Chapter 7

The Science of Cryobiology

T.K. Woodruff and K.A. Snyder (eds.) *Oncofertility*. © Springer 2007

The original publication of this article is available at www.springerlink.com

Steven F. Mullen, PhD and John K. Critser, PhD

Introduction

The demand for effective bio-preservation methods in the medical community continues to increase with advances in transplantation and transfusion medicine [1]. In reproductive medicine, pre-implantation embryo cryopreservation has become an integral component of overall patient care, increasing the success rate per oocyte retrieval cycle [2,3]. Oocyte cryopreservation is becoming increasingly important due to legal restrictions on the creation and transplantation of supernumerary preimplantation embryos as well as ethical considerations surrounding the cryopreservation of pre-implantation embryos [4,5].

Early investigations into the effects of sub-physiologic temperatures on living cells have been reviewed in great detail [6]. The current chapter will attempt to provide a broad overview of cryobiology, and refer to the reproductive biology literature when appropriate. Readers interested in learning more details are directed at several excellent texts and reviews on the various subjects [7–20].

Anatomy of Cryopreservation

Cryopreservation is the successful preservation of the normal function of cells or tissues by a reduction in temperature below which biochemical reactions take place. It is not the long-term storage of cells at these temperatures that is damaging, but the progression to these temperatures and back to normothermia that results in cryoinjury. Cryopreservation nearly always entails the use of one or more compounds that confer protection to cells during freezing. These so-called cryoprotectants are typically very simple, low molecular weight molecules with high water solubility and low toxicity. One feature that is common among these compounds is their ability to interact with water via hydrogen bonding [21]. Application of cryoprotectants is done (in most cases) simply by incubating the cells in solutions into which these compounds have been dissolved. After this exposure, the cells are cooled to a low sub-zero temperature (specimens are typically held at the temperature of liquid nitrogen; −196°C). At the appropriate time, the specimen is warmed, washed free of the cryoprotectants, and used in whatever manner is deemed appropriate. While this seems like a relatively straightforward procedure, many types of injuries can result from any one of the steps; thus numerous lethal effects need to be avoided.

The Effects of Water Precipitation (as Ice) During Cooling

Ice Nucleation, Crystallization, Vitrification, and Devitrification

Figure 7.1 shows a supplemented phase diagram for a generic aqueous solution. The physical transitions of water in solution which occur as a result of cooling and warming are described with such a diagram. The temperature at which these transitions occur depends upon the concentration of solutes in the solution. The curve

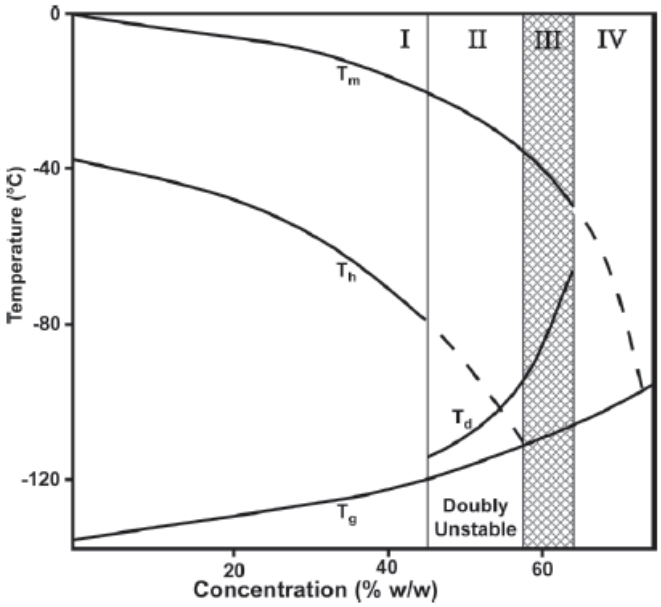


Fig. 7.1 A phase diagram for a hypothetical solution is shown. The concentration and temperature dependent physical transitions, including melting (T_m), homogenous nucleation (T_h), glass formation (T_g), and devitrification (T_d), are described by the respective curves. The use of such diagrams allows the calculation of variables which are important considerations for freezing injury [22]. Reprinted from Fahy et al. (1984) [7] with permission from Elsevier. See text for further details

labeled *T*m describes the melting temperature of the solution (which is also the equilibrium freezing temperature). The dashed extensions of the curves represent extrapolations across hypothetical boundaries. As the solute concentration of the solution increases (moving along the *X*-axis from left to right), the melting temperature of the solution decreases. This is a well known result of the colligative effects of the solutes.

The curve labeled *T*h characterizes the homogenous nucleation temperature. Homogenous nucleation is defined as the nucleation of ice crystals in the absence of nucleating agents. Aqueous solutions will usually crystallize at temperatures much higher than this due to impurities which act as effective ice nucleators. A solution containing pure water (i.e., free of heterogeneous nucleators) will remain liquid down to ~−39°C, at which point the entire solution will crystallize (the point where *T*h intersects the *Y*-axis). The homogenous nucleation temperature decreases with increasing concentration of solutes.

The reason why water can remain liquid below its melting point is because the creation of a crystal entails the creation of a liquid–crystal interface with an associated interfacial free energy.

The size of a thermodynamically-stable crystal (i.e., one that will continue to grow by the addition of water molecules) is dependent upon temperature (smaller crystals are more stable at lower temperatures). So as the temperature is lowered, the probability of formation of a stable crystal increases until *T*h, where the probability is 1.

The curve labeled *T*g represents the glass transition temperature. At this temperature, liquid solutions will transition to a stable glass (vitrify) and remain vitreous upon further cooling.

In region I of this chart (solutions with concentrations <= ~45 wt% (weight/ weight) in this example), achieving true vitrification is nearly impossible. Nucleation (both homogenous and heterogeneous) is essentially unavoidable (at least with practical cooling rates). In region II, the difference between the *T*h and *T*g curves is small enough that vitrification can be achieved with practical cooling rates. However, a solution which does attain a vitreous state in this region is thermodynamically unstable. In the regions marked III and IV, vitrification is easily achievable. Notice that the *T*h curve actually intersects *T*g at the transition between regions II and III. Heterogeneous ice formation will not occur at temperatures below the glass-transition temperature.

If a sample with a composition described by regions II and III is cooled fast enough to vitrify, ice may still form during warming due to nucleation. The temperature at which this happens is described by *T*d, the devitrification temperature. Essentially, between *T*g and *T*m, a solution free of ice is in a metastable state. Above *T*g during warming, the solution is no longer a glass (the glass "melts" to form an unfrozen liquid with the molecules having translational mobility), and nucleation and ice growth can occur. Ice formation and crystal growth during warming is more likely than during cooling, all else being equal.

Why is this so? Consider the following two facts: (1) The probability of ice nucleation increases as temperature decreases (notice that as temperature decreases, the sample will get closer to *T*h; (2) crystal growth, however, being a kinetic phenomenon, will be faster at higher temperatures. Now, imagine the following scenario. A sample with solutes at a concentration of 40% (w/w) is cooled very quickly so that nucleation occurs only at the temperature around *T*h (~−70°C). However, at −70°C, ice crystal growth is very slow (at least relative to higher temperatures). Thus, crystal growth from the nucleation sites will be slow, especially considering that cooling is still taking place. However, during warming, once the sample gets above *T*g (~−120°C), ice crystals can grow from the nucleation sites. However, it will still be slow at low temperatures, but the rate will increase as the sample warms. Because there were no ice nuclei above −70°C during cooling in this example, ice growth at higher temperatures could not happen. However, ice nuclei are present during warming and crystal growth can occur until *T*m. Therefore, if nucleation does occur during cooling, warming must be very fast to avoid crystal growth at temperatures below *T*m. The striking differences between the critical cooling rate (defined as the cooling rate necessary to achieve vitrification) and the critical warming rate (defined as the warming rate necessary to avoid more than 0.2% crystallization during warming) are illustrated by the analysis of Baudot and Odagescu [23]. According to their calculations, for a 40% (w/w) solution of ethylene glycol in water, the critical cooling rate is 569°C/min, but the critical warming rate is 1.08 × 1010°C/min.

Solute Concentration as a Result of Ice Crystallization, The Associated Osmotic Effects, and Cell Death at Supra-Optimal Cooling Rates

As ice forms during cooling, only water molecules comprise the ice crystals. As a result, all other components (salts, etc.) become concentrated in the remaining solution. As the solution concentration increases, the chemical potential of the water in the solution decreases. Water will continue to crystallize until the chemical potential of the water in the liquid phase equals the chemical potential of the water in the solid phase. In other words, the remaining solution will reach its equilibrium freezing point (the curve defined by *T*m). Therefore, the concentration of the remaining liquid phase can also be determined from a phase diagram.

For example, assume that Fig. 7.1 represents the phase diagram of a sodium chloride–water binary solution. If you start with an isotonic saline solution (0.9 wt%) and cool it to −20°C, ice will form until the remaining solution is at its equilibrium freezing point. In this example, the remaining solution will attain a concentration of ~45 wt% (note the point where the *T*m curve reaches −20°C). In this hypothetical example, the unfrozen solution would be roughly 14 mol/L sodium chloride (compared to 0.15 mol/L initially). In reality, sodium chloride will only concentrate to ~4 mol/L at −20°C (the phase diagram for sodium chloride is markedly different than the one shown in Fig. 7.1).

When cells are frozen in suspension, the cells are sequestered in channels of concentrated unfrozen medium. The high concentration of this unfrozen solution establishes an osmotic gradient across the cell membrane, and as a result, water will flow out of the cell via exosmosis. Below a cell's equilibrium freezing point, the cytoplasm is in a supercooled state. If the sample is cooled slowly enough, exosmosis occurs to a sufficient degree to keep the cells in a near-equilibrium state with the extracellular solution. Such a situation will preclude intracellular ice formation. On the other hand, if the cooling rate is relatively rapid, water cannot leave the cell fast enough to maintain a near-equilibrium state with the extracellular solution, and at some point equilibrium will be re-established by intracellular ice formation (see Fig. 7.2).

