity, toxicity and potential ice crystal formation are specific to each cell and tissue type. The choice of appropriate cryoprotectant and freeze-thawing rates thus entails a compromise between oocytes, follicular and stromal cells.

Adequate penetration of cryoprotectant through the stroma, granulosa cells and oocytes is necessary, as a major concern of cryopreservation is the possible intra- and extracellular ice formation during freezing and rewarming. This may be minimized by choosing optimal freezing and thawing rates. Rewarming may cause cell swelling, endothelial cell damage and tissue edema. During thawing, non-permeating cryoprotectants prevent osmotic shock, avoiding excessive intracellular influx of free water and consequent swelling and rupture of cells.

On the basis of current knowledge, the standard method for human ovarian cryopreservation is slow-programmed freezing, using human serum albumin-containing medium, and PrOH, DMSO or ethylene glycol (EG) as a cryoprotectant, with or without sucrose (Hovatta, 2005). Numerous TEM studies have confirmed that these freezing protocols do not markedly affect the ultrastructural quality of follicles, compared with fresh tissue (Cortvrindt *et al.*, 1996; Gook *et al.*, 1999; Nisolle *et al.*, 2000; Abir *et al.*, 2001; Hreinsson *et al.*, 2003; Eyden *et al.*, 2004; Lucci *et al.*, 2004; Rodrigues *et al.*, 2004a,b; Santos *et al.*, 2006; Fabbri *et al.*, 2006a,b; Nottola *et al.*, 2007b; Fauque *et al.*, 2007). As illustrated in **Figure 2**, frozen–thawed human primary follicles show a well-preserved ultrastructure. However, these protocols do not appear to be optimal for the stromal cell compartment (Gook *et al.*, 1999; Hreinsson *et al.*, 2003; Fabbri *et al.*, 2006a). Indeed, it is hard to define a single

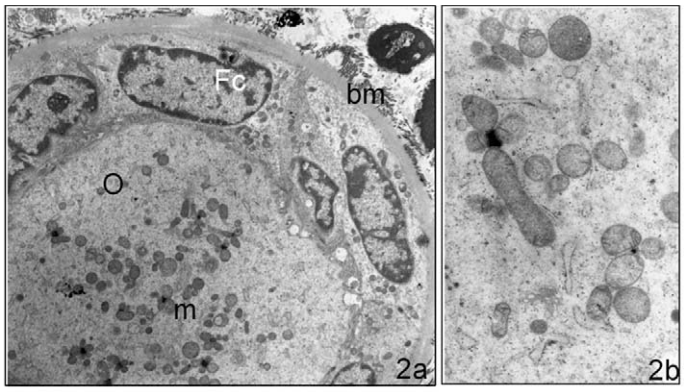

Figure 2. Frozen–thawed human primary follicles. (**a**) A single layer of follicular cells (Fc) along a continuous basal membrane (bm) encloses the oocyte (O). Note the patches of heterochromatin in follicular cells. (**a, b**) The oocyte cytoplasm shows rounded mitochondria with peripheral cristae (m) intermingled with numerous microtubules. Original magnification: (**a**) ×5000; (**b**) ×10,000.

cryopreservation regimen suitable for all tissue cell types.

Inadequate cryoprotectant concentrations or cooling and thawing rates may induce ultrastructural cryodamage at different levels, as detailed below.

Vacuolization

Although a slight degree of vacuolization may be observed in oocytes seen in fresh ovarian tissue (Hreinsson *et al.*, 2003), an increased number of vacuoles is an indicator of cellular degeneration. Vacuolization can be considered a relatively non-specific feature, commonly found in cells responding to injury (Ghadially, 1982). It may represent endoplasmic reticulum or mitochondrial alterations and could be due to ice crystal formation resulting from inadequate dehydration (Fuku *et al.*, 1995b).

Nisolle *et al.* (2000) reported vacuolization in some frozen–thawed human follicles, mainly at the secondary stage. Vacuolization was also occasionally observed in oocytes and/or granulosa cells after thawing (Gook *et al.*, 1999; Abir *et al.*, 2001; Hreinsson *et al.*, 2003; Lucci *et al.*, 2004; Rodrigues *et al.*, 2004a; Santos *et al.*, 2006; Fauque *et al.*, 2007), sometimes associated with mitochondrial damage (Gook *et al.*, 1999; Lucci *et al.*, 2004).

