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Mouse ovarian tissue vitrification on copper electron microscope grids versus slow freezing: a comparative ultrastructural study

Ferda Topal-Celikkan[A], *Sinan Ozkavukcu*[B,E], *Deniz Balci*[C], *Sibel Serin-Kilicoglu*[D] *and Esra Atabenli-Erdemli*[A]

[A]Ankara University Faculty of Medicine, Department of Histology and Embryology, 06230 Sihhiye, Ankara ,Turkey.
[B]Ankara University Faculty of Medicine, Center for Assisted Reproduction, 06100 Dikimevi, Ankara, Turkey.
[C]Ankara University Biotechnology Institute, 06830 Golbasi, Ankara, Turkey.
[D]Ufuk University Faculty of Medicine, Department of Histology and Embryology, 06520 Balgat, Ankara, Turkey.
[E]Corresponding author. Email: sinozk@gmail.com

Abstract. There are many reasons, including cancer therapy, for premature ovarian failure and infertility. Oocyte, embryo and ovarian cryopreservation are current options for fertility preservation. Ovarian tissue cryopreservation is essential in patients whose cancer therapy cannot be delayed, including prepubertal girls, and is mostly performed using slow freezing. In the present study, mouse ovarian tissues were vitrified on copper electron microscope grids ($n = 18$) or conventionally slow frozen ($n = 18$). Post-thaw tissues were examined histologically using light and electron microscopy and compared with the control group. According to light microscopy observations, antral follicles were found to be better preserved with the slow freezing technique rather than vitrification. Electron microscopy revealed swollen mitochondria in the oocyte cytoplasm, condensations in the zona pellucida, breakages in the junctions of granulosa cells and vacuolisation in the extracellular space in pathologic follicles, which were relatively more frequent, in the vitrification group after thawing. These results indicate that ovarian slow freezing is preferable than vitrification on copper electron microscope grids, especially for larger follicles. Conversely, vitrification of ovarian pieces using cooper grids is user-friendly and provided good protection for primordial follicles and stromal cells. There is a need for further studies into advanced tissue vitrification techniques and carriers.

Additional keywords: cryoprotective agents, fertility preservation, follicle, granulosa cells, oocyte, thermal conductivity, ultrastructure.

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Introduction

Over 790 000 women are diagnosed with invasive cancer each year in the US (Siegel *et al.* 2013). Current therapy protocols provided increased rates of survival, up to 90% for certain cancer types (Blatt 1999). The reinstitution of quality of life after cancer treatment is as essential as survival (Partridge *et al.* 2004). Conceiving can be challenging for patients who have undergone chemotherapy and/or radiotherapy, which have been proven to be highly toxic to gonads because follicle damage gives rise to premature ovarian failure (Meirow and Nugent 2001).

Currently, fertility preservation before cancer therapy is critical for overcoming infertility due to toxicity. Fertility of female cancer patients is preserved from the effects of radiotherapy by repositioning the ovaries under the peritoneum in order to protect oocytes (Tulandi and Al-Took 1998). However, this procedure is not applicable for patients receiving chemotherapy. Assisted reproduction and cryopreservation of oocytes and/or embryos for those who have time for controlled ovarian hyperstimulation (COH) is the optimal approach. Nevertheless, gonadotropins being used for ovarian stimulation may induce oestrogen-sensitive cancer cells (Prasath 2008). In case of COH, collected oocytes are either frozen or fertilised with the partner's spermatozoa and the developed embryos are cryopreserved (Revel *et al.* 2004). Conversely, girls who are under the age of puberty and patients without a partner may not be suitable candidates for oocyte collection and/or embryo cryopreservation.

The ovarian cortex nestles hundreds of primordial follicles that are small in size, containing immature oocytes without a

