Cryo-Injury and Biopreservation

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ABSTRACT: **Mammalian cells appear to be naturally tolerant to cold temperatures, but the formation of ice when cells are cooled leads to a variety of damaging effects. The study of cryo-injury, therefore, becomes the study of when and how ice is formed both inside and outside the cell during cooling. Protectant chemicals are used to control or prevent ice formation in many preservation protocols, but these chemical themselves tend to be damaging. Cooling and warming rates also strongly affect the amount and location of ice that is formed. Through careful modification of these parameters successful cold preservation techniques for many cell types have been developed, but there are many more cell types that have defied preservation techniques, and the extension of cell-based techniques to tissues and whole organs has been very limited. There are many aspects to the damaging effects of ice in cells that are still poorly understood. In this brief article we review our current understanding of cellular injury and highlight the aspects of cellular injury during cryopreservation that are still poorly understood.**

KEYWORDS: **cryopreservation; freezing; vitrification; cell injury**

INTRODUCTION

Cellular injury that results from freezing and thawing has been the object of scientific study for more than 60 years.[1] The concept of preserving cells and tissues for long periods of time by freezing them has led to a continual interest in how and why cells are damaged when they are exposed to cold temperatures. Successful preservation of human red blood cells by freezing in the early 1950s made successful organ preservation and possibly even suspended animation appear to be within reach. Researchers increased their efforts to understand the sources of damage to cells that experienced freezing and ways to prevent that damage. It turns out that preventing cellular damage during freezing is far more difficult than most people imagined in 1950. In this brief article we will review our current understanding of cellular injury and highlight the aspects of cellular injury during cryopreservation that are still poorly understood.

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The motivation for cellular preservation by freezing is fairly clear. Many people understand the benefits of being able to freeze blood, and can understand the benefits of being able to store a kidney, heart, or liver for long periods of time. The goal of freezing whole organs for future use remains one of the main goals of the field of cryopreservation, but the many difficulties researchers have encountered in trying to reach that goal have made whole-organ preservation seem like an ever-receding horizon. Techniques and lessons learned from successful preservation of suspended and cultured cells have not translated well to complex tissues. Research into whole-organ preservation continues, and may someday be successful, but no one expects to see a whole liver cryopreserved anytime soon.

The field of cryopreservation has gained renewed momentum and garnered new interest recently, however, because of the emerging interest in cell-based therapies. Cryopreservation has achieved reasonable success in establishing protocols for the frozen preservation of cells in culture; and many new therapies are emerging that make use of living cells as the basis for treatment.[2,3] Tissue engineering, gene therapy, and cellular implantation all rely on the ability to store and transport cells and tissues in order to be clinically successful. While whole-organ preservation remains an important goal for cryopreservation, the ability to preserve and maintain a wide variety of cell types in suspension and culture has grown increasingly important on its own.

It appears to be the case that cells are not damaged by exposure to cold temperatures. Extensive evidence indicates that ice-formation inside or outside of the cell leads to the damage that cells experience when exposed to cold, not the cold temperature itself. This can be contrasted directly with hyperthermic exposure, where the proteins within the cells begin to denature as soon as the cell temperature is raised a few degrees above the physiological norm. Patients undergoing major surgery are routinely cooled 15 to 20 degrees below normal in order to slow physiological degradation during the surgery; and this does not appear to harm any of the cells in the human body. Whole organs are also cooled to hypothermic temperatures in order to store them for a short period of time prior to transplantation. Since it is well established that cooling slows down metabolic and degradation processes in cells and tissues, the fact that cells are not injured "immediately" by cooling suggests that if one cooled them to low enough temperatures one could virtually stop the degradative processes and preserve the cells for long periods of time. This is the concept behind cryopreservation efforts in both medicine and food science.

When cells are cooled below the freezing point of water, however, ice can form. The formation of ice can lead to lethal damage to cells and tissues. It has been observed in countless studies that many cell types can survive freezing, but the number of cells that survive depends critically on the rate at which the cells are cooled and the rate at which they are warmed. This is because the cooling and warming rates critically affect whether ice is formed inside the cell, outside the cell, or is never formed at all. If ice is not formed, then the cells and tissues generally remain undamaged.

