

Article

Theoretical considerations for oocyte cryopreservation by freezing



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Abstract

Attempts to cryopreserve oocytes by freezing have, to date, been based mostly on empirical approaches rather than on basic principles, and perhaps in part for this reason have not been very successful. Theoretical considerations suggest some fairly 'heretical' conclusions. The concentrations of permeating cryoprotectants employed in past studies have probably been inadequate, and the choice of propylene glycol (PG) as a protective agent is questionable. The use of non-penetrating agents, such as sucrose to preshrink oocytes prior to freezing and which, therefore, exacerbate osmotic stress during freezing, may be inappropriate, yet may protect in part by reducing the concentration of PG during freezing. The methods used to add and remove cryoprotectant may be suboptimal, and may be based on an inadequate understanding of the cryobiological constraints for oocyte survival. Given these concerns, it is not surprising that fully satisfactory results have been elusive, but there is every reason to believe that greater success is possible using a more theoretically appropriate approach.

Keywords: cryoinjury, cryoprotectant toxicity, cryotoxicity, minimum cell volume hypothesis, ova, solution effects injury

Introduction

Life is complex, and the responses of living systems to environmental perturbations can be counter-intuitive and even contrary to theoretical expectation. Perhaps in part for this reason, most studies of oocyte cryopreservation have been empirical in nature. However, the introduction of variables, such as the use of sucrose (Fabbri *et al.*, 2001) or choline chloride (Stachecki, 2006), prior to freezing has been pursued for the most part in lieu of an attempt at rational analysis and understanding of the biophysics of oocyte freezing injury. This approach, unfortunately, has not been very successful, and it is suggested here that closer attention to basic theoretical concepts may be helpful for accelerating progress. Here, a few basic and proposed principles of cryobiology will be briefly considered that may allow errors to be avoided and may permit a better understanding of the true needs of the oocyte, which in turn should allow those needs to be better met.

Vitrification is a promising approach to cryopreservation in general (Fahy *et al.*, 1984; Vajta, 2006; Desai, 2007; Fahy,

2007) and oocyte cryopreservation in particular (Fahy *et al.*, 2004a; Kuwayama *et al.*, 2005), but is the subject of a separate contribution to this compendium. Therefore, only the problem of freezing oocytes is considered here.

Freezing and thawing as a form of osmotic stress

Freezing and thawing cell suspensions is, to a first approximation and under most circumstances relevant to the present discussion, believed to result from the indirect effects of ice formation and not from direct effects such as cell crushing by spearing or adhesion to ice (Lovelock, 1953; Mazur, 1984, 1988). The formation of ice in an aqueous solution is well known to involve the formation of crystals that, for all practical purposes, usually consist only of water molecules and exclude all dissolved solutes. Freezing and thawing injury has, therefore, been successfully simulated (Lovelock, 1953; Takahashi and Williams, 1983;

Pegg and Diaper, 1988) by changing the concentration of solutes surrounding cells in suspension so as to simulate the changes in concentration that take place upon freezing, in which liquid water is subtracted from the suspending medium, and then upon thawing, in which water is added back to yield the original pre-freezing concentrations. Furthermore, the effects of freezing and thawing in a wide variety of cryoprotectants have been successfully related to the highest concentrations of non-cryoprotective solute reached during freezing (Lovelock, 1953, 1954; Lovelock and Polge, 1954; Meryman *et al.*, 1977).

The mechanisms that link hypertonic exposure to injury have been investigated primarily in human erythrocytes. The collective works of many authors (Soderstrom, 1944; Lovelock, 1953; Meryman, 1968, 1970; Zade-Oppen, 1968; Farrant and Woolgar, 1970, 1972a,b; Mazur *et al.*, 1970; Takahashi and Williams, 1983; Pegg and Diaper, 1988) have, in the case of red blood cells, supported a link between observed injury after thawing and a failure of membrane semipermeability during freezing that allows solute uptake during freezing and entrapment of the solute upon thawing (dilution) as membrane resealing takes place. This may lead to cell swelling and haemolysis as thawing or dilution is completed (post-hypertonic haemolysis) (Farrant and Woolgar, 1970, 1972b; Meryman, 1970).

