Character, distribution and biological implications of ice crystallization in cryopreserved rabbit ovarian tissue revealed by cryo-scanning electron microscopy

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BACKGROUND: Ovarian tissue banking is an emerging strategy for fertility preservation which has led to several viable pregnancies after transplantation. However, the standard method of slow cooling was never rigorously optimized for human tissue nor has the extent and location of ice crystals in tissue been investigated. To address this, we used cryo-scanning electron microscopy (cryo-SEM) to study ice formation in cryopreserved ovarian tissue.

METHODS: Rabbit ovarian tissue slices were equilibrated in 1,2-propanediol-sucrose solution and cooled at either 0.3–8 °C/min or 3.0–8 °C/min after nucleating ice at −27 °C, or snap-frozen by plunging in liquid nitrogen. Frozen tissues were fractured, etched and coated with gold or prepared by freeze substitution and sectioning for cryo-SEM.

RESULTS: The size, location and orientation of extracellular ice crystals were revealed as pits and channels that had grown radially between freeze-concentrated cellular materials. They represented 60% of the total volume in slowly cooled samples that were nucleated at −27 °C and the crystals, often >30 μm in length, displaced cells without piercing them. Samples cooled more rapidly were much less dehydrated, accounting for the presence of small ice crystals inside cells and possibly in organelles.

CONCLUSIONS: Cryo-SEM revealed the internal structure of ovarian tissue in the frozen state was dominated by elongated ice crystals between islands of freeze-concentrated cellular matrix. Despite the grossly distorted anatomy, the greater degree of dehydration and absence of intracellular ice confirmed the superiority of a very slow rate of cooling for optimal cell viability. These ultrastructural methods will be useful for validating and improving new protocols for tissue cryopreservation.

Key words: cryopreservation / electron microscopy / freezing / ovary / rabbit

Introduction

Low-temperature banking of ovarian tissue is evolving from a purely experimental procedure in animals to become an option for young patients with cancer or other diseases needing fertility preservation (Gosden, 2005; Donnez et al., 2006; Kim, 2006; Jeruss and Woodruff, 2009). The technology can potentially enable natural fertility to be restored after transplanting the thawed tissue and is a practical alternative to oocyte or embryo banking when they are unavailable or inappropriate. To date, six viable pregnancies have been reported after natural or assisted conception (five to former cancer patients) after transplanting frozen–thawed ovarian tissue (Donnez et al., 2004; Meirow et al., 2005; Demeestere et al., 2006; Andersen et al., 2008; Silber et al., 2008), and large numbers of specimens are being stored for patients awaiting treatment. Despite this progress, it is difficult to predict clinical success because the numbers of follicles in transplanted tissue are unknown and the impact on cell viability of freezing, thawing and ischaemia is variable and difficult to quantify. Rapid freezing/vitrification technology is an alternative to conventional slow-freezing methods, but the latter has been used to bank most clinical specimens and given superior results (Isachenko et al., 2007).
The gold standard for evaluating successful cryopreservation is a successful pregnancy after transplantation, but there have been too few cases to judge the reliability of current protocols. The goal is to maximize follicle survival because a direct, quantitative relationship is expected between the size of the follicle reserve and the number of menstrual cycles generated after transplantation. To date, few clinical cases have been followed up long term, and such provide few clues about the nature of any cryoinjury and cannot therefore guide refinements in cryopreservation technique. Microscopy is useful for revealing damage to tissue after thawing (Gook et al., 1999; Nottola et al., 2008), for counting follicles surviving xenografting (Newton et al., 1996) and scoring the fraction that is moribund using live and dead cell stains (Oktay et al., 1997), but none of these methods can reveal the extent, character or location of ice formed in cryopreserved tissue, which are crucial factors affecting cellular viability. During cryopreservation, frozen extracellular water creates a hypertonic environment which dehydrates cells, thereby protecting them from intracellular ice injury ( Fuller and Paynter, 2004; Pegg, 2006), but this process has not previously been observed directly in ovarian tissue.

