22 Vitrification in Tissue Preservation: New Developments

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22.1 INTRODUCTION

Cryopreservation has been notably effective for banking and shipping of isolated cells, but much less so for more complex, integrated multicellular systems. We now have a broad understanding of the mechanisms that injure cells during freezing and thawing, and techniques have been developed that limit or prevent this injury, so that very low temperatures can now be used to preserve, virtually indefinitely, many cell-types with very high recovery rates (Karlsson and Toner, 1996; Taylor, 1984; Mazur, 1984a). These techniques are all aimed at preventing intracellular freezing and minimizing the damaging changes that occur in the remaining liquid phase as a consequence of the separation of water to form ice. In tissues and organs, however, it is not sufficient to maintain cellular viability—it is also important to maintain the integrity of the extracellular structure on both a micro- and macroscale. Techniques that are effective for the cryopreservation of cell suspensions do not always maintain this integrity because highly organized multicellular tissues present a special set of problems; foremost among these is the effect of extracellular ice formation, which disrupts the tissue architecture (Hunt et al., 1982; Karlsson and Toner, 1996; Pegg, 1987; Pegg et al., 1979; Taylor and Pegg, 1982). However, extracellular ice cannot explain all the problems encountered in moving from single cells to tissues—for example, the detachment of the corneal endothelium has a different, and as yet unknown, cause (Taylor, 1986), but prevention of this detachment is crucial for corneal viability. The effects of cryopreservation on the mechanisms of cellular adhesion have not yet been studied widely. To date, there have been very few studies focused on the effects of cryopreservation on anchorage-dependent cells, and in general, these studies have shown that postthaw recovery of adherent cells is lower than comparable cells frozen and thawed as cell suspensions (Ohno, 1994; Hetzel et al., 1973).

As outlined throughout this book, the science of cryobiology has defined many factors that must be optimized for cells to be stored for lengthy periods at low temperatures. Survival of cells through the rigors of freezing and thawing in cryopreservation procedures is only attained by using appropriate cryoprotective agents, and in general, these techniques are applicable to isolated cells in suspension or small aggregates of cells in simple tissues. More complex tissues and organs that have a defined architecture are not easily preserved using conventional cryopreservation techniques; this is principally because of the deleterious effects of ice formation on organized multicellular structures (Karlsson and Toner, 1996; Pegg, 1987; Pegg et al., 1979). The application of cryobiological principles to engineered tissue constructs is likely to be fraught with problems similar to those identified during the course of trying to extrapolate the successes of freezing cell suspensions to organized tissues and organs. Avoidance of ice damage has therefore become the principal focus in research to develop effective storage techniques for multicellular tissues and organs. In this chapter we review some of the underlying principles of the various approaches to "ice-free" cryopreservation, with particular emphasis on vitrification, which has recently been shown to provide a practical solution for the cryopreservation of complex tissues that can not be adequately preserved by freezing/thawing methods.

The aim of this chapter is to deal with "ice-free" cryopreservation of tissues, which at face value might be considered to be a departure from the main theme of a book titled *Life in the Frozen State*. However, frozen systems usually embody a vitreous component, and our objective here is to review the approach to cryopreservation that aims to achieve vitrification at the outset in an attempt to circumvent the hazards of water crystallization as ice formation. The vitreous state is essentially a solidified, amorphous liquid state obtained by specific conditions of cooling and solute concentration that inhibit ice nucleation and growth. During cooling, molecular motions within the liquid are slowed and eventually arrested with extreme viscosity; the "arrested liquid" state is known as a glass. It is the conversion of a liquid into a glass that is called vitrification (derived from *vitri*, the Greek word for glass). The specifics of this process are addressed more completely below.

22.2 BACKGROUND AND HISTORICAL PERSPECTIVE

Readers of this book will be aware that the foundations of modern-day cryopreservation were laid in the middle of the last century following the milestone discovery of the cryoprotective effects of glycerol by Polge et al. (1949; see Chapters by 11 and18). The practical successes for cryopreservation of a wide variety of cells that ensued from these empirical studies led to considerable fundamental work that has defined a number of mechanistic principles underlying the cryopreservation of cells. Critically important mechanisms include the fundamental importance of the total quantity of ice and its location in relation to the cells, the toxicity of cryoprotectants and the temperature dependence of that toxicity, and the extent of osmotically induced changes in cell volume. Because an understanding of these factors is the foundation of cryobiology, a detailed discussion of these mechanistic principles can be found in Chapters 1 and 2, as well as in numerous other literature reviews on the subject.

Following the advent of modern-day cryobiology in the 1950s, it was confidently anticipated that cryopreservation techniques for tissues and organs would quickly ensue from the notable successes with cells in suspension. Unfortunately, this transition proved to be very difficult, and it was quickly realized that there were significant additional hurdles to be overcome in the freezing of mammalian tissues and organs (Smith, 1961). Only recently has it been possible to demonstrate successful cryopreservation of tissues using the vitrification approach that avoids some of the destructive events inherent in freezing; this approach is the focus of this chapter. In the intervening years, both empirical and systematic studies have contributed to a greater understanding of the mechanisms of cryoinjury in multicellular systems; these mechanisms have in turn mandated consideration of vitrification as the most realistic approach to circumvent the problems of which extracellular ice is undoubtedly a primary concern. Nevertheless, avoidance of ice for optimum preservation is not a new idea, as even before the ground-breaking discovery of Polge et al. (1949), Luyet had concluded that ice formation is not compatible with the survival of living systems and ought to be avoided if possible (Luyet, 1937; Luyet and Gehenio, 1940). At that time, the idea of vitrifying biological systems at low temperatures was born, but it was conceptually confined to cooling small living systems at extremely high rates to achieve a vitreous state and avoid ice crystallization (Luyet, 1937; Luyet and Gehenio, 1940). These constraints have been minimized to some extent in the modern era of cryobiology with the introduction of cryoprotective solutes that include the ability to promote vitrification during cooling as one of their inherent properties.

It is important to appreciate that vitrification and freezing (water crystallization) are not mutually exclusive processes and that the crystalline phase and vitreous phase can, and often do, coexist within a system. In fact, during conventional cryopreservation involving controlled freezing of cells, a part of the system vitrifies. This occurs because during freezing, the concentration of solutes in the unfrozen phase increases progressively until the point is reached at which the residual solution is sufficiently concentrated to vitrify in the presence of ice. Conventional cryopreservation techniques are optimized by designing protocols that avoid intracellular freezing. Under these cooling conditions, the cell contents actually vitrify because of the combined processes of dehydration, cooling, and the promotion of vitrification by intracellular macromolecules. In this context, the use of the term "vitrification" in the title of the seminal paper by Polge et al. (1949) was justified, as the spermatozoa would be vitrified in the presence of extracellular ice during cryopreservation with glycerol. However, the term is now used more generally to refer to a process in which attempts are made to vitrify the whole system to avoid any ice formation (Armitage and Rich, 1990; Fahy, 1988, 1989; Fahy et al., 1984; Pegg and Diaper, 1990; Rall, 1987).

The conditions necessary to achieve this objective are discussed more completely late. It will suffice in this section on the historical perspective to record that the current interest in vitrification was spurred by two independent approaches to achieving cryopreservation in the absence of damaging ice. The first has been referred to as the "equilibrium approach," in that it seeks to

preequilibrate the biological specimen with a sufficiently high concentration of cryoprotectants before cooling such that freezing is prevented irrespective of the cooling rate (Elford, 1970; Elford and Walter, 1972a, 1972b; Farrant, 1965; Taylor et al., 1978). The second approach is called the "nonequilibrium approach," as the initial concentrations of solutes in the system are not of themselves sufficient to prevent ice nucleation and growth. This approach is achieved by using rapid cooling with or without the application of increased pressures to promote vitrification (Fahy, 1988, 1998; Fahy et al., 1984; MacFarlane, 1987; MacFarlane et al., 1992). In practice, because of constraints of the maximum tolerated limits of cryoprotective additive (CPA) toxicity, this leads to a metastable condition that is highly dependent on cooling and warming conditions if ice nucleation and crystal growth are to be avoided. Over the last 20 years, interest in this second approach to vitrification has in large part been kindled by the resolve of Fahy et al. to employ this as the only feasible way to achieve the cryopreservation of whole organs (Fahy, 1989; Fahy et al., 1984). Although this milestone has still to be achieved, the basic studies underpinning the research in this area have contributed to a better understanding of the ground rules that will ultimately lead to successful cryopreservation of organs. In the meantime, we have pursued this approach for the cryopreservation of complex tissues that may be regarded as intermediate between cell suspensions and whole organs with considerable recent success, as outlined below. Ten years ago, Pegg and Diaper reviewed the basic principles of freezing vs. vitrification as markedly different approaches to cryopreservation (Pegg and Diaper, 1990). Their review of the status of this technology was summed up with the reminder that at that time, "no system that is susceptible to damage by extracellular ice has yet been successfully vitrified, and all those systems that have been preserved by vitrification (early embryos, monocytes, and pancreatic islets) can equally well be preserved by conventional freeze-preservation methods" (Pegg and Diaper, 1990, p. 68). In such systems a vitrification method is often preferred because of practical benefits of operational simplicity, avoiding the need for expensive cooling equipment. Thus, in 1990 the challenges of vitrifying complex tissues remained formidable but approaches toward ice-free cryopreservation were still regarded as the way forward. Ten years later, this barrier has now been removed and we will review here the developments that have led to the successful vitrification of several tissues, two of which, blood vessels and articular cartilage, were previously refractory to cryopreservation with a high degree of functional survival.

22.3 EVIDENCE THAT EXTRACELLULAR ICE IS HARMFUL

Over the years since the early discoveries of cryoprotectants, attempts to extrapolate from the successful freeze-preservation of a wide variety of cell types to multicellular tissues and organs have been fraught with frustrating failures. We now recognize that the cryopreservation of complex tissues imposes a set of additional problems over and above the known mechanisms of cryoinjury that apply to single cells in suspension. These have been discussed in a number of reviews and will not be recounted here (Karlsson and Toner, 1996; Mazur, 1984b; Pegg et al., 1979; Taylor, 1984). Most important, it is now generally accepted that extracellular ice formation presents a major hazard for cryopreservation of multicellular tissues. As we have stated above, the predominance of this as a primary mechanism of cryoinjury in complex tissues has led to a focus on the development of low-temperature preservation techniques that avoid ice crystallization and *ipso facto* circumvent the associated problems. The evidence for the damaging role of ice in tissue cryopreservation has been outlined in a series of prior publications (Hunt et al., 1982; Jacobsen et al., 1984; Pegg, 1987; Pegg et al., 1979; Taylor, 1984; Taylor and Pegg, 1982). We will review briefly some of the early studies that provided clear evidence for a definitive role of ice *per se* as a principal hazard during cryopreservation of smooth muscle tissue. This is summarized here as a prelude to a review of the approaches to ice-free cryopreservation methods.