Although the biological generality of observations made on erythrocytes can be questioned, studies from Diller's laboratory (Knox *et al.*, 1980) showed a net increase in cell volume in comparison with expectations both during and after thawing in nucleated cells, implying solute inleak during freezing. Lovelock was also able to show that freezing injury in spermatozoa from four different species follows the same predictive rules as freezing injury in red blood cells (Lovelock and Polge, 1954), and spinach chloroplast grana also behave similarly to red cells (Williams and Meryman, 1970). For oocytes, more subtle effects of cell volume reduction, such as significantly increased membrane permeability to calcium (Gardner *et al.*, 2007), might occur well before general loss of membrane semipermeability to ions takes place.

The primary factor that links hypertonic exposure to membrane injury may be cell volume reduction below a tolerated critical minimum value (osmotic stress) (Meryman, 1971, 1974). For example, Meryman and colleagues calculated the osmolality of salts during the freezing and thawing of red cells under a variety of conditions and were able to show consistent relationships between salt osmolality and injury during freezing in the presence of a wide variety of cryoprotectants (Meryman *et al.*, 1977). Meryman proposed that injury is linked to an inability of the cell to shrink below a critical minimum volume, and that this causes a hydrostatic pressure gradient across the cell membrane that causes the increased membrane permeability to solute (Meryman, 1968, 1970, 1971, 1974), but this is not a necessary postulate for linking osmotic stress to freezing injury (Williams and Meryman, 1970; Meryman, 1974). Excessive cell shrinkage can cause extrusion of membrane lipids (Lovelock, 1955; Dowgert and Steponkus, 1984) and proteins (Takahashi *et al.*, 1985) by mechanisms that remain to be fully elucidated. Regardless of the mechanisms, the practical empirical observation is that these events are related to reduced cell volume.

Meryman's minimum cell volume hypothesis, whether including or excluding the concept of a hydrostatic pressure gradient across a rather fragile cell membrane, seems to be the only plausible

way to explain the shape of the curve of cell survival after freezing and thawing versus cooling rate. It is well known that this curve, sometimes called the Oak Ridge curve by those who remember its initial explanation by Peter Mazur in Oak Ridge, Tennessee in 1963 (Mazur, 1963), has the general shape of an inverted U, survival rising with increasing cooling rate until an optimum is reached and then declining with further increases in cooling rate. The descending portion of this curve is linked to the formation of intracellular ice, and the cooling rate at which this descent takes place has been very convincingly linked to the permeability of the cell to water (Mazur, 1984, 1988). Cells that are very permeable to water can lose water osmotically at a high rate during freezing, and therefore it is more difficult to cool them fast enough to leave enough residual supercooled water inside the cells to nucleate into intracellular ice crystals.

Based on this understanding, it is not surprising that the cooling rate needed to reduce cell survival due to intracellular freezing can vary widely from cell type to cell type. But what is all but impossible to explain without Meryman's hypothesis is the fact that the cooling rate required to suppress slow-freezing injury varies in proportion to the cooling rate required to produce intracellular ice. For example, Chinese hamster cells must be cooled ~100 times faster than mouse marrow stem cells to freeze intracellularly when both are protected with 1.25 mol/l glycerol, but they must also be cooled ~100 times faster to attain the same degree of escape from slow-freezing injury (Mazur *et al.*, 1970).

Why should the hamster cell membrane be injured by slow freezing 100 times more rapidly than the mouse marrow stem cell membrane when intracellular ice does not form? One explanation is that the cooling rate dependence of slow-freezing injury, like the cooling rate dependence of intracellular ice formation, is directly related to the transport of cell water across the cell membrane. At cooling rates low enough to avoid intracellular freezing, progressively lowering the cooling rate will allow more and more cell volume reduction. If cell volume reduction is injurious, this would explain why the ascending and descending limbs of the Oak Ridge curve must always be coupled to one another rather than being able to vary independently of one another as one should expect if slow-freezing injury were caused by, for example, a denaturing effect of intra- or extracellular electrolytes, whose time dependence should be independent of membrane water permeability.

