Article

A rational approach to oocyte cryopreservation



Dr Paynter was awarded her PhD entitled 'Corneal Tolerance of Vitrifiable Concentrations of Cryoprotectant' in 1990 from Bristol University, UK. She then investigated cryopreservation of pancreatic islets at Leicester University, UK, and was involved in the first clinical transplantation of pancreatic islets in the UK. Dr Paynter has been working in Cardiff since 1993. There she has studied the permeability characteristics of oocytes in the presence of a range of cryoprotectants. Dr Paynter has also developed protocols for the vitrification of mature murine oocytes producing high levels of development post-thaw, and studied slow cooling of immature oocytes and ovarian tissue.

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Abstract

Reports of clinical pregnancies from cryopreserved human oocytes have been steadily increasing in recent years. However, success in terms of births per thawed oocyte remains poor. A wide variety of freezing techniques has been used lately, but modifications to protocols are made on an empirical basis. Methods of cryopreservation are often poorly described or protocols are not strictly adhered to, resulting in variability of outcome. The first stage of a freezing protocol is exposure to cryoprotectant. If performed inappropriately, such exposure can result in damage due to chemical toxicity and/or osmotic stress. Measurement of cell volume change during exposure to cryoprotectants demonstrates the extent of osmotic stress experienced by that cell. Such measurements have been performed during perfusion of murine and human oocytes with cryoprotectant concentrations commonly used for cryopreservation of these cells. It has been demonstrated that changes in the cryoprotectant type, concentration and temperature of exposure can dramatically affect the extent of cell volume change. Even small changes in duration of exposure to cryoprotectant prior to cooling can result in drastic changes in cellular hydration. Such factors will potentially influence the ability of the cell to survive the stresses experienced during the subsequent stages of the cryopreservation protocol.

Keywords: *cryopreservation, cryoprotectant, oocyte, osmotic stress, propane-1,2-diol*

Introduction

There are several circumstances in which the ability to store oocytes would be advantageous. The potential benefits of long-term low-temperature storage of unfertilized oocytes are probably greatest for young female patients about to lose ovarian function, in particular cancer patients about to undergo fertility threatening chemo- and radiotherapy. Oocyte storage is also of potential benefit to patients undergoing fertility treatment who have excess oocytes, since storage of unfertilized oocytes raises fewer legal and ethical concerns than embryo storage. Oocyte cryopreservation would facilitate banking of oocytes, not only for a patient's own use, but also for possible donation to other patients or for use in research. Banks of oocytes would help alleviate the current shortage of oocytes for donation, and would make donation less complex by avoiding the need to synchronize donor and recipient treatment cycles. More controversially, oocyte storage could also be seen as a means of delaying childbearing. In a broader context, the ability to store oocytes would be of great significance in the fields of conservation and animal husbandry.

Unfortunately, unlike sperm and, where it is permitted, embryo storage, oocyte storage is not widely available in fertility clinics. Reports of live births from frozen oocytes have been steadily increasing since 1997, when the first report of a live birth using a combination of cryopreservation and intracytoplasmic sperm injection (ICSI) occurred (Porcu *et al*., 1997). However, only around 100 babies have been born worldwide following oocyte freezing (Borini *et al*., 2004b). Results are variable and success in terms of live births expressed as a percentage of oocytes thawed is low (Paynter, 2000; Van der Elst, 2003).

Oocytes are particularly susceptible to damage during cryopreservation, for a number of reasons. Oocytes are large single cells, which means they have a low surface area to