Cell shrinkage and nuclear pyknosis

Oocyte shrinkage associated with nuclear pyknosis was reported in goats (Rodrigues *et al.*, 2004b) and sheep (Santos *et al.*, 2006), when ovarian tissue was cryopreserved using high cryoprotectant concentrations (3 mol/l PrOH, DMSO or EG). In humans, Hreinsson *et al.* (2003) reported an increase in granulosa cell pyknosis in frozen–thawed tissue compared with fresh tissue. However, it is difficult to establish whether nuclear condensation results from the cryopreservation treatment or from in-vitro culture.

Mitochondrial and membranous organelle damage

Plasma, nuclear and organelle membranes may be affected by ice crystal formation, osmotic stress and low temperatures. Alterations to mitochondrial membranes, cristae and matrix, as well as swelling, have all been reported as signs of follicular injury after cryopreservation (Lucci *et al.*, 2004, Fabbri *et al.*, 2006a; Santos *et al.*, 2006). Mitochondria with extensive swelling and loss of cristae, associated with an increase in endoplasmic reticulum volume, are considered to be early signs of follicular degeneration (Santos *et al.*, 2006).

The presence of empty cytoplasmic areas and loss of granulosa content were observed in frozen–thawed cow, goat and sheep ovarian tissue, particularly when exposed to high cryoprotectant concentrations (Lucci *et al.*, 2004; Rodrigues *et al.*, 2004a,b; Santos *et al.*, 2006). These alterations may be due to swelling and rupture of cytoplasmic organelles, as a result of osmotic stress.

Impairment of intercellular contacts

Impairment of intercellular contacts between the oocyte and granulosa cells was also observed after freeze-thawing (Lucci *et al.*, 2004; Rodrigues *et al.*, 2004a,b; Santos *et al.*, 2006). Rodrigues *et al.* (2004a,b) and Lucci *et al.* (2004) reported detachment and disorganization of follicular cells with oocyte alterations in goats and cows, respectively. Complete loss of follicular cells, leaving vacuolated intercellular spaces, was also described in sheep (Santos *et al.*, 2006). Cell–cell detachment is probably due to ice crystal formation in intercellular spaces during freezing and rewarming procedures. Furthermore, induction of apoptosis in follicular cells after cryostorage may induce follicular cell degeneration and loss of contact between the follicular layer and the oocyte (Lucci *et al.*, 2004; Fauque *et al.*, 2007).

Vitrification is an emerging technique (Isachenko *et al.*, 2007). Preliminary results on vitrification of ovarian tissue in mice look promising, but this approach needs to be investigated further. Standardization of protocols is necessary, as contradictory results were obtained between studies (Salehnia *et al.*, 2002; Chen *et al.*, 2006). Mazoochi *et al.* (2007) found a well preserved ultrastructure in oocytes and follicular cells from cryopreserved mouse ovarian tissue, while Chen *et al.* (2006), using a conventional vitrification protocol, reported poor follicular ultrastructure, confirmed by viability testing, post-grafting follicular density and pregnancy rates. Direct application of liquid nitrogen to ovarian tissue with a reduction in cryoprotectant concentrations seemed to yield significantly better results (Chen *et al.*, 2006).

Transplantation of ovarian cortical fragments

Ultrastructural studies may be useful to analyse the impact of transplantation on follicular quality. However, information on follicular ultrastructure after grafting is limited. A recent confocal microscopy study by Kim *et al.* (2005) revealed a possible increase in cytoplasmic and nuclear abnormalities in human follicles after xenotransplantation to SCID mice.

In studies conducted in the authors' laboratory, xenotransplantation of frozen–thawed human ovarian tissue to nude mice for 3 weeks did not appear to affect human primordial/primary follicle ultrastructure greatly (Nisolle *et al.*, 2000; Nottola *et al.*, 2007b). Interestingly, all secondary human ovarian follicles found in xenografts showed asynchrony between oocyte and granulosa cell development when observed by TEM, although both oocyte and granulosa cells were morphologically normal. Indeed, the follicles contained an immature oocyte with characteristics of the primordial/primary stage, surrounded by multiple layers of granulosa cells, typical of the secondary stage. These secondary follicles displayed a number of strange features compared with fresh secondary follicles: cytoplasmic organelle topography and characteristics typical of primordial and primary stages, and absence of a regularly structured ZP and of a concentric arrangement of the perifollicular tissue. This may result in functional uncoupling between oocytes and granulosa cells. Further studies are needed to determine the mechanisms underlying this phenomenon and to assess how these secondary follicles will evolve after longer term grafting.