VITRIFICATION

The formation of ice in pure water becomes energetically favorable when water at atmospheric pressure is cooled to below 0°C. In order for ice formation to actually

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occur, however, a group of water molecules has to arrange itself into a stable crystalline nucleus.[4] Once a stable ice nucleus is formed, additional molecules of water will attach and the crystal will grow as long as the temperature remains below the equilibrium melting point. The formation of the initial crystal nucleus is stochastic,[5] resulting from random motion of the liquid molecules. Nucleation becomes increasingly likely as temperature decreases because the number of molecules that have to coordinate to form a stable crystal nucleus decreases with decreasing temperature. Usually the formation of a crystal nucleus is abetted by the presence of a non-water molecule that imposes a certain amount of local order on the surrounding water molecules, thereby increasing the probability that they will form an ordered crystal.[6] These types of objects are heterogenous nucleators, and they usually cause ice to form at temperatures between −10 and −18°C. Even if no heterogeneous nucleators are present, however, a nucleating crystal will form by random action of the water molecules at about −40°C in most circumstances. If the water is able to reach a temperature of about −138°C without crystal formation, then a glass-phase transition occurs and the water will remain in an amorphous solid state for as long as it is held below the glass-phase transition temperature.[7] The ability to reach −138°C without crystal ice formation should provide the perfect cryopreservation method. There are two known methods for reaching cold temperatures without ice formation: one can freeze really fast or one can add solutes and molecular ice inhibitors so that crystals will not form.

The creation of a non-crystalline glass-phase solid from a liquid is called "vitrification."[8] Glass formation is a second-order phase transition in which the specific heat and the viscosity of the substance change significantly. The viscosity of glass is so great that inter-molecular relaxation and diffusion will not occur in ordinary laboratory time scales. In a glass, crystals do not form, even though they are energetically favorable, because the molecules are no longer free to arrange themselves into a crystal structure. In addition, chemical reactions, such as those necessary for cellular degradation, become virtually impossible due to molecular immobility.

For pure water, glass forms at −138 °C. Glass-phase water cannot ordinarily be formed because ice crystals form at temperatures much higher than that, but vitrification of pure water can be achieved by very rapid cooling (10^6 °C/sec) so that ice crystals do not have time to form. Cooling rates this high are achievable for regions up to about 10 microns in depth if the water surface is instantaneously exposed to liquid nitrogen temperatures. The most successful technique for the vitrification of pure water has been supersonic deposition of droplets onto cryogenically cooled surfaces.[9] Cooling rates this high have only rarely been achieved using cells[10,11]; and there does not seem to be any way to achieve cooling rates this high for tissues or whole organs.

Vitrification can be achieved at more modest cooling rates by adding cryoprotectant chemicals that inhibit the formation or growth of ice crystals or increase the glass-phase temperature of the solution. FIGURE 1 illustrates a number of techniques that have been used in conjunction with vitrification-promoting chemicals to achieve vitrification of cells.[12–14] The overall approach is to minimize thermal mass of the holder in order to achieve high cooling rates and thus lower the likelihood of deleterious ice formation. One can see that these techniques are still 100 times too slow to achieve vitrification of pure water, but they can be used to vitrify cryoprotectant solutions. If the concentration of cryoprotectants is high enough, then the system will

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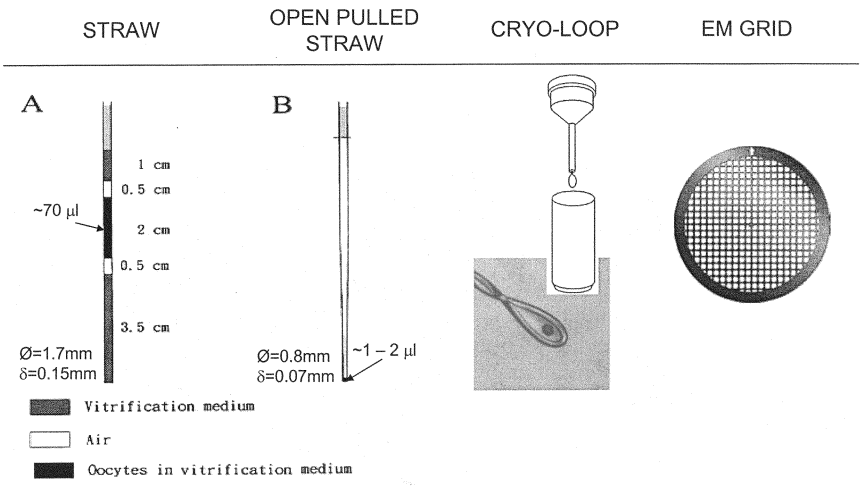


FIGURE 1. Schema of various vitrification techniques. Straw technique is the most commonly used approach for oocyte and embryo vitrification using cryoprotectant cocktails of 5 to 7M in concentration. Typically such high cryoprotectant concentrations are very toxic and difficult to load into and remove from cells. Open pulled straw, cryo-loop, and EM (electron microscopy) grid techniques have been developed to achieve rapid cooling to increase the probability of vitrification at a lower cryoprotectant concentration.

form a glass regardless of cooling rate.[8] Solutions containing 41–50% of propanediol are reported to have heterogenous nucleation temperatures that are below their glass-phase transition temperatures.[15] This means that ice crystals will not form at temperatures above the glass-transition temperature. A non-crystalline glass phase, therefore, can be achieved at any cooling rate, which makes it possible to vitrify large samples. Luyet suggested in 1937 that vitrification might provide an excellent method for cryopreservation,[8] and recent articles have touted its potential for preserving whole organs.[16] If a solution can be put into the glass phase without any possible chance of ice formation, then the ice will not kill the cells. Whole organs or even whole bodies should be possible to preserve.