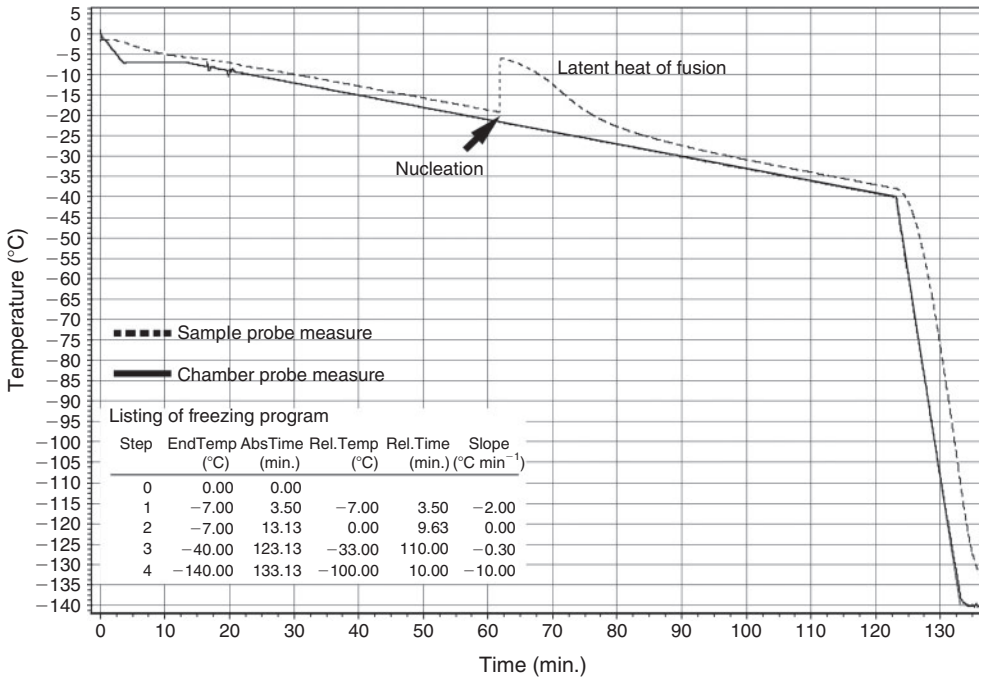

Fig. 1. A test run was performed to determine the nucleation temperature of the cryoprotectant agent.

zona pellucida (ZP; Kim 2006). Because these immature oocytes have a very small volume of cytoplasm and low water content, they are believed to tolerate cryopreservation better (Kim *et al.* 2001). Ovarian cortex cryopreservation is favourable over oocyte and embryo freezing because it can be performed independent of the timing of cancer therapy and patient age (Comboni *et al.* 2008). Moreover, in addition to restoring fertility, retransplantation of ovarian tissue may reinstate the hormone-producing units, thus preventing the side effects of early menopause due to premature ovarian failure.

Vitrification and slow freezing are routinely being used for cryopreservation. Because slow-rate freezing needs lower concentrations of cryoprotectants, it is believed to cause less serious toxicity (Gosden *et al.* 2010). To maintain vitrification, the freezing medium must contain a high concentration of cryoprotectants, which can result in cellular toxicity and osmotic trauma (Ting *et al.* 2011). In cleavage stage embryos, blastocysts and oocytes, vitrification is the key method for cryopreservation, whereas both methods need to be improved for ovarian freezing (Kuwayama *et al.* 2005). The vitrification procedure needs a practical carrying device to keep the tissue safe and to allow a sudden drop in temperature to maintain a glassy state transaction. To date, no efficient carrying device has been developed for tissue vitrification.

The aim of the present study was to compare the effects of cryopreservation after slow freezing and vitrification on mammalian ovarian tissue. To sustain an ultra-rapid drop in temperature and maintain vitrification, we used copper electron microscope (EM) grids to minimise the volume of liquid medium surrounding the tissue and to test whether the grids were a suitable carrying device for vitrification, because the heat transfer coefficient of copper is relatively high (Powell *et al.* 1966).

Materials and methods

In all, 18 pairs of ovaries were collected from 8-week-old female BALB/c mice. Ovaries were transported to the cryobiology laboratory on ice (4°C) from the animal research surgery room within 5 min, in HEPES-buffered Liebowitz-15 medium (L-15; catalogue no. 11415–049; Invitrogen, Carlsbad, CA, USA). In the laboratory, ovaries from each mouse were divided into 0.3 × 0.3 × 0.1 cm pieces (length × width × depth) and divided among the experiment groups evenly in order to compare within-subject differences. Fresh ovarian pieces were fixed in Bouins' solution and glutaraldehyde as the control group for histological examination. All experiments undertaken in the present study were approved by the Ankara University Ethics Committee.

Ovarian tissue cryopreservation

Slow freezing and thawing

Before the experimental studies on tissues, a series of test runs were performed using a controlled freezer (IceCube; SyLab, Neupurkersdorf, Austria) to determine the ice nucleation temperature for the freezing solution. Therefore, initiation of latent heat of fusion was detected at −17°C and subsequently suppressed with an appropriate programmed temperature fall (PTF) during the freezing protocol (Fig. 1; Balci and Can 2013).

Collected ovarian pieces were equilibrated in cryovials on ice for 15 min in freezing solution, which was composed of L-15 supplemented with 1.5 M dimethyl sulfoxide (DMSO), 20% fetal bovine serum (FBS) and 0.1 M sucrose (Oktem *et al.* 2004). After the equilibration period, cryovials were moved into a controlled freezer.