Human ovarian tissue was analysed after cryopreservation and autotransplantation in one patient, and the preliminary results showed no major alterations in follicular ultrastructure (Camboni *et al.*, 2007). Indeed, primordial follicles contained in frozen–thawed human ovarian fragments can survive in autografts for 13 months, maintaining a normal ultrastructure. When observed by TEM, two of these follicles looked perfectly healthy. In the oocyte cytoplasm of these follicles, numerous rounded mitochondria were seen associated with microtubules, and close interdigitations were observed between oocyte microvilli and follicular cell projections. Follicular cells showed a voluminous nucleus containing a well-developed nucleolus, isolated peripheral patches of heterochromatin and well-preserved mitochondria. The stromal cell compartment was viable, but showed poor cellularity by TEM analysis (Camboni *et al.* 2007).

Cryopreservation of whole ovary

The authors' group is currently investigating a slow freezing method to cryopreserve a whole human ovary with its vascular pedicle (Martinez-Madrid *et al.*, 2004a; 2007; Jadoul *et al.*, 2007). To achieve adequate diffusion of cryoprotective agents into large tissue masses and minimize the risk of intracellular ice crystal formation, vascular perfusion was performed via the ovarian artery (Martinez-Madrid *et al.*, 2004a). Moreover, sucrose was added to the thawing solution to avoid reperfusion damage due to osmotic shock.

It was crucially important to assess whether osmotic stress, ice crystal formation or toxic cryoprotectant damage occurred with the protocol before autografting a frozen–thawed whole human ovary. Therefore, in addition to histology, viability assessment and apoptosis detection by both active caspase-3 immunohistochemistry and TdT-mediated dUTP nick end labelling (TUNEL) assays, TEM was also used to analyse ovarian tissue quality and detect any possible fine subcellular alterations resulting from cryodamage (Martinez-Madrid *et al.*, 2004a; Camboni *et al.*, 2005).

Ultrastructural analyses proved that the majority of primordial follicles (96.7%) were intact after cryopreservation, exhibiting normal ultrastructural features (**Figure 3a**), confirming the previous histology and viability findings (Martinez-Madrid *et al.*, 2004a), as well as the results of the apoptosis study (Martinez-Madrid *et al.*, 2007). The follicles showed intact nuclear and cellular membranes, well-preserved cytoplasmic organelles, normally arranged chromatin and close interdigitations at the oocyte–follicular cell interface.

Imhof *et al.* (2004) also demonstrated the feasibility of intact ovary cryopreservation in pigs using a similar protocol, showing a well-preserved structure (84.4%) and ultrastructure of the follicular compartment after cryostorage. These two studies (Imhof *et al.*, 2004; Martinez-Madrid *et al.*, 2007) proved adequate diffusion of the cryoprotectant, indicating that no significant follicular injury occurred using this approach.

However, a major problem of whole organ preservation, which may compromise the subsequent transplantation procedure, is vascular injury resulting from osmotic damage, cryoprotectant toxicity, or ice crystal formation (Yin *et al.*, 2003). Although transplantation of intact fresh human ovaries with their vascular pedicle is feasible (Leporrier *et al.*, 1987; Hilders *et al.*, 2004; Mhatre *et al.*, 2005), vascular integrity after cryopreservation needs to be demonstrated before this approach can be applied to frozen–thawed whole human ovaries.

Endothelial lesions frequently encountered after freeze-thawing may cause vascular thrombosis, compromising graft viability (Zook *et al.*, 1998). Studies on autotransplantation of intact frozen–thawed sheep ovaries with microvascular anastomosis (Bedaiwy *et al.*, 2003; Arav *et al.*, 2005; Imhof *et al.*, 2006) appear to indicate that vascular thrombosis is indeed a factor limiting the success of this approach. Although restoration of ovarian function and one live birth were achieved, a significant proportion of ovaries were lost after transplantation.