Smooth muscle is a good model tissue, intermediate between cells and organs, that requires for its function not only the survival of a high proportion of its cells but also the structural integrity

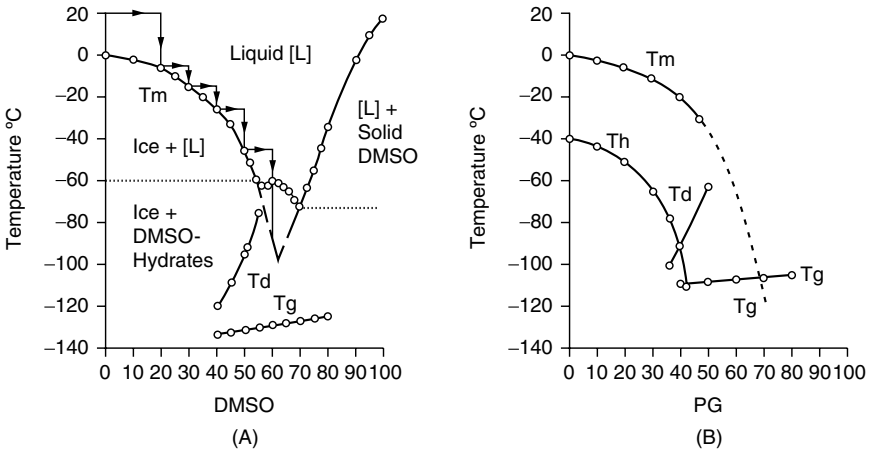

FIGURE 22.1 Supplemented binary phase diagrams for aqueous mixtures of Me_2SO (A) and Propane-1,2-diol (B) showing the principal events and phase changes associated with cooling and heating. A supplemented phase diagram combines nonequilibrium data on a conventional equilibrium phase diagram and serves to depict the important transitions inherent in cooling and warming aqueous solutions of cryoprotective solutes. Details are described elsewhere (Fahy, 1998; MacFarlane, 1987; Rasmussen and MacKenzie, 1968; Taylor, 1987) and in the text. T_m, equilibrium melting point curve (liquidus curve); T_h, homogeneous nucleation curve; T_d, devitrification curve; T_g, glass transition curve. The stepped line above the Me_2SO–H_2O liquidus T_m curve represents a scheme for incremental equilibration of a tissue with sufficient cryoprotective additive such that the system does not freeze during cooling. (See text for details.)

of the tissue so that the contractile response of the cells can remain coordinated. Experiments were designed to discover whether ice formation as such was damaging (Taylor and Pegg, 1982). Strips of muscle were cooled to –21°C in 2.56 *M* dimethyl sulfoxide (Me_2SO), held at that temperature overnight, and then thawed; then the Me_2SO was removed in excess Krebs's solution at 37°C and contractile response to histamine was measured. As illustrated in Figure 22.1, phase diagram data show that medium containing 20% (2.56 *M*) Me_2SO freezes at –8°C and concentrates 1.7 times to 34% (4.35 *M*) Me_2SO at –21°C. Another group of muscles was therefore equilibrated with 35% (4.49 *M*) Me_2SO containing 1.75 times the normal salt concentration and then cooled to and held at –21°C, unfrozen, for the same length of time. It was found that the unfrozen samples gave 72 to 76% recovery whether they were cooled at 2°C min^{-1} or 0.3°C min^{-1}, whereas the frozen samples gave only 21% ± 4% recovery if cooled at 2°C min^{-1} or 53% ± 7% if cooled at 0.3°C min^{-1}. Comparison of the frozen and unfrozen groups cooled at 2°C min^{-1} shows that ice damaged the tissue ($P < .001$), and comparison of the groups cooled at 0.3°C min^{-1} leads to the same conclusion ($P < .05$). In each case, the cooling rate, final temperature, concentration of Me_2SO, and concentration of other solutes was the same: Clearly, solute concentration was relatively innocuous, but ice formation was harmful. However, there was a striking difference between the recovery of tissue frozen at 0.3°C min^{-1} and tissue frozen at 2°C min^{-1} (Figure 22.2A). In the absence of freezing, there was no significant effect of cooling rate, but frozen tissue was more severely damaged when cooled at 2°C min^{-1} than were frozen muscles cooled at 0.3°C min^{-1}. A similar effect of cooling rate was found when muscles were frozen to –60°C (Figure 22.2A).

It seemed likely that, as with cells, the slower cooling rate would have allowed more complete dehydration of the tissue during cooling, and hence less ice in the tissue as a whole. Thus, it was proposed that a reduction in the quantity of extracellular ice could explain the difference in survival. This notion was subsequently confirmed by freeze substitution studies carried out for groups of muscle treated similar to the function studies. As illustrated in Figure 22.2C and D, a noticeable difference was detected in the patterns of ice formation between the two groups. Cooling at 2°C

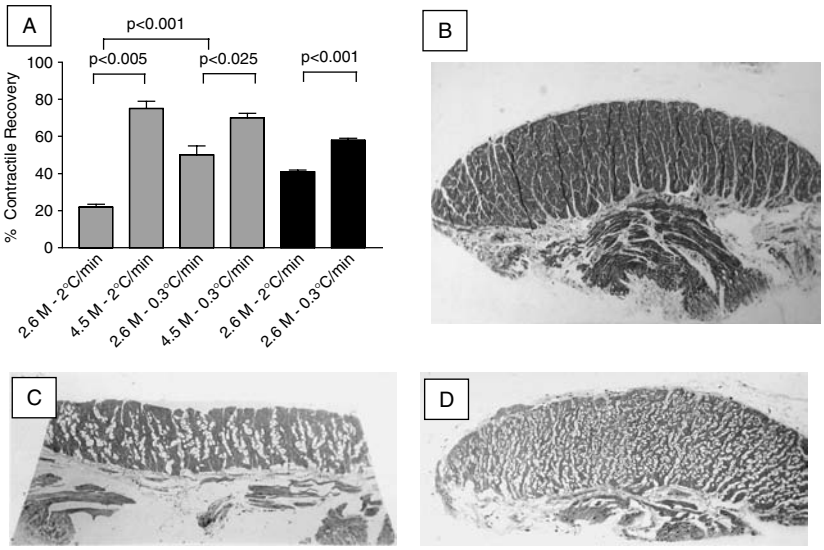

FIGURE 22.2 Structure and function of smooth muscle tissue after cooling to either –21°C, or –60°C in either the frozen or unfrozen state. (A) Histograms of postwarming contractility (mean ± SEM) normalized to the control responses derived before cooling at either –21°C (grey bars) or –60°C (black bars) under the conditions indicated. (B) Light micrograph of a section of nonfrozen control taenia coli smooth muscle showing the normal configuration of the muscle blocks that are the effector units of muscle contraction. (C) and (D) Light micrographs of freeze-substituted taenia coli smooth muscle depicting the location of ice domains after cooling to –21° at either 0.3°C/min (C), or 2°C/min (D). Details have been published elsewhere (Taylor and Pegg, 1982; Hunt et al., 1982) and are described in the text.

min^{-1} (Figure 22.2D) caused a random distribution of ice throughout the muscle tissue, whereas cooling at 0.3°C min^{-1} produced ice cavities that were predominantly in the extracellular matrix separating the muscle bundles and at the periphery of the bundles (Figure 22.2C). Because the muscle bundles are the effector units of muscle contraction, the greater disruption by ice of the muscle fasciae after cooling at the faster rate can explain the functional differences produced by varying the cooling rate (Hunt et al., 1982). The unequivocal conclusion from studies of this type was that the amount and location of extracellular ice has a dramatic effect on the postthaw function of complex tissues and organs. As a result, it is generally thought that cryopreservation of multicellular tissues and organs will mandate that the amount of ice in the system is limited, restricted to harmless sites, or preferably, that ice crystallization is prevented altogether.

22.4 APPROACHES TO ICE-FREE CRYOPRESERVATION

22.4.1 Equilibrium Approach

This approach to the avoidance of freezing during subzero cryopreservation was first proposed by Farrant in 1965. He suggested that if 60% of the cell water was replaced by a cryoprotective solute such as Me_2SO, freezing would be prevented at temperatures as low as –70°C; hence, any damage associated with the formation of ice crystals and the simultaneous rise in the concentration of solutes would be avoided (Farrant, 1965). Actually, Farrant's focus in developing this approach was principally on attenuating problems relating to increased solutes and, notably, electrolytes. The significance of the concomitant benefits of avoiding ice was appreciated more recently. This technique was explored using smooth muscle and involved the progressive mutual exchange of tissue water with Me_2SO during cooling and the gradual removal of the CPA during rewarming to minimize the known toxic effects of the CPA additive. The stepwise addition and removal of CPA

such that the system remained above the equilibrium freezing point at each stage is best appreciated by reference to a phase diagram as illustrated in Figure 22.1A. (Elford, 1970; Farrant, 1965). Although ice crystallization and solute concentration (other than that of the CPA itself) could play no part in cryoinjury sustained by tissue during storage, other factors such as the pH and the anionic composition of the medium in which the tissue was immersed were found to have a profound effect on survival (Elford and Walter, 1972a; Taylor, 1982; Taylor et al., 1978). Freezing could be avoided completely, irrespective of the cooling rate, provided the tissue was fully equilibrated with CPA at each stage. This approach, which was tested using smooth muscle cooled and kept unfrozen at –79°C, failed to provide adequate contractile function until adequate steps were also taken to optimize the ionic composition of the CPA medium (Taylor, 1982; Elford and Walter, 1972a, 1972b). Nevertheless, this equilibrium approach has not been pursued, presumably because the technique demands lengthy periods of exposure to toxic solutes to ensure equilibration and because permeation studies have shown that adequate exchange of tissue water with CPAs at subzero temperatures may not be achievable either in a practical timescale or without exceeding the tolerable limits of solute toxicity in the tissue (Elford, 1970; Elford and Walter, 1972b).

22.4.2 Nonequilibrium Approach—Vitrification

We will now turn our attention to the nonequilibrium approach to the avoidance of ice by focusing on the details of vitrification as it is currently practiced. The basic principles of this approach have been reviewed in great detail by others (Armitage and Rich, 1990; Fahy, 1988, 1989; Fahy et al., 1984; MacFarlane et al., 1992; Pegg and Diaper, 1990; Rall, 1987; see also Chapter 10). Thus, it will only be necessary here to outline the salient points that will enable an understanding of the practical applications to be discussed in this chapter.

Vitrification is the solidification of a liquid without crystallization. This state is achieved in systems that are sufficiently concentrated or that are cooled sufficiently rapidly that the increase in viscosity inhibits molecular rearrangement into a crystalline pattern. As cooling progresses, viscosity increases to the point at which translational molecular motion is essentially halted and the solution becomes a glass. The resultant solid retains the random molecular arrangement of a liquid but has the mechanical properties of a solid (MacFarlane, 1987; MacFarlane et al., 1992). We advisedly use the term "essentially halted" in referring to the attainment of molecular stasis during vitrification because there is a kinetic component to the process. In practical terms, the glass is a liquid that is too cold or viscous to flow, and although it is metastable in a strict thermodynamic sense, it is regarded as possessing pseudostability on the timescale of practical interest for biological preservation.

When considering the physico-chemical and biophysical responses of biological systems to low temperatures, it is important to be aware that events rarely take place under true equilibrium conditions. For example, the phase-change phenomena depicted schematically by the phase boundaries (e.g., T_m in Figures 22.1A and 22.1B) would hold true only for a simple binary system of CPA–H_2O in which supercooling was avoided by ensuring that nucleation occurred at the equilibrium freezing/melting point (T_m) during cooling at a sufficiently slow rate to prevent appreciable temperature gradients. Many interdependent factors determine whether an aqueous system, such as a biological system, approaches the thermodynamic state of lowest free energy during cooling. Metastability is thus often unavoidable, especially in concentrated systems. Many of these nonequilibrium states are, however, sufficiently reproducible and permanent to have been described as pseudoequilibrium states, and conversion of such metastable thermodynamic states to more stable forms may be subject to large kinetic barriers. The prevalence of so-called "unfreezable" or "bound" water in the vicinity of macromolecules is a prime example, where the expected path of thermodynamic stabilization by way of crystallization is prevented by large kinetic restraints (Franks, 1982a, 1982b; Taylor, 1987). A clear understanding of the occurrence and effects of metastable states during the cooling of compartmentalized living systems is complicated by the interaction of

thermodynamic and kinetic factors, which are difficult to separate. Moreover, these complexities are compounded when such systems are cooled rapidly to low subzero temperatures. Nevertheless, some basic principles have been established with the aid of model systems such as aqueous solutions of cryoprotective solutes and other macromolecules that interact with water by hydrogen bonding (Franks, 1977; MacKenzie, 1977). Such studies have permitted some qualitative interpretation of the nonequilibrium phase behavior of the fluids in cells and tissues during cooling and warming. To this end, phase diagram data such as those depicted in Figure 22.1 have proved to be a useful tool in understanding the physico-chemical relationship between temperature, concentration, and change of phase. A detailed discussion of the role and interpretation of solid–liquid state diagrams in relation to low-temperature biology has been given in a previous review (Taylor, 1987). In particular, supplemented phase diagrams that combine nonequilibrium data on conventional equilibrium phase diagrams serve to depict the important transitions inherent in cooling and warming aqueous solutions of cryoprotective solutes. Reference to these major transitions, illustrated in Figure 22.1, can serve to explain and summarize the principles of achieving vitrification as follows.