An alternative explanation for the coupling of faster slow-freezing injury to faster water transport might be that substantial solute loading on cooling is caused by solute transport through aquaporins. In this scenario, cells with high water permeability would have to be cooled rapidly to avoid slow-freezing injury through solute loading through aquaporins at lower cooling rates. Aquaporins are impermeable to salts but they are permeable to cryoprotectants (Edashige *et al.*, 2003; Yamaji *et al.*, 2006). However, because cryoprotectants can move in either direction through aquaporins, their transport into cells during freezing would not be expected to be damaging in the way that salt influx, which is essentially irreversible, is apparently damaging. Some net loading of cells with cryoprotectants might be possible under conditions of slow cooling and rapid thawing, but slow cooling tends to eliminate the transmembrane gradient for cryoprotectant uptake, and net flux rates are quantitatively unlikely to be significant under most circumstances (Fahy, 1981). Therefore, the general coupling of slow-freezing injury

and fast-freezing injury as in the Oak Ridge curve seems unlikely to be explicable on the basis of water and solute cotransport through aquaporins, leaving cell shrinkage as the only apparent explanation.

These observations all suggest that the fate of slowly frozen oocytes depends first on the amount of ice that forms in the solution, which determines the total amount of potential osmotic stress, and second on the cooling rate, which determines how much of that potential osmotic stress is realized. Mathematical models describing how to calculate appropriate cooling rates for avoiding intracellular ice formation have been described elsewhere (Mazur, 1963, 1984, 1988; Mazur *et al.*, 1970; Fahy, 1981) and are well known. Here, this paper will consider only how to estimate the amount of ice that forms during equilibrium freezing, and therefore how to estimate the amount of cryoprotectant needed for adequate cryoprotection.

Cryoprotecting the oocyte

There are several ways of estimating or calculating the amount of ice formed in an aqueous cryoprotectant solution at different temperatures. One of the simplest methods is to make use of estimates based on the behaviour of ideal solutions (solutions that obey Raoult's law). Lovelock set forth such a method in a classic paper (Lovelock, 1954) that related freezing point depression to the mole fraction of dissolved solute:

$$\Delta T = kX_s \quad (1)$$

where ΔT is the difference between the freezing point of pure water (0°C) and the temperature to which the solution has been frozen under equilibrium conditions and X_s is the mole fraction of solute (in his case consisting of Na⁺, Cl⁻, and cryoprotectant). A similar and better known relationship, derived in the same way, relates the freezing temperature to the osmolality of the solution:

$$\Delta T = \theta\pi, \quad (2)$$

where $\theta = 1.86^\circ\text{C}/\text{osmolal}$ and π is the osmolality of the solution (in osmoles per kilogram of water [Osm]). For an ideal solution, the osmolality is equivalent to the molality of the solution when molality is calculated taking the dissociation of species such as NaCl into osmotically active components into account.

From the form of equations (1) and (2) it can be seen that the relationship between freezing temperature and mole fraction is essentially equivalent to the relationship between freezing temperature and osmolality. Therefore, correlations between freezing injury and mole fraction support a relationship between freezing injury and osmotic stress.

Lovelock used equation (1) to calculate the amount of cryoprotectant needed to protect red cells at a given freezing temperature. Let $X_s = 2X_n + X_c$, where $2X_n$ is the corrected mole fraction of non-penetrating solute (NaCl or culture medium consisting mostly of NaCl), and X_c is the mole fraction of permeating cryoprotectant. Let CF_D be the concentration factor, or the fold change in X_s caused by freezing, that is just sufficient to begin to cause cell damage. Then the temperature

descent below 0°C at which damage will just begin to occur, ΔT_D , is given by:

$$\Delta T_D = kCF_D(2X_n^i + X_c^i) \quad (3)$$

where the superscripted *i* refers to the initial (pre-freezing) concentrations of salt and cryoprotectant, and where $2X_n^i$ represents an isotonic solution. Based on this equation, X_c^i , the concentration of cryoprotectant that must be present before freezing to allow undiminished survival just to T_D , can be calculated from k and CF_D if CF_D is known. Lovelock found that for human red cells, $CF_D = 5$, so, given $X_n^i = 0.00286$, the expected concentration needed to protect is:

$$X_c^i = (\Delta T_D - 2kCF_D X_n^i)/kCF_D = (\Delta T_D - 3)/525 \quad (4).$$

Equations (3) and (4) basically define the concept of colligative cryoprotection.