This situation is described schematically in Fig. 7.3. The formation of intracellular ice is usually (but not necessarily) fatal to cells (see below for more details). Direct cryomicroscopic observation of intracellular ice formation in mouse oocytes (similar to that which is seen in Fig. 7.2) and the correlation to cell survival were some of the most convincing data to support the assertion that ice formation was the lethal cause of cell death at supra-optimal cooling rates [24,25].

The rate at which water flows out of a cell is dictated by the cell membrane water permeability. The permeability of cells to water is dependent upon several factors including temperature and the presence of cryoprotectants. For example, in the absence of cryoprotectant, human sperm water permeability is 1.84 μm/min/atm at 22°C, but is reduced to 1.23, 0.84, 0.77 and 0.74 μm/min/atm in the presence of propylene glycol, dimethylsulfoxide, glycerol, and ethylene glycol, respectively [26]. Furthermore, water permeability can vary greatly across cell types. For example, water permeability for human erythrocytes [27] is an order of magnitude higher than the value for human oocytes [28]. Because intracellular ice formation is

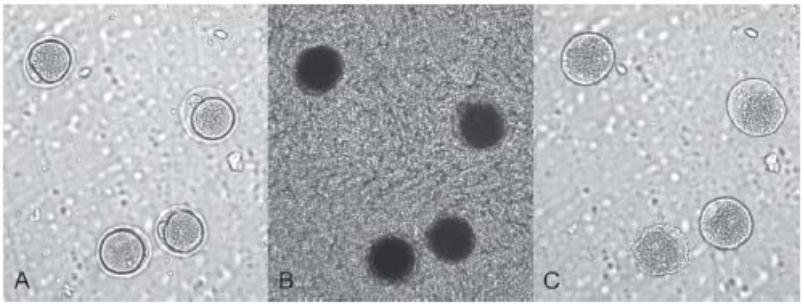


Fig. 7.2 Photomicrographs of intracellular ice formation in mouse oocytes cooled at 100 °C/min in an isotonic solution is shown. In Panel A, intact oocytes are shown prior to ice crystal formation; note the well-defined oolemma within the zona pellucida. As cooling proceeds, ice forms in the extracellular solution and eventually, the intracellular solution. The darker background in Panel B is due to ice, and the "blackening" of the oocytes indicates that intracellular ice formation has occurred. When the solution is warmed and the ice melts (Panel C), the cell membrane within the zona pellucida is no longer visible, indicating cell lysis. The speckled appearance of the background in Panels A and C is due to atmospheric water precipitation on the cold glass surface of the cell chamber, and not due to ice crystals

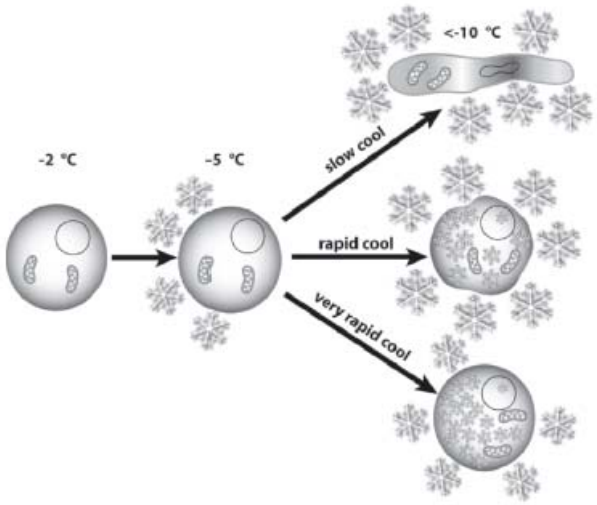


Fig.7.3 The cooling-rate-dependent fate of intracellular water resulting from extracellular ice formation is shown. As the temperature of the solution is cooled below the equilibrium freezing point, ice will form in the extracellular solution. As a result, water is driven out of the cell by an osmotic gradient across the cell membrane. If cooling is slow (upper cell, right side), sufficient water leaves the cell and intracellular ice formation does not occur. If the cooling rate is faster, ice will form inside the cell, and the amount of ice that forms and the size of the crystals will depend upon the cooling rate. Intermediate cooling rates (middle cell, right side) result in partial cell dehydration, larger crystals, and less intracellular ice. Very rapid cooling rates result in virtually no cell dehydration, a greater amount of ice formation, but smaller crystals. If cells are cooled very quickly (lower cell, right side) and warmed slowly, the average crystal size will increase (smaller crystals will tend to melt and larger crystals will tend to grow; a process known as recrystallization). This will be more damaging to the cell compared to very rapid warming. Figure adapted from Mazur, 1977 [29] with permission from Elsevier

dependent upon the degree of supercooling (hence the water content), the rate of cooling which results in intracellular ice formation differs widely across cell types. The theory of cell death due to intracellular ice formation resulting from the interaction of cooling rate and water loss outlined above was developed quantitatively by Peter Mazur [30] (see Mazur [19] for a recent review). The practical benefit of this theory comes from its potential to predict optimal cryopreservation

procedures. For example, if one knew the degree of supercooling that a cell could tolerate during cooling and the membrane permeability, it would be possible to predict the cooling rate which would prevent intracellular ice formation and the temperature at which cooling could stop and the sample could safely be transferred to liquid nitrogen [31]. See the original description of mammalian embryo cryopreservation for a relevant example [32].

Ice formation in the cytoplasm of cells is not necessarily damaging. Studies over the years have investigated the correlation between the morphology of cytoplasmic ice, cooling and warming rates, and survival [33,34]. Results from such studies have shown that larger ice crystal size correlated positively with cell damage. As discussed above for bulk solutions, devitrification and re-crystallization can occur to cell water/ice during warming [35] resulting in the growth of large ice crystals [34]. Re-crystallization is the phenomenon whereby large ice crystals grow at the expense of small ice crystals due to the greater stability of large crystals at a given temperature. According to a more recent study, the formation of small ice crystals may actually be beneficial to cell survival [36]. Such a result is likely due to the reduced level of cell dehydration when water is trapped inside the cell in small ice crystals and the reduced level of osmotic stress and associated water flux during warming. However, the warming needs to be fast enough under such circumstances to avoid re-crystallization as just discussed.

Attempts have been made to explain the mechanism(s) which cause intracellular ice formation, and to date several theories have been put fourth. As explained in more detail by Mazur [19], theories of intracellular ice formation must account for several experimental facts: (1) in order for ice formation to occur in cells above ~−30°C, extracellular ice must be present, and the proximity of the cells and ice is important; (2) extracellular ice is not a necessary precondition for intracellular ice below ~−30°C; (3) intracellular ice formation usually happens immediately if the cells and the surroundings are supercooled −15 to −20°C and extracellular ice is rapidly initiated; (4) if extracellular ice forms near the cell's equilibrium freezing point, intracellular ice usually does not form at slow cooling rates; and (5) the nucleation temperature decreases substantially if the extracellular solute concentration increases.

Several lines of evidence suggest that intracellular ice formation can be triggered by more than one mechanism. It is generally agreed that ice formation below ~−30°C in the absence of extracellular ice is due to the presence of intracellular nucleators (or as a result of homogenous nucleation at lower temperatures). The fact that intracellular ice formation above this temperature requires the presence of extracellular ice strongly suggests that the extracellular ice is acting to nucleate the intracellular ice. As extracellular ice does not nucleate intracellular ice at low degrees of supercooling, an intact plasma membrane effectively blocks the passage of ice into the cell. However, the plasma membrane is implicated mechanistically in the major theories put fourth to explain the initiation of intracellular ice formation above ~−30°C.

Mazur [37] hypothesized that ice crystals can grow through membranes via protein pores like aquaporins. While evidence exists that ice can grow through channels which connect cells (i.e., gap junctions [38–40]), the pore size in these channels is much larger than those in aquaporins, making ice growth more likely. Mazur and colleagues are currently using genetic engineering techniques in oocytes as a means to test this hypothesis directly (see [41–43] for results from initial experiments). Toner and colleagues [44,45] have suggested that ice interaction with the

plasma membrane causes a structural change to the inner membrane surface, resulting in an increase in the efficiency of ice nucleation. Muldrew and McGann [46,47] have put fourth a different mechanism altogether which suggests that ice grows through the membrane after the formation of a lesion as a result of the osmotic pressure gradient and resultant water efflux. A similar argument regarding the formation of a membrane lesion as a prelude to intracellular ice formation had been proposed by Steponkus and colleagues several years earlier [48,49].

Despite the debate as to the nature of intracellular ice nucleation, the evidence that intracellular ice formation is a very damaging (and usually lethal) event is overwhelming. Hence, preventing ice formation in the cytoplasm (at least to a large degree) during cryopreservation is critical.

Cell Death at Sub-Optimal Cooling Rates and the Role of Cryoprotectants in Mitigating Cell Damage

Given that the probability of intracellular ice formation decreases proportionally with cooling rate, an obvious question is: why not just cool cells at very slow rates to prevent damage from intracellular ice? The simple answer is because intracellular ice formation is not the only cause of cell damage during freezing, and some of the other causes are more detrimental at lower cooling rates. When cell survival is plotted as a function of cooling rate, an inverted "U-shaped" curve is generated (Fig. 7.4). The peak of this curve represents the optimal cooling velocity, cell viability being lost at both higher and lower cooling rates.

The shape of this curve has been interpreted to suggest at least two mechanisms of cell damage, each of which is oppositely dependent upon cooling rate. As

Fig. 7.4 The relationship between cooling rates and cell survival, for different cell-types,

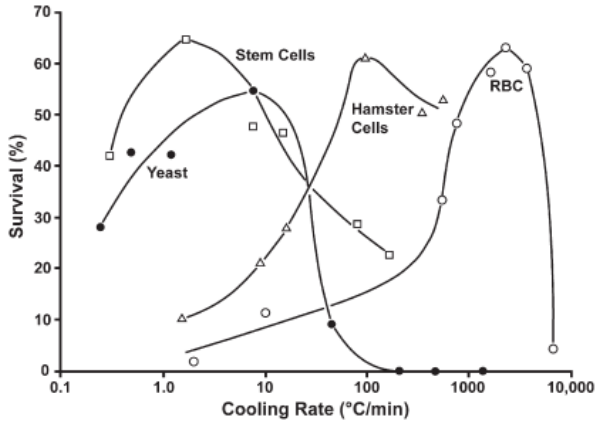


Fig. 7.4 The relationship between cooling rates and cell survival, for different cell-types, is shown. Higher water permeability allows faster cooling rates to be applied without a high probability of cell death due to intracellular ice formation. The specific optimal rate is also dependent upon other factors such as the presence of cryoprotectants and the warming rate. Figure reprinted from Mazur 1970 [50] with permission from Elsevier. See text for more details

described above, damage due to intracellular ice formation can explain the loss of cell viability at cooling velocities higher than optimal (the right side of the curve). The mechanisms responsible for the loss of viability at lower than optimal cooling rates are varied, and we still do not have a comprehensive understanding of the nature of these injuries. Nevertheless, a general idea has

emerged as to the nature of some injuries which occur at slow cooling rates. One of the principal mechanisms of cell injury at sub-optimal rates of cooling is directly tied to the nature of cryoprotectants.