The problem is the incredibly high concentration of cryoprotectants necessary to achieve vitrification at ordinary cooling rates.[15,17,18] Cryprotectants are generally toxic to cells, and at the concentrations typically used for vitrification, almost anything except water is toxic to cells. Even the simple osmotic stresses associated with addition and removal of such massive amounts of additives can be lethal.[15,17,19] The challenge of finding vitrification solutions that do not kill the cells they are intended to preserve, and the challenge of introducing those solutions into every part of a complex tissue without causing severe damage, remain formidable. Vitrification is currently a very promising cryopreservation technique for the possible preservation of whole organs and complex tissues, but its success at this point is limited to a few simple tissues[16,20] and it is not yet clear whether it will become the answer to the ultimate challenges of biopreservation.

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SLOW-FREEZING INJURY

The curve of survival versus cooling rate for many cells takes on the shape of an inverted U, in which a maximum survival is achieved at an intermediate cooling rate.[21–23] This optimal cooling rate depends strongly on cell type and also on the warming rate used to thaw the cells. Studies have revealed that one of the parameters that most strongly effects the optimal cooling rate is the membrane permeability of the cell (often symbolized as L_{pg}) , which is a measure of how quickly water can move in and out of the cell through the cellular membrane. Cells with highly permeable membranes, such as erythrocytes, have very high optimal cooling rates, and cells with relatively low permeabilities, such as oocytes, tend to have low optimal cooling rates.[24]

The inverted U phenomenon has been shown to be a direct function of exactly when and where ice is formed within the system. There do not appear to be any "strong" heterogeneous nucleators present in the cellular interior. This means that if a cell is in isolation, ice will not form inside the cell until the cell reaches a temperature of about –40°C. Ice will usually form outside the cell, therefore, where heterogeneous nucleators are almost always present, before ice forms inside the cell.[25] In most practical approaches, ice is seeded in a controlled manner in the extracellular solution such as by touching with a spatula cooled to cryogenic temperatures. One of the unusual properties of water is its very strong solute rejection.[4] When ice forms, very little solute is incorporated into the ice. Since cells are always frozen in solutions, rather than in pure water, solute rejection occurs as soon as ice is formed and the solute concentration in the remaining water increases. The increased solute

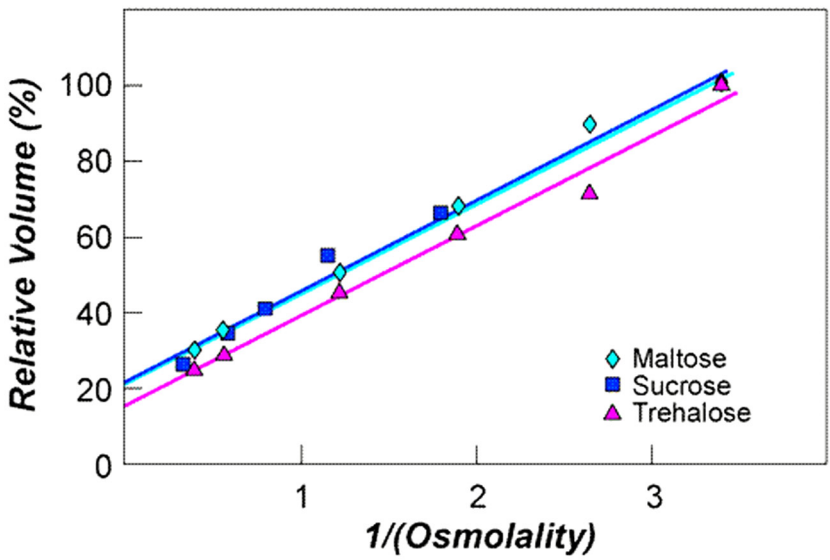


FIGURE 2. Osmometric behavior of human oocytes as a function of increasing extracellular osmolality. (From McWilliams *et al.*[72] Reproduced by permission.)

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concentration has two effects: it lowers the freezing point of the remaining solution and it creates an osmotic pressure gradient across the cellular membrane. FIGURE 2 illustrates the relationship between cellular volume and external osmolality for human oocytes. One can see that increasing the osmolality of the external solution by the addition of sugars (which do not enter the cell) can cause the cells to shrink to about 20% of their initial volume.