Assuming that slow cooling is needed at least to -40°C to prevent intracellular ice formation upon subsequent plunging into liquid nitrogen (Fahy *et al.*, 1984), $X_c^i = 37/525 = 0.0705$. Given a molecular weight of 92.1 for glycerol, a glycerol density of 1.26 g/ml and an initial salt concentration of 0.16 mol/l, this value of X_c^i equates to 3.24 mol/l glycerol, or 30% w/v. In practice, Lovelock found that he needed 24% w/v glycerol, or 2.6 mol/l, to protect his cells to -40°C , which is reasonably close to his approximate theoretical prediction and consistent with the errors expected from dilute solution assumptions.

For comparison, it is typical for investigators to freeze oocytes in only 1.5 mol/l permeating cryoprotectant, but the human oocyte may tolerate much less ice formation than the amount tolerated by red cells, CF_D being perhaps as low as 2.0 (Mullen *et al.*, 2004). Lovelock's work on sperm freezing indicated that spermatozoa, for which $CF_D = 2.0$ to 2.7, require much more than 2.6 mol/l glycerol for full protection during equilibrium freezing to -40°C (Lovelock and Polge, 1954). Furthermore, propylene glycol (PG) has become popular for the freezing of oocytes, but Lovelock found he needed more PG (3.9M), than glycerol to fully protect red cells to -40°C (Lovelock, 1954).

Sucrose, which does not penetrate cells and therefore cannot nominally protect them against osmotic stress, provided little protection to red cells in Lovelock's experience (Lovelock, 1954). Preshrinkage of oocytes with sucrose would be expected actually to exacerbate shrinkage injury during freezing in the presence of a permeating cryoprotectant just as has been found or simulated for red cells and platelets (Meryman, 1970, 1971), yet it has been advocated for the protection of human oocytes by many investigators (see discussion below).

These observations suggest that currently reported attempts to freeze oocytes may have employed approaches that are far from optimal in view of fundamental cryobiological principles.

Equations (1) to (4), while simple and convenient, are approximations based on dilute ideal solution assumptions. More accurate calculations can be made on the basis of explicit phase diagram information when this is available (Fahy, 1980, 1986; Pegg, 1983, 1986; Pegg and Arnaud, 1988) and on the basis of osmotic virial coefficients when it is not (Elliott *et al.*, 2007).

Osmotic stress above 0°C

To use cryoprotectants to prevent freezing injury while minimizing injury from the cryoprotectants themselves, it is necessary to avoid step changes in concentration that correspond to changes in osmolality that are greater than CF_D . The best way to do this is to base cryoprotectant addition and washout protocols on mathematical models of cryoprotectant and water permeation rates (Kleinhans, 1998; Newton *et al.*, 1999; Edashige *et al.*, 2003; Paynter, 2005), but this is often not done, nor is it yet very clear how much oocyte shrinkage is acceptable in shrink–swell curves typically associated with such models. Once the osmotic limits of the oocyte become better established, very high-efficiency loading and unloading protocols may become possible (Meryman, 2007).

Cryoprotectant-associated freezing injury

In 1977, it was pointed out that although glycerol protects human red cells against freezing injury by postponing ice formation to lower temperatures as described above, it also sensitizes the cells to injury once injury begins, the amount of osmotic stress required to kill half the cells, for example, being less in the presence of glycerol than in its absence (Fahy and Karow, 1977). This effect was later confirmed for red cells (Rall *et al.*, 1978; Pegg and Diaper, 1988) and is now known to pertain to many different biological systems and many different cryoprotectants (Fahy, 1986; Pegg and Arnaud, 1988). This type of cryoprotectant-associated freezing injury can be subtle, and may become apparent only upon careful analysis of actual versus expected rates of survival. In other cases, injury from cryoprotective additives such as ethanol or methanol can become more overt after these agents become concentrated by freezing to beyond a critical threshold concentration for cytotoxicity (Meryman *et al.*, 1977). This effect was noticed at least as long ago as 1954, when Lovelock first compared a variety of different cryoprotective agents for the protection of human red cells and found that no concentration of either ethanol or triethylene glycol could protect to -30°C (Lovelock, 1954). Toxic effects of solutes during freezing have been referred to as ‘cryotoxicity’ by some investigators (Hinch *et al.*, 1993).