We have used cryo-scanning electron microscopy (cryo-SEM) to study ice in cryopreserved ovarian tissue. Cryo-SEM is particularly useful for measuring ice crystals and assessing their distribution, and the fraction of tissue in the frozen state and diffusion path lengths can also be estimated. Using freeze fracture and freeze substitution methods with cryo-SEM, we have analysed rabbit ovarian tissue during cryopreservation with a slow cooling protocol originally developed in animals which was later applied for preserving human tissue (Carroll et al., 1990; Newton et al., 1996; Gook et al., 2001). We compared results with this protocol to faster rates of cooling which were expected to create chemical gradients far from equilibrium and thus less compatible with cell survival.

### Materials and Methods

#### Ovarian tissue

Whole ovaries were obtained from adult rabbits which were being euthanized humanely under protocols ethically approved for other experiments (100 mg/kg sodium pentobarbital, i.v.). The organs were transferred within 30 min to the laboratory in a HEPES-buffered salt solution containing 5% w/v human serum albumin at room temperature and cut with a scalpel into strips ~1.0 mm thick.

#### Cryopreservation

The strips of tissue were equilibrated stepwise at 4°C with vigorous agitation in a solution of cryoprotective agents in the HEPES medium for 5 min at each step, as follows: (i) 0.2 M sucrose, (ii) 0.5 M 1,2-propanediol (PROH) + 0.2 M sucrose, (iii) 1.0 M PROH + 0.2 M sucrose and (iv) 1.5 M PROH + 0.2 M sucrose. The freezing solution (iv) had a melting point of −5.8°C and a glass transition temperature (T_g) of −58°C. The samples were transferred to 1.5 ml cryovials (Nunc™ Thermo Fisher Scientific, Rochester, NY, USA) in the same solution and randomized to three freezing protocols.

(i) Samples were cooled in an automated freezer (Planer PLC, Sunbury-on-Thames, UK) at 2°C/min to −7°C, ice nucleation being induced by gripping the vials with forceps cooled in liquid nitrogen and held at this temperature for 10 min. They were then cooled at 0.3°C/min to −40°C, followed by 10°C/min to −140°C before plunging in liquid nitrogen in which they were stored for several weeks.

(ii) Samples were cooled and nucleated as above, but at a faster rate of 3.0°C/min down to −40°C before plunging.

(iii) Samples were plunged directly into liquid nitrogen without controlled ice formation (~300°C/min). A subset of these ultrapidly cooled samples was warmed to −50°C at 1°C/min and then returned to liquid nitrogen to test for the presence of intracellular ice.

The morphology of ice crystals was also studied in the absence of tissue using the same cryoprotective solution (1.5 M PROH + 0.2 M sucrose). Plastic straws were filled with the solution (0.25 ml) and cooled in a liquid nitrogen-free, controlled rate freezer (EF600, Asymptote Ltd, Cambridge, UK) (Morris et al., 2006a) at a rate of 2°C/min to either −7°C or −10°C at which temperature they were held for 5 min before nucleating the solution with a cryopen (Morris et al., 2006a). After holding for a further 10 min, they were cooled at 1°C/min−1 to −80°C and finally plunged in liquid nitrogen.

The T_g of the freeze-concentrated matrix was determined by conductivity measurements during cooling and warming (Morris et al., 2006b). The samples (2.5 ml) were frozen and thawed in a eutectic cell which monitored temperature and resistance at 1 s intervals (Telstar Industrial, Barcelona, Spain). They were cooled at 1°C/min within a Planer Kryo 10/16 controlled rate freezer to −150°C with ice nucleation at −7°C; all samples were warmed to 1°C/min to 0°C. The T_g is the temperature at the inflection point of the logarithmic resistance–temperature curve, i.e. the point at which the second derivative equals zero. The second derivative was calculated directly from the measured data via local curve fits to the logged data and calculated first derivatives.

The melting point of the cryoprotectant solution was determined by measuring the temperature of 1 ml samples using T-type thermocouples (28 SWG) connected to a data logger. The samples were cooled in the same Planer freezer to −7°C and nucleated. The melting temperature of the solution was taken as the stable temperature attained immediately after ice nucleation.