volume ratio, and this, coupled with the fact that they have a low permeability to water, means that they are likely to retain water when frozen, creating intracellular ice which is extremely damaging to cells. The oocyte is also a short-lived cell that must undergo fertilization and development if it is to continue to survive. In order to achieve this, it must retain integrity of its unique structural features (Smith and Silva, 2004). These include the zona pellucida, which if damaged can result in multiple sperm entry and abnormal fertilization, and the cortical granules, premature release of the contents of which results in zona hardening and a block to fertilization. However, the consequences of damage to either of these structures can be overcome by the use of ICSI to achieve fertilization following cryopreservation. The mature oocyte also contains condensed chromosomes arranged on a microtubular spindle that is known to be sensitive to both low temperatures (Pickering and Johnson, 1987; Pickering *et al*., 1990; Sathananthan *et al*., 1992) and cryoprotectants (Johnson and Pickering, 1987; Pickering *et al*., 1991). There is evidence of the ability of the spindle to undergo some degree of repair post-cryopreservation (Rienzi *et al*., 2004; Stachecki *et al*., 2004), and indeed reports of dispersed chromosomes following cryopreservation are rare (Gook *et al*., 1994; Zenzes *et al*., 2001). Danger of damage to the spindle can be avoided if immature oocytes are cryopreserved, since the spindle has not yet formed in such oocytes. However, immature oocytes need to be matured before fertilization can occur and techniques for in-vitro maturation of human oocytes require refinement. Furthermore, attachment of the cumulus cells that surround the immature oocyte is required for successful maturation of the oocyte. Cryopreservation has been shown to result in detachment of cumulus cells from the oocyte (Cooper *et al*., 1998; Ruppert-Lingham *et al*., 2003). In addition, cumulus cells differ greatly in size from oocytes, hence a cryopreservation protocol that is optimal for cumulus cells is likely to differ from that which is optimal for oocytes, making preservation of both cells types difficult. Consequently, live births from frozen immature oocytes are even rarer than births from mature oocytes (Tucker *et al*., 1998b). It is likely that other structures within the oocyte are also damaged during freezing; for example, there are recent reports that mitochondria within the oocyte are adversely affected by cryopreservation (Jones *et al*., 2004).

Cryopreservation procedure

In order to successfully cryopreserve cells, they must undergo a cryopreservation procedure that involves a number of steps (Fuller and Paynter, 2004). First, the cells are exposed to cryoprotectants. Cryoprotectants can be divided into two groups, those that enter the cells and act to replace water therein and those that are non-permeating and act to draw water from within the cell.

Once the cell has been exposed to cryoprotectant, cooling can begin. The rate of cooling is either slow, <1°C/min, or fast, >100°C/min. With slow cooling, ice continuously forms around the outside of the cell as slow cooling proceeds, drawing water from within the cell. In this way the inside of the cell remains ice-free. Slow-cooling rates are employed, typically to temperatures of –40°C or –80°C, after which it is safe to use faster cooling rates to the storage temperature. When fast cooling rates are employed throughout, the aim is to cool the sample so rapidly that ice does not have time to form either within or outside the cell, a process known as vitrification. In practice, vitrification of cells cannot be achieved without the presence of high concentrations of cryoprotectant (around 6–8 mol/l, as opposed to the 1–2 mol/l used for slow cooling).

Samples are usually stored either in liquid nitrogen or in liquid nitrogen vapour. If samples are maintained at temperatures below –140°C, then they can effectively be stored indefinitely.

The rate at which a sample is warmed will depend upon how it was cooled. If the sample was cooled at slow rates to a temperature of around –80°C then warming is usually performed at ~10°C/min. Such slow warming allows rehydration of the sample and time for movement out of the cell of any solute that may have entered during cooling (Griffiths *et al*., 1979). If slow cooling was stopped at a higher temperature, around –40°C, as is usually the case, less dehydration and loading of solute will have occurred on cooling and the sample can therefore be warmed at a faster rate. Such rates of warming can indeed be necessary to prevent freezing of the intracellular contents and subsequent cell damage during warming (Rall and Polge, 1984; Rall *et al*., 1984). These faster warming rates are usually achieved by holding the sample in air or in a water bath. If rapid cooling rates have been used throughout, then the sample will certainly need to be warmed rapidly in order to prevent the growth of any tiny ice nuclei that formed on cooling. Once warmed, the cryoprotectant will need to be removed from the cells.

Exposure to cryoprotectant

It is the first step of the cryopreservation procedure that this paper will concentrate upon, namely exposure to cryoprotectant. The presence of cryoprotectant helps to protect the cell from freezing related injury in two main ways. It replaces or withdraws water from within the cell to prevent intracellular ice formation. In addition, the presence of cryoprotectant within a cell will reduce the increase in concentration of salts that occurs due to the removal of water.

Exposure of cells to cryoprotectants can be damaging even in the absence of freezing, if it is performed inappropriately. Depending on the cryoprotectant used, there can be problems with cellular toxicity, particularly when high concentrations of cryoprotectant are used. Toxicity can be reduced by using a combination of several cryoprotectants (Katayama *et al*., 2003) or by using cryoprotectants with low toxicity for oocytes, such as ethylene glycol (Kuleshova *et al*., 1999).