Cryotoxicity might explain, at least in part, the relatively weak benefits of the inclusion of sucrose in combination with PG for the freezing of oocytes (Chen *et al.*, 2005; Coticchio *et al.*, 2006): extracellular sucrose and intracellular solutes concentrated by the shrinkage induced by sucrose prior to freezing will reduce the extracellular and intracellular concentration of PG at any given temperature during freezing in accordance with equations (1) and (3) with the insertion of a new term for the mole fraction of sucrose, thereby minimizing the toxic effects of PG. Similar effects have been demonstrated in other systems (Ulrich *et al.*, 1979; Withers, 1980), and the existence of cryotoxic effects of PG in particular has been convincingly demonstrated in platelets (Arnaud and Pegg, 1990) and successfully reduced by using glycerol to depress PG concentrations during freezing (Pegg and Arnaud, 1988). Empirical observations on whole kidneys (Pegg *et al.*, 1987) and theoretical observations on kidney slices (Fahy *et al.*, 2004a) also indicate that PG can be a particularly cytotoxic cryoprotectant when it is present in sufficiently

high concentrations despite its compatibility with spindle reassembly after thawing of frozen oocytes (Rienzi *et al.*, 2004; Chen *et al.*, 2005; Coticchio *et al.*, 2006). In fact, 2.5 mol/l PG has recently been shown to kill 80% of mouse oocytes even when osmotic injury from this agent was precluded, whereas 2.5 mol/l ethylene glycol was non-toxic (Mullen, 2007).

Despite the arguments above for cell shrinkage as a primary cause of slow-freezing injury, cryoprotective effects of non-penetrating cryoprotectants are well established and can be powerful, particularly when frozen cells are thawed rapidly (see, for example, Mazur, 1970; Mazur *et al.*, 1970). Shrinking oocytes prior to freezing will remove intracellular water that might otherwise be more liable to freeze, and this argument has been put forth as one reason for the efficacy of sucrose. However, a greater benefit might be obtained in principle by freezing more slowly in the absence of sucrose if lower freezing rates are feasible. Sucrose will also raise the glass transition temperature of the extracellular solution, and may therefore protect by arresting cell volume reduction at a higher temperature (Takahashi *et al.*, 1988). On the other hand, the absence of sucrose will also raise cell volume at any given temperature during freezing, and judicious choice of the temperature from which the cells are transferred into liquid nitrogen would also preclude fatal cell shrinkage. The presence of sucrose also reduces the total amount of ice formed and the concentration of salts during freezing (Farrant and Woolgar, 1970, 1972b) while also raising viscosity, all of which may minimize the influx of sodium chloride should membrane semipermeability be compromised (Farrant and Woolgar, 1970, 1972b). In addition, the added sucrose would provide osmotic support by its presence after thawing, which might reduce post-hypertonic cell swelling and allow more opportunity for extrusion of leaked electrolyte before or after dilution to isotonic conditions. Meryman has also presented evidence that sucrose can stimulate potassium efflux during cell swelling, which would reduce the chances of cell lysis due to post-hypertonic swelling (Meryman, 1970). However, minimizing or compensating for the influx of extracellular solute that arises as a result of a breach in membrane semipermeability may be more risky than preventing the breach in permeability from taking place in the first place (Meryman, 1970; Williams, 1976), particularly for a cell as delicate as the oocyte.

In some systems, cryoprotectant-associated freezing injury can be reduced by using permeating cryoprotectant mixtures that have less intrinsic toxicity (Fahy, 1983, 1984, 1986; Pegg and Arnaud, 1988; Fahy *et al.*, 1990). This supports the existence of directly cryotoxic effects of ordinary cryoprotectants during freezing and at the same time provides at least a partial remedy for this problem. Recent advances in the formulation of minimum-toxicity cryoprotectant formulas for vitrification (Fahy *et al.*, 2004a,b) may offer superior freezing solutions when the same mixtures are used in low enough concentrations to permit freezing.

Theoretical analysis as a learning tool

Oocytes may or may not behave in accordance with theoretical predictions, but only by analysing their behaviour from a theoretical point of view can departures from theory be

identified and new hypotheses of injury be developed. Isolating and reconciling the requirements of the plasma membrane, the spindle, and intracellular targets may be difficult, but careful observations of the dependence of injury on basic cryobiological variables should be instructive (Lovelock, 1953, 1954; Fahy and Karow, 1977; Meryman *et al.*, 1977; Rall *et al.*, 1978; Pegg and Diaper, 1982, 1988; Fahy, 1986; Mazur, 1988; Pegg and Arnaud, 1988) and should be able to provide new directions for research and, hopefully, improved outcomes.