#### Cryo-SEM

Cryovials containing tissue were cut open under liquid nitrogen and the tissue was dissected from the ice using cooled forceps and scalpels with long insulated handles. The ovarian strips were cross-fractured under liquid nitrogen, loaded onto the stage of a cryo-SEM (Oxford Instruments SL30-FIG, Oxford, UK) and warmed from −145°C to −90°C for 6 min to etch the cut surface for removing some of the crystalline water. The samples were recooled to −145°C. Subsequently, they were transferred to a preparation stage for coating with 10–15 nm gold particles and reloaded onto the cryo-SEM stage for examination and capturing digitized images. The maximum dimensions of ice crystals and the total amount of ice in the samples were estimated by image analysis using methods described by Morris et al. (2006b).

#### Freeze substitution

Some samples were refractured to make segments with a maximum dimension of 1 mm and then transferred under liquid nitrogen to automated freeze substitution chambers (Reichert-Leica, Vienna, Austria). The substitution medium contained 2% osmium tetroxide and 1% uranyl acetate in methyl alcohol as contrast agents. Samples were maintained at −90°C for 24 h, warmed to −70°C at 3°C/h and then maintained at −70°C for 24 h. Afterwards, they were warmed to room temperature at 3°C/h, rinsed in methyl alcohol and embedded in Spurr’s epoxy resin. Sections, 0.5 μm thick, were cut with a Reichert Ultracut S and
stained with methylene blue for preliminary inspection and orientation. Samples that had been plunged into liquid nitrogen proved to be the most difficult to cut because the tissue became very fragile.

Assessment of tissue preservation

The histological integrity of rabbit ovarian tissue was assessed after slow-freezing, fast-freezing or plunging in liquid nitrogen using the protocols described above. After holding the tissues in liquid nitrogen for at least 1 day, they were rapidly thawed by swirling in a water bath at 37°C and washed by reversing the step concentrations of cryoprotective agents. The thawed tissues were transferred with fresh ovarian tissue (controls) to multi-well culture dishes and incubated for 24 h at 37°C in α-minimum essential medium containing 10% fetal calf serum and penicillin G (100 U/ml). Afterwards, the tissues were fixed overnight in 4% paraformaldehyde, embedded in paraffin wax and sectioned at 7.0 μm. The dewaxed sections were either stained with haematoxylin and eosin or the Terminal deoxynucleotidyl Transferase-mediated dUTP Nick End Labeling (TUNEL) method according to the manufacturer’s instructions using 3,3’-diaminobenzidine as the substrate (Roche Diagnostics, Indianapolis, IN, USA) to reveal the 3’-OH ends of DNA exposed by internucleosomal cleavage in moribund or dead cells with single- and/or double-stranded DNA breaks. The sections were pre-incubated with proteinase K (additionally with DNAse I for positive controls) and counter-stained afterwards with toluidine blue (0.1%) before examination at ×100 and ×400 by bright-field microscopy.

**Results**

Structure of ice in the cryoprotectant medium

After cross-fracturing and deep etching, the character and distribution of the ice and freeze-concentrated matrix were resolvable in the frozen straws (Fig. 1a). The structure of ice in frozen cryoprotective medium was determined by the temperature at which it was nucleated because when nucleation was induced at −7°C (i.e. 1.2°C of supercooling) with subsequent cooling at 1°C/min, the ice was more-or-less isotropic with branching ‘dendritic’ crystals that were generally uniform in shape and measuring 10–30 μm in cross-section (Fig. 1b). On the other hand, when nucleation was induced further from the melting point (4.2°C of supercooling), the structure of the ice was more chaotic and the crystals were smaller (<10 μm), even after the same rate of cooling. These results underlined the importance of a critical temperature at nucleation for the resulting crystal structure, and this was fixed at −7°C for all experiments.