The slow freezing program was initiated at 0°C. After soaking for another 15 min equilibration (total 30 min;

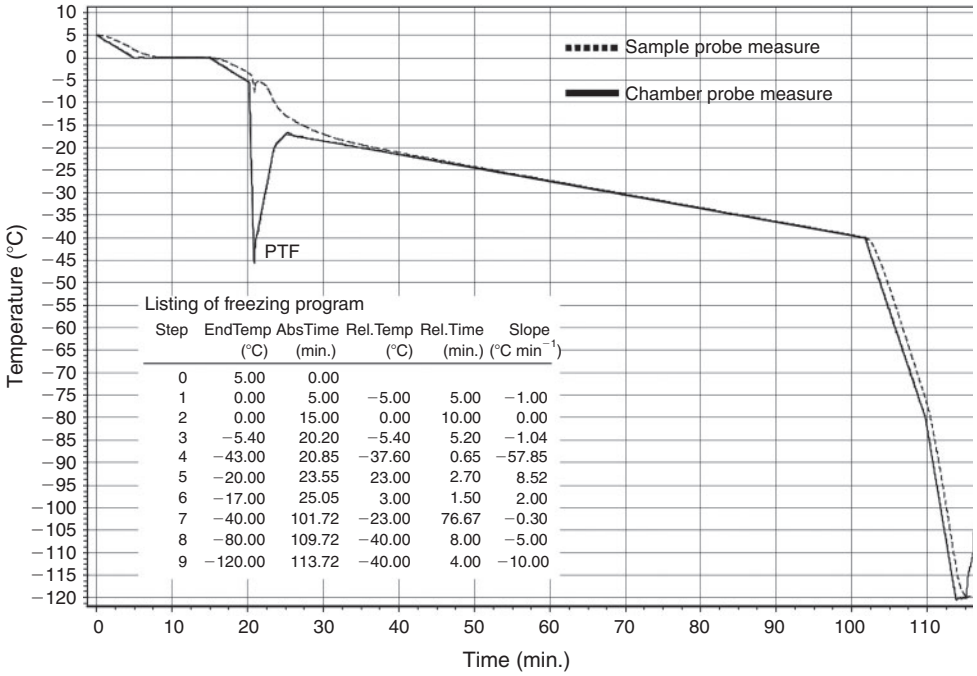

Fig. 2. Summary of the slow freezing protocol used for conventional freezing of ovarian tissue in the present study. The programmed temperature fall (PTF) is fitted according to the nucleation temperature, thus the latent heat of fusion is suppressed.

Fabbri *et al.* 2003; Luz *et al.* 2009) the temperature was lowered by 1°C min^{-1} to −5°C. Using a PTF down to −43°C at a rate of 57.85°C min^{-1}, suppression of latent heat of fusion was accomplished. The slow freezing protocol went on from −17°C following the negative peak of PTF. The next temperature, −40°C, is reached at a rate of 0.3°C min^{-1}, followed by cooling to −80°C at a rate of 5°C min^{-1} (Oktem *et al.* 2004). Cryovials were plunged into liquid nitrogen at the end of the program when the last step was run at 10°C min^{-1} down to −120°C (Fig. 2).

After 1 week, ovarian tissues were removed from the nitrogen storage tank, kept at room temperature for 30 s and then plunged into a 37°C water bath until they melted. The contents of the cryovials were transferred into dishes containing L-15 medium, supplemented with gradually decreasing concentrations of cryoprotective agents (CPA) for 5 min at each concentration, specifically 1.5, 1.0, 0.5 and 0 M DMSO with 0.1 M sucrose, then 0.05 M sucrose only. Finally, tissues were washed in L-15 medium and transferred to fixative solution.

Vitrification and thawing

Tissue pieces were equilibrated in gradually increasing concentrations of CPA with a final concentration of 20% DMSO, 20% ethylene glycol (EG) and 0.4 M sucrose (Kagawa *et al.* 2009). After equilibration, tissues were loaded onto copper electron microscope grids (catalogue no. G300-Cu; Electron Microscopy Sciences, Hatfield, PA, USA) with a diameter of 3.05 mm and thickness of 25.40 µm (Fig. 3*a*), and excess solution was removed using blotting paper. Using fine tweezers, the EM grids were plunged directly into liquid nitrogen (Fig. 3*b*) and then gently put into cryovials under liquid nitrogen and the vial caps sealed.

After 1 week, the EM grids with vitrified ovarian tissues were removed from the cryovials using tweezers while still under liquid nitrogen and then plunged directly into 37°C vitrification medium. After thawing for 30 s, tissues were transferred through a series of gradually decreasing concentrations of CPA for 5 min at each concentration to remove the CPA osmotically (20%, 10%, 5% and 0% for both DMSO and EG with 0.4 M sucrose, concluding with 0.2, 0.1 and 0 M sucrose). Then, tissues were washed in L-15 medium and transferred to fixative solution (Kagawa *et al.* 2009).