The equilibrium freezing curve, labeled T_m, is often described as the liquidus curve and represents the points at which a solution having a particular concentration will freeze (or melt) under equilibrium conditions of temperature change. Hence this curve represents the phase-change boundary for the two-component solution as a function of temperature. Cooling a solution below the liquidus curve will result in ice formation if the conditions are favorable for nucleation, with the result that the remaining liquid phase becomes more concentrated in the solute as defined by the curve. A discussion of the details of nucleation is beyond the scope of this chapter but has been the subject of excellent reviews published in recent years (Fahy, 1998; Mehl, 1996; see also Chapters 1 and 2). In practice, it is well known that freezing is rarely initiated at the liquidus point. Inherently, solutions tend to undercool to varying degrees before nucleation and ice crystal growth proceed at a significant rate. In pure water at temperatures above –38.5°C, ice formation is catalyzed by surfaces, usually particulate impurities, that act as seeds for crystal growth. This is the process of heterogeneous nucleation. If the process is avoided, pure samples of water will self-nucleate at –38.5°C, known as the homogeneous nucleation temperature (T_h; Taylor, 1987). As shown in Figure 22.1B, the temperatures of both heterogeneous and homogeneous nucleation are progressively lowered by increasing concentration of dissolved solutes.

The phase diagram for propane diol (Figure 22.1B) shows that in the region of 0 to 35% freezing will occur at some point 5 to 20°C below T_m, invariably by heterogeneous nucleation. At sufficiently high concentrations and low temperatures, the kinetics of the process become so slow that T_h is difficult to detect and any nucleated crystals that form in the region of T_h remain microscopic. As temperature is lowered further, molecular motion is slowed to the point at which translational and rotational molecular motion is essentially halted and the system is trapped in a high-energy state that resembles a liquid-like configuration, or a vitreous glass (MacFarlane et al., 1992). This glass transition (T_g) is associated with a marked change in physical properties such as specific heat and refractive index and certain mechanical properties such that T_g can be clearly identified. Determination of the transition temperatures that provide data for the construction of supplemented phase diagrams is usually derived from thermograms generated using differential scanning calorimetry or the related technique, differential thermal analysis. The kinetic nature of these transitions means that T_g has to be defined with reference to a particular set of experimental conditions. For example, changing the cooling rate means that the thermal events would occur over a different range of temperatures (MacFarlane et al., 1992; Moynihan et al., 1976).

Reference to Figure 22.1B shows that in the region of 35 to 40% propane diol, it is possible to cool samples through the T_h curve without apparent freezing and to form what have been referred to as doubly unstable glasses (Angell et al., 1981). This term reflects the fact that the vitreous system almost certainly contains ice nuclei and that if warming is not sufficiently rapid, further nucleation and crystallization will occur (devitrification), as signified by the curve T_d. Hence, during cooling, the sample attains the glassy state, but it invariably contains ice nuclei—the growth of

which is arrested along with all other molecular motions in the sample. However, on rewarming, crystallization can be detected, either visibly or by an exothermic event in a thermogram, reflecting the growth of ice by devitrification and recrystallization. The phenomenon of crystallization on warming a glassy sample to temperatures in the vicinity of T_g is often referred to as devitrification of a doubly unstable glass, as it is unstable with respect to both the liquid and solid states (Angell et al., 1981; MacFarlane, 1986). Hence, the process by which a metastable glass, or supercooled liquid obtained by heating the glass above its T_g, forms the stable crystalline phase is generally referred to as devitrification (MacFarlane, 1986). The growth of small existing ice crystals into larger more stable ice forms occurs by recrystallization (Forsyth and MacFarlane, 1986), and the important distinction between devitrification and recrystallization has been discussed in a previous review (Taylor, 1987).

In the higher concentration range of 41 to 50% for propane diol (Figure 22.1B), the T_h curve meets T_g, and in this region it is possible to slowly cool even bulk liquids directly to T_g without experiencing any detectable freezing events. However, it is noteworthy that in this region of the supplemented phase diagram, devitrification still occurs and has been taken as evidence for significant heterogeneous nucleation during cooling. Nevertheless, the amount of ice formed under these conditions is extremely small, and further growth can be prevented by using moderate warming rates, thereby avoiding devitrification. It can also be seen in Figure 22.1B that at concentrations above 50%, devitrification ceases to be detectable even at low warming rates and the system can be regarded as stable, as nucleation is prevented. The intersection of the melting curve and the glass transformation curve at T_g' indicates the minimum concentration of propane diol in aqueous solution that will vitrify irrespective of cooling rate. The concentration at which a glass transition occurs varies according to the nature of the solute. It appears that those systems with the strongest solute–solvent hydrogen bonding provide the best suppression of ice nucleation and promote vitrification (MacFarlane and Forsyth, 1990; MacFarlane et al., 1992). In extreme cases, when appropriate concentrations of cryoprotectant solutions are maintained in the amorphous state, even during slow cooling and warming rates, the biological component should in principle be protected (in the absence of cold shock or osmotic stresses befire cooling) because there would be no phase transition during cooling and warming and the injuries associated with the coexistence of two phases would be avoided.

22.4.2.1 Stability of the Amorphous State

If the physical phenomena outlined above for the attainment of a vitreous state are to have practical value for the cryopreservation of tissues, then the stability of the amorphous state is of paramount importance. Boutron and his colleagues have made comprehensive studies of the stability of the amorphous state for a variety of potentially important cryoprotective mixtures with a view to identifying the most useful compounds for improved cryopreservation without freezing (Boutron et al., 1986). The stability of the amorphous state has been defined empirically in terms of the critical heating rate, V_{cr}, above which there is insufficient time for a vitreous sample to crystallize, even to a limited extent, before T_m is reached. The smaller the value of V_{cr}, the more stable the amorphous state. The dependence of T_d on the rate of warming can be measured, and the difference $T_m - T_d$, corresponding to a given warming rate, has been used to define the stability of the amorphous state (Boutron and Kaufmann, 1979; Boutron et al., 1986). The warming rate for which $T_m - T_d$ is zero is defined as the critical heating rate, V_{cr}, for which the supercooled mixture does not devitrify or recrystallize. On the basis of these considerations, it has been shown that the stability of the wholly amorphous state of aqueous solutions of 1,2-propane diol and its glass-forming tendency are much greater, for the same water contents, than for all other solutions of commonly used cryoprotectants, including glycerol, Me_2SO, and ethylene glycol. Butane-2,3-diol is the only new cryoprotectant to have emerged in recent years with comparable, or slightly better, physical characteristics for vitrification than any of the aforementioned cryoprotective solutes (Boutron,

1990). Nevertheless, solutions of polyalcoholic CPAs, such as propane diol and butane diol, that show the most promise in terms of cooling rates and concentrations necessary for vitrification also required unrealistically high heating rates to avoid devitrification. Moreover, principally because of isomeric impurities that crystallize a hydrate at reduced temperatures, 2,3-butanediol has proved to have an unanticipated biological toxicity at concentrations below that necessary for vitrification (Hunt et al., 1991; Mejean and Pegg, 1991; Mehl and Boutron, 1988; Taylor and Foreman, 1991). This disappointing development led to attempts to use lower concentrations of butane-2,3-diol by adding polymers such as polyethylene glycol to promote the vitreous state (Sutton, 1992), but to our knowledge, successful vitrification of a biological system using such mixtures has not been reported.

Despite developments to devise solutions that would vitrify at practically attainable cooling rates for sizeable biological tissues, achieving the corresponding critical warming rate necessary to avoid devitrification remains a critical challenge. Conceptually, elevated pressures (MacFarlane and Angell, 1981), electromagnetic heating (Robinson and Pegg, 1999; Ruggera and Fahy, 1990; Marsland et al., 1987), and the use of antifreeze molecules (DeVries, 1983) have been proposed as ways to tackle the problem. Nevertheless, each of these approaches presents a set of new problems that must be overcome if practical solutions are to be realized. Control of ice crystal growth using appropriate natural or synthetic molecules is a promising area of research that we discuss in more detail below.

24.4.2.2 Material Properties and Cracking

On a macroscopic scale, instability of the amorphous state can be manifest as fracturing or cracking, with devastating consequences for a biological tissue encased within the glassy matrix (Pegg et al., 1997; Rall and Meyer, 1989). The formation of cracks during the vitrification of glycerol solutions was reported by Kroener and Luyet (1966), and more recently, fracturing has been recognized as a hazard during cryopreservation of a variety of tissues (Pegg et al., 1997; Wassenaar et al., 1995; Wolfinbarger et al., 1991). In addition to the anticipated and observed mechanical destruction of tissues by fracturing, it has been reported that fractures provide an interface for nucleation that can initiate devitrification (Williams, 1989).

In his review of the physical properties of vitreous aqueous systems, MacFarlane (1987) emphasized that information on the material properties of vitreous aqueous solutions does not exist. Material properties such as thermal conductivity and fracture strength of aqueous solutions in the glassy state have many similarities with their inorganic analogues that exist at normal temperatures, for example, window glass and ceramics, but studies of these properties in the context of cryobiology have not yet been made extensively. Nevertheless, some information on thermomechanical stresses in frozen systems has begun to emerge in recent years, paving the way for comparable studies to be undertaken in fully or partially vitrified tissues, as we outline in the following section.

24.4.2.3 Thermomechanical Stress during Cryopreservation of Tissues

Mechanical stress in a material is related to pressure, and it is the force per unit area that either pulls the material apart (tensile stress) or presses it together (compressive stress). The magnitude of stress is related to the deformation of the material, where deformation, or strain, is defined as the change in geometric size relative to the initial size. When a material is at its original length, with no force acting on it, the stress is zero. This stress increases as the material is stretched (strained) while being maintained at a constant temperature. The rate at which stress increases with increasing strain depends on the material; this material stiffness (elastic modulus) is much greater, for example, in steel than in rubber. Some materials exhibit a more complex relation between stress and strain. For some materials that have been stretched and then held at the stretched length, the stress decreases with time; this is called stress relaxation. Biological materials often exhibit such

time-dependent behavior. The rate of stress relaxation in materials generally tends to be lower when the material is held at lower temperatures. Extensive testing is often necessary to determine how the stress depends on straining, time, and temperature.

Changes of temperature produce another independent effect. Any material that is unrestrained will undergo a change in size (thermal strain) when subjected to a change in temperature. Materials in general, and tissues as they are cryopreserved in particular, shrink when they are brought from physiological temperature down to lower temperatures. Extensive testing is necessary to determine how the thermal strain (shrinkage) of a material depends on the temperature and possibly on the rate at which the temperature is changed. Under these unrestrained conditions, when thermal expansion or contraction is free to occur, the stress remains zero; that is, no forces act on the material.

As tissues are cryopreserved, they are externally free to shrink. However, in practice it is impossible to cool a tissue, of realistic size, uniformly; the outside surface decreases in temperature more rapidly than the inside. The outside of the tissue is forced to shrink less and the inside to shrink more. The level of stresses that must arise to accommodate the differential shrinkage is dependent on the stiffness and relaxation of the material. If these stresses are too severe, they have the potential to produce fractures. There are methods of predicting the stresses that arise because of nonuniform changes in temperature. These methods combine mathematical analysis with data that capture both the thermal strain caused by uniform temperature changes (material unrestrained at zero stress) and the time-dependent response of stress to strain as a function of temperature.

The development of stresses in biological tissues during freezing began being investigated after Rubinsky et al. (1980) proposed that mechanical destruction to cell membranes could arise from thermal expansion during freezing (known also as "thermal stress" or "thermomechanical stress"). Calculations of stress in freezing biological tissues have been carried out by Rabin et al. showing that thermal stress can easily reach the yield strength of the frozen tissue, resulting in plastic deformations or fractures (Rabin and Steif, 1998, 2000; Rabin and Podbilewicz, 2000). The driving mechanism of thermal stress is the constrained contraction of the frozen or vitrified tissue. It is commonly assumed that thermal expansion of frozen biological tissues is similar to that of pure water ice crystals (Rabin and Podbilewicz, 2000; Rubinsky et al., 1980), and Rabin et al. (1998) have confirmed this experimentally. Moreover, their studies provided some preliminary insight with regard to the effect of the presence of cryoprotectants on the thermal expansion. Results of pilot expansion tests of rabbit muscle permeated by the cryoprotectants Me_2SO and glycerol solutions, and pig liver perfused with Me_2SO solution, indicated that the cryoprotectants dramatically reduced the thermal expansion at higher temperatures and created a maximum value of thermal expansion within the temperature range of –70° to –100°C. A significant effect of the Me_2SO concentration on the thermal expansion of pig liver was demonstrated, and it appears that the thermal expansion decreases with the increase in Me_2SO concentration. A rapid change in thermal strain was observed in the lobe suspected of attaining the highest concentration of Me_2SO, which could be related to a change in physical properties associated with a glass transition. A more complete understanding of the effect of cryoprotectants on thermal expansion during cooling will require further detailed study in fully equilibrated tissues and with an experimental device designed specifically for vitrified specimens (Y. Rabin, personal communication).