Pattern of ice formation in ovarian tissue

When ovarian tissue was fractured and etched in the frozen state, the cellular and ice compartments became resolvable, indicating how the crystals had grown after nucleation. The surfaces appeared smooth.
after fracturing, apart from occasional particles of ice or other contaminants (Fig. 1c), but at higher magnifications, elongated pits were visible that represented extracellular ice crystals after etching (Fig. 1d). There was a strong impression from the directionality of the pits that ice had crystallized radially (i.e. from periphery to centre), although this was less obvious in fast-cooled and ultrarapidly cooled samples.

In slowly cooled samples (0.3°C/min), a continuous network of large ice crystals had formed but the cells were not visible as discrete entities because they were concentrated into dense masses of dehydrated material between the pits and channels (Fig. 2a and b). No intracellular structures were discernable at the fractured surfaces. When tissue was cooled 10-fold faster after nucleating at the normal temperature, extracellular ice crystals were formed between densely packed cells as before, but their pattern was less uniform than at the slower rate of cooling and the voids were less regular in shape with thread-like cross-bridges of cellular material (Fig. 2c and d). The ice structure was also distinct in ultrarapidly cooled specimens where there had been no control of nucleation (Fig. 2e and f). Small pits were distributed more-or-less evenly across the tissue and the cellular material was not freeze concentrated. Additionally, there were patches of small vesicular structures measuring <1 μm which may represent lipid droplets (Fig. 2f).

The micrographs revealed that the ice fraction external to the freeze-concentrated cellular material was 60% of the total area after slow freezing and somewhat less (56%) after fast freezing. From these estimates, the PROH concentration in the freeze-concentrated cellular material

Figure 2 Cryo-SEM of frozen rabbit ovarian tissue after fracturing and etching. Three samples are shown in pairs at low and high magnifications. The samples were cooled at 0.3°C/min (a and b) or at 3°C/min (c and d) or very rapidly by plunging into liquid nitrogen (~300°C/min) (e and f). The figures are labelled at some former locations of ice crystals which were large in (a and b) but diminutive in (c–f).
was estimated to be \(-3.75 \text{ M}\). The ice fraction after plunging tissue into liquid nitrogen was much reduced (34%) compared with other samples, and the concentration of PROH in the cellular matrix was estimated to be only 2.2 M. Crystals that had formed within the concentrated cell matrix of fast-cooled and ultrarapidly frozen samples (i.e. intracellular ice) were small and could not be quantified accurately because their boundaries were sometimes indistinct.

When samples that had been rapidly cooled were rewarmed at \(18^\circ C/\text{min}\) to \(-50^\circ C\) (i.e. \(T_g\) for the cryoprotectant used in this study) and then returned to liquid nitrogen, ice crystal regrowth was apparent (Fig. 3a). The minute pits observed in samples at high magnification were probably sites formerly filled with intracellular ice, which is incompatible with cell survival (Fig. 3b).

**Freeze substitution**

The freeze substitution technique revealed the presence of intracellular ice in finer detail than could be discerned just from the fractured surfaces. In slowly cooled samples, the dense packing of cellular material was confirmed and it was possible to distinguish the boundaries of some cells and identify lipid droplets, and possibly even mitochondria judging by cristae-like striations, but other organelles were not visible (Fig. 4a and b). At a faster rate of cooling (3°C/min) after nucleating at the same temperature, there was more extensive disruption and shrinkage of the cellular matrix which was separated by ice into smaller blocks and islands connected by thin bridges (Fig. 4c). At higher magnifications, small ice crystals were apparent as clear spaces within cells (Fig. 4d), but they were more widely distributed in the samples that had been cooled by snap-freezing (Fig. 4e and f). Although the micrographs sometimes gave the impression of intracellular ice in mitochondria, its precise intracellular location was generally uncertain (Fig. 4f). Lipid droplets bounded by membranes were visible irrespective of the rate of cooling, some containing clear areas where the contents had apparently been differentially extracted during preparation.