Brightfield microscopy

Fresh and thawed tissues were fixed directly in Bouins' solution for histological examination. After 24–48 h, tissue processing was finalised and paraffin blocks were sectioned serially at 5 µm. Slides were stained with haematoxylin and eosin (HE) and observed under a brightfield microscope (Eclipse E600; Nikon, Tokyo, Japan). At least 200 follicles containing an oocyte with a visible nucleus were examined and counted. One in every five paraffin sections was used to avoid evaluating the same follicle. Ovarian follicles were classified as follows: (1) primordial follicle, with one layer of squamous follicle cells surrounding the oocyte; (2) early primary follicle, with a single layer of cuboidal follicle cells; (3) late primary follicle with a multilayered mass of granulosa cells (differentiated from follicle cells); (4) secondary follicle (antral follicle), with multiple layers of granulosa cells (a thickness of six to 12 cell layers) and

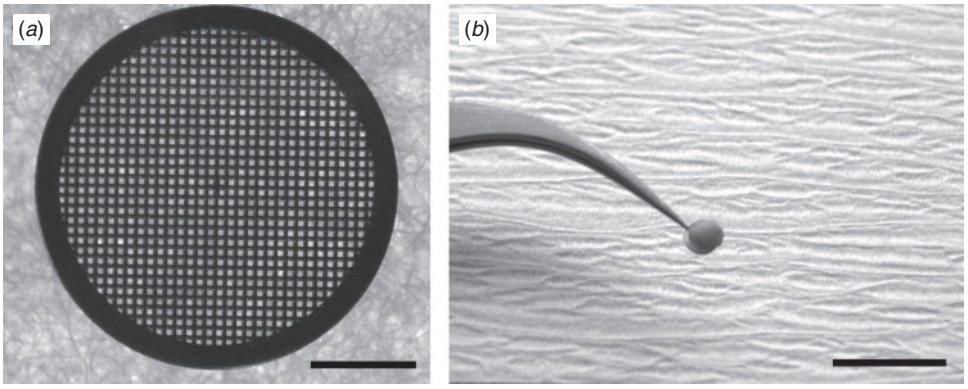

Fig. 3 (*a*) A copper electron microscope grid. Scale bar = 1 mm. (*b*) Equilibrated ovarian tissue on a copper grid to be plunged into liquid nitrogen for vitrification. Scale bar = 1 cm.

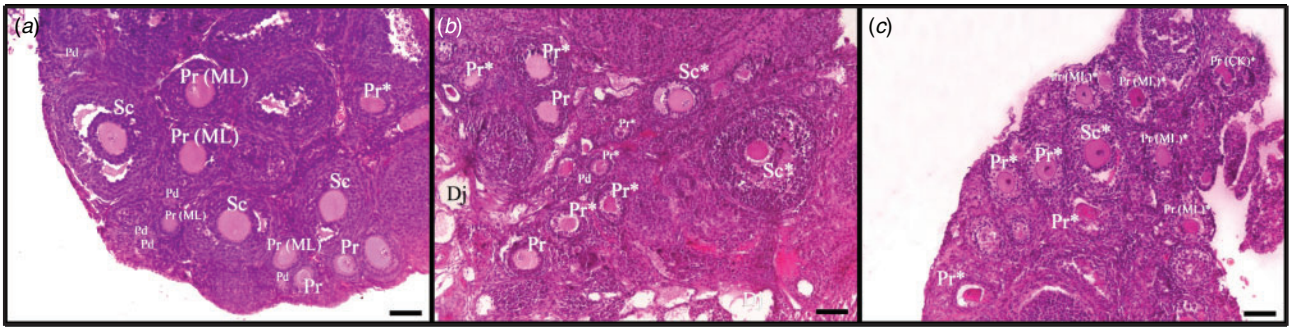

Fig. 4. Histological sections of tissues from (*a*) the control group, (*b*) slow freezing group and (*c*) vitrification group showing: Pd, primordial follicle; Pr, primary follicle; Sc, secondary follicle; ML, multilayered; Dj, tissue disjunctions. Asterisks indicate degenerated follicles. Haematoxylin–eosin staining. Scale bars = 50 μm.

a fluid-containing antrum; and (5) a Graafian follicle, with a large antrum and cumulus oophorus (Ross and Pawlina 2011).

Transmission electron microscopy

For ultrastructural analysis, fresh and thawed tissues were fixed in 0.1 M phosphate buffer, supplemented with 2% glutaraldehyde and 2% paraformaldehyde. After tissue processing, semi-thin (1 μm) and ultra-thin (700 nm) sections were cut; semi-thin sections were evaluated under a brightfield microscope (Eclipse E600; Nikon), whereas ultra-thin sections were observed by transmission electron microscopy (TEM; LEO 906 E TEM 60 kV; Zeiss, Jena, Germany).

Statistical analysis

In total, 1734 follicles were analysed to assess morphology. The effects of cryopreservation on the follicles were evaluated by the Chi-squared test. $P < 0.05$ (two sided) was considered significant.