During freezing the formation of extracellular ice causes an increase in extracellular osmolality. As soon as ice forms outside of a cell in solution, the cell begins to dehydrate. If the cell membrane is highly permeable to water, then the intracellular solution will maintain osmotic equilibrium with the extracellular solution as the temperature continues to drop and progressively more ice is formed. The intracellular and extracellular solution concentrations will continue to increase as temperature drops until a eutectic point is reached and the remaining solution solidifies, or more commonly in cryobiological systems, a glass transition occurs and the remaining unfrozen solution is vitrified. At this point the system can be cooled to liquid nitrogen temperatures without significant changes to the chemical structure inside or outside the cell.

During very slow cooling, the process of gradually dehydrating the cell as ice is slowly formed outside the cell leads to extensive cellular damage. A complete review and analysis of the literature relating to this type of damage is given in a recent review by Mazur.[24]

It has been clearly established that one source of cellular injury in the slow-freezing process is the lethal concentration of electrolytes to which the cell is exposed.[26] Since electrolytes are almost always present both inside and outside the cell, their concentrations will inevitably go up as ice is formed extracellularly. High concentrations of electrolytes, such as those seen by cells during freezing, have been shown to be lethal. The damage seen during slow freezing has been well correlated with the damage seen when cells are exposed to equivalent electrolyte concentrations without freezing.[24,27] This mechanism of damage, usually called solute damage or solute effect, is the one most cryobiologists consider when thinking about the cause of slow-freezing death to cells.

There is evidence, however, that slow-freezing damage is not solely a function of increased electrolyte concentration. Mazur and Cole have shown that the survival of slowly frozen red blood cells depends on electrolyte concentration and the unfrozen fraction of water.[27] The unfrozen fraction is the fraction of water in the system that does not form ice, but instead is incorporated into a eutectic solid or into a glass. By adding glycerol to the cellular solution they controlled the electrolyte concentration independently of the unfrozen fraction. They found that blood cell survival depends strongly on electrolyte concentration as long as there is a sufficiently large fraction of unfrozen water. If the unfrozen fraction is less than 20%, however, the survival depends only on the unfrozen fraction, and survival decreases dramatically as the unfrozen fraction decreases. They believe this damage is caused by the aggregation of the cells into small channels as they are excluded from the ice, and they are forced into some type of adverse cell–cell interaction. Further evidence for adverse cell interactions as a source of cellular injury is provided by studies involving the dense packing of cells during freezing.[28]

Other researchers have suggested that the total volume change the cells experience as they undergo shrinkage during slow freezing may cause membrane damage,

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and still others have proposed that there may be a critical minimum volume for cells, and if cells are shrunken to sizes smaller than that critical level during slow freezing they may suffer irreparable damage.[24] None of the evidence for any one of these hypotheses is conclusive; but it is clear and well established that if cells are frozen slowly without cryoprotectant, they will shrink as ice forms, and they will almost always die.

Excessive cellular shrinkage can be reduced by the addition of penetrating cryoprotectants. Cryoprotectants are chemicals that are added to the cellular solution to try and protect the cell during freezing or vitrification. There are hundreds of cryoprotectants of different types. Some of the most commonly used are dimethyl sulfoxide (DMSO), glycerol, sucrose, and trehalose. There are two fundamental types of cryoprotectant: penetrating cryoprotectants and non-penetrating cryoprotectants. Penetrating cryoprotectants pass through the cellular membrane unaided, and non-penetrating ones do not. When considering slow freezing damage, which seems to be caused by cellular shrinkage and increased electrolyte concentration, the two types of cryoprotectant act very differently. The addition of non-penetrating cryopotectants increases the osmolality of the extracellular solution and causes the cells to shrink before freezing as shown in FIGURE 2. Freezing may be harder to initiate due to the presence of the cryoprotectant (at the very least a higher osmolality will lead to a lower equilibrium freezing temperature), but once freezing is initiated solute rejection will commence and the cell will begin to shrink as the osmolality of the unfrozen solution continues to increase. The liquidus curve of the extracellular solution will depend on its components, so the osmolality of the system as a function of temperature may be different depending on the cryoprotectant used, and the final state of the extracellular unfrozen fraction may be altered. The extracellular solution may form a glass rather than a water and salt eutectic. The composition of the intracellular solution, however, will not be altered by non-penetrating cryoprotectants, so the electrolyte concentration of the intracellular solution and the cell size will both be functions of the extracellular osmolality exactly as if the cryoprotectant were not added. If water is removed from the cell until the intracellular solution forms a glass or eutectic solid, then the end state inside the cell will be the same whether non-penetrating cryoprotectant is added or not. In most cases, unless the cryprotectant dramatically alters the liquidus curve of the extracellular solution, the addition of non-penetrating cryoprotectants will not prevent injury to slowly frozen cells.