Cryoprotectants: Beneficial Effects

Cryoprotectants are defined in a functional manner as compounds that allow a higher degree of cell survival during freezing in their presence than in their absence (see [15,21,51,52] for reviews). Although our knowledge of the mode of action of cryoprotectants is still incomplete, it is likely that the effects of cryoprotectants are multi-factorial, and cryoprotectants of different classes (e.g., alcohols, sugars, diols, amides, large polymers) may act by different mechanisms [15,53]. One of the earliest theories of the mechanism of action of cryoprotectants was developed from a series of experiments investigating the protective action of glycerol on erythrocytes. James Lovelock, a physical chemist by training, studied the effects of salt concentration on hemolysis. In his initial experiment [54] he investigated the relationship between the salt concentrations in partially frozen saline solutions which caused cell damage with the degree of cell damage when cells were exposed to the same salt concentrations without freezing. He determined that the degree of damage could be explained by the increase in salt concentration due to ice precipitation (Fig. 7.5).

Since that time, others have repeated these experiments and confirmed Lovelock's original findings [55,56]. Lovelock proceeded to show that when cells are frozen in solutions containing glycerol, the temperature at which hemolysis began was progressively lower as the amount of glycerol was increased [57]. As discussed above, solutes depress the equilibrium freezing point of a solution. By adding glycerol to a cryopreservation solution, the amount of water that freezes at any given temperature will be reduced. As a consequence, the final concentration of the salts in the remaining solution will also be reduced. Lovelock's experimental results supported the conclusion that the colligative depression of the freezing point and concomitant reduction in salt concentration explained the protective mechanism by which glycerol exerted its effect (Fig. 7.6). Hemolysis always began at the same concentration of sodium chloride (~0.8 mol/L).

Several other modes of action have been proposed for cryoprotectants. One effect includes interacting with water molecules and altering the water structure in a solution, and reducing the ability of water to join the ice phase [58,59]. Polymers can also facilitate vitrification upon cooling and reduce the concentration of permeating cryoprotectants necessary to attain a glassy state [7].

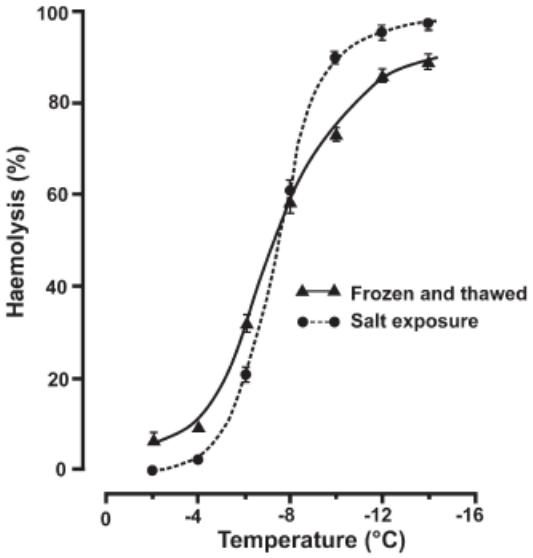


Fig. 7.5 The correlation between hemolysis and salt concentration for cells frozen or only exposed to salt is shown. The striking correlation lead Lovelock [54,57] to conclude that the concentration of salt resulting from ice formation was a primary mechanism of cryodamage. Others have argued that the effect of the salt concentration on cell volume was the true cause of cell damage [60]. Figure reproduced from Pegg 1987 [55] with permission from The Company of Biologists. See text for more details

Cryoprotectants have also been shown to either directly interact with or be preferentially excluded from biosurfaces (e.g., the surface of lipid bilayers or proteins) [61–64]. The apparent opposite nature of these modes of interaction seems to suggest opposite effects. However, each mode of interaction can be beneficial to the stability of these structures. In addition, Rudolph and Crowe [65] have shown that trehalose and proline can prevent freezing-induced fusion of lipid vesicles. For more details on these mechanisms, interested readers are directed to recent reviews [53,66–68] and references therein.

Perhaps it is not surprising that many organisms living in climates where freezing temperatures are encountered have evolved to include the metabolic production of cryoprotectants as a survival strategy. As discussed by Erica Benson [69] and reviewed by Ken Diller [70], the cryoprotective properties of sugars and glycerol in plants were described by Nikolay Maximov in the early 20th century (following on the work of others). The farsighted nature of his conclusions is remarkable considering what has been learned about cryoprotectants and their mechanisms since that time.

A great deal of research has been conducted to understand the response of various members of the animal kingdom to freezing temperatures. The metabolic production of cryoprotectants is also a common strategy in these organisms. Inhibition of freezing at high sub-zero temperatures is one strategy among arthropods and fish,

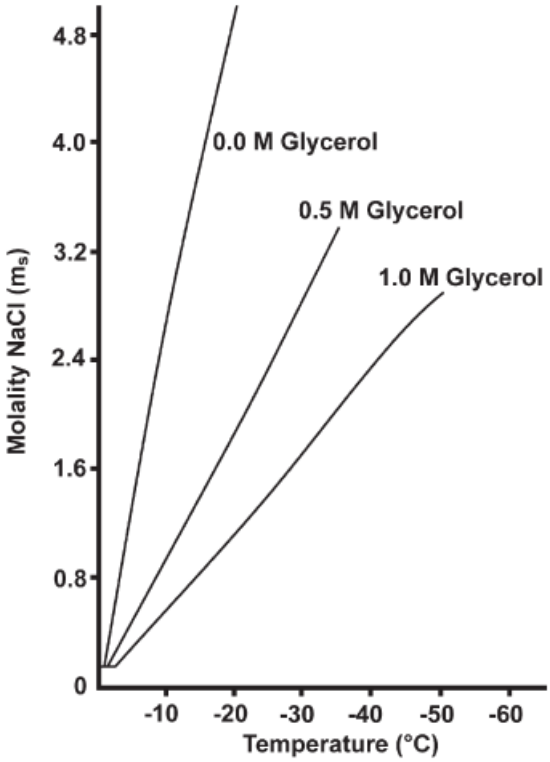


Fig. 7.6 The presence of cryoprotectants reduces the salt concentration at a given sub-zero temperature due to a colligative reduction in the freezing point of the solution (shown here for a glycerol and sodium chloride solution mixture). Notice that, as the concentration of glycerol increases, the salt concentration is significantly reduced. Figure reprinted from [71] with permission from The Biophysical Society

and is accomplished by regulative supercooling. In many instances the freezing point of physiological solutions is regulated by thermal hysteresis proteins [72].
The tertiary structure of these proteins allows them to directly interact with ice crystals due to polar residues along the protein backbone [73]. These proteins lower the freezing point of water without significantly altering the melting point. Thus, their mode of action is not colligative. According to a general model for their activity, these proteins bind to ice crystals and alter the radius of curvature of the growing crystal, which reduces the temperature at which it is thermodynamically favorable for additional water molecules to join the crystalline phase [74,75] (see Raymond et al. [76] for more detail on an early model of the mechanism of fish hysteresis proteins, and Kristiansen et al. [77] for a recent review on the mechanism of action). Overall, these proteins restrict ice growth when the environmental temperature is slightly below the equilibrium freezing temperature of the body fluids.

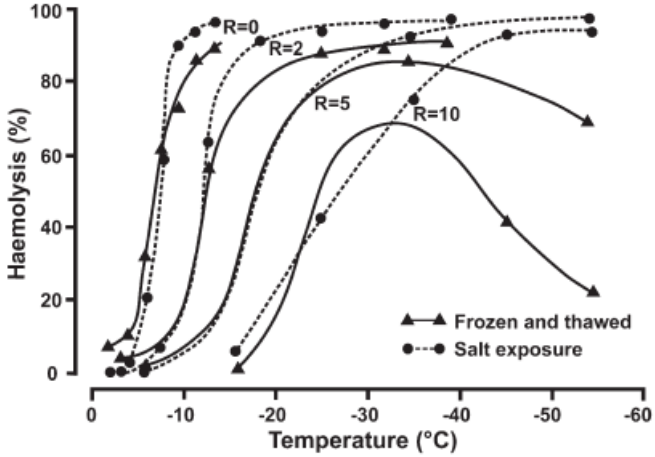
As an alternate strategy, organisms across many phylogenetic groups have developed mechanisms of regulating ice formation in situ. In naturally freeze-tolerant organisms, avoiding the formation of intracellular ice is managed by actively promoting and regulating the formation of extracellular ice. This allows freeze-induced dehydration of the cells and prevents ice from forming in the cytoplasm. As a coupled strategy, mechanisms to avoid the damaging consequences of cellular dehydration and ischemia that accompany freezing have also evolved.

Ken and Janet Storey, in a recent review on the subject [78], described four requirements for the successful freeze tolerance in animals: (1) ice must be confined to extracellular spaces and damage from ice crystals must be minimized; (2) the rate of freezing must be slow and controlled; (3) cell volume reduction beyond a minimum tolerable volume must be avoided; and (4) mechanisms must be present to prevent damage from resulting ischemia. These requirements are often met through both behavioral and physiological adaptations. For example, slow, controlled temperature change is often facilitated by the chosen hybernaculum of the organisms. Controlled ice nucleation can be performed by specific ice nucleating proteins in the blood [79,80]. Cryoprotectant synthesis (e.g., glucose production) in some organisms is initiated by freezing [81] (see the review by Storey and Storey [82] for more details). Membrane adaptations to cold have also been described [83]. As improved cryopreservation methods are sought, it is likely that attention paid to nature's laboratory will provide insights into appropriate means to avoid cryodamage.