Results

Microscopic outcomes

We determined that intact oocyte and granulosa cells were found in normal follicles, whereas a pyknotic oocyte nucleus and irregular granulosa cells were found in damaged follicles. In normal follicles, the nuclei of oocytes were euchromatic and nucleoli were distinct. Between granulosa cells and oocytes, contact patches were intact. In degenerated follicles, there were chromatin condensations in the oocyte nuclei and the cytoplasm of the oocytes became acidophilic. In addition, vacuoles between oocyte–granulosa and granulosa–granulosa cell surfaces and ZP undulation were observed in damaged follicles. Furthermore, there was a loss of contact surfaces between granulosa cells and oocytes. Apoptotic granulosa cells with pyknotic nuclei and apoptotic bodies were also noted.

Control group

In ovarian tissue sections, primordial and primary follicles with good morphology were examined under epitelium germinativum. The oocytes were normal in appearance, with large cytoplasm and euchromatic nuclei. Degenerated follicles that had an undulating oolemma, diminished ZP and irregular architecture of granulosa cells were observed together with normal follicles (Fig. 4*a*).

In thin sections, oocyte nuclei and the distribution of the cytoplasm were intact in non-degenerated primordial and primary follicles (Figs 5*a*, *b*, 6*a*, *b*). The junctions between oocyte–granulosa cells and granulosa–granulosa cells were intact in this group (Fig. 5*c*).