Penetrating cryoprotectants, on the other hand, pass through the cellular membrane. Adding these cryoprotectants, of which DMSO and glycerol are the most common, causes the concentration of electrolytes to decrease for a given temperature during freezing, since for any given osmolality a certain fraction of the solutes inside and outside the cell are cryoprotectants. They also increase the final size of the cell when dehydration due to freezing is complete, because in addition to the usual intracellular components, there is an additional non-water volume component to the intracellular volume. The unfrozen fraction of the solution is also increased, as is the case for non-penetrating cryoprotectants. The effects of DMSO addition on the amount of ice formed and on the electrolyte concentration are illustrated in FIGURE 3. One can see that the addition of DMSO substantially decreases the amount of ice that is formed and the electrolyte concentration as the sample is cooled. Since these are the leading causes of cellular damage during slow cooling it is clear that the addition of DMSO can mitigate this damage; but DMSO is toxic to cells.[19] The amount

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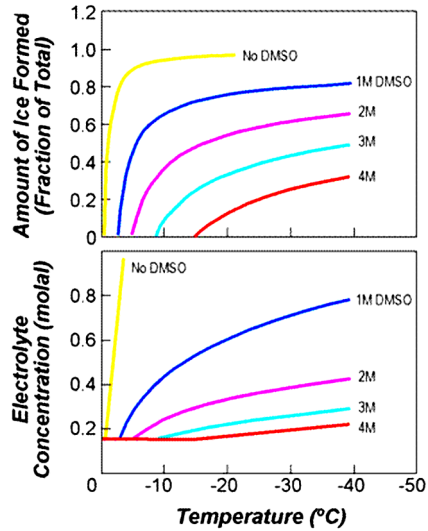


FIGURE 3. Amount of ice and electrolyte in frozen DMSO solutions.

of DMSO that cells can tolerate varies by cell type. Often the amount of DMSO or other penetrating cryoprotectants required to prevent electrolyte damage during slow cooling is lethal to cells.

The end result of a slow-freezing process is one in which the cells are shrunken and trapped in the unfrozen fraction of the extracellular media. With the addition of penetrating cryoprotectants, the interior of cells are usually in a glass-phase solid or in a partially crystallized glassy matrix. During warming the cells are rehydrated as the ice melts. In general the cell survival for slowly frozen cells does not depend strongly on the rate at which they are warmed.[23] Unfortunately most cells die when they are frozen too slowly.

FAST-FREEZING INJURY

In order to avoid solute effects and excessive cellular shrinkage one can freeze the cells more quickly. Since the cellular membrane resists water transport, water can be retained inside cells during cooling if one cools the cells at a high rate. FIGURE 4 illustrates the two possible outcomes for frozen cells: if the cooling is slow, then the cells dehydrate and ice is formed only outside the cells, but if cooling is fast, intracellular water will be retained and freezing will occur both inside and outside the cells. Many studies have shown that the presence of ice outside the cell promotes ice formation inside the cell.[29–31] FIGURE 5 illustrates the two mechanisms that can lead to ice inside a suspended cell. The precise mechanism by which ice outside the cell promotes ice formation inside the cell is unknown: three widely held theories are (1) that ice manages to propagate through pores in the cell membrane; (2) that ice on one

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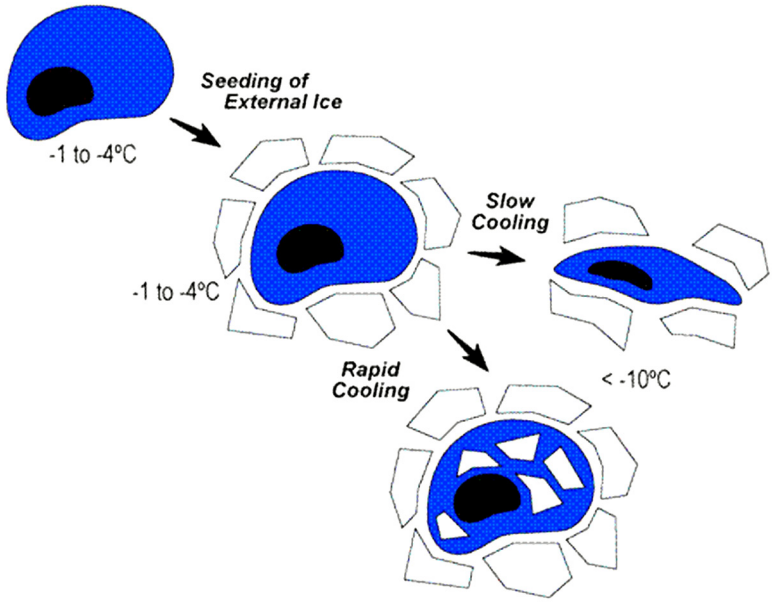


FIGURE 4. Schema of the key events during freezing of cells. Typically, the extracellular ice is seeded between −1 and −4°C, and then the cooling process starts.

side of the membrane causes conformational changes in the membrane or on the water just inside the membrane that make ice formation more likely; or (3) that the cell membrane is damaged by the formation of extracellular ice and ceases to be a barrier to ice propagation into the cell.[25,30,31] Whatever the mechanism, it is clear that ice forms at much higher temperatures inside the cell if there is ice already present outside the cell.