Cryoprotectants: Detrimental Effects

As their name implies, cryoprotectants are beneficial during freezing. Their use, however, is not necessarily benign. Since the time of Lovelock's original work, it has been pointed out [84] and experimentally confirmed [55] that the correlation between the freezing damage in the presence of glycerol and the associated increase in salt concentration is strongest at low levels of hemolysis. In addition, as the concentration of glycerol is increased, the concentration of salt that causes a given degree of hemolysis decreases, suggesting that high concentrations of glycerol contribute to cell damage during freezing (Fig. 7.7). This suggests that high glycerol concentrations (particularly as a result of ice precipitation) contribute to the damage of cells frozen slowly. Similar results have also been shown for dimethylsulfoxide [85,86].

Injury from cryoprotectants is not limited to those which occur during freezing. Exposing cells to solutions containing cryoprotectants prior to cooling can be damaging due to an osmotic effect. Many of the commonly used permeating cryoprotectants have lower plasma membrane permeability coefficients compared to that of water. This relationship results in cells experiencing osmotically driven volume excursions during cryoprotectant addition to and removal from the cell during the course



of a cryopreservation procedure. It has been shown in numerous cell types that damage to cells can occur as a result from volume excursions alone [87–106]. Furthermore, many studies have demonstrated a beneficial effect of prolonging the cryoprotectant addition and/or removal process which reduces the associated volume excursions [107–109].

The rate of water movement across the plasma membrane is determined by several factors and can be described by (7.1):

$$\frac{dV_w}{dt} = -L_p A R T \left(M_s^e + M_n^e - \frac{n_s^i}{V_w} - \frac{n_n^i}{V_w} \right)$$

where d*V*w/d*t* represents the change in the cell water volume over time, *L*p and *A* represent the cell membrane hydraulic conductivity and surface area, respectively, *R* and *T* represent the gas constant and temperature, *M* represents molal concentration, *n* represents the number of moles of solute (collectively, the terms in parentheses represent the concentration gradient across the cell membrane). The letters e, i, s, and n in the super- and subscripts represent the extra- and intracellular compartments, and permeating (s) and non-permeating (n) solutes respectively.

A concentration gradient of permeating cryoprotectants will also result in movement of these compounds across the cell membrane. The rate of change in intracellular cryoprotectant resulting from such a gradient can also be described by an ordinary differential equation (7.2):

$$\frac{dn_s^i}{dt} = P_s A \left(M_s^e - \frac{n_s^i}{V_w} \right)$$

where d*ni s*/d*t* represents the change in the number of moles of intracellular cryoprotectant over time, *P*s represents the membrane permeability, and the remaining variables are equivalent to those in (1). Here we have shown the so-called two-parameter membrane transport model (*L*p and *P*s are the phenomenological parameters defining the permeability of the cell membrane to water and cryoprotectant). A three-parameter model incorporating an interaction coefficient (*s*) was proposed on the basis of irreversible thermodynamics for membranes where water and solute move through a common pathway [110]. It has been argued that the interaction coefficient is not applicable to biological membranes as water and cryoprotectants usually travel through independent pathways. Furthermore, being phenomenological in nature, a three-parameter model is less parsimonious than a two-parameter model. Interested readers can find more details on this debate in a recent review [111].

Osmotic damage is often ascribed to the associated volume reductions [60, 93]. Cell volume response can be controlled during cryoprotectant addition and removal by modifying the procedures for loading and unloading these compounds [112]. As a result, cryoprotectant addition and removal can be accomplished in a manner that prevents injury due to excessive volume excursions. Because the volume response of cells can be modeled on a computer when the parameters in (1) and (2) for the cells are known, one can proactively predict optimal methods for this process (see Gao et al. [26] for a more thorough discussion).

True chemical toxicity is also a concern associated with the use of cryoprotectants [51,113]. This is particularly true for vitrification methods (see below) as very high concentrations of these compounds are necessary to achieve and maintain a vitreous state at practical cooling rates. The precise nature of the toxic effects of cryoprotectants remains, to a large degree, uncertain. Fahy and colleagues have concluded that protein denaturation is not a general effect of cryoprotectants [114]. They offered an argument that effects on membranes could provide an alternate explanation to a direct effect on proteins that would be consistent with some data and proposed models. Cryoprotectants have been shown to alter cytoskeletal components in mammalian oocytes, particularly the filamentous actin network and meiotic spindle [115–117]. Re-polymerization after treatments is common, but the particular organization of the polymers often does not resemble those of untreated oocytes. Frequently, toxicity is argued to be a significant cause of cell death in oocyte cryopreservation studies. However, rarely is the chemical effect isolated from the osmotic effect in such experiments. In a previously unpublished experiment in our laboratory, the osmotic effects associated with exposure to 2.5 mol/L 1,2-propanediol and ethylene glycol were controlled when assessing the effects of exposing mouse oocytes to these cryoprotectants by including a treatment simulating the volume excursions associated with cryoprotectant addition and removal. The results suggested that the damage associated from exposure to 1,2-propanediol was not a result of the osmotic effects, but a true chemical effect. Exposure to the same concentration of ethylene glycol was not detrimental to mouse oocyte survival (Fig. 7.8).

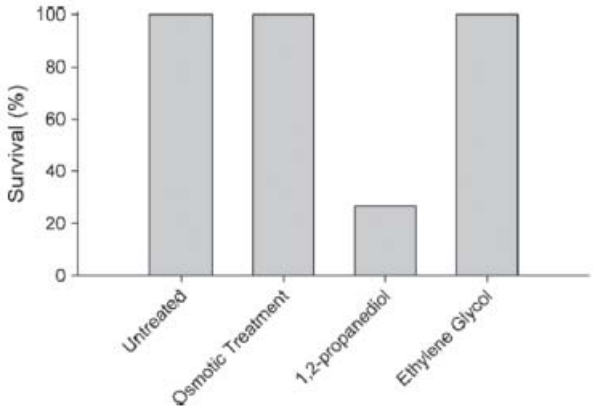


Fig. 7.8 The effect on cell viability of exposing mouse oocytes to 1,2-propanediol or ethylene glycol (2.5 M final with 0.3M sucrose) is shown. A solution containing 0.5 M sucrose which simulates the osmotically-driven volume excursions of the other treatments was included as a control. Neither osmotic stress or ethylene glycol exposure had an effect on oocyte viability after a 6-h incubation. Exposure to 1,2-propanediol resulted in a dramatic loss in cell viability (cell lysis)

It was mentioned earlier that most cryoprotectants have hydrogen bonding capability, and altering water structure is one of the mechanisms by which cryoprotectants are hypothesized to function. It is also recognized that the toxic concentration differs for different cryoprotectants. In a more recent report [118], Fahy and colleagues have determined that a compositional variable they call *qv** is directly associated with the toxic properties of a cryoprotectant when toxicity is non-specific. The proposal these authors make is that *qv** is related to the degree of hydration of a cryoprotectant. In their report, Fahy et al. show that the total concentration of cryoprotectants was not as strong of a predictor of toxicity as was the ratio of the molarity of water (*M*W) in the solution to the molarity of polar groups in the solution (*M*PG; i.e., *qv** = *M*W/*M*PG). Polar

groups were defined as hydroxyl groups (−OH), sulfoxide groups (−S=O), carbonyl groups (−C=O), and amino groups (−NH2) on the cryoprotectant. In their experiment, they initially tested the toxicity of various cryoprotectants with differing *qv** indices using rabbit renal cortical slices and examined the K+/Na+ ratio after exposure. As *qv** increased in the range from 2 to 6, the K+/Na+ ratio decreased from ~80% to ~10% relative to the controls.

The significance of the polar groups is such that they account for the interaction with water molecules, and compounds with a lower *qv** can interact with fewer water molecules. Such compounds are poorer glass formers compared to those with a higher *qv**. Hence, weak glass forming cryoprotectants are less toxic. Using this new information, the investigators were able to predict and confirm that substitution of 1,2-propanediol (a very good glass former) with ethylene glycol (a very poor glass former) in a previously developed vitrification solution (VS41A) would be a superior vitrification solution using rabbit renal cortical slices and mouse oocytes. Others have also shown that the protective potential of cryoprotectants was correlated to the molarity of potential hydrogen bonding groups [119]; (see the review by Mazur [120] for a more complete discussion). As discussed below, reducing cryoprotectant toxicity is one approach to improving vitrification methods.

Cell Death at Sub-Optimal Cooling Rates

Many of the factors which might contribute to cell damage as a result of freezing are interdependent. For example, ice crystal formation may have deleterious mechanical effects on cells in suspension or in tissues [46,55,71,121] and the amount of ice formed and the crystal structure is dependent upon cooling rate, warming rate, and the presence of cryoprotectants. Not surprisingly, the idea of a single optimal cooling rate for a cell is an oversimplification. The cooling rate at which cell survival is highest is dependent upon other factors such as cryoprotectant concentration and warming rate [50,122].

Intracellular cryoprotectants have noticeable effects on intracellular ice formation [50,41,42,123–125]. In general, in the presence of a permeable cryoprotectant, cell water will crystallize at a slower cooling rate compared to the cooling rate resulting in crystallization in the absence of an intracellular cryoprotectant [126]. This effect is likely a result of several factors. One includes the reduction in the water permeability of cell membranes in the presence of cryoprotectant as mentioned above. A second is likely a result of lowering the freezing point of the cytoplasm which causes a general reduction in the temperature at which a given driving force for water efflux is present. Because of the temperature dependence of water permeability, less water can move out of the cell in a given amount of time under such circumstances [124,127]; as discussed in Mazur [19].

