

REVIEW ARTICLE

# Cryopreservation of precision-cut tissue slices

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## Abstract

1. Cryopreservation of precision-cut tissue slices (PCTS) would have many advantages for drug development and would encourage more extensive use of the PCTS preparation.
2. Three methods have been studied to date: slow freezing, fast freezing, and vitrification.
3. Slow freezing can be very effective for some PCTS but is devastating to rat liver PCTS. Fast freezing can be successful for rat liver PCTS but is devastating to renal PCTS and has given inconsistent results even for rat liver PCTS. Vitrification has been effective for some slice systems but less effective for rat liver PCTS. Rat liver PCTS appear to be particularly difficult to cryopreserve well.
4. The general cryobiological principles of slow freezing, rapid freezing, and vitrification are reviewed. The empirical literature on the cryopreservation of PCTS has not taken sufficient account of these principles, and may, for example, include the effects of easily preventable osmotic injury.
5. More attention is needed to the effects of cryopreservation on specific cell types within PCTS and to the general integrity and viability of cryopreserved PCTS. Drug metabolism as a sole endpoint of study can be highly misleading.
6. Better application of cryobiological principles may enable improved results in the future.

**Keywords:** Tissue banking, osmotic stress, cooling rate, warming rate, chilling injury, thermal shock, fracturing, viability assays

## Introduction

### Advantage of precision-cut tissue slices in drug development

Successful drug discovery requires drug screening models that mimic, as effectively as possible, the *in vivo* response to drugs. *In vivo*, cells that come in contact with drugs are generally both viable and functioning in a three-dimensional context that includes important interactions with other cells, and it is well-known that cell-cell interactions play an important role in drug toxicity (Boess et al. 2003). In contrast, much screening work currently is performed, out of necessity, on frozen-thawed hepatocytes that have neither long-term functionality nor normal relationships with other cells and the extracellular matrix. This represents a compromise to drug screening that may be costly to the industry in both money and time.

Precision-cut tissue slices (PCTS), in contrast, highly resemble the organ they are prepared from, as they largely retain the organ architecture, the cellular heterogeneity and the cell-cell and cell-matrix communications found *in vivo* (Lerche-Langrand & Toutain 2000; de Graaf et al. 2010). PCTS from liver, intestine, kidney, and lung from different species have been widely used as *in vitro* models to study the pharmacology (Moreno et al. 2006; van de Bovenkamp et al. 2006), toxicology (Freeman & O'Neil 1984; Fisher et al. 1994; Parrish et al. 1995; de Kanter et al. 2002; Groneberg et al. 2002; Brandon et al. 2003; Vickers & Fisher 2005; Plazar et al. 2007; Elferink et al. 2008) and metabolism of drugs as well as the pathology of disease (de Kanter et al. 2005; van de Bovenkamp et al. 2005; Carranza-Rosales et al. 2010; Groothuis & de Graaf 2012). The preparation and incubation of PCTS, and especially of liver, intestine, kidney and lung PCTS,

has been developed and optimized for decades (de Graaf et al. 2010). Due to the size of slices (e.g. about 250  $\mu\text{m}$  thickness and 5–8mm diameter for liver slices) and the availability of specially-designed commercially available slicers (de Graaf et al. 2010), it is easy to make a large number of slices (e.g. more than 150 slices from one rat liver) from different organs in a short period of time. Thus, several drugs can be studied in organs from the same animal at the same time, thereby reducing the influence of inter-individual differences. In addition, tissue slices from different organs of the same animal have been used to investigate inter-organ interactions (van Midwoud et al. 2010).

The remaining contributions to this special issue provide more details about the advantages of this model, but given general awareness of the theoretical advantages of tissue slices as facilitators of the drug discovery process, it is worth considering why this model is not more popular. It is our conviction that the availability of high quality tissue slice banks would present advantages that could overcome reluctance to use this theoretically superior *in vitro* screening tool.

### Potential advantages of banked PCTS for drug development

The ability to bank fully functionally normal tissue slices would enable significant improvements in laboratory efficiency, would offer the possibility of greater industrial standardization of testing models, would significantly facilitate inter-species comparisons, and, most importantly, would have the potential of enabling far more research on human tissue, both normal tissue and tissue representing numerous specific disease states. Human tissue is scarce and its availability is unpredictable, but when available the amount of tissue is often far beyond the capacity of a single laboratory to process at one time. A single human liver, or even just one lobe of a human liver, for example, could potentially be divided into very large numbers of slices that could be preserved and used for multiple experiments over weeks, months, or years, providing both a long-term reference standard and extensive access to human tissue that otherwise would simply not be possible. The greater accuracy of prediction enabled by human tissue could also be extended by the ability to perform inter-organ-interaction studies with human tissue, something that is effectively impossible without banks of cryopreserved tissues of different kinds. Banked slices would also enable comparisons to be made between human and animal tissue earlier in the drug discovery process.

Fully viable banked organ slices from human and animal sources would also enable routine studies in academic and industrial research laboratories and the use of fewer laboratory animals for drug discovery or even basic biomedical research studies, since a single animal could contribute slices from multiple organs, all of which could then be used at a later time by widely separated

laboratories. Furthermore, the successful commercial development of cryopreservation protocols for PCTS would result in industrial standardization and characterization of banked PCTS in a way that is not likely when many different and independent laboratories are preparing their own material in their own way.

In summary, banked PCTS, available to be drawn upon at any desired time, should provide a reliable resource that would facilitate comparisons between different species, tissues, and laboratories and should eliminate bottlenecks due to variations in animal and human tissue supply and the costs and turnover of personnel skilled in preparing tissue slices.

### Cryopreservation by freezing and by vitrification

Past studies of PCTS or non-precision cut tissue slice cryopreservation have employed both freezing and vitrification. Both methods have been able to give encouraging results, and both continue to evolve. A general consensus has not yet been reached as to which method is most appropriate for drug development purposes, so the basic aspects of both approaches are now briefly reviewed. Our aim in the following review is to consider the subject from a broad perspective that illuminates not just empirical results but also the general cryobiological context of the problems at hand.

#### Effects of freezing on organized tissues

The traditional method of tissue banking relies on freezing, which is the conversion of liquid water into ice. The effects of freezing can act on both the cellular level and the supra-cellular level represented by organized tissues.

#### Freezing and the single cell

The formation of ice in a biologically relevant solution normally begins in the extracellular space because ice formation under normal conditions is catalyzed by impurities (heterogeneous nucleating agents) that are ubiquitous in the extracellular compartment but absent or greatly inhibited inside cells (Rasmussen et al. 1975; Franks et al. 1983). The extracellular nucleation and growth of ice crystals sequesters water into the growing ice mass, but the remaining components of the solution are not incorporated into ice and are therefore concentrated into a smaller total volume of unfrozen liquid water that remains (Lovelock 1953). These concentrated solutes then exert an osmotic effect on the cells between the ice crystals, causing them to lose water and shrink (Mazur 1963). Although the precise mechanisms by which these events lead to cell damage and death remain to be elucidated, the best correlations between the extent of ice formation and the extent of cell damage have been obtained by linking the injurious event to shrinkage of the cells to below a critical threshold (Lovelock 1953; Meryman 1970; Meryman 1974; Meryman et al. 1977; Fahy 2007).

The inclusion of a cryoprotective agent (CPA) that can enter the cell before freezing (referred to as a penetrating CPA or pCPA) depresses the freezing point and resets the isotonic osmolality to a higher value, and for these reasons limits both ice formation (Figure 1A) and the extent of cell shrinkage in response to ice formation in the extracellular space (Figure 1B) and therefore reduces or prevents cell injury and death. Shrinkage also concentrates intracellular osmolytes, thus depressing the freezing point of the cytoplasm and organelles and preventing intracellular supercooling (cooling to below the thermodynamic freezing point in the absence of freezing).

However, cell shrinkage, while rapid, is not instantaneous, and for that reason, it is inhibited as the cooling rate increases and the time for shrinkage is thereby reduced. To a certain extent, this effect is protective, since less shrinkage generally results in less cellular injury (Fahy 2007). However, failure to shrink can result in extensive intracellular supercooling, and this supercooled intracellular water is liable to nucleation by any one of several potential mechanisms, resulting in intracellular ice formation (Mazur 1963), which is often lethal. These phenomena result in the existence of an optimal cooling rate that limits shrinkage on the one hand and minimizes intracellular ice formation (IIF) on the other (Mazur 1970, 1984). Cooling rates that are below the optimum are said to represent “slow” freezing and cooling rates that result in IIF are said to represent “rapid” freezing (Mazur 1970).

The use of pCPAs is the main remedy, along with choice of the cooling rate, for freezing injury on the cellular level. However, this strategy is limited by the toxicity of higher concentrations prior to freezing as well as by the fact that protection at higher concentrations tends to be less complete than predicted on the basis of cell shrinkage responses at higher temperatures (Fahy 1986, 2007). This may be due either to toxicity as the cryoprotectants are concentrated to lethal levels in the frozen state (Figure 1C) or to less robust tolerance of shrinkage at lower temperatures, where the membrane is expected to be more viscous and brittle and may become less tolerant of distortion. Other factors, such as damage due to cooling per se (chilling injury or thermal shock; see below), may also enter into the final outcome as well.