**Assessment of tissue preservation**

The rabbit ovary has a cortico-medullary gradient of follicle development which is typical of mammalian ovaries, but its stroma is denser and more extensive than in small laboratory rodents, and less voluminous than in humans (Fig. 5a). The ovaries were cultured for a day to allow time for the effects of cryoinjury to become manifest. Growing follicles rarely survived the stress of freezing and thawing, the only exceptions being in tissues that had been cooled slowly, and even they were rare and appeared to be damaged (Fig. 5b). Separation of granulosa cell layers sometimes occurred in growing follicles, but this was likely an artefact of histology since it occurred in fresh tissue. Primordial follicles were present in fresh and slowly cooled tissues but were never found in fast- or ultrarapidly frozen samples in which follicles were often just remnants containing a distorted oocyte and infiltrated by other cell types (Fig. 5c and d). Stromal tissue appeared to be similar in all treatment groups, although light microscopy was unable to resolve fine cytological differences. Results of the TUNEL method are not illustrated because they were uninformative. The distribution of TUNEL-positive reaction product was similar in controls and the three treatment groups, although light microscopy was uninformative. The findings being consistent with previous negative results (Demirci et al., 2002; Hussein et al., 2006).

**Discussion**

This is the first reported use of cryo-SEM for ovarian tissue and the first to investigate this tissue in a state of cryopreservation. We used a slow-freezing protocol similar to those in clinical use for patients choosing ovarian banking for fertility preservation. Cryo-SEM has rarely been used in reproductive biology, and only to study gametes (Morris et al., 1999; Hernández et al., 2007; Walther, 2008), although it has been applied extensively in plant biology and in the food and chemical industries. Its chief advantage compared with conventional electron microscopy is in enabling tissues and other materials to be studied while they are frozen, avoiding artefacts from dehydration, fixatives and solvents. The processes of fracturing and etching to reveal the distribution of ice and freeze substitution to resolve fine ultrastructural details provide unique insights into the structure of ice in cryopreserved tissues. Information about the extent of ice formation, the degree of dehydration and diffusion

![Figure 3](image-url) (a) Cryo-SEM of rabbit ovarian tissue that had been cooled by plunging into liquid nitrogen followed by warming to \(-50^\circ C\) and plunging back into liquid nitrogen. (b) Detail of a similar sample showing pits within the cellular material which represent the former location of intracellular ice.
distances for water can be used to draw inferences about the degree and character of cryoinjury sustained by a given cryopreservation protocol before the tissue is thawed.

Our observations using cryo-SEM techniques were interpreted from principles of cryobiology and the findings were broadly consistent with those obtained in our studies of frozen sperm suspensions (Morris et al., 1999). Since the rate of cooling and the temperature of ice nucleation are key variables, their effects on ice formation were at the forefront of investigation, serving as a basis for more exhaustive studies in future aimed at refining cryopreservation protocols to improve cell survival.

Cells are closest to chemical equilibrium with the external solution when they are subjected to the slowest rates of cooling, which was 0.3°C/min in this study. The cellular compartment was greatly shrunken compared with normal tissue, appearing as a complex network of irregular ridges and blocks surrounding pits and channels that had been occupied by ice before etching. The environment must have been sufficiently viscous under these conditions for intracellular vitrification to have occurred since this is a precondition for viability and the morphology of the thawed tissue was relatively normal. Moreover, similar protocols have achieved clinical success after transplanting human ovarian tissue, as summarized above. It is obvious but