During intact organ preservation, vascular endothelium is the

first line of contact with cryoprotectant during perfusion and thus the most likely to suffer toxic damage. Vascular lesions are considered to be one of the main limiting factors in whole organ preservation (Carbognani *et al.*, 1995). Structural alterations to mitochondria, endoplasmic reticulum, nuclei, cytoskeletal fibres and cytoplasmic vacuolization, as well as disruption of cell–cell contacts and focal endothelial cell blebbing, were found in endothelial cells after cold storage (Faggioli *et al.*, 1994; Eberl *et al.*, 1999).

Vitrification can also cause vascular injury. Courbiere *et al.* (2005) reported a higher incidence of vascular fractures after vitrification of whole sheep ovaries and endothelial lesions in 70% of ovarian arteries. Inadequate cryoprotectant perfusion or concentration and an inappropriate cooling rate may cause damage to the vascular compartment in its deepest parts. It was indeed reported that ice crystal formation may induce smooth muscle cell bloating in vascular muscularis (Courbiere *et al.*, 2007). Moreover, inadequate cooling appears to cause irreversible ultrastructural damage, such as mitochondrial dilation and rupture, reticular fragmentation and peripheral nuclear condensation in endothelial cells (Pascual *et al.*, 2004).

In the authors' TEM study (Martinez-Madrid *et al.*, 2007), the vessels appeared totally healthy, with all their vascular components (endothelial cells, basal membrane, smooth muscle cells and pericytes) well preserved (**Figure 3b**). They did not show any loss of endothelial cells or exposed connective tissue, and exhibited continuous endothelium and intact junctions between the endothelial cells. This indicates an adequate rewarming rate, as no macroscopic or microscopic fractures occurred in the vascular compartment during thawing. Moreover, no significant physical (from ice crystals), toxic or osmotic damage was detected, as 96% of the endothelial cells showed well preserved cytoplasmic organelles, cellular membranes and nuclear content (**Figure 3b**) when observed by TEM. Only a few vessels contained endothelial cells with altered features: nuclear and cytoplasmic condensation, swollen and electron-translucent cytoplasm or separations of intercellular contacts. However, these changes are compatible with reversible damage, as reported by Pascual *et al.* (2004). These results are encouraging and lead us to believe that grafting whole frozen human ovaries will be feasible in the future.

Isolation of ovarian follicles

Ovarian follicle isolation has been reported in a wide range of species including mice (Eppig, 1977; Wang *et al.*, 1991; Hreinsson *et al.*, 2003), rabbits (Nicosia *et al.*, 1975), hamsters (Roy and Greenwald, 1985), rats (Grob, 1964), cats (Jewgenow, 1996), sheep (Amorin *et al.*, 2003, 2004; Cecconi *et al.*, 2004), pigs (Greenwald and Moor, 1989), goats (Lucci *et al.*, 1999), cows (Figueiredo *et al.*, 1995; van den Hurk *et al.*, 1998; Lucci *et al.*, 2002) and humans (Roy and Treacy, 1993; Cortvrindt *et al.*, 1996; Osborn *et al.*, 1997; Abir *et al.*, 1999, 2001; Hovatta *et al.*, 1999; Martinez-Madrid *et al.*, 2004b; Dolmans *et al.*, 2006).

Two methods have been described to isolate ovarian follicles: mechanical and enzymatic (Abir *et al.*, 2006). The mechanical method allows isolation of ultrastructurally well-preserved preantral follicles with an intact basal membrane, as demonstrated in goats (Lucci *et al.*, 1999), cows (Lucci *et al.*, 2002) and cats (Jewgenow, 1996). In only one study in cows, mechanically isolated follicles showed a poor ultrastructure, especially in the oocytes, indicative of degeneration (van den Hurk *et al.*, 1998). Mechanical isolation therefore appears to be an efficient way of obtaining good quality isolated follicles in some mammalian species.