#### Freezing on the tissue level

These hazards are significant enough on the single cell level, but on the level of organized tissue, additional factors become important. Tissues are composed of cells and extracellular materials that are all related to one another in specific patterns and by specific molecular connections, all of which are important for the organized function of the tissue and none of which evolved to withstand subtraction of large volume fractions of water. As cells shrink, they inevitably exert forces on one another and on their attachments to extracellular structures, and although most tissue structures can withstand most of these forces to one degree or another, damage is

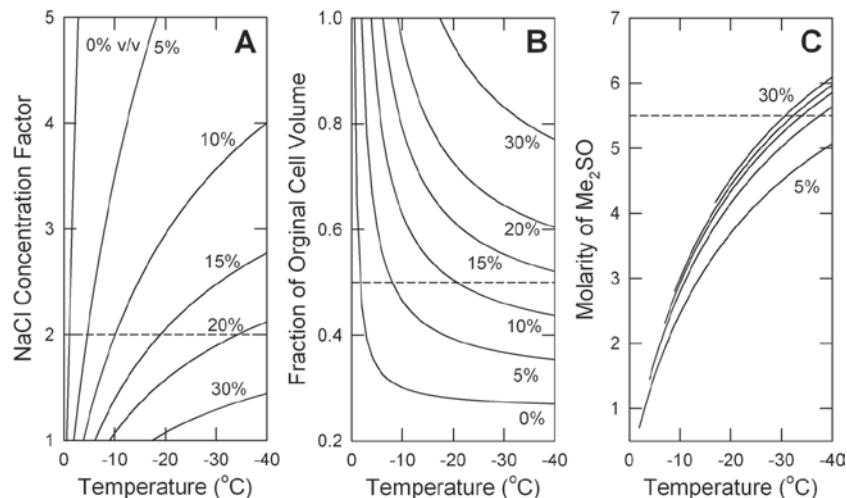


Figure 1. The basis of cryoprotection and the hazard of cryoprotectant toxicity in the frozen state. (A) Suppression of the molar concentration of extracellular non-penetrating solute (modelled as NaCl) during freezing by adding different concentrations of pCPA [modelled as dimethyl sulfoxide (Me<sub>2</sub>SO)] before freezing. The factor by which the extracellular salt concentration is multiplied on a molar basis during equilibrium freezing is equal to the factor by which the liquid volume of the extracellular solution is reduced, and is shown as a function of temperature at different pre-freezing concentrations of Me<sub>2</sub>SO (indicated in percent by volume units next to each curve). A PCTS that is injured primarily by exposure to salt concentrations two or more times greater than normal (threshold indicated by the dashed line) might be expected to experience injury at about -1°C without Me<sub>2</sub>SO, -35°C with 20% Me<sub>2</sub>SO, and at no temperature when 30% v/v Me<sub>2</sub>SO is used. (B) Suppression of equilibrium cell shrinkage by pCPA. The assumed non-osmotic volume of the cell is 25% of the initial cell volume, and the curves are derived from A. A hypothetical PCTS whose cells can tolerate shrinkage to only 50% of their normal volumes should remain injury free down to about -20°C when protected with 10% v/v Me<sub>2</sub>SO and to below -40°C with 15% v/v Me<sub>2</sub>SO. (C) The concentration of Me<sub>2</sub>SO in the unfrozen liquid phase (both intracellularly and extracellularly) during equilibrium freezing. As temperature falls, Me<sub>2</sub>SO concentrations rise and may reach toxic levels in some cases. Hypothetically, a PCTS injured by exposure to 5.5M Me<sub>2</sub>SO independent of exposure temperature would be expected to be damaged only below -40°C when protected with 5% v/v Me<sub>2</sub>SO, but at about -30°C when pre-treated with 30% v/v Me<sub>2</sub>SO. Panel A modified from (Takahashi & Fahy 2012). All data calculated using methods described elsewhere (Fahy 1980a).

still produced. In addition, ice growth can distort tissue architecture independently of osmosis, and ice formation in a confined and dilute space can exert a pressure of as much as about 190 MPa (1 900 atmospheres, or 28 thousand psi) on surrounding structures (Rubinsky et al. 2005). Generally speaking, the distortion of organized tissues in the frozen state, as visualized by freeze substitution studies, is so extreme as to be potentially shocking to those who first see such images (Meryman 1958; Fahy 1981; Brockbank & Song 2004; Pegg 2010), even though, upon thawing, structural changes may not be particularly noticeable upon casual examination (Meryman 1958). Given these problems, it is not surprising that slow freezing methods for rat liver PCTS have yielded worse results than the same methods applied to isolated hepatocytes (Maas et al. 2000b).

As in the case of single cells, these freezing-induced distortions can be reduced in tissue by the use of one or more cryoprotective agents. The same limitations of use of cryoprotectants apply on the tissue level as they do for single cells, but in addition the larger dimensions of tissue slices introduce greater obstacles to the successful addition and subtraction of the cryoprotectant without the induction of osmotic injury from the agents themselves.

Tissue freezing also faces the fact that the different cells within a complicated tissue vary in both their water permeability and their cryoprotectant permeability from cell type to cell type and, potentially, in dependence on their specific relationships to other cells and extracellular structures. This introduces the concern that the optimum cooling rate for one cell type may differ significantly from that for another cell type in the same tissue (Mazur 1988), with the result that some cells may shrink too much and others may not shrink enough. In addition, some cells may have to be over-exposed to cryoprotectants before cooling to enable others enough time to take up these agents.

Despite these theoretical problems, dozens of different types of tissue can be frozen and thawed with results that have been considered satisfactory by the experimenter (Fahy 1980b). Even intact dog intestinal segments have been frozen in liquid nitrogen, thawed, transplanted, and survived (Hamilton et al. 1973; LaRossa et al. 1973), and dog livers (Zimmermann et al. 1971) and even lungs (Okaniwa et al. 1973) have shown some function after freezing to and thawing from  $-60^{\circ}\text{C}$  or  $-25^{\circ}\text{C}$ , respectively. The fact remains, however, that the latter temperatures are not low enough for long term storage (which generally should be at  $-130^{\circ}\text{C}$  or below (Taylor et al. 2004)), and the intestinal segments, while able to repair themselves, were initially damaged far beyond what would be preferred for drug development studies (Hamilton et al. 1973).

For drug discovery and development purposes, the requirements for successful freezing can frequently be less stringent than the requirements for quality survival of the PCTS, and the literature indicates that enzymatic

activity relevant to drug metabolism can persist reasonably well after freezing and thawing (see below). However, qualitative and quantitative changes in the total and relative amounts of drug and substrate metabolizing activity have often been reported (Glöckner et al. 2001; Vanhulle et al. 2001; Vittorelli et al. 2004; Panoutsopoulos 2005; Panoutsopoulos & Beedham 2005), and this is clearly undesirable and could be misleading.

Such discrepancies as well as the ability to assess the direct toxic effects of specific drugs could presumably depend on injury caused by the preservation process, so the gold standard should be a PCTS that resembles fresh tissue to a high degree of fidelity. To date, however, that standard remains to be established, and viability and metabolic capacity after freezing and thawing can at best only be maintained for about 6 h (Glöckner et al. 1998; Maas et al. 2000a). Therefore, there is significant incentive to find a better method of cryopreservation.

### Theoretical advantage of vitrification over freezing for organized tissues

Vitrification is a type of cryopreservation that avoids the formation of ice (Fahy et al. 1984). By replacing a sufficient amount of water with cryoprotectants, cooling results in glass formation rather than ice formation once the temperature reaches the glass transition temperature ( $T_g$ ) of the system. A glass is simply a liquid that is so cold that it is unable to change its structure significantly over time scales that are of interest to the observer. By simply immobilizing cells in a glassy state, the problems of potentially lethal cell shrinkage, tissue distortion by ice, the risk of IIF, and the need to search for compromise optimal cooling rates for mixed populations of cell types, all of which are intrinsic to freezing techniques, are eliminated. As long as cooling and warming are fast enough to avoid ice formation (i.e. faster than the critical cooling rate and the critical warming rate, respectively), careful control of cooling and warming rate is generally unnecessary, although cooling near  $T_g$  may need to be slowed in some cases to avoid fracturing of the sample (de Graaf et al. 2007), and overly rapid cooling could be undesirable in principle for systems susceptible to thermal shock (see next paragraph).

Cells that are sensitive to cooling per se may also be better preserved by vitrification. With vitrification, the ability to cool rapidly without IIF enables some cells such as oocytes to be cooled ultra-rapidly and thereby “out-run” injury otherwise caused simply by brief exposure to low temperatures, which is known as chilling injury (Martino et al. 1996). There is on the other hand another form of cooling injury that is caused more by high rates of cooling than by exposure to lower temperatures per se, and this is known as thermal shock or cold shock (Takahashi & Williams 1983). Although it may or may not be possible to “out-run” thermal shock, the ability to cool at low rates and still avoid ice formation, which was introduced in the early 1980s as a method for cryopreserving whole mammalian organs by vitrification (Fahy & Hirsh



1982; Fahy et al. 1984), potentially offers a method for avoiding thermal shock as well. Therefore, if vitrification is sufficiently robust, it has the potential of allowing the avoidance of both ice-related and cooling-related forms of cryopreservation injury.

## Obstacles to successful vitrification

### *Cryoprotectant toxicity*

The most obvious obstacle to vitrification is cryoprotectant toxicity. Vitrification in comparison to freezing requires replacement of much larger quantities of water with chemical agents, which is intrinsically hazardous. The biochemical effects of these agents have to date been poorly investigated under conditions that are germane to how these agents are actually used for vitrification, so most available studies are difficult to evaluate (for review, see Fahy et al. 1990a).

All common CPAs are relatively inert chemically, which presumably explains why they are as non-toxic as they are. However, glycerol can be phosphorylated by some cells, including hepatocytes and renal cortical cells, resulting in ATP depletion (Burch et al. 1970; Jans & Willem 1988). Ethylene glycol can also be metabolized by some cells, particularly hepatocytes, into reactive byproducts such as glycoaldehyde and glycolic acid (Booth et al. 2004; Leth & Gregersen 2005). Dimethyl sulfoxide ( $\text{Me}_2\text{SO}$ ) can also chemically react with sulfhydryl groups in tissues of all kinds (Snow et al. 1975; Fahy et al. 1995; Fahy 2010) and is known to have many pharmacological effects of its own (Karow 1974; Jacob & Herschler 1986; Fahy 2010). Propylene glycol is generally more toxic than glycerol, ethylene glycol, or  $\text{Me}_2\text{SO}$ , but its direct toxicity, too, is poorly understood.

Although cryoprotectant toxicity is inadequately understood, it can be controlled in a variety of ways. For example, all of the above biochemical problems are diminished by CPA administration at lower temperatures. To provide one chemical illustration of this point, although 3.1 M  $\text{Me}_2\text{SO}$  can convert dissolved cysteine to insoluble cystine relatively quickly at room temperature or body temperature, this reaction remains 99% incomplete even after 1 h at 2°C (Fahy et al. 1995), and 1 h is a long exposure period for a vitrification protocol.

Other common methods for controlling toxicity include optimizing the temperatures of administration of various steps of concentration increase or decrease, optimizing exposure times to attain vitrification without exceeding safe exposure limits, and using the minimum possible concentration consistent with preventing or minimizing ice formation at the fastest possible cooling and warming rates. In addition, the use of specific mixtures of pCPAs has reduced toxicity (de Graaf 2003; Fahy et al. 2004a, 2004b; Kuwayama et al. 2005) by mechanisms discussed in more detail below.

By definition, pCPAs have access to intracellular sites that may be vulnerable targets for toxic effects, but vitrification can also be aided by the inclusion of non-penetrating CPAs (npCPAs), which do not have the same

access and therefore tend to be far less toxic. Because cells contain proteins and other water-perturbing molecules such as DNA, all of which contribute to vitrification tendency, cytoplasm can vitrify even when cells are exposed to a pCPA concentration that, by itself, does not entirely vitrify. This effect can be exploited by including npCPAs to make the total vitrification tendency extracellularly sufficient to permit vitrification, the npCPAs effectively balancing the effects of natural intracellular solutes (Fahy et al. 1984).