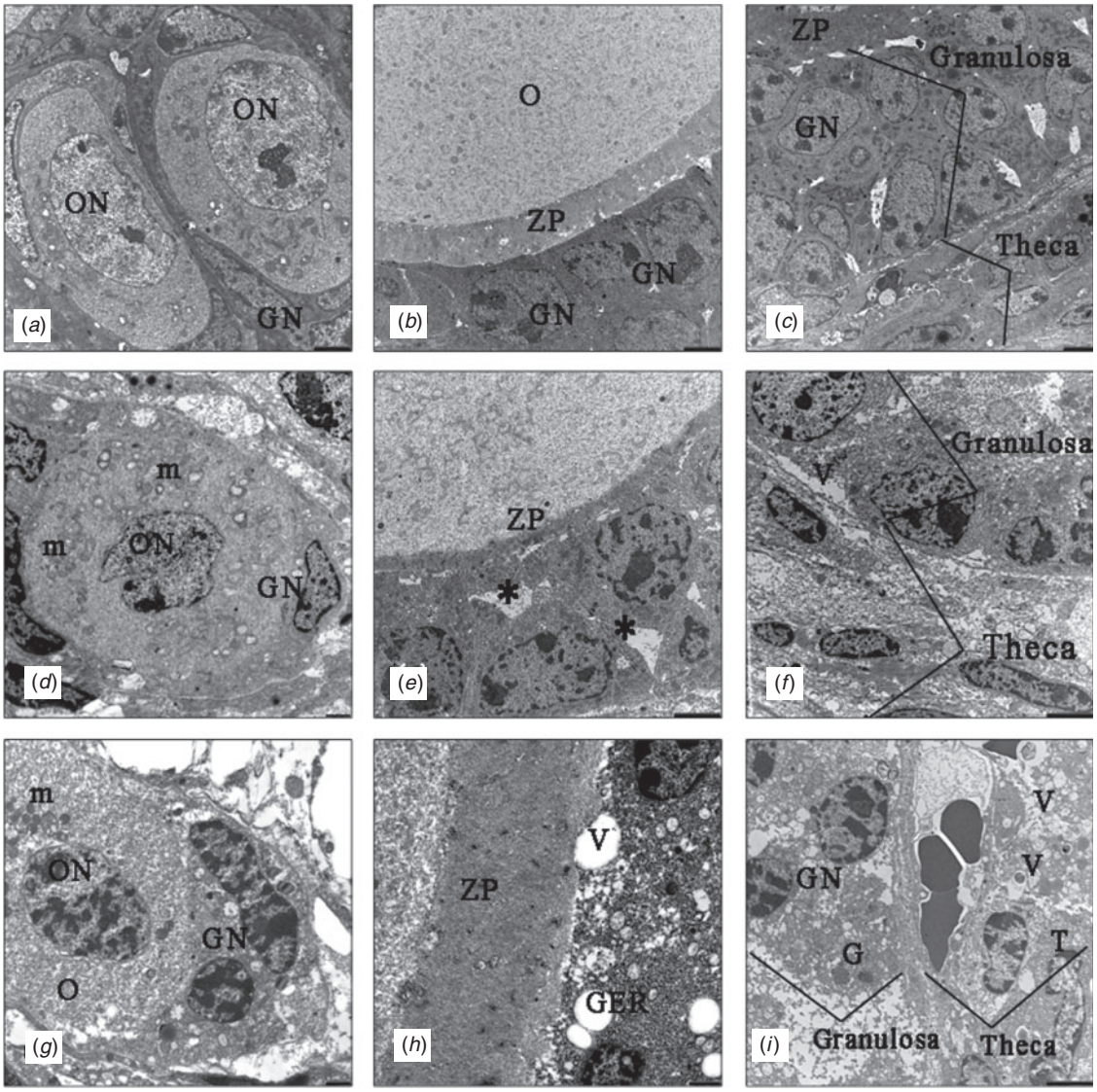

Fig. 5. Transmission electron microscopy of tissue sections from the (*a–c*) control group, (*d–f*) slow freezing group and (*g–i*) vitrification group showing: ON, oocyte nucleus; GN, granulosa nucleus; O, oocyte; ZP, zona pellucida; T, theca; m, mitochondrion; v, vacuole; GER, granular endoplasmic reticulum. Asterisks indicate intercellular spaces. Scale bars = 2.5 μm.

Slow freezing group

Normal primordial and primary follicles were also observed in the slow freezing group. The nucleus and cytoplasm of oocytes were normal. Disjunction was evident between some of the oocyte and granulosa cell borders. In the ovarian tissue, parenchymal splits were noted as a result of the freezing process. Degenerated follicles, with oocytes that had a pyknotic nucleus, acidophilic cytoplasm, deregulated oolemma and ZP, were also seen (Fig. 4*b*).

In normal follicles, the nucleus and organelles of the oocytes were conserved in the thin sections. The linkages of follicle cells between each other and oocytes were preserved. The ZP, composed of glycoproteins, was normal in appearance (Figs 5*d*, *e*, 6*c*, *d*). The basal lamina between granulosa and theca cells was regular. Deterioration in the integrity of the oocyte membrane in atretic follicles was observed. Spaces between granulosa cells and vacuolisation inside the cytoplasm of granulosa cells were noted (Fig. 5*e*, *f*).

Vitrification group

Although primordial and primary follicles were in normal appearance, atretic follicles were also observed. Undulation of the ZP, non-uniform oocyte shape and condensation of the nucleus were seen in semi-thin sections. Vacuolisation was evident in the interstitium and between granulosa cells (Fig. 4*c*).

In thin sections, particularly in early primary follicles, the distribution of the oocyte cytoplasm and the structure of ZP were normal. Shrinkage in granulosa cells and an increase in condensation were noted. Junctions between oocytes and degenerated granulosa cells disappeared and plasmalemma of

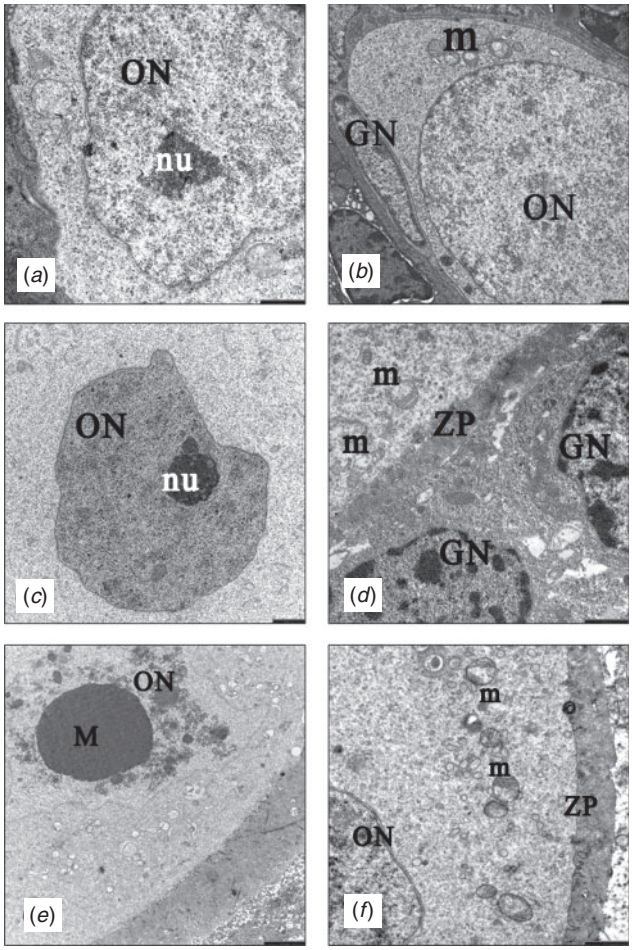

Fig. 6. Transmission electron microscopy of tissue sections from the (*a*, *b*) control group, (*c*, *d*) slow freezing group and (*e*, *f*) vitrification group showing: ON, oocyte nucleus; GN, granulosa nucleus; nu, nucleolus; GN, granulosa nucleus; O, oocyte; m, mitochondrion; ZP, zona pellucida; M, dark mass of nuclear remnant. Scale bars = 1 μm (*a*, *b*, *d*, *f*); 2.5 μm (*c*, *g*).