In humans, large preantral follicles (100–400 μm) have been obtained by mechanical procedures (Abir *et al.*, 1997) while primordial and primary follicles have been isolated from their surrounding connective tissue by combining microdissection and enzymatic digestion (Cortvrindt *et al.*, 1996; Abir *et al.*, 1999, 2001) or by enzymatic treatment using collagenase alone or in combination with deoxyribonuclease (Roy and Treacy, 1993; Cortvrindt *et al.*, 1996; Osborn *et al.*, 1997; Abir *et al.*, 1999, 2001; Hovatta *et al.*, 1999; Martinez-Madrid *et al.*, 2004b; Dolmans *et al.*, 2006).

The main drawback of the enzymatic procedure is that the protocol is difficult to standardize because of the variability between batches of crude collagenase preparations (Dolmans *et al.*, 2006). The use of purer collagenase enzyme preparations such as Liberase Blendzyme 3™ (Roche, Indianapolis, USA), a purified enzyme blend with low endotoxin levels, may be helpful to overcome this problem (Dolmans *et al.*, 2006). As illustrated in **Figure 4**, follicles isolated by Liberase digestion showed a well-preserved ultrastructure and well-maintained oolema–follicular cell interface, with only occasional signs of atresia present (Dolmans *et al.*, 2006). Oktay *et al.* (1997) obtained isolated human follicles of good quality after collagenase treatment, as evidenced by viability assessment of fully isolated follicles and TEM analysis of partially isolated follicles. In three studies on rodents, rabbits and hamsters, collagenase-isolated follicles exhibited a well preserved ultrastructure with an intact basement membrane, as demonstrated by TEM (Nicosia *et al.*, 1975; Roy and Greenwald, 1985, 1996).

By contrast, TEM revealed an increase in lipid droplets in granulosa cells from collagenase-isolated follicles, although this did not appear to affect their ability to grow (Abir *et al.*, 2001). In addition, inadequate enzymatic digestion may cause detachment between follicular cells and oocytes, as well as fine subcellular alterations typical of early atresia (Dolmans *et al.*, 2006). Collagenase-isolated pig primordial follicles were shown to contain a number of small oocytes with an incomplete layer of pregranulosa cells (Greenwald and Moor, 1989). Partially denuded oocytes (with detachment of some follicular cells) and totally denuded oocytes (without a follicular cell layer) were also observed in collagenase-isolated human follicles by means of LM and TEM (Dolmans *et al.*, 2006). Other studies revealed alterations in the basal lamina of collagenase-isolated follicles in mice, rats and pigs (Greenwald and Moor, 1989; Eppig, 1994; Roy and Greenwald, 1996).

It has been reported that the presence of an intact basal membrane is of crucial importance to protect follicles from damage during isolation (Figueiredo, 1995), allowing maintenance of follicular morphology and adhesion capacity during in-vitro culture (Lucci *et al.*, 2002). A major problem for in-vitro development of primordial follicles is the breakdown of basement membrane material and other intrafollicular components during enzymatic isolation (Gosden *et al.*, 2002). This erosion causes the

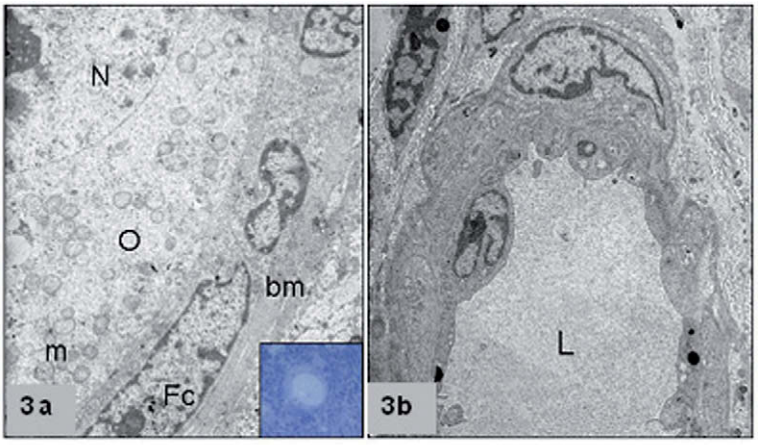

Figure 3. Primordial follicle in frozen–thawed whole human ovary. (**a** and inset) The oocyte (O) is surrounded by a single layer of flattened follicular cells (Fc) on a continuous basal membrane (bm). Note the presence of rounded mitochondria (m) with a pale matrix and peripheral cristae in the oocyte cytoplasm. Follicular cells show indented nuclei containing peripheral patches of heterochromatin and numerous rod-shaped mitochondria in the cytoplasm. N, oocyte nucleus. (**b**) A well-preserved arteriole in frozen–thawed human ovary. L, vascular lumen. Original magnification: light microscopy: ×400; transmission electron microscopy: ×4400 (**a**); ×3000 (**b**).