A particularly powerful and novel example of the use of npCPAs is the use of npCPAs that are able to specifically block ice nucleation and crystal growth in a non-colligative (chemically specific) manner in addition to reducing ice formation in the usual colligative manner (see below for more details). The use of these “ice blockers” significantly reduces the amounts of other CPAs required for vitrification (Wowk et al. 2000; Wowk & Fahy 2002; Fahy et al. 2004b).

### *Devitrification*

Vitrified solutions freeze upon rewarming (devitrify) much more rapidly than liquid solutions freeze during cooling (de Graaf & Koster 2001; Fahy & Rall 2007), a fact that is often overlooked. Devitrification can often be survived (Fahy 1987; Fahy & Rall 2007), but tends to be lethal if warming rates are not high (Rall & Fahy 1985; de Graaf & Koster 2001). Some vitrification solutions are so concentrated that they make devitrification relatively easy to avoid (Fahy et al. 2004a, 2004b), but most laboratories prefer to use more dilute solutions to minimize toxicity, and therefore devitrification should always be kept in mind as a possible limiting factor for complete recovery.

### *Fracturing*

A glass is unable to quickly relieve thermal contraction stress by changing its structure, and therefore rapid cooling or warming through  $T_g$ , which leads to large contraction and expansion stresses, respectively, can cause cracking (fracturing or shattering) of the glass and of any tissue embedded in it (Fahy et al. 1990b; de Graaf et al. 2007). The fracturing of vitrified PCTS was reported to reduce them to small fragments (of undoubtedly non-uniform size) and to be associated with a loss of viability (de Graaf et al. 2007). Even without a loss of viability, fragmentation would defeat the purpose of making precision-cut as opposed to hand-cut slices in the first place, and render the slice fragments awkward or impossible to evaluate (to get an impression of the intensity of fracturing that can take place, see (de Graaf et al. 2007) and (Fahy et al. 1990b)). It was not established whether fractured slices were injured by a direct effect of fracturing itself, by measurement/assessment difficulties as noted above, or by an effect of high cooling rate unrelated to fracturing. However, a direct negative effect on viability is certainly possible because, in addition to cutting more cells directly, fracture faces are strong ice nucleating sites (Williams & Carnahan 1990). Fortunately, fracturing can

be avoided even in a 10-gram organ by careful cooling and warming near and below  $T_G$  (Fahy 2005).

#### ***Thermal contraction and expansion within closed containers***

A relatively unexplored potential obstacle to vitrification is the fact that the coefficient of thermal expansion (or contraction) of vitrification solutions is quite significant (Fahy et al. 1990b; Eisenberg et al. 2012), which implies that cooling macroscopic samples in rigid and closed containers that do not include a substantial air space will generate large negative pressures (tension) within the sample as the sample attempts to but is not permitted by the container to contract toward its equilibrium volume even at temperatures well above  $T_G$ . The concern is that under some conditions, the tension developed might affect cells or tissues within the sample either directly or as a result of cavitation even when the strains involved are insufficient to lead to outright fracturing. Other forms of “thermomechanical” stress involving edge effects have also been suggested (Fahy et al. 2009), but in the context of PCTS, contraction and expansion within a confined space is the most relevant possibility. So far, no direct experimental data bear on this question, but it remains a question for future investigation.

#### ***Chilling injury***

As noted above, chilling injury is damage caused by cooling per se. It can take place in natural, non-cryoprotected systems cooled to sub-physiological temperatures that are still above zero Celsius (Sonna et al. 2002; Al-Fageeh et al. 2006), but it can also take place in the context of vitrification (Mazur et al. 1992), although the underlying mechanisms may be very different in these different contexts. A major problem with chilling injury is that little is known about it in systems relevant to drug discovery, and demonstrations of methods that can inhibit it are few in number. Possible mechanisms of, and remedies for, chilling injury are discussed below.

#### ***Osmotic injury***

The need for high concentrations of CPAs for vitrification introduces the hazard of inducing osmotic injury from overly rapid addition or removal of these agents. Osmotic injury arises because water crosses cell membranes far more rapidly than do pCPAs, and overly large step changes in pCPAs can therefore cause excessive cell shrinkage during pCPA addition as intracellular water rushes out of the cell to dilute the extracellular pCPA and excessive cell swelling during pCPA elution as extracellular water rushes into the cell to dilute intracellular pCPA. To prevent these hazards, the rate of pCPA addition and removal needs to be consistent with the rate of permeation of the pCPAs across cell membranes.

There are few careful studies of the permeabilities of PCTS to different CPAs as a function of concentration and temperature, without which the addition and removal of these agents is an empirical process, the results of which may be confused with cryoprotectant toxicity. However,

the permeability of rabbit and dog renal cortical slices to glycerol, ethylene glycol (EG),  $\text{Me}_2\text{SO}$  (Fahy 1980b; Clark et al. 1984; Tsonev et al. 1994), and 2,3-butanediol (Tsonev et al. 1994) has been studied in detail, and permeability to formamide, acetamide, N-methylformamide, propylene glycol, and dimethylformamide was studied more cursorily (Fahy et al. 1987). The behaviour of both EG and  $\text{Me}_2\text{SO}$  showed peculiar features (Clark et al. 1984), but 20 min steps for exponential increases and decreases in concentration at 0°C based on these findings have evidently enabled osmotic stress to be avoided even at concentrations relevant to vitrification (Fahy 1984; Fahy et al. 1987; Fahy et al. 1990a; Fahy 2010).

In the absence of direct information, it is possible to investigate the role of osmotic injury empirically by determining how overall injury responds to changes in the size of applied concentration steps and/or to the chosen exposure times at each step. If smaller concentration steps or longer exposure times at different concentrations reduce injury, this can be interpreted as a sign of osmotic injury with the previous protocol. Another sign of osmotic damage is a reduction in damage when an osmotic buffer is used. Osmosis is generally more hazardous during removal of pCPAs than during their addition due to the fact that cell swelling is generally less well tolerated than cell shrinkage (Meryman 2007). To protect against this problem, non-penetrating solutes such as sucrose, mannitol, or sometimes trehalose are generally used during pCPA washout to raise the extracellular osmolality and thus create a tendency for cell shrinkage to offset the tendency for cell swelling that is induced by removing pCPAs (Meryman 2007). Osmolytes used in this way are therefore sometimes called osmotic buffers, osmotic buffering agents, or osmotic antagonists.

One illustration of the empirical approach to evaluating osmotic damage is provided by a recent study by Guan et al., whose results suggest that, at least in the case of hepatic PCTS (PCLS), osmotic issues may be less significant than usual. Using a specially designed apparatus to gradually and continuously add pCPA to and then remove it from rat liver PCLS, it was found that despite reduced osmotic stress as compared to step-wise methods, viability as indicated by ATP content was not improved (Guan et al. 2012c). This suggested that rat PCLS are not very sensitive to osmotic changes, and based on this observation, the step-wise CPA loading/unloading protocol was successfully reduced from 8 steps to 4 steps.

Other investigators have made similar and even extreme observations of minimal osmotic responsiveness in liver PCTS, the most telling of which relate to the pCPA removal process. Despite the sensitivity of most cells to the osmotic effects of pCPA dilution and despite the general importance of osmotic buffering, it was possible to load human liver PCTS with 4.8M 1,2-propanediol in three 5 min steps at 0°C and to wash it out by exposure to CPA-free media without any osmotic buffer (nearly a 17-fold osmotic dilution factor, compared to survival of only a 2-fold effective osmotic dilution by many cells

(Meryman 2007)) and still at least retain significant metabolism of 7-ethoxycoumarin (Wishnies et al. 1991). Similarly, it was possible to recover 80% of cells within rat liver PCTS in a histologically intact state in comparison to controls (controls retained just 75% intact cells due to damage to surface cells owing to the sectioning process itself, and treated PCTS retained 60% intact cells) after loading a 7.5M vitrification solution (VS4) by initial exposure to 1.9M CPA (over a 7-fold increase over the initial tonicity of the medium) and unloading the solution using a final jump from 1.9M to 0M with no osmotic buffer (de Graaf & Koster 2001), which would again be an extreme osmotic stress for most cells or tissues.

Although hepatocytes appear to be extremely permeable to pCPA compared to most cells, PCTS of extra-hepatic organs should be exposed to more moderate steps of concentration and will often benefit from the use of osmotic buffers. However, over-use of such osmotic buffering agents can in principle lead to additional unnecessary injury because once the pCPA has been removed, inappropriately prolonged exposure to the osmotic buffer could cause cell shrinkage injury (hyper-tonic injury).

#### Processing steps

A purely practical obstacle to successful vitrification is the simple need to follow a protocol that will generally involve several discreet concentration steps or a continuous program of concentration increase and then decrease. While this is feasible in a cryobiological research laboratory, the process may be hazardous and time-consuming for non-specialists to carry out, and these drawbacks may reduce enthusiasm for adopting the protocol. The answer to this problem is to develop automated equipment that, at least in the laboratory of the end user, can rewarm the banked PCTS and remove the pCPAs in a quick, safe, and efficient manner, without direct technician involvement. Equipment of this nature is currently under development, and is being tested for perhaps the most delicate of all tissue slice systems, the adult hippocampal slice preparation, with encouraging results (Figure 2).