The generic term “solution effects” has been coined to collectively describe the various forms of injury to cells cooled slowly enough to preclude damaging intracellular ice formation [128]. This term reflects a notion that the damage results from the solution conditions created by ice formation as described above. Meryman and colleagues suggested that damage was a physical and not a biological event [128], resulting either from the osmotic dehydration of the cells and the resulting stress placed upon the cell membrane due to cell volume reduction, or a direct osmotic effect on the membrane itself. In earlier work, Meryman described a hypothesis of

cryoinjury based upon the cell reaching a minimum critical volume [129]. This later work supports this theory.

Another interesting hypothesis has been put fourth to explain solution effects damage in relation to the formation of ice. In a series of studies, Mazur and colleagues investigated the effects of the fraction of the solution which remained unfrozen on cell damage [71,130–132]. Their data showed a strong correlation between survival and the unfrozen fraction when the unfrozen fraction was low (5–15%). They proposed that as the unfrozen fraction was reduced, the cells were damaged by mechanical effects of the ice and/or close apposition with other cells. When the unfrozen fraction is increased, damage was less strongly dependent on that variable and more on the salt concentration until the effects of the unfrozen fraction were lost. When the unfrozen fraction is not a damaging mechanism, the loss was attributed to the osmotic effect of the solute (both during exposure and dilution) [133].

The interpretation of these data came under scrutiny as Pegg and Diaper [134] pointed out that the unfrozen fraction variable was confounded by the treatments used to change the unfrozen fraction (changing the initial osmolality of the solution). Such treatments would systematically alter the volume excursions which the cells would undergo during the experiment, and this difference could also result in the outcome seen. Mazur [19] goes into greater detail about this debate and adds additional evidence for the unfrozen fraction hypothesis.

Perhaps the most important message to get from this particular debate is the difficulty in designing experiments to isolate the effect of a single variable on cell damage during freezing when numerous potential variables are interdependent (see [55,134] for an elaboration). Another good example of this is the challenge to the explanation for slow-cooling injury resulting from increased salt concentration. As discussed above, concentrated solutes cause exosmosis and result in a reduction in cell volume. Thus, either high salt concentration or volume reduction could explain the damage (it could also be an interaction of the two factors). The minimum volume hypothesis was strongly supported by the results of Williams and Shaw with erythrocytes [93] following up on earlier work by Meryman [60].

In more recent years, the molecular mechanisms of cryodamage, particularly the induction of apoptosis, have been investigated [135]. John Baust and colleagues have suggested that the trigger for apoptosis is not necessarily an immediate effect of the cryopreservation stresses, but can be delayed for several hours as the cells try to recover from these stresses [136]. Clearly, at the present time we are far from understanding all of the mechanisms which result in cryodamage.

Cooling and Cooling Injury/Cold Shock

Even in the absence of ice, cold temperatures have profound effects upon cells. Injury from cooling is often differentiated by the degree to which the rate of cooling causes the specific event. Injuries from rapid cooling are usually categorized as cold shock injuries. These types of injuries occur quickly after cooling, and are generally independent of the warming rate. In the context of cryopreservation, a significant body of literature has been produced which describes the effects of cold shock on cell membranes, particularly for spermatozoa [18,137].

A description of the liquid crystalline model of cell membranes can be found in a standard cell biology text [138]. In general, amphipathic lipid molecules form a bilayer structure with various proteins being integrated throughout. At physiologic temperatures, the membrane is fluid such that molecular mobility is high and many of the proteins and lipids are free to diffuse laterally within the bilayer (however, opposite faces of the bilayer are not identical, and moving from one face of the bilayer to the other is energetically unfavorable). The structure that lipids can take in solution is more diverse than just a simple bilayer configuration. A lamellar (i.e., bilayer) structure is common, but micelles, inverted micelles (micelles within the bilayer), hexagonal-II, and cubic-phase structures can occur (see Fig.1 in the review by Quinn [139]). The particular arrangements lipids take is dependent upon factors such as water activity, temperature, pH, salt concentration, and interactions with other molecules (e.g., proteins).

When membranes are cooled, they exhibit thermotropic behavior; that is to say they tend to undergo phase transitions. As membranes are cooled, the lipids tend to transition from a liquid-like state to a gel-like state, with the molecules being arranged in an orderly, crystalline fashion with a characteristic hexagonal arrangement [140]. Due to the complexity of biological membranes, a transition is not like a crystallization event in a simple solution (i.e., a rapid precipitation), but more like a (relatively) slow lateral separation of membrane lipids into distinct domains (see Fig. 7.9). Nevertheless, this transition is a distinct change from the usual lipid arrangement, and can have significant effects on membrane function.

The temperature at which this transition occurs is dependent upon several factors, including the length of the hydrocarbon chain in the lipid group, the presence and location of *cis*-unsaturated bonds (transition temperatures decrease as the position

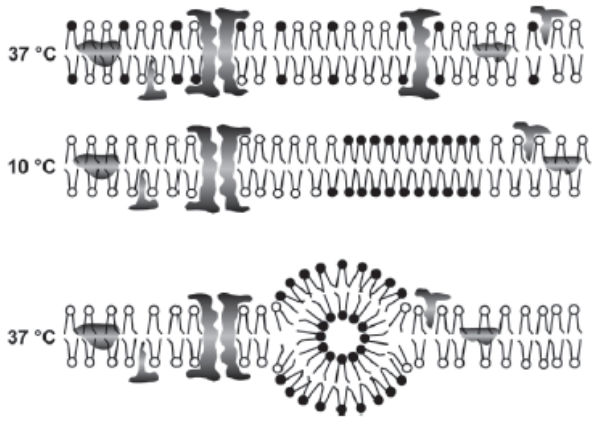


Fig. 7.9 A model of temperature-induced phase changes to membranes is described in this figure. In the upper portion of the figure, a typical biomembrane is shown, with various integral membrane proteins and lipid species. As the temperature is reduced from physiologic to hypothermic (10 °C in this instance), lateral redistribution of the various molecules occurs, with lamellar-forming lipid species (represented with white polar groups) and hexagonal-II-forming lipid species (represented with black polar groups) separating into distinct domains. Upon warming, an inverted micelle structure is created by the hexagonal-II-forming lipids. Such a configuration could result in a significant disruption of the membrane selective permeability, and the possibility of membrane failure and cell death. Figure adapted from Parks, 1997 [141], which was adapted from Quinn 1985 [139]

of the bond moves away from the polar group and toward the middle of the chain), and the concentration and valence of cations in the solution. An increase in the concentration of polyvalent cations increases the phase transition temperature, whereas monovalent cations increase lipid fluidity and decrease the phase transition temperature. The presence of cholesterol in a membrane can also affect phase transition behavior by (1) altering the ability of lipid species to transition to a gel-like configuration; and (2) increasing the disorder of the gel phase.

The propensity to develop a lamellar or hexagonal-II structure varies across lipid species. Different species tend to aggregate into domains during the phase change, and the creation of inverted micelles (hexagonal-II structures) within a bilayer can occur as a result (Fig. 7.9) [142]. Rearrangements such as these can alter the selective permeability of membranes, resulting in the loss of cell homeostasis.

Changes in the biochemistry of spermatozoa as a result of cold shock have been examined. A reduction in anaerobic glycolysis and respiration, ATP levels, Cytochrome *C* loss from the mitochondria, and release of numerous intracellular enzymes have all been described (reviewed in [18,143]). Furthermore, changes in the distribution of intracellular ions have also been noted.

Numerous compounds have been shown to confer protection to spermatozoa from cold shock. Protective agents include glycerol, phosphatidylserine, egg yolk, lecithin, milk, and albumin. The low density lipoprotein fraction of egg yolk is particularly effective at preventing cold shock injury [144], with phosphatidylcholine being a particularly active component [145]. The results from Quinn and colleagues [145] suggest that the effect is a result of interactions with the surface of the membrane, and not as a result of components intercalating within the lipid bilayer. The mechanisms of these compounds are not fully understood, but one model for the effect of adhering cryoprotectants on phase separations of membranes has been put fourth [4] and is shown schematically in Fig. 7.10.

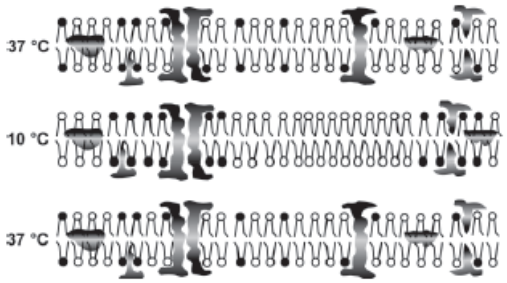


Fig. 7.10 A model of the effect of cryoprotectants on preserving biomembrane stability as described in Quinn 1985 [139] is shown. In the presence of cryoprotectants (not shown schematically), the hexagonal-II –forming lipid species preferentially associate with membrane proteins during cooling (compare middle panel to middle panel in Figure 7.9) and only lamellar-forming lipid species segregate into distinct domains. When the cell is warmed, the hexagonal-II-lipid – protein interactions prevent a non-lamellar transition, promoting the return to a normal bilayer configuration

Studies during recent years have focused on the genetic regulation of the response to cold shock [146–148]. While it seems a bit ironic, members of the heatshock protein family are often up-regulated as a response to cold stress. Other proteins that have been referred to as cold-shock

proteins are also up-regulated as a response to cold temperatures. Compared to that which is known in prokaryotes, our knowledge of the genetic response to cold temperatures in eukaryotic cells is just developing. For further review of these topics, see [149–152] and references therein.

Vitrification as an Alternate to Equilibrium Cryopreservation

As has been alluded to several times in this chapter, vitrification can be employed as an alternative to equilibrium freezing. The obvious benefit of this approach is that the damage due to intracellular ice formation can be completely avoided. Unfortunately, other kinds of damage are more likely to occur when using this method.