Conclusions

The biology of the oocyte provides many challenges for cryopreservation by either freezing or vitrification. Although recent advances in oocyte vitrification have been encouraging (Fahy *et al.*, 2004a; Kuwayama *et al.*, 2005), cryopreservation by freezing remains a viable though not a well-realized option. Perhaps greater attention to the fundamental cryobiological needs of the oocyte can facilitate and add direction to ongoing efforts at oocyte cryopreservation by both freezing and vitrification.

Declaration

The author is an officer and shareholder in a company that sells cryopreservation products and services.

References

- Arnaud FG, Pegg DE 1990 Cryopreservation of human platelets with propane-1,2-diol. *Cryobiology* **27**, 130–136.
- Chen SU, Lien YR, Chen HF *et al.* 2005 Observational clinical follow-up of oocyte cryopreservation using a slow-freezing method with 1,2-propanediol plus sucrose followed by ICSI. *Human Reproduction* **20**, 1975–1980.
- Coticchio G, de Santis L, Rossi G *et al.* 2006 Sucrose concentration influences the rate of human oocytes with normal spindle and chromosome configurations after slow-cooling cryopreservation. *Human Reproduction* **21**, 1771–1776.
- Desai N 2007 Cryoloop vitrification of human day 3 cleavage-stage embryos: post-vitrification development, pregnancy outcomes and live births. *Reproductive BioMedicine Online* **14**, 208–213.
- Dowgert MF, Steponkus PL 1984 Behavior of the plasma membrane of isolated protoplasts during a freeze–thaw cycle. *Plant Physiology* **75**, 1139–1151.
- Edashige K, Yamaji Y, Kleinans FW *et al.* 2003 Artificial expression of aquaporin-3 improves the survival of mouse oocytes after cryopreservation. *Biology of Reproduction* **68**, 87–94.
- Elliott JAW, Prickett RC, Elmoazzen HY *et al.* 2007 A multisolute osmotic virial equation for solutions of interest in biology. *Journal of Physical Chemistry B* **111**, 1775–1785.
- Fabbri R, Porcu E, Marsella T 2001 Human oocyte cryopreservation: new perspectives regarding oocyte survival. *Human Reproduction* **16**, 411–416.
- Fahy GM 2007 Vitrification: An overview. In: Liebermann J, Tucker MJ (eds) *Vitrification in Assisted Reproduction: A User's Manual and Troubleshooting Guide*. Informa Healthcare, London (in press).
- Fahy GM 1986 The relevance of cryoprotectant 'toxicity' to cryobiology. *Cryobiology* **23**, 1–13.
- Fahy GM 1984 Cryoprotectant toxicity reduction: specific or nonspecific? *Cryo-Letters* **5**, 287–294.
- Fahy GM 1983 Cryoprotectant toxicity neutralizers reduce freezing damage. *Cryo-Letters* **4**, 309–314.
- Fahy GM 1981 Simplified calculation of cell water content during freezing and thawing in nonideal solutions of cryoprotective agents and its possible application to the study of 'solution effects' injury. *Cryobiology* **18**, 473–482.
- Fahy GM 1980 Analysis of 'solution effects' injury: equations for calculating phase diagram information for the ternary systems NaCl–dimethylsulfoxide–water and NaCl–glycerol–water. *Biophysical Journal* **32**, 837–850.
- Fahy GM, Karow AM, Jr 1977 Ultrastructure-function correlative studies for cardiac cryopreservation. V. Absence of a correlation between electrolyte toxicity and cryoinjury in the slowly frozen, cryoprotected rat heart. *Cryobiology* **14**, 418–427.