Addition and removal of the cryoprotectants can be damaging, since it can result in extreme cell shrinkage or swelling. This paper will review data on the cell volume change of murine and human oocytes on exposure to the most commonly used cryoprotectants. The effect of temperature and stepwise addition of cryoprotectant will be discussed, as well as the use of combinations of permeating and non-permeating cryoprotectants, in particular, the combination of propane-1,2-diol (PrOH) with various concentrations of sucrose. Such cryoprotectant combinations have been used recently for the cryopreservation of human oocytes.

Materials and methods

Source of oocytes

Murine oocytes were obtained from virgin 6- to 8-week-old CBA/Ca × C57BL/6 female mice that were superovulated (as detailed in Paynter *et al*., 1997). Oviducts were removed from killed mice. Oocytes surrounded by the cumulus mass were released from the oviduct into 150 IU/ml hyaluronidase (Sigma, Poole, Dorset, UK) contained in phosphate-buffered medium plus 4 mg/ml bovine serum albumin (Sigma) (PB1). When the cumulus cells had dispersed (1–2 min), mature metaphase II oocytes were washed three times in PB1. Mature oocytes from two or three mice were pooled and held in PB1 at 37°C until required, a maximum of 2 h.

Human oocytes were obtained from patients, with informed consent, undergoing treatment for infertility at Cardiff Assisted Reproduction Unit, Tecnobios Procreazione, Bologna or Department of Obstetrics and Gynaecology, Vita-Salute University, HS Raffaele, Milan. Approval for use of the oocytes donated had been obtained previously from the local Institutional Review Board. The oocytes used were surplus material donated by patients undergoing fertility treatment and were qualitatively comparable with those used for treatment. Oocytes were retrieved from follicles following treatment with ovulatory drugs and were placed into Fertilization Medium at 37°C. The oocyte cumulus complexes were then placed into Oocyte Wash Medium containing 80 IU/ml hyaluronidase (Sigma-Aldrich, Milan, Italy), at 37°C for less than 60 s, followed by four washes in hyaluronidase-free Wash Medium (Cook IVF, Brisbane, Australia) and gentle pipetting to remove the cumulus cells more closely connected to the zona pellucida. The denuded oocytes were washed twice and held in Cleavage Medium (Cook IVF) at 37°C until required. All oocytes were mature (with an extruded first polar body) at the time of use, which was 4–6 h after retrieval.

Microperfusion

The method for microperfusion of oocytes has been described previously (Paynter *et al*. 1997). Briefly, a single oocyte was placed in a 5-µl droplet of PB1 (murine oocytes) or for human oocytes, Dulbecco's phosphate-buffered solution (PBS) (Gibco, Life Technologies, Paisley, UK) supplemented with 20% (final concentration, 10 mg/ml) of plasma protein solution (PPS) (Baxter AG, Vienna, Austria). The dish containing the oocyte was placed on the stage of a Nikon Diaphot 200 inverted microscope or Axiovert 35M (Carl Zeiss Ltd., Welwyn Garden City, Herts, UK). A holding micropipette (Cook IVF) with a 1.5 µm diameter tip opening was used to hold the oocyte in the correct position during perfusion. The micropipette was filled with PBS and positioned adjacent to the oocyte using a Narishige or Zeiss micromanipulator. The pipette was then used to hold the oocyte by negative pressure generated by a Narishige IM-5A injector applied to the outer zona pellucida, care being taken not to deform the inner oolemma (measurement of the magnitude of negative pressure was not conducted in these experiments). The oocyte was then perfused by adding carefully 1 ml of perfusate by means of an air displacement pipette. The time taken between placing the oocyte in the 5-µl droplet and flushing it with the perfusate was minimized to reduce evaporation from the droplet. The perfusates contained dimethyl sulphoxide (Me_2SO), PrOH, glycerol, ethylene glycol or sucrose (all obtained from Sigma).

The experiments were performed at room temperature (~20°C), 10°C or 37°C. The temperature of the perfusate was controlled at 37°C by a heated stage and control unit (TRZ 3700; Zeiss) and at 10°C by a brass stage containing circulating cooled water (RTE-5DD; Neslab Instruments Inc., Newington, NH, USA).