Recalling the initial discussion of phase transitions in solutions during cooling, it was mentioned that with a sufficiently high solute concentration, ice formation could be avoided altogether. The easiest way to achieve vitrification would be to use a solution that has a concentration as indicated in section IV of Fig. 7.1. Water in solutions with such a composition will not crystallize nor devitrify even at cooling rates applicable to equilibrium freezing methods. Unfortunately, the toxicity of such solutions is too high to render them practical. Similarly, solutions with concentrations around 60 wt% (Region III) are often too toxic to be useful, although they can also be cooled slowly without crystallization. Various strategies have been described to counter the potential toxicity of solutions, and include: (1) the use of a combination of solutes, each of which is below a concentration that is very toxic, yet in combination will facilitate vitrification; (2) the substitution of polymers in the extracellular medium for the smaller permeating agents; (3) the application of hydrostatic pressure; (4) the use of compounds which counteract the toxicity of other agents (e.g., acetamide with dimethylsulfoxide [114]); and (5) reducing the time for which and/or the temperature at which the biomaterial is exposed to high concentrations of cryoprotectants. Hydrostatic pressure has an effect by shifting the Th (lower) and Tg curves (higher) such that their intersection point occurs at lower concentrations of solutes.

As discussed above, when solution concentrations are reduced, the likelihood of devitrification during warming increases. Hence, the warming rate is an especially important consideration when designing vitrification strategies. In the first report of successful vitrification of mammalian embryos, Rall and Fahy [153] used a combination of these strategies (1, 4, and 5) to overcome the toxicity associated with the vitrification solution (VS1). Mouse embryos (8-cell) could survive exposure to the solution for up to 15 min at 4°C; survival dropped precipitously with increasing time. Furthermore, loss of viability occurred when samples were cooled slowly compared to rapidly when the concentration of VS1 used was reduced by 25%, and when the warming rate was reduced to 10°C/min from 300 or 2,500°C/min.

These results support the idea that vitrification at moderate solute concentrations (region II in Fig. 7.1) can be precarious. This region is labeled “doubly unstable” on the chart, as the use of solutions in this range are likely to be highly nucleated upon cooling, and prone to devitrification and re-crystallization upon warming. Reducing the solute concentrations in combination with higher cooling rates is a strategy being currently employed for vitrification of mammalian oocytes [154– 156]. However, modification of the cooling and warming rate is not the only strategy that might lead to improvements [157]. As has been discussed throughout this chapter,

several types of cryoinjury exist, and the development of optimal cryopreservation strategies will require that all of these factors are taken into account, along with the specific cryobiological properties of the cells under study [158].

Acknowledgments We would like to thank Howard Wilson and Don Conner for their contribution to the development of the figures in the present volume. Han Xu is also acknowledged for providing the photos in Fig. 7.2.

References

1. Komender J. Cell and tissue preservation and storage for transplantation. Present and future. Ann Transplant 2004;9(1):88–90.
2. Toner JP, Brzyski RG, Oehninger S, Veeck LL, Simonetti S, Muasher SJ. Combined impact of the number of pre-ovulatory oocytes and cryopreservation on IVF outcome. Hum Reprod 1991;6(2):284–289.
3. Schnorr JA, Muasher SJ, Jones HW, Jr. Evaluation of the clinical efficacy of embryo cryopreservation. Mol Cell Endocrinol 2000;169(1–2):85–89.
4. Benagiano G, Gianaroli L. The new Italian IVF legislation. Reprod Biomed Online 2004;9(2):117–125.
5. Bankowski BJ, Lyerly AD, Faden RR, Wallach EE. The social implications of embryo cryopreservation. Fertil Steril 2005;84(4):823–832.
6. Luyet BJ, Gehenio PM. Life and death at low temperatures. Normandy, MI: Biodynamica; 1940.
7. Fahy GM, MacFarlane DR, Angell CA, Meryman HT. Vitrification as an approach to cryopreservation. Cryobiology 1984;21(4):407–426.
8. Meryman HT, ed. Cryobiology. New York: Academic Press; 1966.
9. Fuller BJ, Lane N, Benson EE, eds. Life in the Frozen State. Boca Raton: CRC Press; 2004.
10. Bowler K, Fuller BJ, eds. Temperature and Animal Cells. Cambridge: The Company of Biologists, Ltd.; 1987.
11. Baust JG, Baust JM, eds. Advances in Biopreservation. Boca Raton: Taylor and Francis; 2007.
12. Mazur P. Freezing of living cells: mechanisms and implications. Am J Physiol 1984;247 (3 Pt 1):C125–C142.
13. Karlsson JO, Toner M. Long-term storage of tissues by cryopreservation: critical issues. Biomaterials 1996;17(3):243–256.
14. Fuller B, Paynter S. Fundamentals of cryobiology in reproductive medicine. Reprod Biomed Online 2004;9(6):680–691.
15. Fuller BJ. Cryoprotectants: the essential antifreezes to protect life in the frozen state. Cryo-letters 2004;25:375–388.
16. Karow AM, Critser JK, eds. Reproductive Tissue Banking. San Diego: Academic Press; 1997.
17. Walters EM, Benson JD, Woods EJ, Critser JK. History of Sperm Cryopreservation. In: Pacey A, Tomlinson MJ, eds. Practical Guide for Sperm Banking. Cambridge: Cambridge University Press (In Press).
18. Watson PF, Morris GJ. Cold shock injury in animal cells. In: Bowler K, Fuller BJ, eds. Temperature and animal cells. Cambridge: The Company of Biologist Limited; 1987:311–340.
19. Mazur P. Principles of Cryobiology. In: Fuller BJ, Lane N, Benson EE, eds. Life in the Frozen State. Boca Raton: CRC Press; 2004:3–65.
20. Muldrew K, Acker JP, Elliott JAW, McGann LE. The water to ice transition: implications for living cells. In: Fuller BJ, Lane N, Benson EE, eds. Life in the Frozen State. Boca Raton: CRC Press; 2004:67–108.
21. Ashwood-Smith MJ. Mechanism of cryoprotectant action. In: Bowler K, Fuller BJ, eds. Temperature and Animal Cells. Cambridge: The Company of Biologists, Ltd.; 1987:395–406.
22. Cocks FH, Brower WE. Phase diagram relationships in cryobiology. Cryobiology 1974;11(4): 340–358.
23. Baudot A, Odagescu V. Thermal properties of ethylene glycol aqueous solutions. Cryobiology 2004;48(3):283–294.
24. Leibo SP, McGrath JJ, Cravalho EG. Microscopic observation of intracellular ice formation in unfertilized mouse ova as a function of cooling rate. Cryobiology 1978;15(3):257–271.
25. Toner M, Cravalho EG, Karel M, Armant DR. Cryomicroscopic analysis of intracellular ice formation during freezing of mouse oocytes without cryoadditives. Cryobiology 1991;28(1):55–71.
26. Gao D, Mazur P, Critser JK. Fundamental Cryobiology of Mammalian Spermatozoa. In: Karow AM, Critser JK, eds. Reproductive Tissue Banking, Scientific Principles. San Diego: Academic Press; 1997:263–328.

27. Terwilliger TC, Solomon AK. Osmotic water permeability of human red cells. J Gen Physiol 1981;77(5):549–570.
28. Hunter J, Bernard A, Fuller B, McGrath J, Shaw RW. Plasma membrane water permeabilities of human oocytes: the temperature dependence of water movement in individual cells. J Cell Physiol 1992;150(1):175–179.
29. Mazur P. The role of intracellular freezing in the death of cells cooled at supraoptimal rates. Cryobiology 1977;14(3):251–272.
30. Mazur P. Kinetics of water loss from cells at subzero temperatures and the likelihood of intracellular freezing. J Gen Physiol 1963;47:47–69.
31. Karlsson JO, Cravalho EG, Borel Rinkes IH, Tompkins RG, Yarmush ML, Toner M.
Nucleation and growth of ice crystals inside cultured hepatocytes during freezing in the presence of dimethyl sulfoxide. Biophys J 1993;65(6):2524–2536.
32. Whittingham DG, Leibo SP, Mazur P. Survival of mouse embryos frozen to –196 degrees and –269 degrees C. Science 1972;178(59):411–414.
33. Shimada K, Asahina E. Visualization of intracellular ice crystals formed in very rapidly frozen cells at –27 degree C. Cryobiology 1975;12(3):209–218.
34. Bank H. Visualization of freezing damage. II. Structural alterations during warming.
Cryobiology 1973;10(2):157–170.
35. Karlsson JO. A theoretical model of intracellular devitrification. Cryobiology 2001;42(3): 154–169.
36. Acker JP, McGann LE. Protective effect of intracellular ice during freezing? Cryobiology 2003;46(2):197–202.
37. Mazur P. The role of cell membranes in the freezing of yeast and other single cells. Ann N Y Acad Sci 1965;125:658–676.
38. Acker JP, Elliott JA, McGann LE. Intercellular ice propagation: experimental evidence for ice growth through membrane pores. Biophys J 2001;81(3):1389–1397.
39. Acker JP, Larese A, Yang H, Petrenko A, McGann LE. Intracellular ice formation is affected by cell interactions. Cryobiology 1999;38(4):363–371.
40. Irimia D, Karlsson JO. Kinetics of intracellular ice formation in one-dimensional arrays of interacting biological cells. Biophys J 2005;88(1):647–660.
41. Mazur P, Pinn IL, Seki S, Kleinhans FW, Edashige K. Effects of hold time after extracellular ice formation on intracellular freezing of mouse oocytes. Cryobiology 2005;51(2):235–239.
42. Mazur P, Seki S, Pinn IL, Kleinhans FW, Edashige K. Extra- and intracellular ice formation in mouse oocytes. Cryobiology 2005;51(1):29–53.
43. Guenther JF, Seki S, Kleinhans FW, Edashige K, Roberts DM, Mazur P. Extra- and intra- cellular ice formation in Stage I and II *Xenopus laevis* oocytes. Cryobiology 2006;52(3):401–416.
44. Toner M, Cravalho EG, Huggins CE. Thermodynamics and kinetics of intracellular ice formation during freezing of biological cells. J Appl Physiol 1990;69:1582–1593.
45. Toner M, Cravalho EG, Stachecki J, et al. Nonequilibrium freezing of one-cell mouse embryos. Membrane integrity and developmental potential. Biophys J 1993;64(6):1908–1921.
46. Muldrew K, McGann LE. Mechanisms of intracellular ice formation. Biophys J
1990;57(3):525–532.
47. Muldrew K, McGann LE. The osmotic rupture hypothesis of intracellular freezing injury. Biophys J 1994;66(2 Pt 1):532–541.
48. Dowgert MF, Steponkus PL. Effect of Cold Acclimation on Intracellular Ice Formation in Isolated Protoplasts. Plant Physiol 1983;72(4):978–988.
49. Steponkus PL, Dowgert MF, Gordon-Kamm WJ. Destabilization of the plasma membrane of isolated plant protoplasts during a freeze-thaw cycle: the influence of cold acclimation. Cryobiology 1983;20(4):448–465.
50. Mazur P, Leibo SP, Farrant J, Chu EHY, Hanna Jr MG, Smith LH. 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. London: J and A Churchill; 1970:69–58.
51. Fahy GM. The relevance of cryoprotectant “toxicity” to cryobiology. Cryobiology 1986; 23(1):1–13.
52. Karow AM, Jr. Cryoprotectants – a new class of drugs. J Pharm Pharmacol 1969;21(4): 209–223.
53. Acker JP. The use of intracellular protectants in cell biopreservation. In: Baust JG, Baust JM, eds. Advances in Biopreservation. Boca Raton, FL: Taylor & Francis; 2007:299–320.
54. Lovelock JE. The haemolysis of human red blood cells by freezing and thawing. Biochim Biophys Acta 1953;10:414–426.
55. Pegg DE. Mechanisms of freezing damage. In: Bowler K, Fuller BJ, eds. Temperature and Animal Cells. Cambridge: The Company of Biologists, Ltd.; 1987:363–378.