- Fahy GM, Wowk B, Wu J *et al.* 2004a Improved vitrification solutions based on predictability of vitrification solution toxicity. *Cryobiology* **48**, 22–35.
- Fahy GM, Wowk B, Wu J *et al.* 2004b Cryopreservation of organs by vitrification: perspectives and recent advances. *Cryobiology* **48**, 157–178.
- Fahy GM, Lilley TH, Linsdell H *et al.* 1990 Cryoprotectant toxicity and cryoprotectant toxicity reduction: in search of molecular mechanisms. *Cryobiology* **27**, 247–268.
- Fahy GM, MacFarlane DR, Angell CA *et al.* 1984 Vitrification as an approach to cryopreservation. *Cryobiology* **21**, 407–426.
- Farrant J, Woolgar AE 1972a Human red cells under hypertonic conditions; a model system for investigating freezing damage. I. Sodium chloride. *Cryobiology* **9**, 9–15.
- Farrant J, Woolgar AE 1972b Human red cells under hypertonic conditions; a model system for investigating freezing damage. II. Sucrose. *Cryobiology* **9**, 16–21.
- Farrant J, Woolgar AE 1970 Possible relationships between the physical properties of solutions and cell damage during freezing. In: Wolstenholme GEW, O'Connor M (eds) *The Frozen Cell*. J and A Churchill, London, pp. 97–114.
- Gardner DK, Sheehan CB, Rienzi L *et al.* 2007 Analysis of oocyte physiology to improve cryopreservation procedures. *Theriogenology* **67**, 64–72.
- Hincha DK, de Vries AL, Schmitt JM 1993 Cryotoxicity of antifreeze proteins and glycoproteins to spinach thylakoid membranes – comparison with cryotoxic sugar acids. *Biochimica et Biophysica Acta* **1146**, 258–264.
- Kleinans FW 1998 Membrane permeability modeling: Kedem–Katchalsky vs a two-parameter formalism. *Cryobiology* **37**, 271–289.
- Knox JM, Schwartz GS, Diller KR 1980 Volumetric changes in cells during freezing and thawing. *Journal of Biomechanical Engineering* **102**, 91–97.
- Kuwayama M, Vajta G, Kato O *et al.* 2005 Highly efficient vitrification method for cryopreservation of human oocytes. *Reproductive BioMedicine Online* **11**, 300–308.
- Lovelock JE 1955 Haemolysis by thermal shock. *British Journal of Haematology* **1**, 117–129.
- Lovelock JE 1954 The protective action of neutral solutes against haemolysis by freezing and thawing. *Biochemical Journal* **56**, 265–270.
- Lovelock JE 1953 The mechanism of the protective action of glycerol against haemolysis by freezing and thawing. *Biochimica et Biophysica Acta* **11**, 28–36.
- Lovelock JE, Polge C 1954 The immobilization of spermatozoa by freezing and thawing and the protective action of glycerol. *Biochemical Journal* **58**, 618–622.
- Mazur P 1988 Stopping biological time. The freezing of living cells. *Annals of the New York Academy of Science* **541**, 514–531.
- Mazur P 1984 Freezing of living cells: mechanisms and implications. *American Journal of Physiology* **247** (Cell Physiology 16), C125–C142.
- Mazur P 1970 Cryobiology: the freezing of biological systems. *Science* **168**, 939–949.
- Mazur P 1963 Kinetics of water loss from cells at subzero temperatures and the likelihood of intracellular freezing. *Journal of General Physiology* **47**, 347–369.
- Mazur P, Leibo SP, Farrant J *et al.* 1970 Interactions of cooling rate, warming rate and protective additive on the survival of frozen mammalian cells. In: Wolstenholme GEW, O'Connor M (eds) *The*