The changes in cell volume were recorded on videotape. Images were then captured at set intervals during the perfusion and cell volume calculated from triplicate measurements of oocyte diameter. For some data, best-fit plots to the measured data were generated using computer software (DIFFCHAM) designed by Professor J. McGrath (McGrath *et al*., 1992). This software generated combinations of values for water permeability (L_p) and cryoprotectant permeability (P_s) according to the Kedem–Katchalsky model of the movement of solutes across cell membranes (Kedem and Katchalsky, 1958).

Statistics

Pairwise comparisons of coefficients for L_p or P_s were performed using the Mann–Whitney *U*-test with a 95% confidence interval.

Results

Effect of cryoprotectant type

The volume change of mature murine oocytes on exposure to 1.5 mol/l cryoprotectant at room temperature is depicted in **Figure 1**. Time following addition of cryoprotectant was plotted against the volume of mature murine oocytes that had been normalized to the volume of each oocyte just prior to perfusion. Measurements were performed in the presence of the four most commonly used cryoprotectants, namely Me_2SO, PrOH (Paynter *et al*., 1997), ethylene glycol (Paynter *et al*., 1999a) or glycerol (Paynter *et al*., 1999b). In each case, the oocytes initially shrank. The volume of the oocyte then increased as cryoprotectant began to permeate the cell. The response was similar in the presence of Me_2SO and PrOH. A similar degree of shrinkage occurred prior to permeation of the cryoprotectants at a similar rate. There were no significant differences in permeability coefficients generated in the presence of these two cryoprotectants (see Paynter *et al*., 1997). The permeation of ethylene glycol was slower, with slightly greater shrinkage prior to cryoprotectant entry. Values for P_s were significantly less in the presence of ethylene glycol than in Me_2SO or PrOH, whilst there were no significant differences in the corresponding values for L_p (see Paynter *et al*., 1999a). Glycerol, on the other hand, resulted in extreme shrinkage of the cell – far greater than for the other datasets – with little recovery of cell volume, indicating that the cryoprotectant hardly permeated the oocyte. It should be noted that whilst statistical analysis was performed on the permeability coefficients, it was not on the cell volumes; clearly the error bars for glycerol data do not overlap with those of other data sets so any comparison with other data in **Figure 1** would be significant.

Effect of temperature

The effect of temperature on the volume change of mature murine oocytes on exposure to the cryoprotectant Me_2SO is shown in **Figure 2**. The higher the temperature, the quicker the water moved out of the cell initially and the quicker the cryoprotectant began to move in (see Paynter *et al*., 1997).

Similar perfusions have been performed at 30, 20 and 10°C in the presence of ethylene glycol (Paynter *et al*., 1999a). At 30°C oocytes shrank to a greater extent in the presence of ethylene glycol than in the presence of Me_2SO, whereas at 10°C the volume change was almost identical. In each case, the coefficients for water and cryoprotectant permeability were determined and are given in **Table 1**. At 30°C the water permeability (L_p), and hence the degree of initial shrinkage, was significantly greater in the presence of ethylene glycol than with Me_2SO and the cryoprotectant permeability (Ps), was significantly lower for ethylene glycol. At 20°C, the difference between the two cryoprotectants was less pronounced and at 10°C the coefficients were not significantly different, being practically identical.

Comparison of human and murine oocyte permeability

Figure 3 shows a comparison of the cell volume change of murine and human oocytes exposed to PrOH at room temperature. The human oocytes shrank to a greater extent but permeation of the cryoprotectant occurred at a similar rate for the two cell types. **Table 2** depicts the permeability coefficients for mouse and human oocytes generated at a range of temperatures in the presence of PrOH. At 20°C, human oocytes had a significantly greater permeability to water, hence the greater shrinkage, but there was no significant difference in cryoprotectant permeability between the two cell types. However, at both 30°C and 10°C, human oocytes had a greater permeability to water and to cryoprotectant than did murine oocytes. The overall effect of this was that human oocytes underwent lesser volume excursions than murine oocytes in the presence of PrOH. At 30°C human oocytes reached a minimum volume of 70% of initial volume (Paynter *et al*., 2001) compared with 65% in mouse oocytes (Paynter *et al*., 1997) and at 10°C human oocytes reached 60% of their initial volume compared with 55% in mouse oocytes.