Further insight into the mechanical properties of frozen soft biological tissues has also been provided by measuring the response of frozen liver, kidney, and brain to externally applied compressive stresses (Rabin et al., 1996, 1997). The mechanical properties under study in this work were the compressive strength and the elastic modulus. A new load chamber for measuring the stress–strain relationship of frozen biological tissues in the cryogenic temperature range was designed and constructed to enable such measurements. It was found that the stiffness of the frozen tissues is of the same order of magnitude as that of sea ice and that the yield strength of frozen tissues is up to one order of magnitude higher than that of sea ice; sea ice data are widely available in the literature.

A unique response of frozen biological tissues to compression was observed. We found elastic behavior up to rather small strains, on the order of 0.005, and a sawtooth pattern of stress thereafter, featuring a series of sudden stress drops followed by a linear return to a roughly constant upper level of stress. It was suggested that the stress drops are associated with the formation of micro cracks, which steadily accumulate until final failure. The highly heterogeneous nature of this material may allow such cracks to appear, but not to propagate. Complete unloading leaves the material with a permanent plastic strain; continued microcracking only resumed when the stress was returned to the previous level at which microcracking occurred. Hence, it appears that the mechanical response of frozen tissues can usefully be idealized by elastic perfectly plastic models (Rabin et al., 1997). It was argued that the relationship between the thermal expansion coefficient and the strength-to-stiffness ratio is the dominant factor for fractures to occur, as it represents the relationship between the driven source and the consequential mechanical response in the frozen material.

Rubinsky et al. (1980) were the first to suggest a model for prediction of thermal stress in the context of cryobiology. Unfortunately, predictions of mechanical stresses were inconsistent with one important observation of tissue destruction; namely, in cryobiological applications, severe fractures often form at the early stages of thawing and not, as commonly expected, during cooling. This phenomenon has recently been observed in cryopreserved blood vessels (Pegg et al., 1996). Comparable observations in the context of cryosurgical applications have also been reported (Rabin and Steif, 2000). This inconsistency prompted Rabin et al. to reexamine the assumptions underlying the models of freezing tissues presented to date, as it is now thought that behavior at the freezing front has not been properly modeled heretofore (Y. Rabin, personal communication). Specifically, the deviatoric stress should be zero at an advancing freezing front. Parenthetically, the deviatoric stress is the total stress minus the hydrostatic pressure; excessive deviatoric stress is known to be linked with the likelihood of fracture formation in the theory of solid mechanics. Any volume-preserving strain that occurs while the material is still in the liquid state cannot contribute to the deviatoric stress. Therefore, material that has just solidified at an expanding freezing front must start with zero deviatoric stress.

This new approach for thermal stress modeling of freezing tissues has been investigated recently by Rabin and Steif (Rabin and Steif, 1998, 2000). Typical cryopreservation procedures were analyzed by simulating an inward freezing of a sphere (Rabin and Steif, 1998), and a typical cryosurgical protocol was analyzed by simulating an outward freezing of a sphere (Rabin and Steif, 2000). In both cases, closed-form solutions were obtained, and it was shown that simulation results qualitatively follow experimental data. It was shown that for cryopreservation involving crystallization, the attendant potential for tissue destruction are unavoidable regardless of how slowly the freezing is carried out, provided there is a substantial expansion associated with phase transition. It was noted that the phase transition temperature may significantly decrease during the cryopreservation process, because of the elevated hydrostatic pressure in the unfrozen region. This line of research has not yet been expanded for the case of high cryoprotectant concentration and of the very high cooling rate applicable to vitrification.

In summary, attention to the thermomechanical aspects of cryopreservation in recent years has identified the key physical phenomena that contribute to mechanical stress and fracture formation. These include the thermal expansion, which is the driving mechanism of the process; the stress–strain relationship, which represents the behavior of the material under mechanical load; and the strength of the material, a mechanical stress threshold above which tissue destruction or fractures will occur. These phenomena are affected by many factors such as the temperature, the cooling rate, the warming rate, and the cryoprotectant type and concentration. It is anticipated that systematic study of these parameters and models will generate a more complete understanding of the conditions necessary to avoid thermomechanical stresses during cryopreservation. In the meantime, practical experience has shown that fracturing can generally be avoided by cooling and warming slowly below the T_g. Studies in both frozen (Pegg et al., 1997) and vitrified (Song et al., 2000b; Taylor et al., 1999)

blood vessels have shown that relatively slow warming to –100°C, at which temperature the vitreous material has softened, reduces the thermomechanical stresses and avoids macroscopic fractures.

22.4.3 Alternative Strategies for Achieving Vitreous Cryopreservation

It is clear from the foregoing discussion that the principal objective for achieving "ice-free" cryopreservation is to limit the nucleation and growth of ice during cooling and warming. Although there is now a considerable understanding of the physical processes involved in achieving this objective, achievement in bulky biological samples remains a challenge principally because of the constraints of heat transfer. Hence, successful application of this approach to the cryopreservation of tissues calls for innovative ways to either limit ice nucleation and growth or restrict ice formation to harmless sites within the tissue, as we have outlined above. It is worth emphasizing that ice *per se* is not always synonymous with cryoinjury, provided that its growth is constrained within appropriate limits (Mazur, 1988).

In view of this, it has been suggested that paradoxically, the promotion of ice nucleation might provide an alternative approach to controlling ice damage. The basic premise is that the intentional induction of very large numbers of nucleation events in the extracellular space would be less destructive than an equivalent total amount of ice organized as sizeable ice crystals. Conceptually, it might be feasible to induce nucleation on a massive scale in the region of a single temperature and thereby avoid the consequences of large ice crystal formation, provided of course, that recrystallization is also prevented. MacFarlane's group (Forsyth and MacFarlane, 1986) demonstrated that cooling conditions can be manipulated to produce an extremely high number density of small crystallites that, even after growth is complete, remain sufficiently small (<0.5 μm) to cause insignificant, observable scattering of ordinary light. For example, a 39% by weight solution of propane diol was annealed (held at constant temperature) near T_g for 2 h and remained visibly transparent. This experiment was attempted on the basis of previous observations that the nucleation rate increases with decreasing temperature in aqueous solutions such as these, whereas the growth rate, being a function of the transport properties, falls continuously as temperature is lowered. Thus, growth of the individual crystallites will be slow and nucleation of any new crystallites can continue such that the low-temperature annealing produces a sample containing a much higher density of ice nuclei than a comparable sample would contain during a simple cool/warm cycle. The concept is to anneal a vitrified tissue near T_g to provide for maximum nucleation and thereby prevent further growth during warming, as most of the freezable water would have already been consumed in the formation of the nuclei. It was also noted in these studies that the concentrated solutions (39%) did not appear to recrystallize (Forsyth and MacFarlane, 1986). By inference, ice crystallites smaller than the resolution of light might be expected to be innocuous to the integrity of tissues in which they form. However, this assumption remains to be validated by direct experimentation in cryopreserved tissues.

Another approach to produce maximally nucleated samples is to spike the solutions with special agents that promote nucleation. Products such as freeze-dried *Pseudomonas syringae* (commercially available as Snowmax for the snow-making industry) are potent ice nucleators that have been used experimentally to minimize supercooling in frozen samples (Fahy, 1998). It has been proposed that combining efficient ice nucleators with an abundance of antifreeze compounds, such as antifreeze peptides, might be a way to facilitate the formation and stabilization of myriads of supposedly harmless ice nuclei (Fahy, 1998; see also Chapter 5 for a discussion of similar principles in plants). This concept also awaits specific investigation as a practical approach to vitreous cryopreservation of tissues.

22.4.4 Ice-Growth Inhibitors

Through evolution, nature has produced several families of proteins that help animals (e.g., fish and insects) and plants survive cold climates (see Chapters 3, 5, and 7). These proteins are known collectively as antifreeze proteins (AFPs). AFPs have the ability to modify ice structure, the fluid

properties of solutions, and the response of organisms to harsh environments. The natural AFPs found in polar fish and certain terrestrial insects are believed to adsorb to ice by lattice-matching (Davies and Hew, 1990) or by dipolar interactions along certain axes (Knight and Duman, 1986). These molecules actually bind to the forming face of ice nuclei. By default, when temperature is lowered sufficiently, growth occurs preferentially in the *c*-axis direction (perpendicular to the basal plane) in a series of steps. This abnormal growth mode produces long ice needles, or spicules, that are much more destructive to cells and tissues than normal ice (Mugnano et al., 1995). Regardless, these molecules confer a survival advantage on certain animals. These observations led to the hypothesis that naturally occurring antifreeze molecules might be improved on by synthesis of molecules that will bind either to other ice nuclei domains or on stable ice crystals.

Discovery of new ice-inhibiting cryoprotectants for use in either classical cryopreservation or in molecular ice control techniques and vitrification has become an important focus of the research program of Organ Recovery Systems, (Des Plaines, IL and Charleston, SC). Chou (1992) mentioned an intention to specifically design ice crystal growth inhibitors. However, his interest was confined to minor modifications of existing naturally occurring AFPs and did not include preparation of *de novo* synthetic nonprotein antifreeze molecules. Historically, serendipity has been responsible for most discoveries of cryoprotectants. A major focus of our research has been the intentional design of synthetic ice blockers, which will combine with conventional cryoprotectants, and possibly naturally occurring antifreeze compounds, to minimize ice nucleation and growth during deep subzero cooling and subsequent warming. Two proprietary synthetic ice blockers have already demonstrated exceptional ice-blocking capabilities in our preliminary studies, as described below.

A complementary approach has recently been published by Wowk et al. (2000), who described the enhancement of vitrification solutions using synthetic polyvinyl alcohol. The mechanism of action of this compound is not clear, but based on visual observations and calorimetry, Wowk et al. suggest that polyvinyl alcohol blocks ice primarily by inhibition of heterogeneous nucleation. Such compounds might therefore be classified as antinucleating agents, as opposed to "ice blockers" that bind in some way to an ice nucleus and prevent or slow its growth into a damaging ice crystal.

Ice blockers are compounds that interact directly with ice nuclei or crystals to modify their structure or rate of growth. Examples of naturally occurring compounds include the antifreeze peptides and glycoproteins. Examples of synthetic compounds include the cyclohexanetriols and cyclohexanediols. Their properties are distinct from those of other cryoprotective solutes that lower the freezing point of solutions on a colligative basis. The latter are independent of chemical nature, whereas the former are highly dependent on chemical structure.

22.4.5 Preliminary Physical Studies on the Effect of Synthetic Ice Blocking Molecules

We have used molecular modeling techniques to identify molecular conformations that might complement the atomic spacing of hydrogen-bonding sites on the prism face of an ice crystal. Hypothetically, these structures might be expected to hinder the growth of ice by lattice-matching with available sites on the basal plane surface of an ice crystal, as illustrated in Figure 22.3. Such considerations revealed that 1,3,5 cyclohexanetriol or its -diol derivatives possess the required bond angles and distances to conform with this hypothesis and were selected as lead compounds in preliminary physical studies to determine their efficacy in controlling ice growth (Fahy, 2001). A variety of related molecular structures were tested, but 1,3 cyclohexanediol (1,3 CHD) and 1,4 cyclohexanediol (1,4 CHD) were found to demonstrate significant ice-blocking capability and proved to be more soluble than 1,3,5 cyclohexanetriol, which was impractical to use at concentrations greater than 3% (0.2 *M*).