## Recent conceptual and technological advances

### Understanding cryoprotectant toxicity

#### Toxicity neutralization in different tissues

Our laboratories are pursuing the development of solutions for vitrification that are based on a number of relatively novel principles, the cornerstone of which is the phenomenon of toxicity neutralization. When certain comparatively toxic CPAs, particularly the amides formamide, urea, and to a lesser extent, acetamide, are combined with  $\text{Me}_2\text{SO}$ , their toxicity can be reversed or neutralized (Fahy et al. 1995; Fahy 2010), which enables them to contribute to the overall vitrification tendency of the solution without contributing as much to the

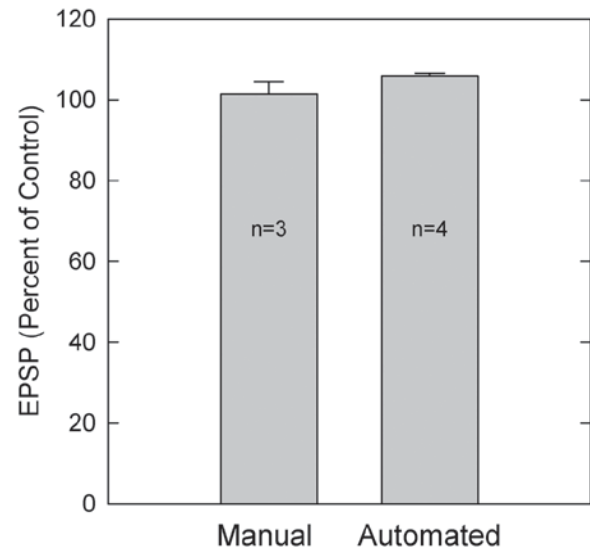


Figure 2. The ability of automated methods of CPA (VM3) addition and subtraction to achieve functional preservation of adult rabbit hippocampal slices equivalent to the function that could be obtained using manual methods based on recovery of normal excitatory post-synaptic field potentials (EPSP) upon normal electrical stimulation. Previously unpublished results.  $n$  = number of separate experiments.

toxicity of the solution as do more standard CPAs (Fahy et al. 2004a, 2004b; Fahy 2010). This phenomenon, known as cryoprotectant toxicity neutralization or CTN, is illustrated in Figure 3, and can allow total CPA concentrations of 50% w/v or even more to be tolerated with little or no toxicity. Of these amides, the most useful currently appears to be formamide, because it contributes more to vitrification tendency than does urea, does not have the very weak *in vivo* carcinogenicity of acetamide (Weisburger et al. 1969), and, having a molecular mass of only 45 daltons, penetrates kidney slices more rapidly than any other useful pCPA (Fahy et al. 1987).

CTN has been found in almost every cell type examined so far. Figure 4 shows previously unpublished examples for rabbit liver and brain slices, and murine osteoblasts have also been found to display CTN,  $\text{Me}_2\text{SO}$  reversing the toxicity of formamide (S.A. Unhale, V. Veerasamy, and J.J. McGrath, unpublished observations). However, CTN may not apply to human oocytes or early stage human embryos (S.F. Mullen, unpublished observations). As of the present time, the mechanisms of CTN remain unknown.

#### Non-specific toxicity depends on tissue hydration

By composing many different vitrification solutions to have widely different compositions but equal vitrification tendency (Fahy et al. 1987), it has been possible to identify a common variable that can explain the toxicity of most vitrification solutions, at least in rabbit kidney slices (Fahy et al. 2004a). The compositional variable,  $qv^*$ , is the ratio of the number of moles of water in the vitrification solution to the number of moles of hydrogen



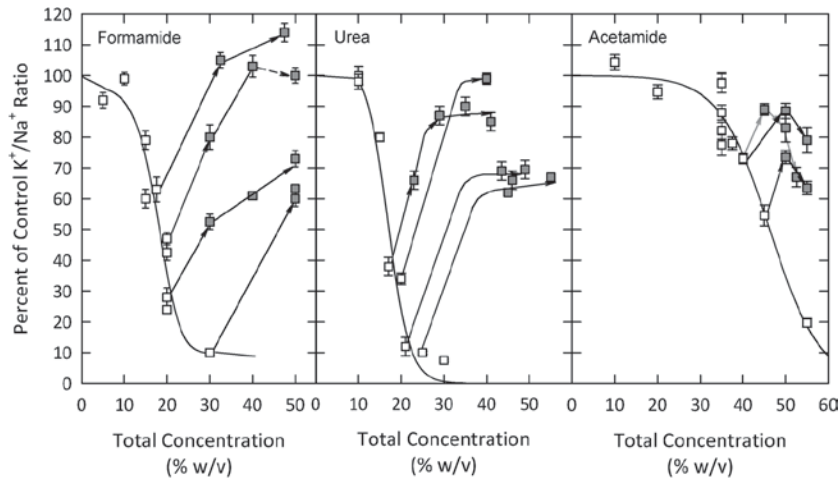


Figure 3. The phenomenon of cryoprotectant toxicity neutralization (CTN) in rabbit renal cortical slices. For formamide, urea, and acetamide, the toxicity of the amides alone (white points) can be reversed by addition of  $\text{Me}_2\text{SO}$  to result in much higher yet less toxic total concentrations (gray points, arrows). This principle has been used to develop highly concentrated vitrification solutions of minimal toxicity (Fahy et al. 2004a, 2004b). Modified and interpreted from curves in (Fahy 2010).

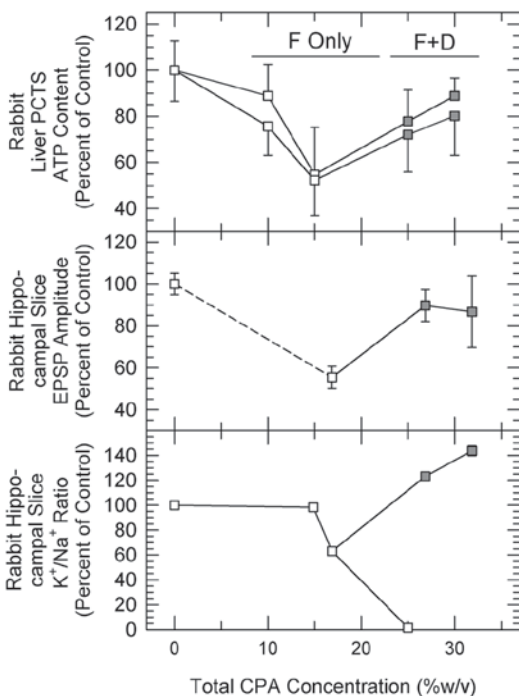


Figure 4. CTN in rabbit liver PCTS (top) and in rabbit brain (hippocampal) slices (bottom two panels). White points: slices exposed to no CPA or to formamide (F) only. Gray points: slices exposed to formamide plus dimethyl sulfoxide (F+D). As in Figure 3, the F+D curves branch off from the F only curves at the specific concentrations of F to which  $\text{Me}_2\text{SO}$  was added. In the panels for hippocampal slices, 14.86, 16.86, and 25% F was used, vs. 10 or 15% F in the upper panel; the added concentrations of  $\text{Me}_2\text{SO}$  were 10% and 15% w/v in all cases. The concentration of F in VM3 and M22 is 12.86% w/v, which is well below the toxic concentration of formamide in brain even without CTN (lower panel). EPSP (excitatory post-synaptic potential amplitude) and  $\text{K}^+/\text{Na}^+$  ratio determined 1 h after CPA washout; ATP content determined 3 h after CPA washout. The curves shown for rabbit liver PCTS represent the results of two independent experiments. Each point in the lower two panels is the result of a separate experiment. Previously unpublished data. Error bars represent  $\pm 1$  SEM unless the error is smaller than the points as drawn.

bonding groups on the pCPAs of the solution when the total concentration of hydrogen bonding groups is just sufficient to enable vitrification under standardized testing conditions. As  $qv^*$  increases, fewer hydrogen bonding groups are responsible for the vitrification of the solution, which means that they are each bonding water more tightly than in a solution with a lower value of  $qv^*$ . The essential observation (Figure 5) is that high  $qv^*$  – i.e. less concentrated – vitrification solutions are more toxic than low  $qv^*$  – that is, more concentrated – vitrification solutions, indicating that it is the strength of hydrogen bonding between water and the pCPAs that is the main driver of non-specific pCPA toxicity, and that this factor is more important than the total amount of water in the solution, which is lower in the less toxic solutions. Although this relationship can only be pushed so far, given that cells require at least some water to maintain their viability, it has enabled particularly promising vitrification solutions to be developed to date, and may help to enable still more efficacious formulae in the future. The correlation between  $qv^*$  and viability has thus far been shown only for rabbit renal cortex, but may be more broadly applicable (see below).

#### *Protein denaturation as a potential contributor to toxicity*

By comparing the concentration of pCPA needed to denature proteins to the concentration needed to induce toxicity in organ slices, it was possible to show that commonly used pCPAs are not likely to be toxic as a result of protein denaturation (Fahy et al. 1990a; Fahy 2010), and in fact, an early investigation of this question showed a positive correlation rather than an inverse correlation between viability and vitrification solution denaturation tendency (Fahy et al. 1990a). However, high concentrations of some rarely-used pCPAs (particularly N-methylformamide, dimethylformamide, and possibly acetamide and N-methylacetamide) may well be toxic because of a protein denaturing effect, and combining



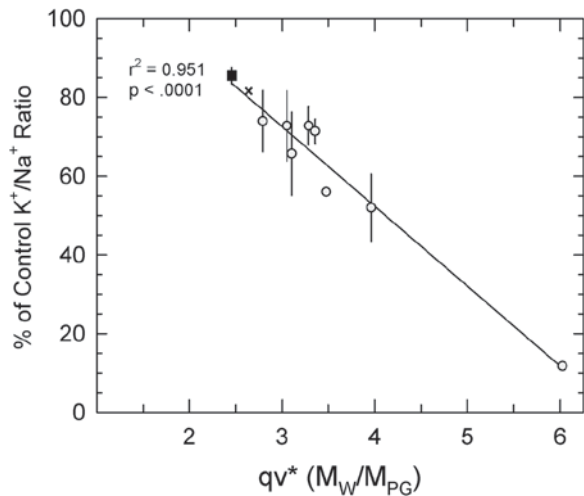


Figure 5. Dependence of rabbit renal cortical slice viability on  $qv^*$ , which is the average number of molecules of water rendered vitrifiable per pCPA hydrogen bonding group in a vitrification solution ( $M_w$ , molarity of water;  $M_{pG}$ , total molarity of polar groups on pCPAs). Two outliers, not shown, independently correlated with  $qv^*$  with an  $r^2$  value of 1.000 and a  $p$  value of 0.0005 (Fahy 2010). For discussion, see text and (Fahy 2010). Modified from Fahy et al. (2004a) and Fahy (2010). Error bars represent  $\pm 1/2$  the range of observed replicate group means.

near-denaturing concentrations of such agents with  $Me_2SO$  tends to cause more injury rather than CTN, perhaps due to exacerbation of denaturation (Fahy 2010). The question of whether strongly vitrifiable combinations of nominally non-denaturing concentrations of individual pCPAs might in fact have denaturing properties overall has not been investigated.

The question of protein denaturation by cryoprotectants may need re-examination in the light of chilling injury. A lack of denaturation at the temperature of administration and removal still leaves open the possibility that subsequent cooling of the system could cause denaturation by a summation of the effect of the cryoprotectants and of the effect of cooling. Evidence bearing on this possibility is discussed below.