Stepwise exposure of human oocytes to 1.5 mol/l PrOH

Cell volume change of human oocytes was measured in the presence of 0.75 mol/l PrOH. Oocytes shrank to 75% of initial volume. Oocytes that had been placed in 0.75 mol/l PrOH for 7.5 min and then perfused with 1.5 mol/l PrOH, all at room temperature, reached 84% of initial volume and returned to initial volume within 7.5 min (Paynter *et al*., 2005).

Exposure of human oocytes to 1.5 mol/l PrOH plus sucrose

Human oocytes that had been exposed to 1.5 mol/l PrOH for 10 min at room temperature were then perfused with 1.5 mol/l PrOH plus either 0.1 mol/l, 0.2 mol/l or 0.3 mol/l sucrose (**Figure 4**). Oocytes continuously shrank in these solutions. After 10 min in these solutions, the oocytes had reached 79, 67 or 55% of initial volume in 0.1, 0.2 or 0.3 mol/l sucrose respectively (see Paynter *et al*., 2005).

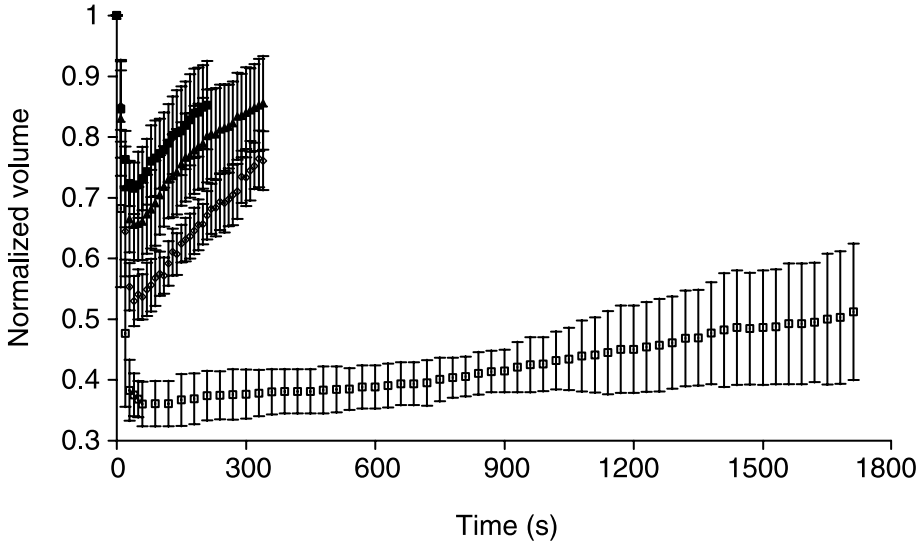


Figure 1. Mean ± SD (n = 10) normalized volume of mature murine oocytes during exposure to 1.5 mol/l Me_2SO (▲), propane-1,2-diol (■), ethylene glycol (◊) or glycerol (□) at room temperature.

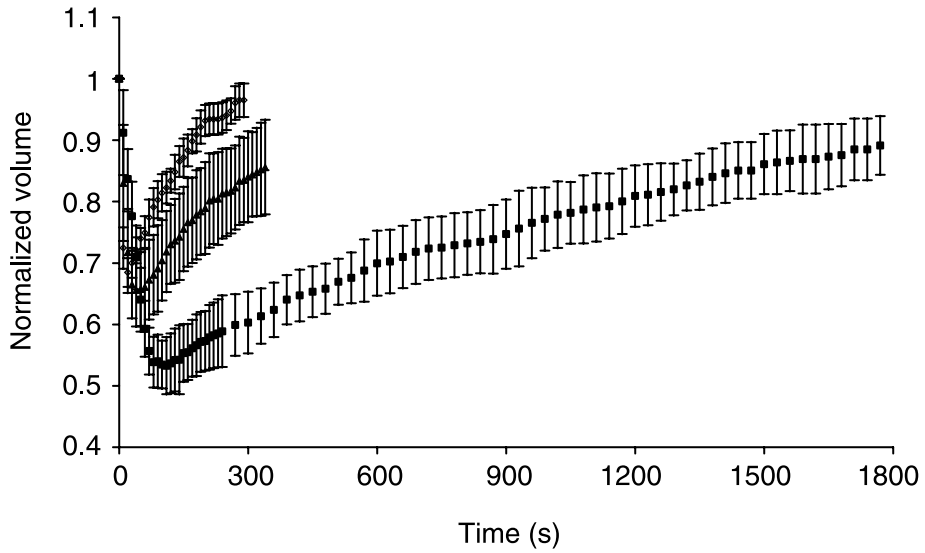


Figure 2. Mean ± SD normalized volume of mature murine oocytes during exposure to 1.5 mol/l Me_2SO at 10°C (■, n = 11), 20°C (▲, n = 10), or 30°C (◊ n = 10).