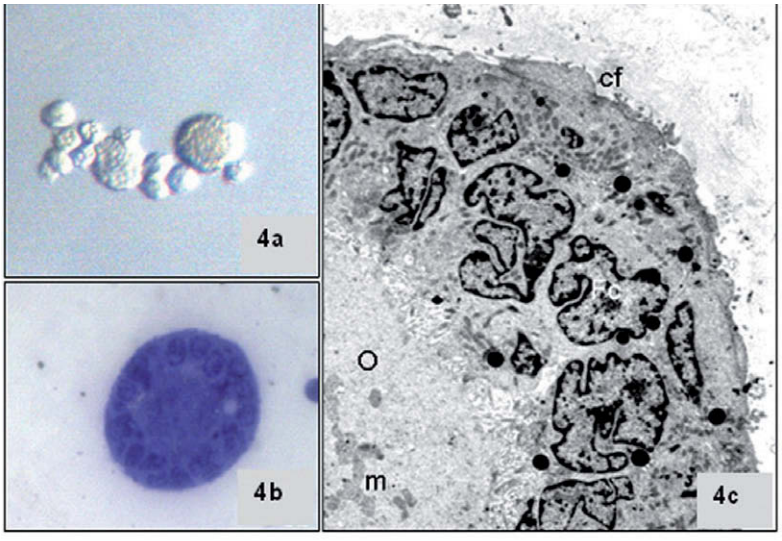

Figure 4. Liberase-isolated follicles. **a** Human ovarian follicles 30–100 μm in size fully isolated by Liberase as seen under a microscope. (**b, c**) Liberase-isolated human follicle at the primary stage. Note the fibres of connective tissue (cf) at the periphery of the follicle. O, oocyte; m, mitochondria; Fc, follicular cells. Original magnification: light microscopy: ×200 (**a, b**); transmission electron microscopy: ×2000 (**c**).

pregranulosa cells to round up and detach from the oocyte, which is an irreversible process that produces oocytes incapable of further development, although they may remain viable for several days (Bachvavora *et al.*, 1980). Hovatta *et al.* (1999) reported premature oocyte extrusion in follicles in culture that had been partially isolated using collagenase.

Both collagenase- and Liberase-isolated human preantral follicles can grow (Dolmans *et al.*, 2007) and reach the antral stage (Dolmans *et al.*, 2008) after xenotransplantation, which heralds a promising future for this approach. However, in order to enhance the chances of follicular survival and development for reproductive function restoration, enzymatic digestion procedures for human ovarian tissue need to be further optimized and standardized (Dolmans *et al.*, 2006).

Conclusions and perspectives

During the last decade, new technologies in reproductive medicine have emerged to preserve the fertility of women suffering from cancer, whose gonadal function is threatened by premature menopause or gonadotoxic treatments. To offer an individualized approach to these patients, different experimental procedures are under investigation, including oocyte cryopreservation prior to treatment followed by IVM and IVF after disease remission or cryopreservation of ovarian tissue before treatment and subsequent transplantation in the form of cortical fragments, whole ovary or isolated follicles.

This review shows that TEM, combined with other in-vivo and in-vitro analysis techniques, can be used to optimize and establish experimental procedures to preserve fertility in women. In particular, TEM has proved useful to assess the impact of cryopreservation, transplantation and enzymatic isolation on cellular and tissue integrity and to set up new protocols. Despite these promising results, relatively few researchers apply TEM studies to the evaluation of the impact of grafting on ovarian tissue integrity. The authors' results clearly show that TEM is required to evidence subtle changes occurring in follicular structure after grafting, not revealed by histological studies.

Although TEM is technically difficult, time-consuming and expensive, it is recommended as a technique of choice, in association with functional and molecular studies, as it provides essential information on cellular and tissue quality.

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