### Next-generation vitrification solutions

A number of "next generation" vitrification solutions have been developed based on the principles discussed above (Fahy et al. 2004a, 2004b). Although the underlying principles were worked out using only rabbit renal cortical slices as the model tissue, next generation solutions have been used successfully for the vitrification of rat (Pichugin et al. 2006) and rabbit (see below) brain slices, rabbit liver slices (unpublished results), rat renal cortical and medullary slices (de Graaf et al. 2007), rabbit renal cortical slices (Fahy et al. 2004b), mouse oocytes (Fahy et al. 2004a), an entire rabbit kidney (Fahy et al. 2009), human corneas (Ge et al., in preparation), and human and porcine cartilage (unpublished results). Earlier versions of these solutions were used successfully to preserve jugular veins (Song et al. 2000a, 2000b), small-diameter arteries (Taylor et al. 2004), heart valves (Brockbank & Song 2004),

mouse embryos (Rall & Fahy 1985), and cartilage (Song et al. 2004a, 2004b).

The next-generation solution known as M22 (Fahy et al. 2004b) is intended to be used at  $-22^\circ C$  (as the name suggests), at which temperature it is comparatively low in toxicity (Fahy et al. 2004b). M22 remains ice-free when cooled at about  $0.1^\circ C/min$  and rewarmed at about  $0.4^\circ C/min$  (Wowk & Fahy 2005), and resists ice formation more effectively than any other documented vitrification solution. Even 2-liter quantities can be cooled to below  $T_G$  without visible ice formation (Wowk & Fahy 2005). M22 incorporates toxicity neutralization, mutual dilution of pCPAs (to prevent any specific toxic effects of any one pCPA from being realized by keeping its concentration low enough to avoid such effects), the use of pCPAs that act through different mechanisms of toxicity (Fahy 2010), the use of npCPAs, including the inclusion of two different polymers (based on polyvinylalcohol-polyvinylacetate copolymers (Wowk et al. 2000) and polyglycerol (Wowk & Fahy 2002)) that have specific and complementary non-colligative anti-nucleation activities (ice blockers), the use of a novel CPA that can interact with water without interacting with itself (Wowk et al. 1999), and chilling injury protection through tonicity adjustment (see next section). The more dilute VM3 vitrification solution (Fahy et al. 2004a) is safe for use at  $0^\circ C$ , has a critical warming rate of only  $3.8^\circ C/min$ , and, at least in rabbit renal cortical slices and intact rabbit kidneys, prevents chilling injury during cooling from  $0^\circ C$  to  $-22^\circ C$  and has therefore been used as a way of transitioning to and from M22 at  $-22^\circ C$  (Fahy et al. 2004b).

In an attempt to make M22 less toxic at  $0^\circ C$ , the possibility that the PVP component of M22 might be a significant contributor to overall solution toxicity was investigated (Guan et al. 2012a, submitted for publication). By replacing PVP gram for gram with sucrose, it was confirmed that the resulting solution, CPR, was able to prevent ice-crystal formation in rat liver PCTS at moderate cooling and warming rates, could be cooled to below the glass transition temperature without the development of fractures, was significantly less toxic to rat liver PCTS than M22 at  $0^\circ C$ , and improved recovery after vitrification and rewarming as well (Guan et al. 2012a, submitted for publication). In rabbit liver PCTS at  $0^\circ C$ , similar results were obtained pertaining to toxicity, but the difference between M22 and CPR was not statistically significant in initial experiments ( $p > 0.1$ ), perhaps because recovery of ATP is normally in the vicinity of 90% even with M22 (unpublished results). In addition, M22 preserved the viability and functionality of rat liver PCTS Kupffer cells better than did CPR (Guan et al. 2012a).

### Understanding chilling injury

#### *Tissue specificity of chilling injury*

Chilling injury, like CTN, is a cell and tissue-specific phenomenon. Thus, while kidney (Fahy et al. 2004b) and some liver (de Graaf et al. 2007) PCTS are highly susceptible to chilling injury, rat and rabbit hippocampal slices

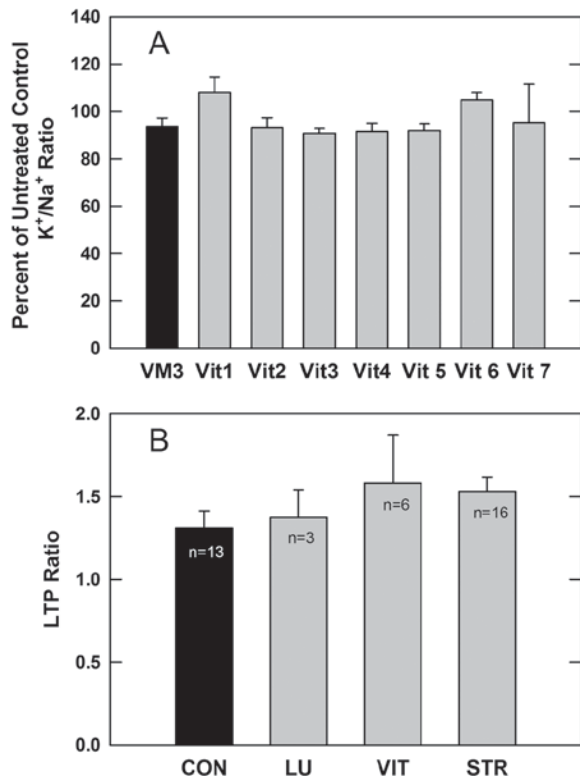


Figure 6. Lack of chilling injury in vitrified rat (A) or rabbit (B) adult brain (hippocampal) slices, which both show 100% recovery after vitrification and rewarming in VM3 compared to the effect of VM3 exposure alone. A. VM3 controls compared to 7 separate example vitrification experiments, which average less than zero injury attributable to cooling to below  $T_g$ . Drawn from previously published tabular data (Pichugin et al. 2006). B. Lack of effect of vitrification on the long term potentiation (LTP) response, a form of neurophysiological “memory” which consists of a permanent increase in the magnitude of the response to a given CA3 cell stimulation (recorded in this case as the amplitude of the excitatory post-synaptic field potentials at the Schaffer collateral-CA1 dendrite junction) as a result of prior “training” (intensive stimulation) of the involved synapses. Control brain slices increased their field EPSP response to about 30% above the baseline response amplitude (LTP ratio of about 1.3) in response to prior “training.” The same basic result was also seen after loading and unloading of VM3 (LU); after loading of VM3, vitrification, rewarming, and unloading of VM3 (VIT); and after storage of vitrified slices for days to months below the glass transition temperature (STR; storage time had no effect on the results obtained). *n* values represent the number of independent experiments represented by each bar. Previously unpublished data.

are either unaffected or less affected, at least based upon the routine recovery of essentially all structural and measured functional features after vitrification and rewarming (Figure 6, and below), although in these studies, it is possible tonicity adjustment was by chance perfect for recovery (see next paragraph). Monkey liver PCTS, too, displayed very little injury after cooling and warming at 5,000°C/min (Fisher et al. 2002) using a concentration of propylene glycol that is just sufficient for vitrification at that cooling rate (Fahy & Rall 2007), implying that they were not injured by either chilling injury or thermal shock, and little cooling-related injury was seen also in rat renal cortical PCTS whether cooled to  $-20^{\circ}\text{C}$

or vitrified (de Graaf et al. 2007). By comparing the biochemistry and molecular biology of tissues and cells that are strongly affected by chilling to those that are affected only minimally or not at all, additional insights into the factors required to avoid this serious source of injury may emerge.

#### Interaction of chilling injury with tonicity

Although excessive cell shrinkage may underlie slow-freezing injury and hypertonic osmotic injury, mild cell shrinkage has been shown to be highly protective against chilling injury in rabbit kidney slices (Fahy et al. 2004b) and porcine embryos (McGrath 1989). In the case of rabbit renal cortical slices, 80–95% recovery of  $K^+/Na^+$  ratio was obtained following cooling to  $-100^{\circ}\text{C}$  or below (maximum chilling injury) using a variety of different vitrification solutions provided the nominal tonicity of the solution was adjusted to the optimum range of 1.35–1.5 times isotonic before cooling to below  $-22^{\circ}\text{C}$  (Fahy et al. 2004b). For cooling to  $-22^{\circ}\text{C}$ , cooling under isotonic conditions reduced viability by 30–40% but tonicities of 1.2–1.5 times isotonic were capable of eliminating all chilling injury. Cooling at tonicities higher than 1.5X reduced viability as a linear function of tonicity, and the latter phenomenon was also seen when cooling was to  $-100^{\circ}\text{C}$  and below (Fahy et al. 2004b), which cautions against the use of excessive npCPA in vitrification solutions for PCTS. In the case of porcine embryos, cooling to an unspecified temperature caused a 100% loss of viability under isotonic conditions, but only an 8–26% loss when 0.5M trehalose was added to the medium before cooling (McGrath 1989). Further investigations of the applicability of this intervention to PCTS are in order.

#### Origins of chilling injury

Chilling injury has been proposed to be the consequence of membrane lipid phase transitions from the liquid crystalline to the gel phase as a result of cooling (Lyons & Raison 1970; Lyons 1972), and both chilling injury and thermal shock have been linked to membrane lipid phase transitions. In the special case of thermal shock in red blood cells, injury is seen only when cooling is combined with cell shrinkage over a specific temperature interval that may define a phase transition temperature of the membrane, no injury being observed if cooling precedes shrinkage (Takahashi & Williams 1983). Lipid phase transitions have been detected in the membranes of many living cells and linked to chilling injury in those cells (Crowe et al. 1989; Zeron et al. 2002) as well as being linked to leakage of liposome-encapsulated markers induced by cooling (Hays et al. 1996; Hays et al. 2001). Modification of membrane phase transitions by modification of cell membrane composition has provided some protection against chilling injury for some animal cells (He et al. 2001; Zeron et al. 2002; Horvath & Seidel 2006), and certain antifreeze proteins have inhibited chilling injury in keeping with their ability to interact with cell membranes (Tomczak et al. 2002) or liposomes (Hays

et al. 1996) and modify their phase transition behaviour. Prokaryotes (Wada et al. 1990) and plants (Murata et al. 1992) have been protected by genetically engineering them to have less saturated membrane lipids, which lowers membrane phase transition temperatures. However, for cryopreservation, prevention rather than depression of the membrane phase transition event might be needed to preclude chilling injury.

Although membrane phase transitions are frequently implicated in chilling injury, chilling injury takes place in many cases when no membrane phase transition appears to take place (McGrath 1989). In addition, in the specific case of liver and kidney PCTS, the phase transition temperatures are above 4°C (Lyons 1972; Hui & Parsons 1976; Kirpatovskii et al. 1991), yet cooling to 4°C is well known not to induce chilling injury in either of these organs, as evidenced, for example, by recovery of essentially full function of PCTS after 1 or more days of storage at 0–4°C (Wishnies et al. 1991; Olinga et al. 1998; Fisher et al. 2002; Plazar et al. 2007). Further, chilling injury in rabbit renal cortex seems to be an approximately linear function of temperature between 0°C and –100°C (Fahy et al. 2004b), which does not match the expected effects of specific phase transitions at discreet temperatures.