Table 1. Permeability coefficients of murine oocytes exposed to 1.5 mol/l cryoprotectant (mean ± SD).

Temperature of exposure	*30°C*		*20°C*		*10°C*	
Coefficient	L_p	P_s	L_p	P_s	L_p	P_s
Me_2SO	0.64 ± 0.09	0.37 ± 0.05	0.41 ± 0.13	0.16 ± 0.06	0.20 ± 0.05	0.03 ± 0.01
Ethylene glycol	0.91 ± 0.16	0.24 ± 0.05	0.51 ± 0.08	0.09 ± 0.02	0.18 ± 0.03	0.03 ± 0.01

L_p = water permeability (μm min^{-1} atm^{-1}); P_S = solute permeability (μm s^{-1}).

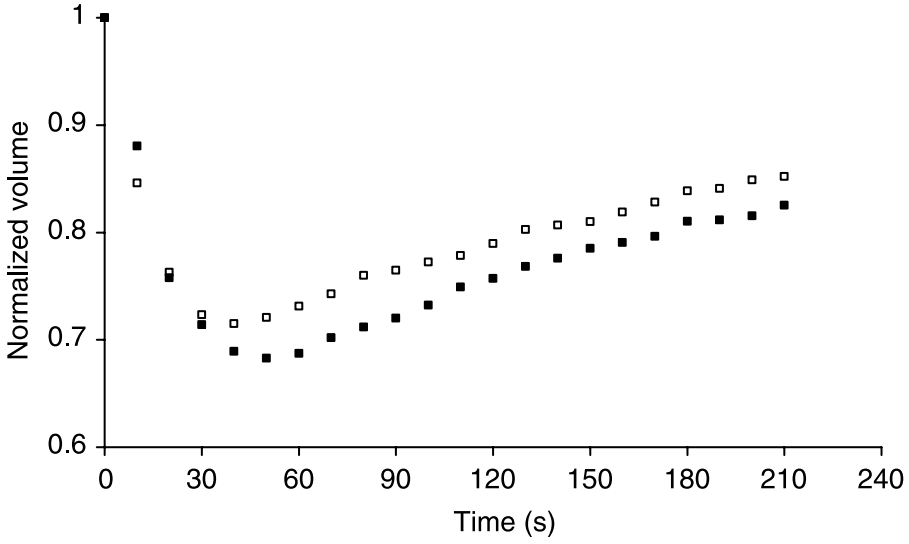


Figure 3. Mean volume of human (■, n = 9) or murine (□, n = 10) oocytes during exposure to 1.5 mol/l propane-1,2-diol at room temperature.

Table 2. Permeability coefficients of oocytes exposed to 1.5 mol/l propane-1,2-diol (mean ± SD).

Temperature of exposure	*30°C*		*20°C*		*10°C*	
Coefficient	L_p	P_s	L_p	P_s	L_p	P_s
Mouse	0.53 ± 0.05	0.43 ± 0.12	0.36 ± 0.13	0.24 ± 0.13	0.15 ± 0.02	0.04 ± 0.01
Human	1.92 ± 0.68	1.08 ± 0.32	0.53 ± 0.11	0.28 ± 0.06	0.41 ± 0.23	0.13 ± 0.04

L_p = water permeability (μm min^{-1} atm^{-1}); P_S = solute permeability (μm s^{-1}).