Whether or not ice is formed inside the cell is more complicated when dealing with tissues rather than with suspended cells. Studies of intracellular ice formation (IIF) for confluent mammalian cells in culture have shown that ice nucleated in one cell can propagate into adjacent cells.[32,33] Recent studies by Irimia and Karlsson have enabled quantification of the rate of intracellular ice propagation from one cell to another.[34] Using microfabrication they were able to precisely control the degree of cell–cell interaction experienced between cells and to study its effect on ice formation. They found that cell–cell interactions did not affect the rate at which ice nucleation occurred within an independent cell, but that intracellular ice did propagate from one cell to another. The rates of cell-to-cell ice propagation were greater for cells that had been in contact with each other for longer periods of time. These studies suggest that intracellular ice is much more likely to form in complex tissues than in suspended cells, because once ice forms in any cell of the tissue that ice can propagate into adjacent cells. This means that freezing protocols that seek to prevent intracellular ice formation may be very difficult to implement for large tissues.

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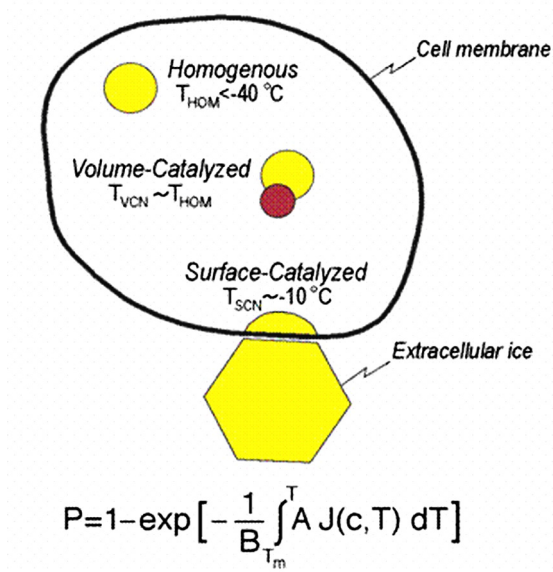


FIGURE 5. Schematic of multi-modal mechanisms of ice nucleation in cells.

A hydrated cell, therefore, will experience ice formation inside the cell during freezing. Most cryobiologists use as a working hypothesis that intracellular ice kills cells. In almost all studies it has been found that if cells are frozen fast enough to form intracellular ice, the cells die. This leads to the famous inverted "U" curve for cell survival versus cooling rate which is illustrated in FIGURE 6. The figure illustrates Mazur's two-factor hypothesis[35]: freezing too slowly kills cells owing to one effect and freezing too quickly kills cells owing to another, but cooling just right may get them to live.

FIGURE 7 illustrates the cooling rates for a wide variety of cells that lead to 50% cell survival and 50% of the cells having intracellular ice. One can see that the cooling rates necessary to get 50% survival vary by three orders of magnitude, and that there is a very strong correlation between the 50% IIF points and 50% survival points. Data such as these clearly suggest that IIF is lethal.

Most successful fast-freezing protocols require that the cells be dehydrated by some means prior to initiating fast freezing so that intracellular ice can be avoided.[36] Two-step methods rely on the seeding of extracellular ice. In this step an ice crystal is formed at a particular temperature by adding an ice crystal, an ice nucleator, or simply touching the sample with something small and very cold. This causes ice to form at a precise temperature rather than at a randomly determined temperature and allows the extracellular ice to cause cellular dehydration so that intracellular ice can be avoided. Optimal freezing protocols cool the cells quickly enough so that some water is retained inside the cell in order to avoid slow-freezing damage, but slowly enough for some cellular dehydration to occur so that intracellular ice will not form.

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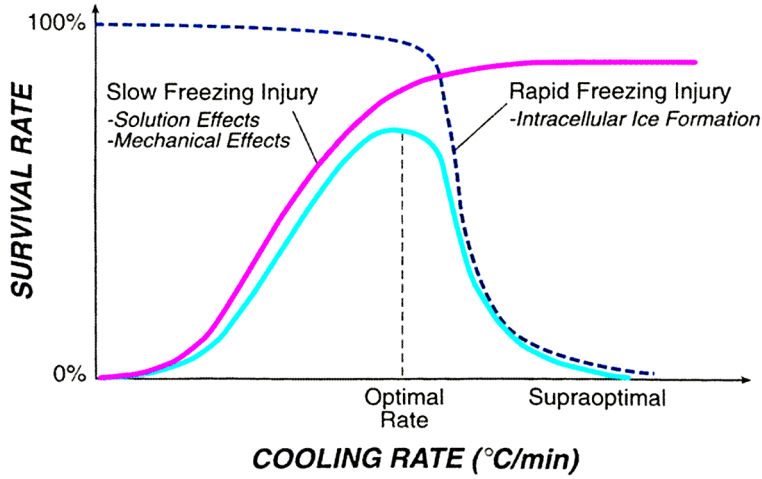


FIGURE 6. Inverted-U hypothesis of cell injury during freezing of cells.