56. Pegg DE, Diaper MP. On the mechanism of injury to slowly frozen erythrocytes. Biophys J 1988;54(3):471–488.
57. Lovelock JE. The mechanism of the protective action of glycerol against haemolysis by freezing and thawing. Biochim Biophys Acta 1953;11:28–36.
58. Nash T. Chemical constitution and physical properties of compounds able to protect living cells against damage due to freezing and thawing. In: Meryman HT, ed. Cryobiology. New York: Academic Press; 1966:179–211.
59. Korber C, Scheiwe MW, Boutron P, Rau G. The influence of hydroxyethyl starch on ice formation in aqueous solutions. Cryobiology 1982;l9(5):478–492.
60. Meryman HT. Osmotic stress as a mechanism of freezing injury. Cryobiology 1971;8(5):489–500.
61. Anchordoguy TJ, Cecchini CA, Crowe JH, Crowe LM. Insights into the cryoprotective mechanism of dimethyl sulfoxide for phospholipid bilayers. Cryobiology 1991;28(5):467–473.
62. Xie G, Timasheff SN. The thermodynamic mechanism of protein stabilization by trehalose. Biophys Chem 1997;64(1–3):25–43.
63. Carpenter JF, Crowe JH. The mechanism of cryoprotection of proteins by solutes. Cryobiology 1988;25(3):244–255.
64. Anchordoguy TJ, Carpenter JF, Cecchini CA, Crowe JH, Crowe LM. Effects of protein perturbants on phospholipid bilayers. Arch Biochem Biophys 1990;283(2):356–361.
65. Rudolph AS, Crowe JH. Membrane stabilization during freezing: the role of two natural cryoprotectants, trehalose and proline. Cryobiology 1985;22(4):367–377.
66. Crowe JH, Crowe LM, Tablin F, Wolkers W, Oliver AE. Stabilization of cells diring freezedrying: the trehalose myth. In: Fuller BJ, Lane N, Benson EE, eds. Life in the Frozen State. Boca Raton: CRC Press; 2004:581–601.
67. Crowe JH, Crowe LM, Carpenter JF, et al. Interactions of sugars with membranes. Biochim Biophys Acta 1988;947(2):367–384.
68. Crowe JH, Carpenter JF, Crowe LM. The role of vitrification in anhydrobiosis. Annu Rev Physiol 1998;60:73–103.
69. Benson EE. Cryoconserving algal and plant diversity: historical perspectives and future challenges. In: Fuller BJ, Lane N, Benson EE, eds. Life in the Frozen State. Boca Raton: CRC Press; 2004:299–328.
70. Diller KR. Pioneers in cryobiology: Nikolay Aleksandrovich Maximov (1890–1952). Cryo- Letters 1997;18:81–92.
71. Mazur P, Rall WF, Rigopoulos N. Relative contributions of the fraction of unfrozen water and of salt concentration to the survival of slowly frozen human erythrocytes. Biophys J 1981;36(3):653–675.
72. Barrett J. Thermal hysteresis proteins. Int J Biochem Cell Biol 2001;33(2):105–117.
73. Knight CA, DeVries AL, Oolman LD. Fish antifreeze protein and the freezing and recrystallization of ice. Nature 1984;308(5956):295–296.
74. Hew CL, Yang DS. Protein interaction with ice. Eur J Biochem 1992;203(1–2):33–42.
75. Wilson PW. A model for thermal hysteresis utilizing the anisotropic interfacial energy of ice crystals. Cryobiology 1994;31:406–412.
76. Raymond JA, Wilson P, DeVries AL. Inhibition of growth of nonbasal planes in ice by fish antifreezes. Proc Natl Acad Sci USA 1989;86(3):881–885.
77. Kristiansen E, Zachariassen KE. The mechanism by which fish antifreeze proteins cause thermal hysteresis. Cryobiology 2005;51(3):262–280.
78. Storey KB, Storey JM. Freeze tolerance in animals. Physiol Rev 1988;68(1):27–84.
79. Storey KB, Baust JG, Wolanczyk JP. Biochemical modification of plasma ice nucleating activity in a freeze-tolerant frog. Cryobiology 1992;29(3):374–384.
80. Wolanczyk JP, Storey KB, Baust JG. Ice nucleating activity in the blood of the freezetolerant frog, *Rana sylvatica*. Cryobiology 1990;27(3):328–335.
81. Vazquez Illanes MD, Storey KB. 6–Phosphofructo-2-kinase and control of cryoprotectant synthesis in freeze tolerant frogs. Biochim Biophys Acta 1993;1158(1):29–32.
82. Storey KB, Storey JM. Natural freeze tolerance in ectothermic vertebrates. Annu Rev Physiol 1992;54:619–637.
83. Hazel JR. Effects of temperature on the structure and metabolism of cell membranes in fish. Am J Physiol 1984;246(4 Pt 2):R460–R470.
84. Fahy GM, Karow AM, Jr. 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 1977;14(4):418–427.

85. Kahn RA. Biochemical changes in frozen platelets. In: Greenwalt TJ, Jamieson GA, eds. The blood platelet in transfusion therapy. New York: Alan R. Liss; 1978:167–180.
86. Fahy GM. Analysis of "solution effects" injury: rabbit renal cortex frozen in the presence of dimethyl sulfoxide. Cryobiology 1980;17(4):371–388.
87. Armitage WJ, Mazur P. Osmotic tolerance of human granulocytes. Am J Physiol 1984;247 (5 Pt 1):C373–C381.
88. Armitage WJ, Parmar N, Hunt CJ. The effects of osmotic stress on human platelets. J Cell Physiol 1985;123(2):241–248.
89. Agca Y, Liu J, Rutledge JJ, Critser ES, Critser JK. Effect of osmotic stress on the developmental competence of germinal vesicle and metaphase II stage bovine cumulus oocyte complexes and its relevance to cryopreservation. Mol Reprod Dev 2000;55(2):212–219.
90. Pukazhenthi B, Noiles E, Pelican K, Donoghue A, Wildt D, Howard J. Osmotic effects on feline spermatozoa from normospermic versus teratospermic donors. Cryobiology 2000;40(2):139–150.
91. Blanco JM, Gee G, Wildt DE, Donoghue AM. Species variation in osmotic, cryoprotectant, and cooling rate tolerance in poultry, eagle, and peregrine falcon spermatozoa. Biol Reprod 2000;63(4):1164–1171.
92. Mazur P, Schneider U. Osmotic responses of preimplantation mouse and bovine embryos and their cryobiological implications. Cell Biophys 1986;8(4):259–285.
93. Williams RJ, Shaw SK. The relationship between cell injury and osmotic volume reduction: II. Red cell lysis correlates with cell volume rather than intracellular salt concentration. Cryobiology 1980;17(6):530–539.
94. Zieger MA, Woods EJ, Lakey JR, Liu J, Critser JK. Osmotic tolerance limits of canine pancreatic islets. Cell Transplant 1999;8(3):277–284.
95. Gao DY, Chang Q, Liu C, et al. Fundamental cryobiology of human hematopoietic progenitor cells. I: Osmotic characteristics and volume distribution. Cryobiology 1998;36(1):40–48.
96. Men H, Agca Y, Mullen SF, Critser ES, Critser JK. Osmotic stress on the cellular actin filament organization of in vitro produced porcine embryos. Reproduction, Fertility, and Development 2004;12(1,2):177.
97. Koshimoto C, Gamliel E, Mazur P. Effect of osmolality and oxygen tension on the survival of mouse sperm frozen to various temperatures in various concentrations of glycerol and raffinose. Cryobiology 2000;41(3):204–231.
98. Songsasen N, Yu I, Murton S, et al. Osmotic sensitivity of canine spermatozoa. Cryobiology 2002;44(1):79–90.
99. Mullen SF, Agca Y, Broermann DC, Jenkins CL, Johnson CA, Critser JK. The effect of osmotic stress on the metaphase II spindle of human oocytes, and the relevance to cryopreservation. Hum Reprod 2004;19(5):1148–1154.
100. Agca Y, Liu J, Mullen S, et al. Osmotic tolerance and membrane permeability characteristics of Rhesus (*Macaca mulatta*) spermatozoa. Cryobiology 2004;49(3):316–317.
101. Walters E, Men H, Agca Y, Mullen S, Critser E, Critser J. Osmotic tolerance of mouse spermatozoa from various genetic backgrounds. Cryobiology 2004;49(3):344.
102. Walters EM, Men H, Agca Y, Mullen SF, Critser ES, Critser JK. Osmotic tolerance of mouse spermatozoa from various genetic backgrounds: Acrosome integrity, membrane integrity, and maintenance of motility. Cryobiology 2005;50(2):193–205.
103. De Loecker R, Penninckx F. Osmotic effects of rapid dilution of cryoprotectants II. Effects on human erythrocyte hemolysis. Cryo-Letters 1987;8:140–145.
104. Agca Y, Mullen S, Liu J, et al. Osmotic tolerance and membrane permeability characteristics of rhesus monkey (*Macaca mulatta*) spermatozoa. Cryobiology 2005;51(1):1–14.
105. Men H, Agca Y, Mullen SF, Critser ES, Critser JK. Osmotic tolerance of in vitro produced porcine blastocysts assessed by their morphological integrity and cellular actin filament organization. Cryobiology 2005;51(2):119–129.
106. Adams SL, Kleinhans FW, Mladenov PV, Hessian PA. Membrane permeability characteristics and osmotic tolerance limits of sea urchin (*Evechinus chloroticus*) eggs. Cryobiology 2003;47(1):1–13.
107. Shaw PW, Fuller BJ, Bernard A, Shaw RW. Vitrification of mouse oocytes: improved rates of survival, fertilization, and development to blastocysts. Mol Reprod Dev 1991;29(4):373–378.
108. Isachenko V, Montag M, Isachenko E, Nawroth F, Dessole S, van der Ven H. Developmental rate and ultrastructure of vitrified human pronuclear oocytes after step-wise versus direct rehydration. Hum Reprod 2004;19(3):660–665.
109. Fiéni F, Beckers JP, Buggin M, et al. Evaluation of cryopreservation techniques for goat embryos. Reproduction, Nutrition, Development 1995;35(4):367–373.
110. Kedem O, Katchalsky A. Thermodynamic analysis of the permeability of biological membranes to non-electrolytes. Biochim. Biophys. Acta 1958;27:229–246.