both cells were not observed (Fig. 5*g*, *i*). In addition, in some follicles, oocyte degeneration, ZP damage, separation, granulated endoplasmic reticulum (GER) vacuolisation and dilated sacs were observed (Fig. 5*h*, *i*). The nucleus of oocytes was described as a 'dark mass' that probably represented fragmented nuclear remnants (Fig. 6*e*). What distinguished this group was that the number of degenerated follicles was greater than that of normal follicles. Degeneration was characterised by separation of the follicular (granulosa) cells, and shrinkage, condensation or vacuolisation in oocyte cytoplasm. Crista damage and condensation of mitochondria were present in oocytes and granulosa cells (Fig. 6*e*, *f*). Dilated GER sacs were also observed in granulosa cells (Fig. 5).

All types of follicles were counted and analysed except for tertiary follicles, because there were few of these and so they were excluded from analysis. Normal primordial follicles were found at a rate of 98.9%, 98.0% and 78.9% in the control, slow freezing and vitrification groups, respectively. There was no difference between the control and slow freezing groups in terms of primordial follicles ($P = 0.261$). The percentage of large (preantral and antral) follicles in the control group was significantly higher than in the vitrification group ($P = 0.006$). When all types of follicles were compared, the percentage of normal follicles was 93.8%, 86.1% and 68.2% in the control, slow freezing and vitrification groups, respectively. Based on these results, no significant difference between the control and slow freezing groups was observed.

Discussion

Ovarian cryopreservation is a challenging procedure because of the heterogeneity of the tissue (Fuller and Paynter 2004). Finding the best protocol for freezing, the most appropriate CPAs and determining the CPA concentrations are the ultimate goals. Nevertheless, studies on ovarian cryopreservation are far from determining the optimum procedure because many contradictory results have been published so far.

This present study investigated, in a mouse model, whether using a copper EM grid during vitrification could improve the morphological outcomes of ovarian cryopreservation compared with slow freezing. For successful vitrification, the final volume of the vitrification solution to be loaded onto the carrier must be reduced as much as possible to maintain a high rate of temperature drop. Many systems have been used, including plastic straws (Isachenko *et al.* 2009; Amorim *et al.* 2011*b*), metal strips (Kagawa *et al.* 2009), cryotops, glass tubes and needles (Amorim *et al.* 2011*a*), and although most were successful in reducing the final volume, not all had a suitable structure to sustain a sudden heat drop to allow solid state transition. Thick walls and non-conductive materials negatively affect the cooling rate. Copper has good thermal conductivity compared with other metal objects (Powell *et al.* 1966). It was thought that the porous structure of the EM grids would allow the withdrawal of excess CPA covering the tissue, leaving a thin film layer before plunging. The only report on the vitrification of ovarian fragments on an EM grid was published by Choi *et al.* (2007), who compared vitrification of isolated preantral follicles (PF) with that of ovarian tissue; the IVM of thawed PF and PF from thawed ovaries was evaluated. Choi *et al.* (2007) concluded that based on the penetration capacity of the CPA, the thawed PF yielded a better IVM outcome than PF obtained from thawed tissues. Ethylene glycol was the only CPA used in that study; however, the combination of CPA (DMSO and EG) as in the present study, may lead to better permeability and lower toxicity to cells. Due to the high concentrations of CPAs, detrimental effects may be initiated long before the vitrification process, during equilibration. We need to test less toxic CPA in controlled studies, such as in the study of Bagis *et al.* (2008), who demonstrated the protective features of antifreeze proteins in a transgenic mouse model. Isolation and vitrification of PF may be a good alternative to tissue freezing (Kagawa *et al.* 2007). The penetration of the CPA and thermal conduction would be maintained better, but many potential oocytes and hormone-producing units would be discarded while restructuring the ovaries to obtain PF.

The cooling rate is extremely crucial in vitrification; similarly, the warming rate plays a critical role in survival. In addition to high rates of cooling, faster warming rates are needed to ensure

that follicles survive. Courbiere *et al.* (2006) have observed ice formation during fast warming after vitrification and detected the risk of fracture formation due to glass fragility.

Obviously larger follicles would be more vulnerable to those changes because of their water content compared with primordial follicles. The results of the present study indicate a lower protection rate of larger follicles in the vitrification protocol, which may be due to this physical phenomenon. In addition, a vitrification procedure using copper grids had good protection for primordial follicles and stromal cells, probably because of their small volume, where similar results have been reported in previous studies (Huang *et al.* 2008; Wang *et al.* 2008; Keros *et al.* 2009).