Tests were conducted with concentrations up to 0.5 *M* (6%) of the new agents added to one of our preferred cryoprotectant mixtures. This solution (designated V49) is a slightly diluted version of the VS55 baseline vitrification solution comprising Me_2SO (2.75 *M*), 1,2-propane diol (2.0 *M*),

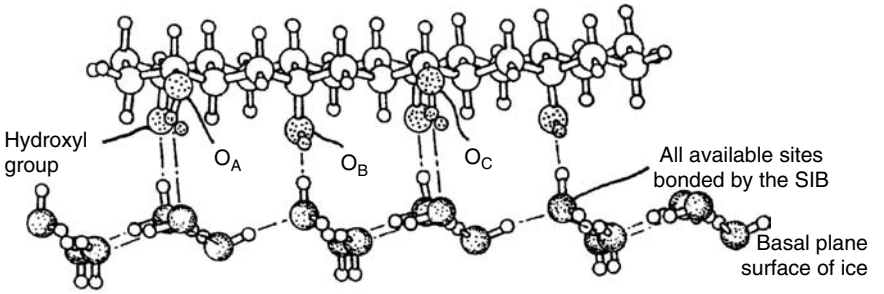

FIGURE 22.3 Molecular-modeling representation of the orientation of a synthetic ice blocker (SIB) with the basal plane of ice to demonstrate lattice matching. The model illustrates a remarkable coincidence between the spacing of strategically located hydroxyls on the SIB backbone and the 4.5 and 7.4 Å spacing of forward-projecting oxygen atoms of ice.

TABLE 22.1
Bulk Phase Ice Crystallization Measurements from Image Analysis

Cryoprotectant Solution	Number of Ice Crystals	Total Area Occupied by Ice (%)
V49 (7.5M CPAs)	Indefinite	100
VS55 (8.4M CPAs)	50 ± 6	1.3 ± 0.1
V49 + NaCl (6%)	609 ± 104	22.2 ± 1
V49 + Sucrose (6%)	5 ± 2	99.8 ± 0.1
V49 + 1,3 CHD(6%)	173 ± 76	2.3 ± 0.7
V49 + 1,4CHD (6%)	107 ± 66	1.7 ± 0.5
DP6 (6.0M CPAs)	2300 ± 385	39 ± 8
DP6 + 1,3 CHD (6%)	0 ± 0	0 ± 0
DP6 + 1,4CHD (6%)	6 ± 3	0.1 ± 0.1

Note: CPA, cryoprotective additive; 1,3 CHD, 1,3 cyclohexanediol; 1,4 CHD, 1,4 cyclohexanediol.

and formamide (2.75 *M*) and would not be expected to vitrify at low cooling rates under 1 atm pressure (Fahy et al., 1995). When cooled to temperatures below –34°C at slow rates (<3°C/min), V49 freezes with extensive ice crystallization throughout the sample (see Table 22.1). Cooling tests with V49 were performed in the presence of single synthetic ice blocking (SIB) compounds (6% w/v) or alternative control solutes of the same concentration known to have high colligative activity. The purpose was to identify specific ice-blocking activity compared with the more general colligative freezing-point depression function of additive solutes such as sodium chloride and sucrose. The data in Table 22.1 show that the presence of these SIBs caused a dramatic reduction of ice crystal formation and growth in bulk samples (75 mL) cooled to –100°C under slow cooling conditions.

Combinations of V49 and colligative solutes (e.g., NaCl and sucrose) yielded ice crystals in more than 20% of the volume (nearly 100% in the case of sucrose). By contrast, the SIBs were effective in reducing ice formation and growth such that the total ice volume in the bulk samples was less than 2%. In the case of our newer vitrification medium (DP6), which is a modification of VS55 that omits formamide (demonstrated to be a toxic component for several cell types; Campbell et al., 1999) and contains only 6.0 total CPA solutes (3 mols/l each of Me_2SO and Propanediol), the addition of the SIBs was effective in reducing the amount of ice from approximately 40% to negligible levels (data summarized in Table 22.1).

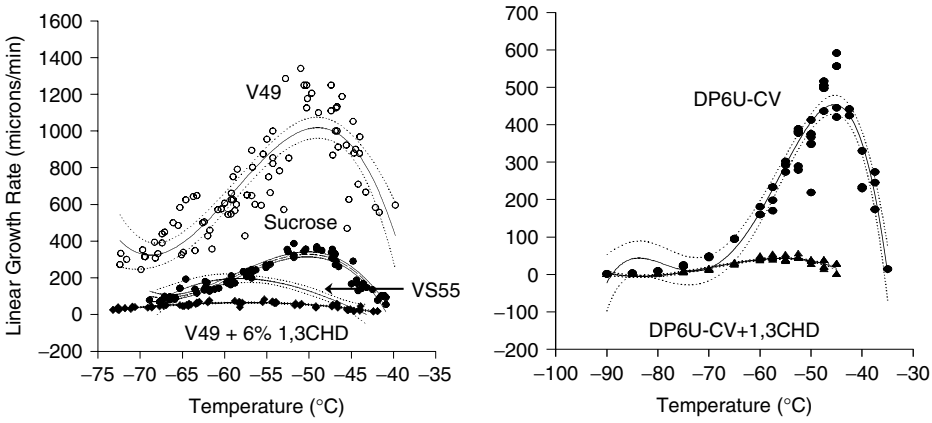

FIGURE 22.4 Kinetics of linear ice crystallization growth in vitrification solutions as a function of temperature. Solid lines represent the third-order regression curves fitted to the data with 95% confidence limits shown as dotted lines. DP6U-CV is DP6 solution prepared in Unisol (Organ Recovery Systems, Des Plaines, IL) cryoprotectant vehicle solution (Taylor et al., 2001; see text for details).

In additional experiments, the most effective ice-blocking compounds were evaluated for their ability to affect the kinetics of ice growth using cryomicroscopy. Figure 22.4 shows the linear ice growth rate measurements in both V49 and DP6 solutions alone and in solutions containing 6% sucrose or 1,3 CHD. Similar to the results in bulk solutions, the SIB molecule was more effective at slowing the rate of ice crystal growth in both solutions compared with sucrose at equal concentrations. 1,3 CHD has been shown to be highly effective for reducing the rate of growth to a very low level over the entire temperature range below the freezing point of V49 or DP6. Importantly, this combination of solutes proved more effective at controlling ice crystal growth than the baseline VS55 vitrification solution.

In comparable experiments using AFPs and antifreeze glycoproteins (AFGP), there was no statistical reduction in ice crystal growth rate by addition of either AFP III or AFGP to the V49 solution. This indicates that the small synthetic compounds exert their effect by a different mode of action to the much larger AFPs. Traditionally, the physical properties of AFP in solution have been quantitatively evaluated by nanoliter osmometry to determine the degree of thermal hysteresis. This is the difference between the freezing and melting points of a solution containing so-called "thermal hysteresis proteins" and typically amounts to a fraction of a degree depending on concentration (Barrett, 2001; Clarke et al., 2002; Duman et al., 1993; Ewart et al., 1998; Tyshenko et al., 1997). Using this technique, we have undertaken some preliminary experiments to examine the effect of our synthetic compounds on the thermal hysteresis (TH) of AFGP. Figure 22.5 illustrates the thermal hysteresis for AFGP as a function of concentration measured using a nanoliter osmometer (Clifton, Hartford, NY). Addition of 0.5 *M* 1,4-CHD (60 mg/mL) to AFGP had no significant effect, but in preliminary experiments using a similar concentration of 1,3-CHD, TH of the glycoprotein appears to be increased by ~35% at every protein concentration increment (Figure 22.5B).

Another highly significant finding was the changes seen in the ice crystal shapes as they formed in the presence of AFGP and 1,3-CHD. It is well documented that when an AFP solution is cooled to subzero temperature, the protein binds to the prism surface of hexagonal ice crystals and limits the growth along the *a*-axis (basal plane), but it does not affect the growth of the crystal along the *c*-axis (perpendicular to the basal plane). The result is the formation of needle-shaped ice spicules that are far more damaging to the frozen tissues and cells than normal hexagonal ice crystals (Mugnano et al., 1995). Addition of 1,3-CHD to the AFGP solution not only retarded the growth of ice crystals (at very low temperatures), it also changed the shapes of the forming ice crystals to

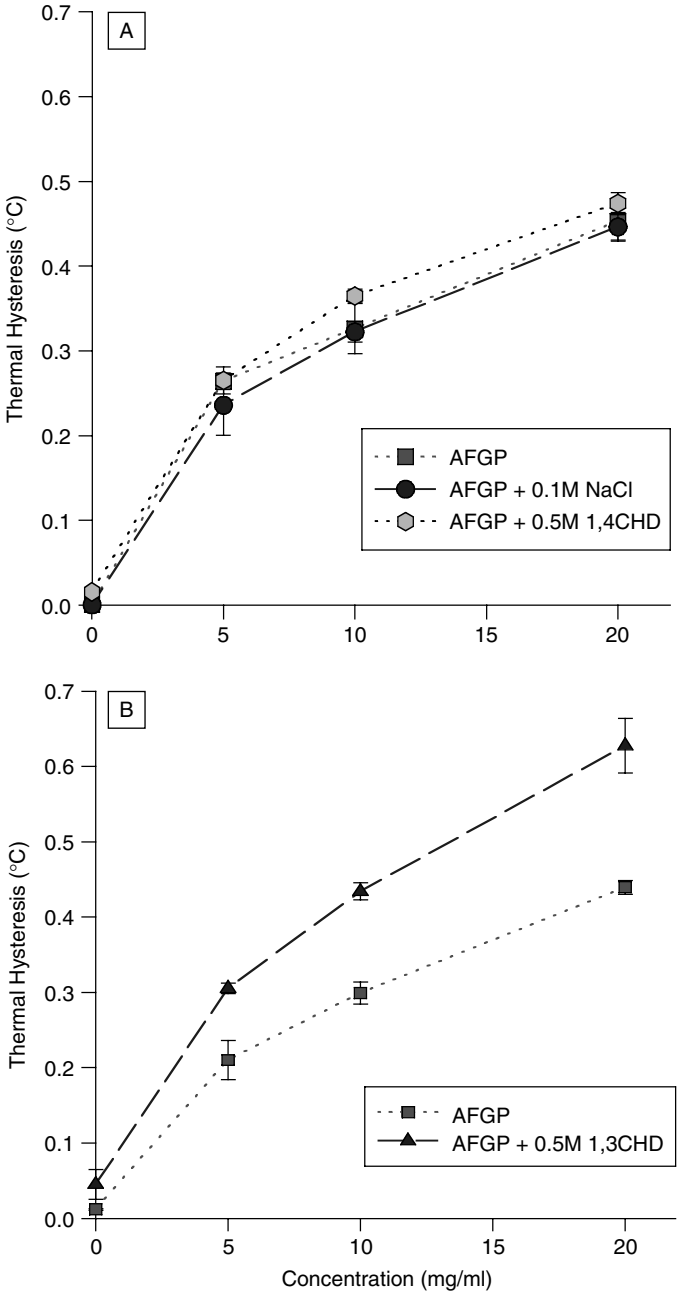

FIGURE 22.5 Thermal hysteresis of antifreeze glycoprotein (AFGP) and SIB molecules. Addition of 1,4 cyclohexanediol (0.5 *M*) or NaCl (0.1 *M*) to AFGP did not affect thermal hysteresis of the protein (A). A similar concentration of 1,3 cyclohexanediol potentiated thermal hysteresis of AFGP by 35% at various protein concentrations (B).

hexagonal, rectangular, or trapezoid forms as detected in the nanoliter osmometer. These observations could have a significant effect on designing new preservation media for freezing and storage of biological samples.

22.5 SYNOPSIS OF VITRIFICATION COMPARED WITH FREEZING, AND THE ADVANTAGES OF VITREOUS CRYOPRESERVATION

Conventional cryopreservation techniques, which require the substitution of up to 30% of cell water by a cryoprotective compound permit storage of many types of cells at deep subzero temperatures (typically <–100°C). When the rate of cooling is low enough, ice forms exclusively outside the cells and the external osmolality rises, dehydrating the cells. In fact, the ice is external to the system that it is desired to conserve, namely the cell, and the concentrated cell contents eventually solidify as an amorphous glass; that is, the cells vitrify. If cooling is too rapid to permit dehydration, and the cell contents actually freeze, the cell is invariably destroyed. It is noteworthy that this result shows that cells can tolerate the vitreous state. It has now been established beyond any doubt that the principal problem in attempting to cryopreserve tissues and organs is that ice forms within the system that it is desired to preserve, albeit outside the cells, and destroys both structure and function (Pegg, 1989; Taylor, 1984; Taylor and Pegg, 1982). It is clear that more than cell survival is needed in tissue preservation; complete structural integrity is vital. We have shown that some tissues and organs are severely damaged by extracellular ice and a mechanism that is adequate to account for the effect of extracellular ice in vascularized tissues – the rupture of capillaries by accumulating ice – has been demonstrated (Pegg, 1987; Pollock et al., 1986; Rubinsky and Pegg, 1988).