Figure 4 The effects of different rates of cooling on the ultrastructure of frozen rabbit ovarian tissue as revealed by freeze substitution. The samples were cooled at 0.3°C/min (a and b) or 3°C/min (c and d) or ultrarapidly by plunging in liquid nitrogen (e and f). Some of the spaces formerly occupied by large ice crystals are labelled (a, c and d). Small intracellular ice crystals were abundant in plunged samples [see arrow in (f)] but absent in slowly frozen samples (a and b). Vesicular structures with smooth contours probably represented lipid droplets from which most of the material had been extracted during sample preparation.
nevertheless noteworthy that cryo-SEM is inappropriate for ‘vitrification technology’ in which it is assumed that the entire tissue and its surrounding medium form an anisotropic, glass-like solid, devoid of ice. Thus, cryo-SEM cannot throw much light on the debate about the relative merits of different methods, for which there is conflicting evidence whether cryopreservation is superior (Gandolfi et al., 2006; Isachenko et al., 2009) or equivalent (Li et al., 2007) or inferior to vitrification (Keros et al., 2009). It would be valuable to apply the techniques of cryo-SEM or freeze substitution to rapidly cooled ovarian tissue in order to verify that vitrification has occurred or whether micro-crystalline ice exists within the tissue. However, in order to detect the presence of ice by cryo-SEM, it is necessary to etch the sample and this requires warming, under reduced pressure to temperatures of −100°C. Similarly, the first step in conventional freeze substitution is transfer to a substitution medium at −90°C. Both cryo-SEM and freeze substitution would require warming the ‘vitrified’ sample to a temperature above its glass transition temperature, which would be expected to lead to de-vitrification and the formation of ice.

When tissue was cryopreserved, it became compartmentalized into freeze-concentrated medium forming large crystals between strands and islands of shrunken cellular material. Considering the striking differences in structural appearance of fresh tissue versus tissue in the frozen state, it is remarkable that experimental and clinical studies have not only shown follicles surviving after thawing but also restoring normal ovarian function and even ovulating developmentally competent oocytes (Donnez et al., 2006). The ovary probably withstands disruption to its anatomical architecture by ice crystals because it is a plastic organ, continually renewing the spectrum of follicles from a reserve of primordial stages. Contrary to popular assumptions about cryopreservation, there were no sharp, icicle-like crystals that might pierce cell membranes in the frozen tissue. Instead, the crystals had grown with smooth contours by recruiting liquid water from the adjacent environment, progressively displacing cells and dehydrating them at the same time. Freeze fracture studies confirmed the absence of intracellular ice after slow freezing, but since this method only revealed surface topography, fine details of cell structure were not apparent except by freeze substitution.

The structure of extracellular ice was determined by the nucleation temperature rather than by the rate of cooling, which is responsible for the degree of ice formation (Searles et al., 2001). When nucleation was induced close to the melting point of the cryoprotective medium (−7.0°C), ice grew into large, uniform crystals, whereas nucleation at a lower temperature created crystals that were less uniform and generally smaller. These findings were consistent with theoretical predictions and the standard practice of nucleating samples (‘seeding’) to avoid an unstable, supercooled state which can precipitate intracellular ice formation. In the absence of controlled nucleation by plunging samples in liquid nitrogen, lethal intracellular ice became more abundant. For these reasons, the temperature chosen for nucleating samples was fixed at −7.0°C, consistent with data showing significant loss of viability when mouse embryos were nucleated at lower temperatures (Whittingham, 1977).

Judging from the orientation of channels that had been etched after nucleation of the cryovials, extracellular ice crystal growth had been propagated across the surface and radially inside the tissue towards its centre. There were some deviations from this pattern between and within samples which may have been due to either local supercooling before nucleation or from a non-homogeneous matrix which could affect the rate of ice propagation.

After extracellular water begins to freeze, water is drawn out of neighbouring cells down the osmotic gradient to add to the growing network of ice. At slow rates of cooling, the growth of ice crystals and the accompanying cellular dehydration leads to the cells within the tissues becoming compacted into dense masses separated by large crystals. Since the shrinking cellular compartments probably contained different numbers of cell layers, water had to traverse variable path lengths before reaching and becoming incorporated into existing crystals of ice. Slow cooling would give more time for near-equilibrium conditions to be attained and facilitate a more complete and uniform dehydration. However, when samples were cooled faster at 3°C/min, there was less time for water to leave the cells, and the risk of intracellular ice formation was correspondingly higher with incomplete dehydration. Samples cooled ultrarapidly by snap-freezing in liquid nitrogen without controlled ice nucleation contained a network of small crystals because there was too little time for cellular dehydration.
and consequently less extracellular ice formed, as predicted by a theoretical plot of the equilibrium phase. In consequence, many cells in these samples were expected to contain ice. Cryobiological characteristics vary between cell types, the viability of ovarian cells likely depending on the fraction and location of ice formed compared with sperm subjected to ultrapid freezing in which the surviving percentage was determined mainly by osmotic stress (Morris, 2006).