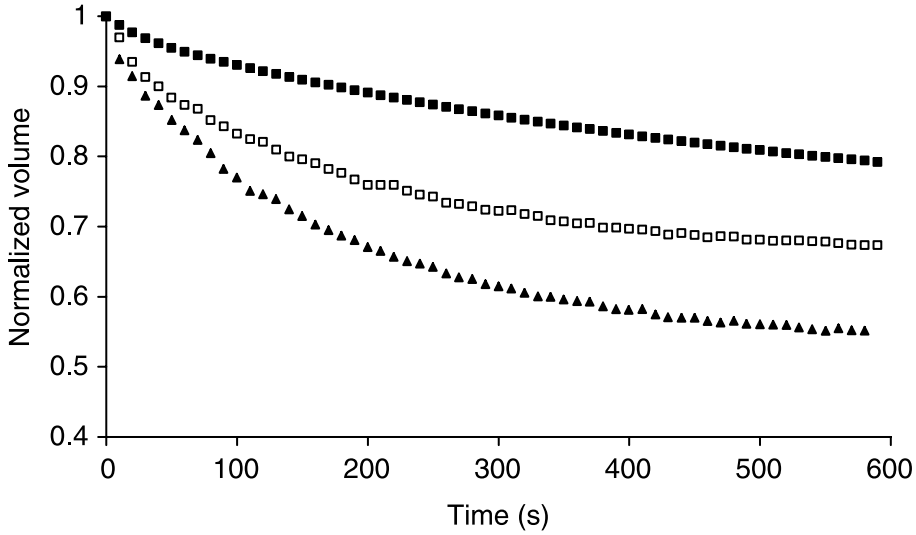


Figure 4. Mean volume of human oocytes during exposure to 1.5 mol/l propane-1,2-diol (PrOH) plus 0.1 mol/l (■, $n = 1$), 0.2 mol/l (□, $n = 12$) or 0.3 mol/l (▲, $n = 13$) sucrose, having previously been exposed to 1.5 mol/l PrOH for 10 min at room temperature. One set of data points is for a single oocyte, hence there can be no error bars. Therefore error bars were not included for other data sets in this figure.

Discussion

In the presence of permeating cryoprotectant oocytes initially shrink. This is because the membrane is more permeable to water than to cryoprotectant, and the first thing that happens is that water leaves the cell. Cell volume then increases as cryoprotectant permeates the cell. The extent of this shrink/swell response varies for each cell type depending on its permeability characteristics, size and also depending upon the cryoprotectant used. The shrink/swell response of murine oocytes was similar in the presence of the cryoprotectants Me_2SO and PrOH. Ethylene glycol permeated the cells slightly less readily, but glycerol resulted in extreme shrinkage and little permeation of the cryoprotectant. Therefore glycerol is not deemed a suitable cryoprotectant for preservation of mature oocytes. The large variability in cell volume after 15–20 min exposure to 1.5 mol/l glycerol probably indicates damage to the membrane of the cell. Thus, not all cryoprotectants are suitable for all cell types.

The extent of the shrink/swell response varies with temperature. The higher the temperature the less the shrink response of the oocytes and the less the osmotic stress. However, the higher the temperature, the greater are the potential effects of cryoprotectant toxicity. Thus, these two factors need to be balanced in designing protocols for exposure to cryoprotectant. In addition, the extent of the effect of temperature can differ for each cryoprotectant, as demonstrated by the differences in permeability of murine oocytes to ethylene glycol and Me_2SO at a range of temperatures.

Murine oocytes are often used as a model for human oocytes, since mouse oocytes are readily available for research, unlike fresh unfertilized human oocytes. The response of these two cell types in the presence of 1.5 mol/l PrOH at room temperature was found to be remarkably similar, particularly given the huge size discrepancy that exists between the two cell types. However, differences in the response to

cryoprotectant exposure are evident at different temperatures, hence results should be verified using human oocytes.

Results following cryopreservation of human oocytes are highly variable. The most commonly used method of cryopreservation for these cells currently is slow cooling in the presence of 1.5 mol/l PrOH plus sucrose. Oocytes are generally exposed to 1.5 mol/l PrOH for around 10 min at room temperature and then to 1.5 mol/l PrOH plus sucrose prior to cooling. On exposure to 1.5 mol/l PrOH at room temperature, human oocytes shrank by slightly greater than 30% of their initial volume before re-expanding. Shrinkage of 30–40% of original volume has been shown to be damaging to sea urchin eggs (Adams *et al*., 2003) and umbilical cord blood cells (Hunt *et al*., 2003). In the current study, with the possible exception of oocytes exposed to glycerol, oocytes appeared intact at the end of the perfusion period. However, oocytes were not returned to isotonic conditions neither was their viability formally assessed. It has been shown that human oocytes retain integrity of the cell membrane following shrinkage to ~40% of their initial volume in the presence of saccharides (McWilliams *et al*., 1995). However, deformation of the microtubular spindle has been reported following exposure of human oocytes to hypo- and hyperosmotic conditions (Mullen *et al*., 2004) and developmental capacity of mature bovine oocytes has been reduced by relatively brief exposure (10 min) to hyperosmotic conditions (Agca *et al*., 2000). Even if 30–40% shrinkage is not damaging to human oocytes *per se*, if the amount of stress created on addition of cryoprotectant can be minimized, this may reduce the cell's susceptibility to damage during the traumatic events of freezing and thawing. In an attempt to reduce the extent of cell volume change on equilibration with 1.5 mol/l PrOH, oocytes were exposed to 1.5 mol/l PrOH in a stepwise fashion, being first equilibrated in 0.75 mol/l PrOH and then moved into 1.5 mol/l PrOH. Stepwise as opposed to one-step addition of PrOH resulted in less extreme cell shrinkage, but a slightly longer exposure time was required in order to achieve full equilibration with 1.5 mol/l PrOH. Similar effects have been shown in the presence of the cryoprotectant Me_2SO (Newton *et al*., 1999).