22.5.1 Avoidance of Ice

If a sufficiently high concentration of CPA could be used, the formation of ice would be avoided completely. The rates of cooling and warming are then unimportant because there is no driving force for transmembrane water movement and no ice to recrystallize during warming. The concentration of CPA necessary to avoid freezing is very high (typically ~60%) and "compatibility" (the absence of deleterious effects of the solute itself) is the essential problem such that the concentration of solute required is unattainable at suprazero temperatures. By taking advantage of the temperature dependence of most toxic actions, it is possible to increase the concentration progressively as the temperature is reduced. It was shown some years ago that by using this approach to increase the concentration of Me_2SO in a stepwise manner to remain above the equilibrium freezing point during cooling, it was possible to recover smooth muscle tissue with a high degree of stimulated contractile function (Taylor et al., 1978; Elford and Walter, 1972b).

More recently, an alternative approach has been explored, based on dynamic features, to reduce the amount of ice by selecting sufficiently high cooling rates to prevent ice nucleation. This approach produces a metastable state that is at risk of devitrifying (recrystallization) during warming, and ice formation during warming is just as injurious as during cooling. Nevertheless, vitrification procedures by this technique have been developed and shown to provide effective preservation for a number of cells, including monocytes, ova and, early embryos and pancreatic islets (Bodziony et al., 1994; Fahy, 1988; Jutte et al., 1987a, 1987b; Rall, 1987; Rall and Fahy, 1985; Takahashi et al., 1986). Vitrification refers to the physical process by which a concentrated solution of CPAs solidifies during cooling without crystallization. The solid, called a glass, retains the normal molecular and ionic distributions of the liquid state and is therefore usually considered to be an extremely viscous supercooled liquid. The difference between conventional cryopreservation and vitrification lies not in the occurrence of vitrification in only the latter method, but in the means by which vitrification is produced—by extracellular freezing and progressive cell dehydration during cooling in conventional preservation, and by achieving a vitrifiable system at the outset in vitrification (Pegg and Diaper, 1990).

When materials are vitrified, no ice forms, even at cryogenic temperatures. The formation of ice is prevented by the presence of high concentrations of chemicals that interact strongly with water and, therefore, prevent water molecules from interacting to form ice. It has been shown that depressing the homogeneous nucleation temperature until it equals T_g permits vitrification of

macroscopic biological systems. Prevention of freezing means that the water in a tissue remains liquid during cooling. As cooling proceeds, however, the molecular motions in the liquid permeating the tissue decrease. Eventually, an "arrested liquid" state known as a glass is achieved. A glass is a liquid that is too cold or viscous to flow. A vitrified liquid is essentially a liquid in molecular stasis. Vitrification does not have any of the biologically damaging effects associated with freezing because no appreciable degradation occurs over time in living matter trapped within a vitreous matrix. Vitrification is potentially applicable to all biological systems.

22.5.2 Advantages of the Vitrification Approach

Cryopreservation by the complete vitrification of the tissue suspension offers several important advantages compared with procedures that allow or require crystallization of the suspension. First, complete vitrification eliminates concerns for the known damaging effects of intra- and extracellular crystallization. Second, tissues cryopreserved by vitrification are exposed to less-concentrated solutions of CPAs for shorter periods of time. For example, during a typical cryopreservation protocol involving slow freezing to –40° or –70°C, cells are exposed to solutions whose concentration increases gradually to 21.5 and 37.6 osmolal, respectively. In contrast, cells dehydrated in vitrification solutions are exposed for much shorter periods of time to less than 18 osmolal solution, although the temperature of exposure is higher (Rall, 1987). Third, unlike conventional procedures that employ freezing, vitrification does not require controlled cooling and warming at optimum rates—cooling and warming need only be rapid enough to prevent crystallization, and this can generally be achieved without the need for specialist equipment. It is widely anticipated, therefore, that for many integrated multicellular tissues, vitrification may offer the only feasible means of achieving cryopreservation without ice damage, and for some tissues such as pancreatic islets that appear to partially withstand cryopreservation by freezing, vitrification offers a number of practical advantages that will be attractive in tissue engineering, as indeed they have been for embryo banking (Rall, 1987). On this basis we have committed to pursuing vitrification techniques for complex tissues, as we discuss in the remainder of this chapter.

22.6 APPLICATION TO VIABLE TISSUES

In a recent editorial article in the journal *Science* (Kaiser, 2002), "New Prospects for Putting Organs on Ice" were discussed with the focus on the need for ice-free cryopreservation methods. The consensus opinion is that viable tissues such as blood vessels, corneas, and cartilage that have proved refractory to cryopreservation using conventional freezing methods can only be successfully preserved with an adequate degree of poststorage function if steps are taken to prevent or limit the amount of ice that forms during the cooling and warming. Some of the recent work that has contributed to this consensus will be summarized here.

Kaiser's 2002 editorial article in *Science* accurately summarizes the state-of-the-art of tissue and organ cryopreservation in that year. Clearly, ice-free approaches have taken preeminence over the futile prospects of fine-tuning conventional freezing techniques to yield adequate methods for cryopreserving structured tissues and organs. The historical background to the ideals of a vitrification approach has already been outlined earlier in this chapter, and much of the current-day impetus for pursuing this approach is credited to Fahy's dedication over two decades to attempt vitreous cryopreservation of kidneys (Fahy, 1989; Fahy and Hirsch, 1982; Fahy et al., 1990). Although his ultimate objective remains elusive, the basic science that he and his collaborators have generated along the way has contributed significantly to the recent advances in this field. The successful application of vitrification as an alternative method for cryopreservation of embryos (Paynter et al., 1997; Rall, 1987; Rall and Fahy, 1985) and pancreatic islets (Bodziony et al., 1994; Jutte et al., 1987a, 1987b) provided enthusiasm for the prospect of applying these techniques to other tissues such as corneas, blood vessels, and cartilage that cannot be adequately preserved using freezing methods.

22.6.1 Corneas

Armitage et al. (Armitage and Rich, 1990; Armitage et al., 2002; Hall and Armitage, 1999; Rich and Armitage, 1991) focused principally on the formulation of the cryoprotectant solutions that would promote vitrification of rabbit corneas. Their work in this area culminated in a demonstration that corneas equilibrated in a solution containing 50% (v/v) propane-1,2-diol, 0.25 *M* sucrose, 6% (w/v) polyethylene glycol, and 2.5% (w/v) chondroitin sulfate could be vitrified, as judged by the absence of visible ice, when cooled to –110°C at approximately 6°C/min. Nevertheless, under these conditions devitrification was reported to be a persistent problem, indicating that the cooling and warming conditions have not yet been sufficiently optimized to avoid ice crystallization altogether.

Similar problems were encountered by Bourne and Nelson (1994) during their attempts to vitrify human corneas using a solution containing 3.1 *M* Me_2SO, 3.1 *M* formamide, and 2.2 *M* 1,2-propane diol in a corneal storage solution with 2.5% (w/v) chondroitin sulfate. It was demonstrated in these studies that increasing the time of exposure of each step in the cryoprotectant addition protocol from 10 to 25 min at 0°C permitted sufficient CPA permeation into the tissue to avoid any detectable ice in the corneas during cooling at ~10°C/min. However, this prolonged CPA-loading protocol led to unacceptable damage to the corneal endothelium, with the conclusion that further advances for cryopreservation of corneas using a vitrification technique will require careful optimization of the loading and unloading protocols for the cocktails known to vitrify at practical cooling rates (Bourne and Nelson, 1994). It is clear, therefore, that significant advances have been made toward ice-free cryopreservation of corneas, giving encouragement that this remains a reasonable approach for the development of improved methods of cryopreservation.

22.6.2 Vascular Grafts

In recent years, an increasing and sometimes urgent need has developed for prosthetic blood vessels for arterial bypass surgery. These prostheses are required for graft replacements in redo procedures and when autologous vessels are not available. In the present era of arterial replacement, at least 345,000 to 485,000 autologous coronary grafts (either arteries or veins; American Heart Association, 1996; British Cardiac Society, 1991) and over 200,000 autogenous vein grafts (Callow, 1983) into peripheral arteries are performed each year. A recent marketing report indicated that at least 300,000 coronary artery bypass procedures are performed annually in the United States, involving in excess of 1 million vascular grafts (Frost and Sullivan, 1997). Many of these patients do not have autologous veins suitable for grafts because of preexisting vascular disease, vein stripping, or use in prior vascular procedures. It has been estimated that as many as 30% of the patients who require arterial bypass procedures will have saphenous veins unsuitable for use in vascular reconstruction (Edwards et al., 1966).

Cryopreserved allogeneic veins are being used clinically (Brockbank et al., 1992; McNally et al., 1992). However, *in vivo* studies using these grafts in both animal models and patients have demonstrated poor long-term patency rates (Müller-Schweinitzer et al., 1998; Stanke et al., 1998; Almassi et al., 1996). These grafts also demonstrate reduced endothelial cell functions and impaired smooth muscle contractility after cryopreservation (Brockbank, 1994). In light of this, we have entertained the hypothesis that prevention of ice formation in blood vessels by an alternative cryopreservation approach, vitrification, will optimize cell functions and minimize extracellular matrix damage, resulting in more effective, durable grafts. We have recently completed studies on the vitrification of both veins and arteries (described in the next section).

22.6.2.1 Vitrification of Veins

A study was designed to evaluate a vitrification approach to storing a vascular tissue model (rabbit jugular vein) compared with a standard commercial method employing slow cooling with dimethyl

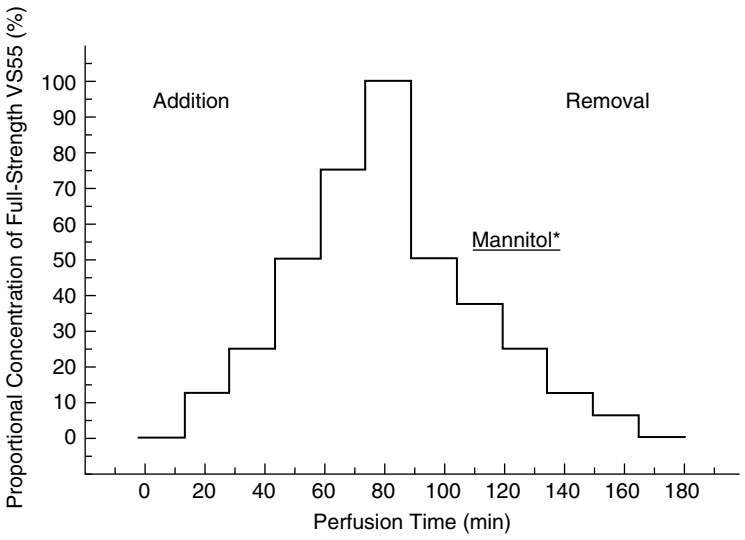

FIGURE 22.6 Incremental steps for the addition and removal of VS55 solution in the baseline vitrification process. During dilution the EuroCollins vehicle solution was supplemented with 300 m*M* mannitol as an osmotic buffer.

sulfoxide (Me_2SO) and chondroitin sulfate as the cryoprotective agents (Brockbank, 1994; Brockbank et al., 1992; McNally et al., 1992). This method was developed during several years of optimization studies by Brockbank and his colleagues for CryoLife, Inc., and remains the most widely used method of cryopreservation for clinical vascular grafts (Brockbank et al., 1992; McNally et al., 1992) because of CryoLife's dominance in the U.S. marketplace.

Following Brockbank's method for conventional cryopreservation involving freezing, the tissue was initially immersed for 20 min in HEPES-buffered Dulbecco's Modified Eagle's Medium (DMEM) containing 1 *M* Me_2SO, 2.5% chondroitin sulfate, and 10% fetal calf serum at 4°C. Samples were then cooled at a controlled rate of 1.0°C/min to –80°C and finally transferred to liquid nitrogen for storage. Thawing was accomplished by immersing the containers in a water bath controlled at 37°C until all ice had visibly disappeared, whereupon the containers were transferred to an ice-bath for elution of the CPA. This was achieved in sequential steps in which the tissue samples were transferred to DMEM containing 0.5 *M*, 0.25 *M*, and finally 0 *M* mannitol as an osmotic buffer.