The ultrastructural appearance of ice crystals within cells was verified under conditions in which ice was expected to form, namely by slowly warming snap-frozen samples above $T_g$ to encourage crystal growth. These results also confirmed the obvious importance of maintaining cryopreserved cells below this threshold temperature throughout storage.

One of the key physical parameters determining the degree of cellular dehydration for effective cryopreservation is the diffusion distance for water, which varies with viscosity for a given temperature (Morris et al., 2006). At a cooling rate of 1 °C/min, this diffusion distance has been calculated to be >150 μm for cryoprotective solutions containing glycerol, but is only 15 μm at much faster rates of cooling (>100 °C/min) (Morris et al., 2006b). Propanediol was used in the present study because it is superior to glycerol as a cryopreservation agent for ovarian tissue (Newton et al., 1996). Although the viscosity of both freezing solutions are similar (Morris et al., 2006b), sucrose increases viscosity and hence the diffusion distance, theoretically requiring a slightly slower rate of cooling for dehydrating tissue to the same degree and avoiding supercooling. Nevertheless, the data obtained from models using glycerol provide approximations to the diffusion distances expected in the present experiments, which were evidently not seriously limiting at a rate of 0.3 °C/min cooling judging from experimental and clinical transplantation data. But, cooling at a rate 10-fold faster allowed too little time for water to diffuse from all cells to the primary ice front. Intracellular ice was expected to form after supercooling because freeze-concentrated cellular material varied in cross-section up to 50 μm, whereas the diffusion distance at the same cooling rate was estimated to be <25 μm. This is a significantly shorter path length than in the cryoprotective solution itself, which could be due to higher intracellular viscosity and/or variations in membrane permeability to water. The diffusion distance within tissues is also likely to vary between cell types and with the density of extracellular fibre material, which could explain why the ovarian stroma was more vulnerable to cryoinjury than follicular cells (Oktay et al., 1997; Keros et al., 2009). Models are needed for estimating diffusion and related physical parameters for a given cryoprotective solution and nucleation temperature, but they will be very difficult to develop for heterogeneous tissues like the ovary.

In conclusion, both the rate of cooling and the nucleation temperature have profound effects on the character, extent and distribution of ice crystal formation in the extra- and intracellular compartments of cryopreserved ovarian tissue. Slow cooling rates were associated with more dehydration and, although there was greater disruption of tissue structure, cellular viability after thawing was improved, consistent with empirical studies showing fertility can be restored by transplanting thawed tissue. Rates of cooling faster than 0.3 °C/min or ice nucleation at temperatures lower than −7 °C are less likely to be compatible with survival because of diffusion-limited dehydration and intracellular ice formation. As for vitrification of tissue, ice is absent by definition. The physical state of water in tissue following ‘ultrapid freezing’ or ‘snap freezing’ would be expected to be intermediate in structure between treatment #3 in this study and vitrified samples, but it is not possible to confirm this with the techniques used in this study.

The results of cryo-SEM provide confirmation of the status quo for ovarian cryopreservation protocols in clinical practice, which are mainly based on a cooling rate of 0.3 °C/min, but it should be noted that this rate cannot be regarded as optimal without more detailed studies for verification. Moreover, the standard protocol originated from studies of mouse oocytes whose cryobiological properties are likely to be different from human ovarian tissue (Carroll et al., 1990). Since follicles succumbing to cryoinjury limit the functional lifespan of transplants, successful cryopreservation (or vitrification) is vital for fertility preservation. But advances in low-temperature preservation cannot depend only on clinical follow-up after transplantation because there have been few cases so far and data are slowly accumulating. Hence, there is a need for the effects of cooling and rewarming to be investigated directly in tissues, and the present studies indicate that cryo-SEM promises to be helpful in revealing the internal frozen state and predicting success rates with cryopreservation protocols.

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