For highly permeable cells such as erythrocytes, the optimal cooling rate is more than 1000°C/min, but for relatively impermeable stem cells the optimum is about 1°C/min.

Determination of optimal cooling rates has been a highly fruitful area of research both empirically and theoretically.[37] The original concept for defining an optimum was proposed by Mazur[36] as the fastest cooling rate possible that would not promote intracellular ice formation. He proposed that intracellular ice would not form if the cell had less than 10% of its initial water when it entered the ice nucleation zone of temperature. Bischof and co-workers refined these values to 5% of the initial water content when the cell reached −30°C.[38] On the basis of this definition and water transport equations originally developed by Mazur,[36] Thirumala and Devireddy developed a theory for determining optimal cooling rates for cell survival.[38] They found they could predict optimal cooling rates that agreed very well with experimental values for a wide variety of cells solely based on knowledge of the cell membrane permeability and the cell's initial surface area to volume ratio. This provides strong evidence that the main sources of cellular injury during freezing are a result of the kinetics of membrane water transport. If too little water is removed, intracellular ice forms, and if too much is removed, the cells die from dehydration effects.

The role of cryoprotectants in preventing injury during fast cooling is very different from their role in slow freezing. Non-penetrating cryoprotectants can be used to partially dehydrate the cell prior to freezing, thereby reducing the chances that ice will nucleate in the cellular interior. Cryprotectants can raise the glass-phase transition temperature so that the cells have a greater chance to reach glass phase without nucleating intracellular ice.[39] Cryoprotectants also change fluid properties and can thereby directly reduce the chances for ice nucleation.[40,41,36]

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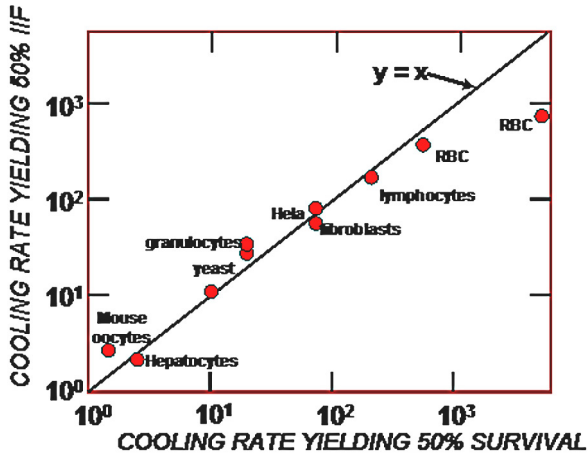


FIGURE 7. Relationship between the cooling rate yielding 50% survival and the cooling rate yielding 50% intracellular ice formation (IIF).

While it is extremely clear from countless studies that the formation of intracellular ice usually leads to cell death, it is not at all clear that the formation of intracellular ice causes cell death.[42,43,44] When one observes cells that have been frozen very rapidly so that intracellular ice has formed, one sees cells that look perfectly intact, although they are encased in ice. During warming, many cells with intracellular ice suddenly lyse. Many studies indicate that the survival of rapidly frozen cells, cells that have been cooled at rates faster than their optimum, depends strongly on the rate of warming.[22,45–48] The faster the cells are warmed, the more of them survive. A lot of evidence seems to suggest that the lethal damage caused by intracellular ice is not caused when the ice is formed, but by some process that occurs if intracellular ice is present as the cell is warmed.[10,49,50] An elegant set of studies by Rall *et al*. showed that damage caused by IIF in rapidly frozen and slowly warmed mouse embryos was not inflicted until the mouse embryos were warmed to a temperature of about –65°C.[51,52] This was after the point at which ice recrystallized from vitrified regions of the sample at about –85°C. No mechanism has been precisely identified for this damage during rewarming, but cells in which IIF could be clearly seen at –85°C could be successfully recovered if they were warmed rapidly through the critical –65°C range. This proves that the formation of IIF during warming was not the lethal event, and that IIF is not necessarily lethal.