A method for vitrification of vein rings and segments was developed in which a baseline vitrification medium (designated VS55 to reflect that it comprises 55% [w/v] total cryoprotective solutes, but previously designated as VS41A by its originators [Mehl, 1993]) was used to replace at least 50% of the tissue water with a combination of CPAs. The VS55 solution consisted of 3.1 *M* Me_2SO, 3.1 *M* formamide, and 2.2 *M* 1,2-propane diol in EuroCollins solution (Fahy, 1988; Rall and Fahy, 1985); the full strength mixture was added, and removed in stepwise manner as outlined in Figure 22.6. This protocol was introduced as a baseline technique, and no attempt to optimize the method was attempted at this stage (Song et al., 2000b; Taylor et al., 1999). In the vitrification experiments with vein rings, the tissue was immersed in vitrification solutions in glass vials at each step. Experiments with vein segments were carried out using a perfusion technique as follows.

The external jugular vein was perfused *in situ* to remove blood from lumen. A 4- to 5-cm length of vein was cannulated *in situ* at its distal end, and perfusion was performed for addition and removal of vitrification solution in these isolated veins. The perfusion system consisted of a reservoir (a 60-cc syringe) connected to the cannula with three-way stopcock. The reservoir was adjusted to

TABLE 22.2
Maximal Physiological Responses of Rabbit Jugular Veins

Drug	Control (g)	Frozen (g)	%	Control (g)	Vitrified (g)	%
Histamine	2.08 ± 0.15	0.52 ±0.05*	25.0	1.78 ±0.19	1.55 ±0.27*	87.1
Bradykinin	1.70 ±0.18	0.50 ±0.05*	29.0	1.75 ±0.18	1.49 ±0.15*	85.1
Angiotensin II	0.94 ±0.09	0.17 ±0.04*	18.0	0.58 ±0.06	0.49 ±0.09*	84.5
Norepinephrine	0.87 ±0.19	0.13 ±0.03*	14.9	0.99 ±0.12	0.83 ±0.14*	83.8

Note: Data are expressed in grams of maximal tension generated. Values are means ± S.E.M. **%,** percentage of corresponding fresh controls; frozen, vein rings cryopreserved with 1.0 *M* Me_2SO (n = 28–32); vitrified, vein rings vitrified with vitrification solution (n = 26); control, fresh vein rings (control for cryopreserved vein rings, n = 12–16; control for vitrified vein rings, n = 11–15).

* $P < .0001$ vs. fresh controls.

provide physiologic pressure by adjusting its height to provide a hydrostatic pressure of 80 to 100 mmHg at the cannula. The vein was placed in a Petri dish containing vitrification solution precooled to 4°C, and the dish was placed on ice during the perfusion process. Vitrification solution was added in six steps and removed in seven steps as shown in Figure 22.6.

Vitrification was achieved by cooling the samples rapidly (43° ± 2°C/min) to –100°C, followed by slow cooling (3° ± 0.2°C/min) to –135°C, whereupon they were transferred to a –135°C freezer for at least 24 h. Rewarming was accomplished in two stages: initially, samples were warmed slowly to –100°C (30° ± 2°C/min) and then warmed rapidly (225° ± 15°C/min) to melting, whereupon the vitrification solution was eluted in a stepwise manner as shown in Figure 22.6. Finally, all preserved samples were returned to physiological DMEM medium in preparation for viability testing or transplantation (Song et al., 2000b; Taylor et al., 1999).

In vitro function of fresh control tissue from each rabbit or preserved rings was assessed using a physiological organ-bath technique (Song et al., 1994, 1995). Each vein ring segment was mounted between two stainless steel wire hooks suspended in a custom organ bath (Radnoti, Monrovia, CA) containing 5 mL Krebs'-Henseleit solution, which was gassed continuously with 95% O_2/5% CO_2 at 37°C. One hook was fixed to the base of the organ chamber and the other was connected to a force transducer (Myograph F-60, Narco Bio-Systems, Houston, TX). Isometric contractile tensions were measured by adding a variety of agonists and antagonists to the tissue in the organ baths and recording the changes in developed tension relative to baseline values. The panel of drugs used in this study included histamine, bradykinin, angiotensin II, norepinephrine, and sodium nitroprusside. Because baseline responses can vary between different freshly isolated veins, the experimental design included paired controls for each preserved vein by testing the contractile responses of fresh untreated sample rings from each jugular vein harvested for the preservation studies.

The maximal contraction of fresh, frozen, and vitrified vein rings in response to the panel of agonists is shown in Table 22.2 (The data has been published in graphical form elsewhere; Song et al., 2000b). It can be seen that the maximum contractions achieved by the vitrified blood vessel rings in response to all four agonists were greater than 80% of fresh matched controls. In marked contrast, the maximum contraction index for frozen rings was less than 30% of fresh matched controls. Smooth muscle relaxation tests using sodium nitroprusside as the agonist drug showed that vitrified veins produced maximum relaxation of the precontraction. This response was similar to that of fresh control veins. In contrast, cryopreserved veins reached only 66% relaxation ($P <$ .01). Moreover, the dose response curves showed that the vitrified vessels demonstrated similar, if not slightly enhanced, drug sensitivities compared with untreated controls, whereas frozen vein rings exhibited decreased drug sensitivities (Song et al., 2000b).

22.6.2.2 *In Vivo* Studies

22.6.2.2.1 Graft Patency

Autologous vein implantation studies showed that the patency of fresh and vitrified rabbit jugular veins was not significantly different after 2 and 4 weeks, with both groups exhibiting short-term patency rates of ~90% (Song et al., 2000a).

22.6.2.2.2 Graft Histology

Graft rupture, aneurysm, thrombosis, or inflammatory infiltration was not noted in any of the patent grafts. Vitrification had not altered the pathophysiological cascade of events that occur when a vein graft is inserted into the arterial system. The vitrification process did not appear to induce any adverse effects locally or systemically *in vivo*. Morphological studies at both the light and ultrastructural level confirmed that vitrification had preserved endothelial cell and smooth muscle cell integrity posttransplantation. Details of these studies have been published elsewhere (Song et al., 2000a).

The demand for cryopreserved allogeneic veins is growing despite the well-documented immune response to these grafts and the low clinical patency rates. Between 1985 and 1992, approximately 3000 cryopreserved allogeneic vein segments were used for arterial bypass (McNally et al., 1992); however, the allograft veins cryopreserved using conventional cryopreservation methods produced less satisfactory results. Walker et al. (1993) reported that the cumulative survival rate was 14% and the cumulative secondary patency rate was 37% at 18 months. The grafts demonstrated reduced endothelial cell functions and impaired smooth muscle contractility after cryopreservation *in vitro* and poor long-term patency rates *in vivo* (Field et al., 1969; Gelbish et al., 1986; Jackson and Abel, 1972; Sellke et al., 1991; Showlater et al., 1989; Stephen et al., 1978; Tice and Zerbino, 1972). Our recent studies using an autologous animal model clearly demonstrate a significant benefit of vitrification for preservation of graft function.

22.6.2.3 Effects of Storage Temperature and Duration on the Stability of Vitrified Blood Vessels

As discussed above, a major concern regarding vitrified tissue storage has been glass stability at very low storage temperatures. Because of this, it has proved advisable for vitrified tissue samples to be stored a few degrees below the solution glass transition point (T'_g; –123°C for the VS55 solution used as a baseline solution in our studies). We further tested the hypothesis that vitrified materials would be stable at less than –160°C (±10°C) compared to –130°C. Rabbit jugular veins were vitrified using our standard vitrification protocol. The stability of the glass (the absence of ice crystallization) during storage and on rewarming was verified by visual inspection. Cell viability was assessed using the smooth muscle physiology method. There were five groups in this study: group 1, fresh control; group 2, vitrified veins stored at –130°C for 4 weeks; group 3, vitrified veins stored at –130°C for 4 months; group 4, vitrified veins stored at the temperature less than –160°C (vapor-phase liquid nitrogen) for 4 weeks; group 5, vitrified veins stored at the temperature less than –160°C for 4 months. The results showed that there was no ice formation in the vitrified samples during storage at either –130°C or less than –160°C. *In vitro* function of fresh control tissue from each rabbit or preserved rings was assessed using a panel of drugs as before. The maximum contractions achieved by the vitrified blood vessel rings stored at either –130°C or less than –160°C for either 4 weeks or 4 months were similar to veins stored for 24 h. The responses of stored samples were not significantly different to fresh controls for three of the four agonists. Smooth muscle relaxation tests using sodium nitroprusside (endothelium-independent) as the agonist drug showed that vitrified veins produced maximum relaxation of the precontraction. This response was similar to that of fresh control veins. Endothelium-dependent smooth muscle relaxation was tested using acetylcholine. No deterioration was observed at –160°C or over time. Dose response curves showed that the stored vessels demonstrated similar drug sensitivities compared with untreated fresh controls. This study demonstrated that the glass stability of vitrified samples

can be retained during vapor-phase liquid nitrogen storage and that the cell viability is maintained for at least 4 months.

22.6.2.4 Vitrification of Arteries

Additional studies focused on arteries to determine whether the methods effective for veins also work for arteries. There is an extensive literature indicating that autologous internal mammary, gastroeploic, and radial arteries are superior in terms of patency to autologous venous grafts. The autologous internal mammary artery has superior long-term patency when it is employed with a pedicle so that its vasa vasoral blood supply is uninterrupted (Barner et al., 1985; Loo et al., 1986; Zeff et al., 1988). Autologous arterial conduits used as free grafts, including internal mammaries and gastroeploic, radial, and inferior epigastric arteries, have similar or better patency rates than autologous saphenous veins (Acar et al., 1992; Loop et al., 1986; Mills and Everson, 1989; Puig et al., 1990).

A study was therefore undertaken to evaluate the feasibility of vitrification for arterial graft preservation of small-caliber arteries (<6 mm internal diameter) for clinical implantation. For this, our baseline vitrification method developed for veins was applied to arterial segments. However, there have not been any studies of small-diameter cryopreserved or fresh allogeneic arteries to compare with the studies of cryopreserved allogeneic saphenous veins for either coronary or peripheral artery bypass. Larger diameter allogeneic arteries may have been used as arterio-venous shunts for dialysis or in association with pulmonary or aortic allograft heart valves. Justification of research on small-diameter artery preservation based on the need for peripheral and coronary grafts is inappropriate and misleading, as allogeneic internal mammary arteries, gastroeploic arteries, and redial arteries are not being used clinically.

During rewarming of vitrified samples, a transparent glassy vitrification solution could be visualized at the early stage of warming, providing visible evidence that, at least by visual criteria, vitrification of the samples had been achieved. As with the prior vein study, cryosubstitution was used to confirm there was no detectable ice in the vitrified arteries, but extensive ice cavities were prevalent throughout the arterial specimens cryopreserved by the traditional method of freezing.

After rewarming and removal of cryoprotective agents, vessel function was evaluated using an *in vitro* contractility test. The maximum contractions achieved by the vitrified blood vessel rings in response to norepinephrine and phenylephrine were similar to those of fresh controls. In marked contrast, the maximum contraction index for frozen rings was less than 30% of fresh control arteries. In addition, vitrified arteries did not show significantly different sensitivity to the agonists compared to controls, whereas frozen arteries were significantly less sensitive to the agonists compared to control arteries ($P < .05$; Figure 22.7). Smooth muscle relaxation tests using sodium nitroprusside (endothelium independent) showed that vitrified and fresh control arteries relaxed to a similar degree. In contrast, frozen arteries reached only 9% relaxation ($P < .001$). Endothelium-dependent smooth muscle relaxation was tested using the calcium ionophore A23107. Although the maximum response in vitrified samples was compromised when compared with fresh controls (37 vs. 68%), the mean response was superior to the frozen vessels, which achieved only 28% relaxation. As with the veins, it was clear that prevention of extracellular ice formation improved vascular smooth muscle function.

Our current study outlined above demonstrated that vitrification is superior to conventional cryopreservation methods in preservation of rabbit carotid arteries. The VS55 solution preserved vascular smooth muscle function, but endothelial functions were not significantly better than in conventionally preserved arteries. VS55 was originally designed for vitrification of kidney slices (Mehl, 1993), and its composition may not be optimal for preservation of arterial endothelial function. Earlier studies had demonstrated significant improvements in endothelial function of vitrified veins compared with conventionally frozen veins (see previous section). Modification of the formulation of the vitrification cocktail and process are being investigated to improve arterial functions.