Therefore, on the basis of the results of the present study, using our vitrification protocol and slow freezing procedure in a mouse model, the latter preserves late stage ovarian follicles better than vitrification. Similarly, Oktem *et al.* (2011) evaluated human ovaries and compared both morphology and functionality after vitrification and slow freezing. The authors concluded that the number of primordial follicles was decreased significantly after vitrification compared with slow freezing (Oktem *et al.* 2011). More interestingly, functional analysis of follicle survival by measurement of anti-Müllerian hormone (AMH) and oestradiol following tissue culture revealed significant differences among the slow freezing, control and vitrification groups. Compared with fresh and slow-frozen ovaries, vitrified ovaries produced significantly less AMH *in vitro* (Oktem *et al.* 2011). This finding supports our results of better survival of larger follicles (preantral and antral follicles) using the slow freezing method because AMH is predominantly secreted from the preantral and growing follicles (Visser *et al.* 2006). Aerts *et al.* (2008) have also shown the superior preservation of the secondary follicles after slow freezing compared with vitrification in a murine model. Recently, in a study based on the comparison of two different vitrification methods in a macaque model, Ting *et al.* (2013) published promising results on the survival of secondary follicles after closed system vitrification using glycerol and EG with a two-step thawing procedure. The use of different carrying systems and CPA combinations needs to be tested in the vitrification of ovaries.

Copper EM grids are convenient when loading the tissue and become a good carrying device throughout the storage and thawing processes. In their study, Rho *et al.* (2002) compared the vitrification of bovine oocytes either within glass capillaries or as droplets on copper EM grids and found that bovine oocytes can be cryopreserved by vitrification within small droplets using copper grids but that damage to microtubules and mitochondria may be involved in the reduced viability of these cells. In another study in which different cryodevices, such as nylon mesh, EM grids and microcapillary tips for vitrification of isolated follicles, were compared, it was found that microcapillary tips resulted in poor immediate post-warming survival, but there were no differences in the subsequent *in vitro* development characteristics between different cryodevices (Desai *et al.* 2011). Recently, Hashimoto *et al.* (2013) undertook an experiment comparing copper plates and graphite sheets in the vitrification of porcine ovaries. The graphite sheets group had a higher rate of survival of follicles compared with the copper carrier group, with the data highlighting the importance of thermal conductivity. The present study is one of the few studies investigating whether copper EM grids are a user-friendly carrier device for ovarian tissue vitrification, in addition their acceptable thermal conductivity. We found that small follicles and stromal cells were protected in terms of both their ultrastructure and morphology.

The pool of primordial follicles determines the ovarian reserve and has the ability to mature further even after retransplantation of thawed tissue. Both vitrification on EM grids and slow freezing seem to preserve primordial follicles effectively. Even though morphological protection is evident with both methods, it has been reported that the IVF outcome is better when ovarian tissue is slow frozen, rather than vitrified, in mammals (Kim *et al.* 2011). Slow freezing is routinely used to freeze ovaries, and numerous papers have indicated the advantages of this technique over vitrification (Gandolfi *et al.* 2006; Isachenko *et al.* 2009; Kim *et al.* 2011). The use of low concentrations of CPA and the simple protocol compared with vitrification are the most important benefits of the slow freezing technique.

Ultrastructural evaluation is essential to understand any injury to organelles, membranes and cytoplasmic structures. Comboni *et al.* (2008) showed organelle damage after cryopreservation, such as injured contact surfaces, increased intracellular vacuolisation, loss of crista in mitochondria and condensation of the ZP. Keros *et al.* (2009) reported ultrastructural findings under TEM: their results after vitrification using propanadiol and EG were better than those after slow freezing. Similarly, Salehnia *et al.* (2002) detected few ultrastructural changes after vitrification. In contrast, Isachenko *et al.* (2009) found that the slow freezing method was morphologically, endocrinologically and molecularly better than the vitrification method.

Ovarian tissue cryopreservation has some advantages over embryo and oocyte cryopreservation, especially in cancer patients. First, primordial follicles, constituting 70%–90% of all follicles, are resistant to the damage caused by freezing and thawing. The reasons for this tolerance include the volume of these follicles, their low metabolic activity and the lack of a ZP and cortical granules. Second, ovarian tissue can be obtained at any time without having to consider the menstrual cycle, a patient's age and the start of cancer therapy. Third, the endocrine function of an ovary can be reconstituted by tissue cryopreservation and retransplantation; this is not possible with cryopreserved oocytes or embryos.

Using the EM grid as a carrier provides good thermal conduction and proper handling for ovarian tissue. Small ovarian cortical pieces are placed on the EM grids and excess CPA is removed using a tissue, thus enabling optimal vitrification after plunging the grids directly into liquid nitrogen. In addition, EM grids can be stored within cryovials and can easily be placed into cryostorage tanks. We think that such porous or cribriform material with a good heat transfer capacity would be a promising device for tissue vitrification.

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