The mechanism by which intracellular ice damages cells is not clear. A complete review of the literature relating to the damage caused by intracellular ice is provided by Muldrew *et al*.[53] If the damage actually occurs during warming and not during freezing, then the most likely mechanism involves recrystallization, as proposed by Mazur.[24] When ice is formed intracellularly during rapid freezing, many nucleation sites are formed, as there is not much time during freezing for crystal growth. The frozen cell is filled with many tiny crystals. During warming the small crystals

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reorganize into larger crystals, which may damage organelles or other cellular structures. How quickly this happens is not known; but if lethal damage to cells with intracellular ice occurs during warming it is probably the formation of large crystals inside the cell that leads to the damage. Indirect evidence in support of the theory that recrystallization may play a major role in cellular injury is provided by naturally freeze-tolerant organisms, some of which generate recrystallization inhibitors in response to freezing stress.[54,55]

Some of the newest and most interesting findings involving intracellular ice come from a series of papers by Acker *et al*.,[32,56–58] who show that intracellular ice propagates from cell to cell in confluent layers of V79W cells, and that although cells that nucleate intracellular ice die, cells into which ice propagates from an adjacent cell do not die. A mechanism for why this would be true has not been determined. One speculation is that nucleated ice may form in all regions of the intracellular space, whereas propagated ice may form in specific non-lethal regions. Another possibility is that some membrane-damaging event leads to the initiating ice nucleating events. Whatever the mechanism, these papers indicate once again that not all intracellular ice is lethal.

SUMMARY OF CELLULAR INJURY DURING CRYOPRESERVATION

If ice can be avoided it appears that cellular injury can be avoided. Unfortunately the solutes and chemicals that prevent the formation of ice are typically toxic at high concentrations. Ice can also be avoided by very rapid cooling, but the cooling rates required to vitrify pure water or unadulterated cytoplasm seem impossible to achieve for samples that are larger than a single cell. The study of cellular injury during vitrification, therefore, is the study of limiting toxicity and osmotic stress caused by cryoprotectants in order to enable vitrification at achievable cooling rates. A nontoxic penetrating glass-former would be the ideal solution.

The type of cellular injury induced during freezing is a function of water transport. Slow-freezing injury definitely results when electrolyte concentrations reach damaging levels as the formation of extracellular ice dehydrates the cell. There are also damaging effects that seem to result from the constriction of cells into the small unfrozen fraction that can exist during some freezing protocols. Penetrating cryoprotectants can reduce both of these damaging effects, but as in the case of vitrification, the cryoprotectants are toxic. Slow freezing and vitrification are almost identical from a physiochemical perspective. In both cases the cells do not experience freezing and they end up in a vitrified state. The difference is that slow freezing uses the solute-exclusion property of ice to drive up solute concentrations inside and outside the cell. Vitrification protocols add more cryoprotectant, both penetrating and nonpenetrating, to control the dehydration and solute concentration increase.

Cellular injury that results from intracellular ice formation is not well understood. It is clear that the formation of intracellular ice usually leads to cell death; but that damage can be mitigated by warming very rapidly. In addition there is a lot of evidence that the lethal damage occurs owing to recrystallization during warming, not during ice formation, which would explain why very rapid warming might mitigate the damage. No understanding yet exists for why ice that propagates from adjacent cells in cell monolayers is not damaging and no one knows what it is about

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recrystallization that causes the damage. A possible future for cryopreservation research may lie in the development of cryopreservation protocols that manage intracellular ice so that it is non-lethal, rather than attempt its prevention. If we could understand more fully why some intracellular ice is damaging and other ice is not, this may become possible.

EMERGING TECHNIQUES IN BIOPRESERVATION

Efforts continue to avoid intracellular ice without damaging the cell. One type of glass-former that is relatively non-toxic is sugar. Many sugars are excellent glass-formers, and cells tolerate them to a high degree. Unfortunately most sugars are non-penetrating, which means they cannot promote glass formation inside the cell, nor prevent the concentration build-up of electrolytes within the cell during freezing. Techniques to get non-permeating sugars inside cells, however, have been developed recently,[59,60,61] and they have been very successful in promoting cell survival after freezing even at concentrations low enough that the cells can be cultured without removing the cryoprotectant.[62,63] A non-metabolizable glucose has also been developed that is actively transported inside the cell by the glut transporters. This sugar (3OMG) has also provided substantial survival benefit during freezing at relatively low concentrations.[64]

Freezing under high pressure has long been recognized as a possible method for decreasing ice nucleation.[65] A recent study by Rubinsky and co-workers has revisited the idea of using isochoric (constant volume) freezing to increase pressure in the system as ice forms.[66] He has shown that by means of this method the solute concentrations as a function of temperature can be decreased by almost an order of magnitude. This would allow for the use of significantly less cryprotectant in slow-freezing protocols.

Finally, the recognition that slow-freezing protocols are actually a method for dehydrating cells, in combination with the observation that many natural organisms survive dehydration, has led to a flurry of research into dried preservation of cells and tissues.[61,67–71] In these studies glass-forming sugars are introduced into the cells by means of a variety of techniques before the cells are air- or freeze-dried.[67] Research into the mechanisms of cellular injury for cells during drying and storage has just begun.

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