McGrath proposed that chilling injury takes place because membrane lipids contract in response to temperature decreases more than does the cytoplasm they contain, resulting in tension in the plane of the membrane and membrane failure, and proposed that this is why hypertonicity protects against chilling injury (McGrath 1987; McGrath 1989). This hypothesis is more consistent with a linear increase in injury in kidney slices down to –100°C than the membrane phase transition model.

In addition to these observations, chilling has also been linked to the production of specific “cold shock proteins” (VanBogelen & Neidhardt 1990; Schindelin et al. 1993) whose production is triggered by events that involve alterations in ribosomal function (VanBogelen & Neidhardt 1990). Heat shock before chilling can protect against chilling injury (Burton et al. 1988), and chilling can stimulate the production of heat shock proteins (Burton et al. 1988), suggesting that protein denaturation could be involved. Proteins are known to be generally destabilized by temperature reduction for basic thermodynamic reasons (Lopez et al. 2008) even at temperatures close to 0°C (Dias et al. 2010), and accumulation of cold-denatured proteins beyond a certain limit would be expected to lead to apoptosis consistent with the unfolded protein or ER stress response (Corwin et al. 2011). Moreover, ER stress might accelerate oxidative stress by, for example, consuming intracellular reduced glutathione (Harding et al. 2003).

Perhaps the first DNA microarray study of chilling injury in liver PCTS (PCLS) has recently begun (Guan et al. 2012b). Rat PCLS were loaded with different CPAs, exposed to –15 °C for 10 min, washed free of CPA, and incubated for 3 h at 37°C before extracting mRNA for

expression profiling. In these studies, other damaging events such as CPA toxicity, osmotic stress, and ice-crystal formation were avoided or minimized based on minimal alteration of PCTS ATP content in non-chilled controls exposed only at 0°C.

A broad spectrum of changes in gene expression profiles was discovered including cell-cycle transition phase arrest, up-regulated immune response, down-regulation of unsaturated fatty acid and cholesterol biosynthesis and cholesterol transport and fatty acid oxidation, up-regulation of chaperones/heat shock proteins, and activation of the ER stress/unfolded protein response in the endoplasmic reticulum as indicated by the up-regulation of *ddit3* and its upstream gene *ATF4* (Okada et al. 2002; Marciniak et al. 2004) (Guan et al., submitted for publication). The activation of intracellular signalling cascades, especially mitogen-activated protein kinase pathways and pathways related to immune responses, might be interpreted as a response to cope with the sudden presence of unfolded proteins by initiating survival or apoptosis pathways.

Because of the multifaceted nature of chilling injury as revealed by these preliminary results even after cooling only to –15°C, comprehensive surveys such as those enabled by microarray technology may be necessary for the elucidation of the initiating events that are responsible for the observed injury. The need for such high-throughput methods is underscored by our more recent observation that chilling injury in rat liver PCTS depends also on time at different temperatures as well as on the final temperature to which the PCTS are cooled below –15°C (Guan et al. 2012b). But separating cause from effect will be essential, since many observed changes in gene expression are undoubtedly compensatory responses while others may be maladaptive secondary effects and yet others may be direct rather than indirect effects of chilling. Still, for the first time a broad view of chilling injury is beginning to emerge in a relevant model of PCTS cryopreservation, and this is bound to help the development of adequate solutions to the “final frontier” problem of chilling injury during PCTS vitrification.

## Empirical results

The practical results of cryopreserving PCTS either by freezing or by vitrification were comprehensively and carefully reviewed by De Graaf in 2003, and the reader is referred to this review for a more detailed evaluation of the literature up to that time (de Graaf 2003). An important point made in this review and elsewhere (Maas et al. 2000b) is that different ways of assessing viability or metabolic integrity can give widely divergent results, which implies that the meaning of the results depend upon the endpoint chosen, and this must be kept in mind in evaluating various reports of successful cryopreservation. For example, 2 h after cryopreservation, Glöckner et al. obtained only 40% of normal potassium content in rat liver PCTS, but a net of 150% of control



7-ethoxycoumarin (7-EC) 0-deethylation (Glöckner et al. 1998), and Maas et al. found 80% recovery of MTT staining but an ATP content of just 5–15% and no living cells by histological criteria (Maas et al. 2000b), while Glöckner et al. showed induction of CYP1a1, CYP2b1, and CYP3a1 mRNA and protein expression in cryopreserved rat PCLS despite the fact that viable cells were only observed at the edges of the slices or around bigger vessels (Glöckner et al. 2002; Lupp et al. 2002). de Graaf suggested that many such potentially misleading discrepancies can arise as a result of artificially adding potentially lost cofactors needed for enzymatic assays, increased membrane permeability leading to greater uptake of added substrates and cofactors, membrane permeabilization insufficient for enzyme leakage but sufficient for potassium and small molecule leakage, etc. and suggested histomorphology, potassium retention, maintenance of ATP and reduced glutathione (GSH) content, and phase II biotransformation of model compounds as some of the most adequate indices of PCTS integrity (de Graaf 2003).

Most studies have focused on liver and kidney slices from various species (particularly rat, guinea pig, rabbit, dog, monkey, and human). Three cryopreservation methods for PCTS have been investigated: slow freezing, fast freezing, and vitrification. At this time no generally accepted and preferred method has yet been determined, and we therefore now review all three.

## Recovery after freezing

### *Rapid versus slow freezing*

Considering that avoiding IIF is a major goal of most freezing protocols (Mazur 1970), it is unusual that the literature on the freezing of liver PCTS focuses to a large extent on the use of fast freezing, as defined above. However, slow freezing of rat liver PCTS was found to result in devastating injury, even though slow freezing was more effective than rapid freezing for isolated hepatocytes (Maas et al. 2000b), and direct comparisons between slow and rapid freezing of PCTS have indicated that rapid freezing was better than freezing at 0.5°C/min (Maas et al. 2000a), 1°C/min (de Kanter & Koster 1995), or 1.5°C/min (Glöckner et al. 1996) for rat liver PCTS.

One possible explanation for the advantage and even the necessity of fast freezing for rat liver PCTS is suggested by an analytical study of cultured hepatocytes that indicated that these single cells were expected to undergo extensive supercooling below –40°C even when cooled at 1°C/min [“the implication is that to prevent completely the nucleation of ice crystals inside cultured hepatocytes, one must either cool at extremely slow rates (i.e. much less than 1°C/min) or use high concentrations of CPA”] (Karlsson et al. 1993). By comparison, isolated rat hepatocytes have an optimal cooling rate of about 3°C/min based on LDH retention (Chesné & Guillouzo 1988), meaning that severely damaging IIF is expected at and above this cooling rate (unless the cooling rate is much faster (Boutron & Arnaud 1984)), and the cooling rate required for IIF would be expected to be lower in

liver PCTS than in isolated cells (Fahy 1981), so it may be that freezing that has been presumed to be “slow” is actually not “slow” freezing at all as it results in IIF in rat liver PCTS. Should this be the case, it could explain the superiority of higher cooling rates, since injury from IIF would be expected to be less severe at high cooling rates than at low rates (Mazur 1984). On the other hand, the intriguing possibility also exists that slowly frozen rat liver PCTS are simply injured by chilling injury.

Additional evidence for IIF with fast freezing of rat liver PCTS is that even though it was better than slow freezing for rat liver PCTS, in the cited comparison, it was still devastating to slice integrity: with fast freezing, the fraction of viable cells was 5–50% after 4 h and 5–25% after 8 h in initial studies, and in a more systematic series of experiments cell integrity averaged 10% and did not exceed 35% (Maas et al. 2000a). However, increasing the Me<sub>2</sub>SO concentration to 18% v/v significantly improved these results, up to 60–70% of the cells appearing to be alive based on histological appearance (de Graaf et al. 2000; de Graaf et al. 2007) and with similar recovery of ATP (de Graaf et al. 2007). Oddly, recovery was worse with 30% Me<sub>2</sub>SO rather than 18% (de Graaf & Koster 2001), perhaps because cytosolic recrystallization is more inhibited with lower intracellular pCPA concentrations. The freezing of rapidly cooled rat liver PCTS has been directly demonstrated and found to be just as extensive as in a tissue-free Me<sub>2</sub>SO solution of the same 18% v/v concentration, so PCTS preserved by this method do not vitrify (de Graaf & Koster 2001).

Both the need for rapid freezing and the apparent ability to survive IIF are unusual features of rat hepatocytes in PCTS. Rat renal cortical and medullary PCTS were largely destroyed by rapid freezing (de Graaf et al. 2007), presumably because they are less tolerant of IIF, and kidney PCTS from other species tend to do well after presumably slow freezing at 12°C/min (Fisher et al. 1993, 1996, 2002). Human liver slices behave in a more conventional way as well, showing more injury as cooling rate is increased from 1°C/min to 12°C (Fisher et al. 1991, 1993), and recovered only 33% of normal protein synthetic capacity after rapid freezing although potassium content 2 h after rewarming was 70% of normal (Glöckner et al. 1999). Cynomolgus monkey liver PCTS frozen at 0.5, 1, or 12°C/min recovered much more fully after cooling at 0.5°C/min than at the higher rates (Fisher et al. 2002). Porcine liver PCTS also sometimes show a trend for improved survival as the cooling rate is lowered below 1°C/min (Fisher et al. 1991).

In fact, perhaps the most generally encouraging results for freezing have been reported using slow freezing of non-rat PCTS. Simple freezing of human liver and kidney PCTS at 1°C/min (for liver) or 12°C/min (for kidney) with 10% v/v Me<sub>2</sub>SO yielded high degrees of functional recovery (78–80% retention of K<sup>+</sup> and 71–77% retention of protein synthesis, for kidney and liver, respectively), with similar recoveries of urea synthesis and gluconeogenesis in the liver and PAH and TEA uptake by kidney



PCTS (Fisher et al. 1993). Similarly, dog liver and kidney slices frozen at 0.5°C/min and 12°C/min, respectively, in 10% v/v Me<sub>2</sub>SO attained 60–70% retention of PCTS potassium content and protein synthetic capacity (Fisher et al. 1996), which are both energy-intensive endpoints (de Graaf 2003). When monkey liver slices were frozen at 0.5°C/min with 10% Me<sub>2</sub>SO, 84% of control potassium retention and 91% recovery of protein synthesis was observed (Fisher et al. 2002).