- Frozen Cell*. J and A Churchill, London, pp. 69–85.
- Meryman HT 2007 Cryopreservation of living cells: principles and practice. *Transfusion* (in press).
- Meryman HT 1974 Freezing injury and its prevention in living cells. *Annual Review of Biophysics and Bioengineering* **3**, 341–363.
- Meryman HT 1971 Osmotic stress as a mechanism of freezing injury. *Cryobiology* **8**, 489–500.
- Meryman HT 1970 The exceeding of a minimum tolerable cell volume in hypertonic suspension as a cause of freezing injury. In: Wolstenholme GEW, O'Connor M (eds) *The Frozen Cell*. J and A Churchill, London, pp. 51–64.
- Meryman HT 1968 Modified model for the mechanism of freezing injury in erythrocytes. *Nature* **218**, 333–336.
- Meryman HT, Williams RJ, Douglas MSJ 1977 Freezing injury from 'solution effects' and its prevention by natural or artificial cryoprotection. *Cryobiology* **14**, 287–302.
- Mullen SF 2007 The science of cryobiology. In: Woodruff T, Snyder K (eds) *Oncofertility*. Springer, New York (in press).
- Mullen SF, Aqca Y, Broermann DC *et al.* 2004 The effect of osmotic stress on the metaphase II spindle of human oocytes, and the relevance to cryopreservation. *Human Reproduction* **19**, 1148–1154.
- Newton H, Pegg DE, Barrass R *et al.* 1999 Osmotically inactive volume, hydraulic conductivity, and permeability to dimethyl sulphoxide of human mature oocytes. *Journal of Reproduction and Fertility* **117**, 27–33.
- Paynter SJ 2005 A rational approach to oocyte cryopreservation. *Reproductive BioMedicine Online* **10**, 578–586.
- Pegg DE 1986 Equations for obtaining melting points and eutectic temperatures for the ternary system dimethyl sulphoxide/sodium chloride/water. *Cryo-Letters* **7**, 387–394.
- Pegg DE 1983 Simple equations for obtaining melting points and eutectic temperatures for the ternary system glycerol/sodium chloride/water. *Cryo-Letters* **4**, 259–268.
- Pegg DE, Arnaud FG 1988 Equations for obtaining melting points in the quaternary system propane-1,2-diol/glycerol/sodium chloride/water. *Cryo-Letters* **9**, 404–417.
- Pegg DE, Diaper MP 1988 On the mechanism of injury to slowly frozen erythrocytes. *Biophysical Journal* **54**, 471–488.
- Pegg DE, Diaper MP 1982 The mechanism of cryoinjury in glycerol-treated rabbit kidneys. In: Pegg DE, Jacobsen IA, Halasz NA (eds) *Organ Preservation, Basic and Applied Aspects*. MTP Press, Ltd, Lancaster, pp. 389–393.
- Pegg DE, Jacobsen IA, Diaper MP *et al.* 1987 Perfusion of rabbit kidneys with solutions containing propane-1,2-diol. *Cryobiology* **24**, 420–428.
- Rall WF, Mazur P, Souzu H 1978 Physical–chemical basis of the protection of slowly frozen human erythrocytes by glycerol. *Biophysical Journal* **23**, 101–120.
- Rienzi L, Martinez F, Ubaldi F *et al.* 2004 PolScope analysis of meiotic spindle changes in living metaphase II human oocytes during the freezing and thawing procedures. *Human Reproduction* **19**, 655–659.
- Soderstrom N 1944 Hemolysis by hypertonic solutions of neutral salts. *Acta Physiologica Scandinavica* **7**, 56–68.
- Stachecki J 2006 Cryopreservation of unfertilized human oocytes. *Reproductive BioMedicine Online* **13**, 222–227.
- Takahashi T, Hirsh A, Erbe E *et al.* 1988 Mechanism of cryoprotection by extracellular polymeric solutes. *Biophysical Journal* **54**, 509–518.
- Takahashi T, Inada S, Pommier CG *et al.* 1985 Osmotic stress and the freeze–thaw cycle cause shedding of Fc and C3b receptors by human polymorphonuclear leukocytes. *Journal of Immunology* **134**, 4062–4068.
- Takahashi T, Williams RJ 1983 Thermal shock hemolysis in human red cells. I. The effects of temperature, time, and osmotic stress. *Cryobiology* **20**, 507–520.
- Ulrich JM, Finkle BJ, Moore PH *et al.* 1979 Effect of a mixture of cryoprotectants in attaining liquid nitrogen survival of callus cultures of a tropical plant. *Cryobiology* **16**, 550–556.
- Vajta G 2006 Are programmable freezers still needed in the embryo laboratory? Review on vitrification. *Reproductive BioMedicine Online* **12**, 779–796.
- Williams RJ 1976 A proposed mechanism for PVP cryoprotection. *Cryobiology* **13**, 653.
- Williams RJ, Meryman HT 1970 Freezing injury and resistance in spinach chloroplast grana. *Plant Physiology* **45**, 752–755.
- Withers LA 1980 The cryopreservation of higher plant tissue and cell cultures – an overview with some current observations and future thoughts. *Cryo-Letters* **1**, 239–250.
- Yamaji Y, Valdez DM, Jr, Seki S *et al.* 2006 Cryoprotectant permeability of aquaporin-3 expressed in *Xenopus* oocytes. *Cryobiology* **53**, 258–267.
- Zade-Oppen AM 1968 Posthypertonic hemolysis in sodium chloride systems. *Acta Physiologica Scandinavica* **73**, 341–364.

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