111. Kleinhans FW. Membrane permeability modeling: Kedem–Katchalsky vs a two-parameter formalism. Cryobiology 1998;37(4):271–289.
112. Gao DY, Liu J, Liu C, et al. Prevention of osmotic injury to human spermatozoa during addition and removal of glycerol. Hum Reprod 1995;10(5):1109–1122.
113. Baxter SJ, Lathe GH. Biochemical effects of kidney of exposure to high concentrations of dimethyl sulphoxide. Biochem Pharmacol 1971;20(6):1079–1091.
114. Fahy GM, Lilley TH, Linsdell H, Douglas MS, Meryman HT. Cryoprotectant toxicity and cryoprotectant toxicity reduction: in search of molecular mechanisms. Cryobiology 1990;27(3):247–268.
115. Johnson MH, Pickering SJ. The effect of dimethylsulphoxide on the microtubular system of the mouse oocyte. Development 1987;100(2):313–324.
116. Vincent C, Johnson MH. Cooling, cryoprotectants, and the cytoskeleton of the mammalian oocyte. Oxf Rev Reprod Biol 1992;14:73–100.
117. Vincent C, Pickering SJ, Johnson MH, Quick SJ. Dimethylsulphoxide affects the organization of microfilaments in the mouse oocyte. Mol Reprod Dev 1990;26(3):227–235.
118. Fahy GM, Wowk B, Wu J, Paynter S. Improved vitrification solutions based on the predictability of vitrification solution toxicity. Cryobiology 2004;48(1):22–35.
119. Doebbler GF, Rinfret AP. The influence of protective compounds and cooling and warming conditions on hemolysis of erythrocytes by freezing and thawing. Biochim Biophys Acta 1962;58:449–458.
120. Mazur P. Cryobiology: the freezing of biological systems. Science 1970;168(934):939–949.
121. Sjostrom M. Ice crystal growth in skeletal muscle fibres. J Microsc 1975;105(1):67–80.
122. Mazur P, Schmidt JJ. Interactions of cooling velocity, temperature, and warming velocity on the survival of frozen and thawed yeast. Cryobiology 1968;5(1):1–17.
123. Rall WF, Mazur P, McGrath JJ. Depression of the ice-nucleation temperature of rapidly cooled mouse embryos by glycerol and dimethyl sulfoxide. Biophys J 1983; 41(1):1–12.
124. Myers SP, Pitt RE, Lynch DV, Steponkus PL. Characterization of intracellular ice formation in Drosophila melanogaster embryos. Cryobiology 1989;26(5):472–484.
125. Harris CL, Toner M, Hubel A, Cravalho EG, Yarmush ML, Tompkins RG. Cryopreservation of isolated hepatocytes: intracellular ice formation under various chemical and physical conditions. Cryobiology 1991;28(5):436–444.
126. Diller KR. Intracellular freezing of glycerolized red cells. Cryobiology 1979;16(2):125–131.
127. Karlsson JO, Cravalho EG, Toner M. A model of diffusion-limited ice growth inside biological cells during freezing. J Appl Physiol 1994;75:4442–4450.
128. Meryman HT, Williams RJ, Douglas MS. Freezing injury from “solution effects” and its prevention by natural or artificial cryoprotection. Cryobiology 1977;14(3):287–302.
129. Meryman HT. The exceeding of a minimum tolerable cell volume in hypertonic suspensions as a cause of freezing injury. In: Wolstenholme GEW, O′Connor M, eds. The Frozen Cell. London: J and A Churchill; 1970:51–64.
130. Mazur P, Cole KW. Influence of cell concentration on the contribution of unfrozen fraction and salt concentration to the survival of slowly frozen human erythrocytes. Cryobiology 1985;22(6):509–536.
131. Mazur P, Cole KW. Roles of unfrozen fraction, salt concentration, and changes in cell volume in the survival of frozen human erythrocytes. Cryobiology 1989;26(1):1–29.
132. Mazur P, Rigopoulos N. Contributions of unfrozen fraction and of salt concentration to the survival of slowly frozen human erythrocytes: influence of warming rate. Cryobiology 1983;20(3):274–289.
133. Zade-Oppen AM. Posthypertonic hemolysis in sodium chloride systems. Acta Physiol Scand 1968;73(3):341–364.
134. Pegg DE, Diaper MP. The effect of initial tonicity on freeze/thaw injury to human red cells suspended in solutions of sodium chloride. Cryobiology 1991;28(1):18–35.
135. Baust JM, Van B, Baust JG. Cell viability improves following inhibition of cryopreservation – induced apoptosis. In Vitro Cell Dev Biol Anim 2000;36(4):262–270.
136. Baust JM, Vogel MJ, Van Buskirk R, Baust JG. A molecular basis of cryopreservation failure and its modulation to improve cell survival. Cell Transplant 2001;10(7):561–571.
137. Morris GJ, Watson PF. Cold-Shock injury – a comprehensive bibliography. Cryo-Letters 1984;5:352–372.
138. Alberts B, Johnson A, Lewis J, Raff M, Roberts K, Walter P. Molecular biology of the cell. 4th ed. New York: Garland Science; 2002.

139. Quinn PJ. A lipid-phase separation model of low-temperature damage to biological membranes. Cryobiology 1985;22(2):128–146.
140. Chapman D. Phase transitions and fluidity characteristics of lipids and cell membranes. Q Rev Biophys 1975;8(2):185–235.
141. Parks JE. Hypothermia and Mammalian Gametes. In: Karow AM, Critser JK, eds. Reproductive Tissue Banking, Scientific Principles. San Diego: Academic Press; 1997:229–261.
142. Sen A, Brain AP, Quinn PJ, Williams WP. Formation of inverted lipid micelles in aqueous dispersions of mixed sn-3–galactosyldiacylglycerols induced by heat and ethylene glycol. Biochim Biophys Acta 1982;686(2):215–224.
143. Watson PF. The effects of cold shock on sperm cell membranes. In: Morris GJ, Clarke A, eds. Effects of low temperatures on biological membranes. New York: Academic Press; 1981:189–218.
144. Pace MM, Graham EF. Components in egg yolk which protect bovine spermatozoa during freezing. J Anim Sci 1974;39(6):1444–1449.
145. Quinn PJ, Chow PY, White IG. Evidence that phospholipid protects ram spermatozoa from cold shock at a plasma membrane site. J Reprod Fertil 1980;60(2):403–407.
146. Phadtare S, Alsina J, Inouye M. Cold-shock response and cold-shock proteins. Curr Opin Microbiol 1999;2(2):175–180.
147. Rieder CL, Cole RW. Cold-shock and the Mammalian cell cycle. Cell Cycle 2002;1(3):169–175.
148. Fujita J. Cold shock response in mammalian cells. J Mol Microbiol Biotechnol 1999;1(2):243–255.
149. Al-Fageeh MB, Marchant RJ, Carden MJ, Smales CM. The cold-shock response in cultured mammalian cells: harnessing the response for the improvement of recombinant protein production. Biotechnol Bioeng 2006;93(5):829–835.
150. Al-Fageeh MB, Smales CM. Control and regulation of the cellular responses to cold shock: the responses in yeast and mammalian systems. Biochem J 2006;397(2):247–259.
151. Inouye M, Phadtare S. Cold shock response and adaptation at near-freezing temperature in microorganisms. Sci STKE 2004;2004(237):pe26.
152. Phadtare S, Inouye M. Genome-wide transcriptional analysis of the cold shock response in wild-type and cold-sensitive, quadruple-csp-deletion strains of *Escherichia coli*. J Bacteriol 2004;186(20):7007–7014.
153. Rall WF, Fahy GM. Ice-free cryopreservation of mouse embryos at –196 degrees C by vitrification. Nature 1985;313(6003):573–575.
154. Kuwayama M, Vajta G, Kato O, Leibo SP. Highly efficient vitrification method for cryopreservation of human oocytes. Reprod Biomed Online 2005;11(3):300–308.
155. Lane M, Gardner DK. Vitrification of mouse oocytes using a nylon loop. Mol Reprod Dev 2001;58(3):342–347.
156. Martino A, Songsasen N, Leibo SP. Development into blastocysts of bovine oocytes cryopreserved by ultra-rapid cooling. Biol Reprod 1996;54(5):1059–1069.
157. Otoi T, Yamamoto K, Koyama N, Tachikawa S, Suzuki T. Cryopreservation of mature bovine oocytes by vitrification in straws. Cryobiology 1998;37(1):77–85.
158. Woods EJ, Benson JD, Agca Y, Critser JK. Fundamental cryobiology of reproductive cells and tissues. Cryobiology 2004;48(2):146–156.