Having equilibrated the oocyte with 1.5 mol/l PrOH, the next step is to expose the oocyte to 1.5 mol/l PrOH plus sucrose with the aim of using the presence of sucrose to withdraw water from the cell prior to freezing. The presence of sucrose was shown to result in continuous shrinkage of the oocytes indicating that sucrose did not permeate the oocyte membrane. Live births have been reported using a combination of PrOH and 0.1 mol/l sucrose (Polak de Fried *et al*., 1998; Borini *et al*., 2004a). The osmotic response of an oocyte PrOH plus 0.1 mol/l sucrose, having been equilibrated with 1.5 mol/l PrOH for 10 min resulted in shrinkage by around 15% of the cell volume at the end of equilibration in PrOH alone (Paynter *et al*., 2001).

Recently, studies have shown that survival post-cryopreservation can be improved if the concentration of sucrose is increased to 0.2 (Fabbri *et al*., 2001; Chen *et al*., 2004) or 0.3 mol/l sucrose (Fabbri *et al*., 2001). It is thought that the beneficial effect is due to a greater degree of dehydration being achieved in the higher concentrations of sucrose and hence less intracellular freezing damage. It has been demonstrated here that the extent of shrinkage in these higher concentrations of sucrose is indeed increased.

Studies of human oocyte cryopreservation show variability in success both within and between studies. What also varies within and between these studies is the time of exposure to the sucrose-containing solutions. In many of these studies, the timing of exposure to the sucrose-containing solutions is not stated (Gook *et al*., 1993; Young *et al*., 1998), is said to be brief (Fosas *et al*., 2003) or is said to vary considerably (Tucker *et al*., 1996, 1998a; Fabbri *et al*., 2001). The time of exposure to sucrose-containing solutions has been stated to vary from 30 s to 15 min, depending upon the number of oocytes to be cryopreserved (Fabbri *et al*., 2001). The main purpose of exposing oocytes to these solutions is to achieve a degree of dehydration prior to cooling, but the degree of dehydration varies considerably during the first 5 min of exposure, particularly in the presence of the higher concentrations of sucrose. After 5 min the response levels off, hence there seems little benefit, in terms of the degree of dehydration achieved, in exposure times of greater than 5 min. However, exposure to PrOH plus 0.2 mol/l sucrose for 10–15 min has been reported to yield better survival post-cryopreservation than shorter exposure times (Fabbri, 2001). There are suggestions of additional benefits of the presence of sucrose during cryopreservation as it is reported to stabilize membrane lipids (Crowe *et al*., 1998).

Therefore, in the presence of 0.2 mol/l sucrose it would seem that exposure times of at least 5 min are to be recommended. An optimum exposure time for oocytes to 0.3 mol/l sucrose is unknown. The tolerated limits of cell shrinkage are unknown for human oocytes as are any potentially toxic effects of the higher sucrose concentration. Adverse effects of 0.5 mol/l sucrose have been shown in cat oocytes, where exposure for 5 min significantly reduced development compared with exposure for 1 min (Murakami *et al*., 2004).

The diversity of protocols used for cryoprotectant exposure may to some degree explain the variability seen in results. The quality of the oocytes prior to cryopreservation is the main factor determining outcome, but if protocols for cryoprotectant exposure are defined and strictly adhered to, this may reduce variability of results. This is particularly the case where high concentrations of cryoprotectant are used, i.e. for vitrification protocols.

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