A difficulty with evaluating fast freezing methods applied to liver PCTS has been that the endpoints used to evaluate recovery have tended to be focused more on the metabolism of added model compounds or on other less stringent tests such as urea synthesis or enzyme retention than on potassium retention, ATP or GSH content, histomorphology, or Phase II biotransformation, which, as noted above, are better indicators of true PCTS integrity (de Graaf 2003). However, of 14 studies on liver PCTS from specific species that were reviewed in 2003, ten included at least one of these assays (de Graaf 2003). Of these 10 studies, just four tissues, reported in three papers, showed consistently more than 50% recovery compared to controls (Glöckner et al. 1996; Day et al. 1999; de Graaf et al. 2000). In addition, good recoveries of various metabolic capabilities (alanine aminotransferase content, urea production, and testosterone metabolism) of rhesus and cynomolgus monkey liver slices (de Kanter & Koster 1995) and the same indices plus N-deethylation of lidocaine and total O-deethylation of 7-EC in the case of human liver slices (de Kanter et al. 1998) have been reported after rapid freezing. A solitary paper on rapid freezing of human thyroid tissue using 15% glycerol also showed promising results (Tanaka-Matsuura et al. 1984).

#### **Freezing studies from 2001–2012**

We have found 19 papers on the freezing of PCTS published from 2001–2012 that were not reviewed in 2003. All appear to involve rapid freezing (with or without a cryoprotectant) of liver PCTS and 11 employ endpoints indicative of viability or Phase II metabolism.

Of the latter papers, only one successfully demonstrated good retention of viability (around 70% recovery of ATP and morphological integrity in rat liver PCTS (de Graaf et al. 2007)). Two earlier reports attained less than 50% recovery of PCTS histomorphology and ATP (de Graaf & Koster 2001; de Graaf et al. 2002) under most conditions, and these rapidly frozen PCTS gained water and lost about 30% of their potassium content, although incubating them at 15°C for 15 min enabled recovery of 70% of fresh control intact cell numbers (de Graaf et al. 2002). Worse results were reported by other labs at around the same time. Lupp et al. rapidly cooled rat liver PCTS using the sub-optimal 30% concentration of Me<sub>2</sub>SO, added and removed in one step without an osmotic buffer (osmotic step factor, 15-fold) (Lupp et al. 2002) and found “remarkable cell damage already after 2 h of incubation affecting about 30–70% of the tissue” and, after 24 h, “viable cells . . . corresponding to [only] about

5–20% of the total tissue in the individual slices.” Another lab reported loss of viability based on a Tox-8 test and strong impairment of apoptotic responses and alleged reduction of Kupffer cell proliferation although without a clear method for identifying Kupffer cells (Kasper et al. 2011). Another report disclosed frank impairment of ATP, K<sup>+</sup>, glycogen, and mitochondrial homeostasis (Vanhulle et al. 2003). Interestingly, although damage to rapidly frozen rat liver PCTS was not blocked by a caspase inhibitor or elevated potassium concentrations in the incubation medium, reduced cell lysis and other improvements were obtained by adding 100 mM LiCl to the incubation medium (Vanhulle et al. 2006).

Rapid freezing permitted 53% retention of 7-hydroxycoumarin glucuronidation (but not sulfation) in human liver slices and good formation of both conjugates in rat liver slices (Granhall et al. 2002). However, it was also shown that phase I and II metabolism can be well maintained in 5 species despite LDH leakage and declining ATP levels, again indicating that the retention of selected biochemical pathways is no guarantee of good overall integrity (Martignoni et al. 2004).

Finally, of the 8 papers on rapid freezing not reviewed in 2003 and that focused primarily or only on metabolic endpoints rather than general viability, just two have shown results that are reproducibly consistent with non-cryopreserved PCTS endpoints (Axelsson et al. 2003; Martignoni et al. 2004).

#### **Recovery after vitrification**

Vitrification has been attempted in liver, kidney, and ovarian (Huang et al. 2008) tissue slices. Given the present focus on tissue models used for drug discovery and development, the following discussion focuses on the first two tissue systems.

Perhaps the first report of attempted organ slice vitrification was published as part of a review of the effects of vitrification and devitrification in 1987 (Fahy 1987). Rabbit renal cortical slices were vitrified with the VS1 solution of Rall and Fahy (Rall & Fahy 1985) and Takahashi et al. (Takahashi et al. 1986) or a modification employing 3% w/v more 1,2-propanediol and 3% w/v less PEG 8000 at a cooling rate of 75–210°C/min and rewarmed at 100–200°C/min. VS1 exposure reduced viability by about 50%, and vitrification reduced viability by another factor of two, either due to devitrification or chilling injury or both. Using the same methods on rabbit liver slices gave no damage after exposure to the vitrification solution but 0% recovery after rewarming (Fahy 1987).

Wishnies et al. in 1991 used metabolism of 7-EC and of its primary metabolite, 7-hydroxycoumarin (7-HC), by human liver PCTS as the main endpoints of the study, examining both Phase I and Phase II biotransformation (Wishnies et al. 1991). Using very rapid introduction of 4.8M 1,2-propanediol as the cryoprotectant, vitrification and rewarming at ~5,000°C/min, and one-step CPA washout, vitrified PCTS were compared to untreated controls (no non-vitrified CPA controls). After vitrification

for 2–10 min, adding 7-EC resulted in about half as much or less of the amount of 7-HC-glucuronide formation in vitrified as in control PCTS, about twice as much added 7-EC remained as unconjugated 7-HC, and formation of 7-HC sulfate was reduced by about half or more as well, consistently for PCTS formed from three human donors (with one additional unexplained exception). When 7-HC was added, similar results were obtained. PCTS stored longer than this gave results that indicate errors or changes in the processing of those slices, which makes the results uninterpretable. There were no direct tests of tissue viability in this paper, and the results of vitrification may have been influenced by osmotic damage, pCPA toxicity, and/or devitrification, none of which were carefully evaluated.

Analogous results were obtained for dog liver PCTS in 1996 using the same method except for the use of slightly less 1,2-propanediol (4.7M) and slightly longer loading steps (8 vs 5 min) (Ekins et al. 1996b). Added 7-EC, rather than being processed to 7-HC glucuronide or 7-HC-sulfate, was largely converted to 7-HC (5.7–8.8 times more 7-HC accumulation than in controls), the Phase II reaction products being reduced by 80–85%. 7-EC metabolism was better maintained with rat liver PCTS, but processing of 7-HC was reduced by 50% and protein synthesis was reduced by 65–97% (Ekins 1996). Adding antifreeze proteins produced only limited benefits (Ekins et al. 1996a), and ultrastructural changes in mitochondria were documented.

Using VS4, a 7.5M vitrification solution that normally devitrifies unless warmed more rapidly than 1570°C/min, the histological recovery obtained when rat liver PCTS were vitrified and rewarmed at ~800°C/min was 78% of VS4 control slices, apparently because devitrification in slices could be avoided at lower warming rates (less than about 640°C/min) (de Graaf & Koster 2001). In these experiments, rather abrupt methods of adding and removing VS4 were used, but gentler methods later gave apparently worse results (de Graaf et al. 2002).

Fisher et al. exposed cynomolgus monkey liver PCTS to propylene glycol (PG; 1.2, then 2.4, then 4.0M, 5 min at each step), plunged them into liquid nitrogen, and warmed them by immersion in 37°C fetal calf serum (one step washout, no osmotic buffer), which is the usual method used by this laboratory with the exception of using a lower than customary final concentration of PG. The results were recovery of 93% of control K<sup>+</sup> content and 88% of control protein synthesis after 4 h of culture, the results being very similar to the effect of PG addition and removal alone (Fisher et al. 2002).

In 2003, de Graaf et al. reported that rat liver PCTS vitrified with the then-undescribed VM3 at a cooling rate of 1.5°C, warmed at an average rate of 40°C/min, and washed free of VM3 using 300 mM trehalose as an osmotic buffering agent recovered 71% of control histological integrity and 58% of control ATP (de Graaf 2003). Some of the loss in recovery was attributed to exposure to the 8.4M VM3 solution, although VM3 was less toxic

than the previously studied 7.5M VS4. The use of trehalose during washout was beneficial. Ironically, given the preference of frozen rat liver PCTS for rapid cooling rates, cooling at 1.5°C/min was better than cooling at 200 or 800°C/min, but this was in retrospect probably an artifact of fracturing of the VM3 glass at the higher rates of temperature change (de Graaf et al. 2007), particularly given that cooling and warming at 800°C/min had previously been shown to give good results and therefore not to be intrinsically damaging (de Graaf & Koster 2001). No investigation of rates between 1.5°C/min and 200°C/min or faster than 800°C/min was attempted.

When the same methods were applied to rat renal cortical PCTS, 86% histological recovery and complete ATP recovery were obtained. Rat renal medullary PCTS yielded 79% histological recovery and 68% of normal ATP content. As was observed for liver PCTS, some of the losses observed were attributed to VM3 exposure (de Graaf 2003).

In 2004, Fahy et al. introduced the first “next generation” vitrification solutions, including VM3, showing low toxicity for hand-cut rabbit renal cortical slices at concentrations high enough for vitrification at low cooling rates (Fahy et al. 2004a, 2004b). Although comparatively low in toxicity at high concentrations, they permitted 30–50% losses of viability (as measured by the intracellular K<sup>+</sup>/Na<sup>+</sup> ratio after 90 min of recovery) upon cooling either to –22°C or to below –100°C unless chilling injury was addressed by optimization of the pre-cooling tonicity, an intervention that permitted up to 95% recovery after cooling to –100°C or below (Fahy et al. 2004b). VM3 was actually found to be sub-optimum for renal cortical slice vitrification, since its tonicity of 1.7 times isotonic was too high and for this reason permitted a 33% decline in K<sup>+</sup>/Na<sup>+</sup> ratio after cooling to below –70°C (Fahy et al. 2004b).

Nevertheless, when applied to adult rat hippocampal slices, the use of VM3 permitted 94% recovery of K<sup>+</sup>/Na<sup>+</sup> ratio without vitrification and 91.5–107.9% (mean, 96.5%) of control K<sup>+</sup>/Na<sup>+</sup> ratio after vitrification and rewarming ((Pichugin et al. 2006); see also Figure 6). Spectacular preservation of both histology and ultrastructure was also demonstrated. In these experiments, VM3 was added in 8 steps and removed in 8 steps, with 300 mM mannitol as an osmotic buffering agent.

The first detailed report of the application of VM3 to rat kidney PCTS was published in 2007 (de Graaf et al. 2007). VM3 was introduced using a comparatively gentle, exponentially increasing and decreasing series of steps with 300 mM trehalose during the washout steps, including a final step in which no pCPA was present. Despite this, rat renal cortical PCTS showed about a 35% loss of histological integrity and a 20–25% loss of ATP content, which is slightly worse than reported preliminarily in 2003 (de Graaf 2003) (see above), for reasons that are not clear. The results also differ from those typically obtained with rabbit renal cortical slices, for which VM3 exposure is not damaging based on assays used for that species (Fahy et al. 2004a, 2004b). The difference may relate to different

methods of VM3 administration and washout, the rabbit slices being treated with typically four or five 20-min loading steps and six or seven 20-min washout steps (Fahy et al. 2004b), compared to 4 asymmetric 15 min loading steps and just four asymmetric 10 min washout steps for rats. In any case, vitrification and warming, both at 60°C/min (except for cooling and warming at 5°C/min near  $T_G$  to avoid fracturing), resulted in little additional damage (de Graaf et al. 2007). Medullary PCTS were not significantly affected by VM3 morphologically and recovered about 70% of their normal ATP content, and after vitrification and rewarming, medullary slices showed about 80–85% recovery of morphological score and 60% recovery of ATP. Rat liver PCTS exposed to VS4 showed 90% of control histological integrity, but vitrification reduced this to about 65% of normal histological score. Exposure to VS4 reduced ATP content to approximately 75% of control, and vitrification lowered it further, to only 45% of normal ATP. Therefore, rat liver was much more sensitive to cooling and warming than was rat kidney.

## The road ahead

### Freezing versus vitrification

At this point in time, an argument can be made for both freezing and vitrification as the best way forward. Slow freezing has allowed good recovery of PCTS of human liver and kidney (Fisher et al. 1993), dog liver and kidney (Fisher et al. 1996), and monkey liver (Fisher et al. 2002). However, this method is inapplicable to rat liver PCTS (Maas et al. 2000b), which is perhaps the most convenient *in vitro* PCTS model, and slow freezing is an intrinsically time-consuming process. Fast freezing, on the other hand, can work for rat and other liver PCTS (Day et al. 1999; de Graaf 2003; de Graaf et al. 2007) but not for kidney PCTS (de Graaf et al. 2007), and results have not been uniformly good even for rat liver PCTS. Therefore, there is no current prospect for a universally applicable freezing method for all PCTS, and if freezing is to be the preferred method, at least two different versions of the method will likely be needed.

Vitrification using various methods has been most successful for kidney (de Graaf 2003; Fahy et al. 2004b; de Graaf et al. 2007) brain (Pichugin et al. 2006) and monkey liver (Fisher et al. 2002) slices and has been reasonably successful for other PCTS, and therefore seems to have the potential to be developed into a more or less universal method.

The main limiting factors for further success with vitrification are important to clearly define, and may vary from organ to organ. For rat renal cortex and medulla, CPA toxicity was more important than chilling injury (de Graaf et al. 2007), but this apparent toxicity might reflect osmotic damage that can be avoided (see above). Toxicity is important for rat liver PCTS also, but perhaps this could be mitigated by using lower concentrations and higher cooling rates (de Graaf & Koster 2001; Fisher et al. 2002) (but still avoiding glass fracturing), and

recent modifications of M22 have also overcome toxicity (see above). On the other hand, published data based on recovery of ATP and morphological integrity as well as ongoing microarray (Guan et al. 2012b) and morphological studies of chilling injury in rat liver PCTS (Guan et al., manuscript in preparation) indicate that, at least in this tissue, chilling injury is a more serious and fundamental obstacle. And yet, the same obstacle does not seem to exist for hepatic and renal PCTS of other species, which, as just noted, can tolerate both rapid and slow cooling without overwhelming injury, with or without the presence of ice. In addition, kidney slices (Fahy et al. 2004b) and a whole rabbit kidney (Fahy et al. 2009), both of which are susceptible to chilling injury, as well as rat and rabbit brain slices and monkey liver PCTS (Fisher et al. 2002), which may not be susceptible to chilling injury, can all be vitrified successfully, and for at least one of these the histology and ultrastructure is known to be unaltered by cooling and warming (Pichugin et al. 2006). For the rest, ultrastructural investigations after vitrification or freezing are certainly in order, and expression profiling would also be highly informative.

For systems that are damaged by chilling, possible remedies include chilling injury control by tonicity optimization and adjustment of membrane composition to prevent membrane phase changes or their consequences. The successful use of cholesterol-loaded-cyclodextrin (CLC) to improve the cryosurvival of spermatozoa (Pamornsakda et al. 2011; Movassaghi et al. 2009) and oocytes (Horvath & Seidel 2006), for example, is consistent with our expression profiling findings of alterations in lipid metabolism and has given positive results for renal cortical slices in our hands (Fahy et al., unpublished observations). The strong possibility that cold denaturation of proteins is involved points to the need to block maladaptive responses to protein denaturation and to develop better protection against protein denaturation and better means of renaturing proteins after rewarming. All of these interventions are technologically feasible, but await direct investigation. In the meantime, increasingly better understanding of the biochemical origins of chilling injury should also help with the design of additional interventions to prevent chilling injury.

### Model refinements

One of the theoretical advantages of PCTS, the fact that they contain not just normal tissue structure but also all of the normal cell types associated with tissue *in vivo*, has barely been studied in past experiments on cryopreservation. Our studies have shown that the behaviours of hepatocytes and other cell types in liver PCTS, including Kupffer cells, endothelial cells, and stellate cells, tend to differ (Guan et al. 2012a) and highlight the need for an explicit focus on this heterogeneity of response in future studies.

Another new area for future investigation is the interaction between the CPA and the non-CPA solutions the



PCTS are exposed to in determining the degree of functional recovery that can be obtained after removing the CPA. For example, recovery of ATP content after addition and washout of variants of M22 was incomplete unless the livers were washed free of blood using an organ preservation solution (TransSend™ tailored for use with liver tissue) prior to slice preparation, but washing out the blood with this solution resulted in full recovery of ATP after CPA washout (Guan et al., 2012d), even though the carrier solution for the CPA itself was the same in either case. Previous studies have shown that the toxicity of pCPAs is highly dependent, in currently unpredictable ways, on the composition of the solution in which the pCPAs are dissolved (Clark et al. 1984; Pegg et al. 1986; Fisher et al. 1991), but the idea that the way in which the PCTS are initially prepared also affects their response to pCPAs provides additional opportunities for improvement of overall outcomes.

Our studies have also emphasized the fact that the npCPAs of vitrification solutions for PCTS should not be neglected. Although nominally of negligible toxicity, the npCPAs can actually have a significant effect on the overall toxicity of the solution and its resistance to ice formation ((Guan et al. 2012a; Guan et al., in preparation), and their contributions to toxicity still require evaluation for each species.

### Need to define success

DNA microarray studies are beginning to bring to light changes in the expression of hundreds to thousands of genes as a result of every aspect of a cryopreservation process. Even the act of removing an organ from the body and replacing its blood with a cold organ preservation solution undoubtedly changes the organ in many ways, and yet no *in vitro* screening method can avoid such a perturbation of normal metabolism, so some approach to accommodating such changes seems necessary. If the goal of zero change to the PCTS compared to the *in vivo* state cannot be attained, then what should the goal of a preservation method be?

Presumably it should be to obtain acceptable results for what is important and relevant and to ignore the rest. If what is important for drug discovery and development is drug metabolism, and eventually drug toxicity, is it acceptable to focus attention on, for example, just those endpoints or just those changes in gene expression that bear directly on those endpoints, and ignore the rest? That is, effectively, what has usually been done prior to the development of microarray studies and other high-throughput diagnostic methods, and it is possible that for practical purposes, this is all that is necessary.

On the other hand, the more unaltered the PCTS is compared to the *in vivo* state, the more likely it is to faithfully reflect the overall metabolic capacity of the organ from which it was taken, and therefore to correctly metabolize a broader range of added drugs, making the preparation more generally useful. This suggests that meaningful endpoints for judging the success of PCTS

cryopreservation should include endpoints related to overall integrity and viability. But many changes in gene expression, for example, do not have a direct relationship to viability and metabolic functions related to drug processing in the *in vivo* state, so the definition of success might be simplified by excluding such changes from consideration.

Another simplifying approach to defining at least a ranking of the success of different methods might be to use comprehensive tools such as expression profiling for comparative purposes, and reject the methods that cause greater deviations from normalcy overall, whether those deviations are obviously germane to drug metabolism or not. Yet another approach to defining success might be to study banked PCTS specifically at their points of maximum recovery after cryopreservation, when imperfections in preservation will have their smallest and most negligible effects, and ignore results at other time periods, selecting methods that optimize peak recovery in particular.

For many studies, the definition of success will ultimately also have to include acceptable preservation of the influences of non-parenchymal cell types in the organ. For example, to predict human metabolism *in vivo*, phase I and II metabolic capacities of human liver slices may often be a sufficient focus of attention, but for studies of drug-induced liver toxicity, the immune responses mediated by Kupffer cells (Roberts et al. 2007) are sometimes involved, making the viability and functions of these cells important. Both Kupffer cells and endothelial cells are involved in inflammatory reactions, whereas stellate cells are involved in fibrosis, both of which are relevant to different aspects of drug development.

### Conclusion

The need for robust cryopreservation methods for PCTS has been recognized for many years, and many attempts have been made to develop these methods using both freezing and vitrification approaches. Although many encouraging results have been obtained, a completely satisfactory method that is applicable with minor modifications to all PCTS from all species remains to be established. Nevertheless, it is encouraging that a method as simple as freezing at 1°C/min or 12°C/min with 10% v/v Me<sub>2</sub>SO has yielded high degrees of human liver and kidney functional recovery, respectively (Fisher et al. 1993), that rapid freezing can provide useful preservation of rat liver PCTS (de Graaf et al. 2007), and that vitrification, while currently faced with the newly emerging obstacle posed by the effects of cooling per se, continues to offer promise and has yielded very good results using widely different methods (Fisher et al. 2002; Fahy et al. 2004b). Overall, we see no reason why the many facts that have been learned and the many possibilities for improvement that remain cannot yet enable realization of the goal of providing the drug development process with the needed and vital benefits of banks of reliable and well-preserved PCTS of many kinds.



## Declaration of interest

G. M. Fahy is the Chief Scientific Officer of 21st Century Medicine, Inc., which develops solutions and methods for cryopreservation of cells, tissues, and whole organs. Y. Tan and L. Griffin are employees of 21st Century Medicine. The remaining authors report no declaration of interest.

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