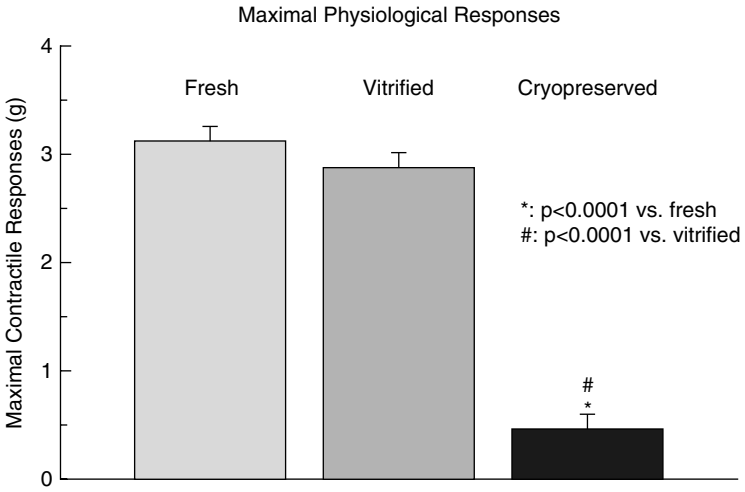

FIGURE 22.7 Maximal contractile responses of fresh, vitrified, and cryopreserved rabbit carotid arteries to noradrenaline. Arteries were either vitrified with VS55 solution or cryopreserved at a controlled rate (1°C/min) with 15% Me_2SO. All values are means ± SEM (n = 8). The *P* values were calculated using an unpaired Student's *t*-test.

22.6.3 Articular cartilage

Fresh osteochondral allografts have proven to be effective and functional for transplantation. However, the limited availability of fresh allograft tissues necessitates the use of osteoarticular allograft banking for long-term storage (Bakay et al., 1998; Marco et al., 1992; Malinin et al., 1985; Ohlendorf et al., 1996). Although cryopreservation by means of freezing is currently a preferred method for storing tissue until needed, conventional protocols result in death of 80 to 100% of the chondrocytes, along with damage to the extracellular matrix as a result of ice formation. These detrimental effects are the main obstacles preventing successful clinical outcome of osteochondral allografts (Ohlendorf et al., 1996; Stone et al., 1998; Tomford et al., 1984) and commercial success of tissue-engineered cartilage constructs. Allogeneic cartilage is generally considered immunologically privileged because of an absence of both blood vessels and a lymphatic system. Recipient immunosurveillance cells do not, therefore, come into contact with the graft's chondrocytes, and immunotherapy is not required for graft function. As a consequence, the search continues for a better preservation method.

Isolated chondrocytes can be preserved using conventional cryopreservation methods involving freezing; however, chondrocytes embedded in their natural matrix are extremely difficult to preserve. Studies using a variety of animal articular cartilage models (Ohlendorf et al., 1996; Marco et al., 1992; Muldrew et al., 1994; Wu et al., 1998) and human cartilage biopsies (Stone et al., 1998) have revealed no more than 20% chondrocyte viability following conventional cryopreservation procedures employing either Me_2SO or glycerol as cryoprotectants. Ohlendorf et al. (1996) used a bovine articular cartilage, osteochondral plug model to study their clinical cryopreservation protocol. This protocol employed slow-rate cooling and 8% Me_2SO as the cryoprotectant. They observed loss of viability in all chondrocytes except those in the most superficial layer at the articular surface. Muldrew et al. (1994) previously investigated chondrocyte survival in a similar sheep model. These researchers observed cells surviving postcryopreservation close to the articular surface and deep at the bone/cartilage interface. The middle layer was devoid of viable cells. More recently, Muldrew et al. (2001) demonstrated improved results using a step-cooling cryopreservation protocol, but cell survival posttransplantation was poor, and again there was significant loss of cells in the midportion of the graft. The reason for lack of cell survival deeper than the superficial layers

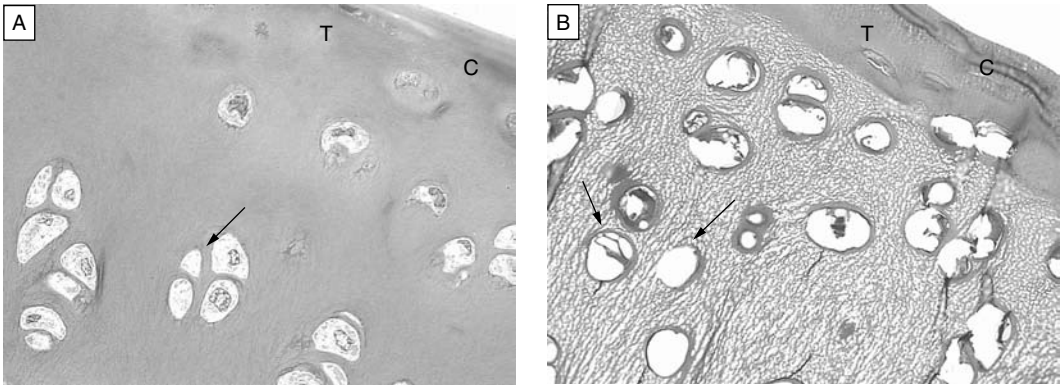

FIGURE 22.8 Light microscopy of vitrified and frozen cryosubstituted articular cartilage. (A) Vitrified cartilage. The matrix shows little evidence of ice; the tangential layer (T) reveals an elliptical chondrocyte (C) within its lacuna. The deeper chondrocytes (arrow) show some shrinkage. (B) Frozen cartilage. The chondrocytes within the ice-filled lacunae (arrows) appear totally disrupted; the deep matrix has considerable ice formation (white spaces). The chondrocytes (C) within the superficial and tangential layer (T) appear to have less ice. (100×).

of articular cartilage is most likely multifactorial and related principally to heat and mass transfer considerations (Karlsson and Toner, 1996). Surface cells freeze and thaw more rapidly than cells located deep within the matrix. This phenomenon could result in a greater opportunity for ice to form, both within cells and in the extracellular matrix, deeper within the articular cartilage. Furthermore, typically employed concentrations of Me_2SO (8 to 20%) may not penetrate adequately to limit intracellular ice formation.

In view of the consistently poor outcome of classical cryopreservation of articular cartilage reported by several groups, we hypothesized that vitreous cryopreservation would provide improved recovery. This hypothesis was tested by using the baseline vitrification method that had proved to be effective for the improved cryopreservation of vascular grafts (described above; Song et al., 2000a, 2000b). Refrigerated cartilage was used as a positive control for viability.

Cryosubstitution studies of frozen and vitrified articular cartilage samples revealed negligible ice in the vitrified specimens (Figure 22.8A) and extensive ice formation, both in the extracellular matrix and deeper lacunae, in frozen specimens (Figure 22.8B) by light microscopy. Some cell shrinkage was observed in the lacunae of vitrified specimens (Figure 22.8A), which was most likely related to high concentrations of cryoprotectants.

Viability was assessed using two methods: fluorescent microscopy using a live/dead stain, and a metabolic assay employing alamar blue. The fluorescence studies allowed observation at 30 to 50 μm deep into the tissue, and the metabolic assay gave an overview of cell viability for the entire implant. Figure 22.9 is representative of the three experiments performed in a preliminary study. Figure 22.9A depicts a quantitative estimate of the relative fluorescent intensity readings using calcein (viable) stain and indicates vitrified tissue had approximately 80% of the viability of fresh controls, whereas the frozen cryopreserved tissue was less than 13% viable. Moreover, the oxidation-reduction indicator, alamar blue assay indicated that the metabolic activity of vitrified samples was approximately 85% of fresh samples (Figure 22.9B) (Song et al., 2004b).

These studies combine to demonstrate that the vitrification process results in ice-free preservation of rabbit articular cartilage tissue and that between 80 and 85% of the cells were alive following rewarming. Frozen samples contained ice within the cells and the matrix, with the exception of the articular surface, where some viable cells were observed. For an *in vivo* assessment of survival and function of osteochondral allografts, tissue samples were randomly assigned to the three treatment groups: fresh, vitrified, and frozen. The O'Driscoll Score (O'Driscoll et al., 1985)

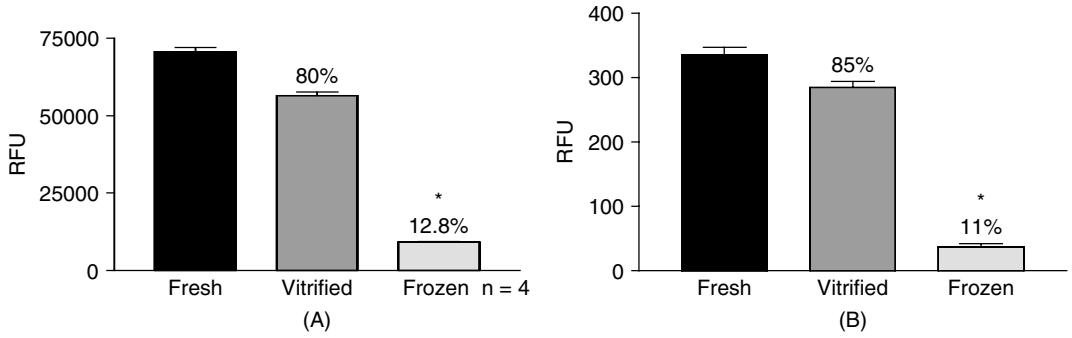

FIGURE 22.9 Viability assessments of articular cartilage samples using two fluorescence assays. (A) shows the mean (± sem) of four experiments using Calcein AM (measures intracellular esterase activity). (B) shows the mean (±sem) of ten samples of articular cartilage assayed using alamarBlue. Viability is expressed as relative fluorescence units (RFU) relative to the dry weight of each articular cartilage sample. *p<0.05 by one-way ANOVA, using the Kruskal-Wallis Test, Dunns Post-test.

is a composite of multiple factors that allows comparisons of overall graft survival between the three treatment groups. Analysis of variance showed that there was a significant treatment effect (P = .037), and a significant time effect (P = 0.031). The difference between the fresh and the vitrified samples was not significant ($P > 0.05$), but the differences between the fresh and the frozen ($P = 0.05$) and the vitrified and the frozen were significant ($P < 0.01$) at the 95% level of confidence.

The histology of fresh (4°C preservation) and vitrified explants was essentially the same, although the cells were less well organized within the explants when compared with unoperated control cartilage (Figure 22.10). The frozen cryopreserved explants were devoid of chondrocytes, and only fibroblast-like cells were present (Figure 22.10D) (Song et al., 2004a).

These data make a strong case for cryopreservation of cartilage by vitrification. The vitrification process protected the cartilage from damage associated with ice and resulted in 80 to 85% retention of cell viability. Moreover, fresh refrigerated and vitrified cartilage plugs performed similarly *in vivo* and were statistically superior to frozen plugs.

22.6.4 The Emerging "80/20" Rule

In the process of collating the information on tissue cryopreservation for this chapter, an interesting observation was made that warrants brief mention in summary of this section on the vitrification of tissues. Table 22.3 summarizes the viability data contrasting the outcome of vitrification with conventional cryopreservation involving freezing. Interestingly, the functional survival of vitrified tissues was approximately 80% or higher, whereas the frozen counterparts yielded less than 20% survival. This marked contrast (~80% vs. 20 to 30%) appears consistent irrespective of the nature of the tissue or the method of assay. This phenomenon serves to emphasize that avoidance of large amounts of ice in organized tissues can improve the outcome of cryopreservation from a meager 20% survival to a respectable 80%. It will be interesting in future studies to see whether the magnitude of this difference is also manifest in other tissues and whether the maximum recovery can be further improved by optimization of the vitrification protocols.

In addition, a better understanding of molecular signaling pathways involved in cell injury caused by cryopreservation is required. It is likely that both inhibition of apoptotic cell death and stabilization of cell membranes during cryopreservation may have significant benefits. The idea that there is a preservation threshold for the cryopreservation of viable tissues is depicted schematically in Figure 22.11. This schematic was constructed from the data given in Table 22.3, which shows for a variety of tissues that survival after cryopreservation could be increased from ~20% to ~80% by employing ice-free techniques. Althogh this represents a marked improvement that

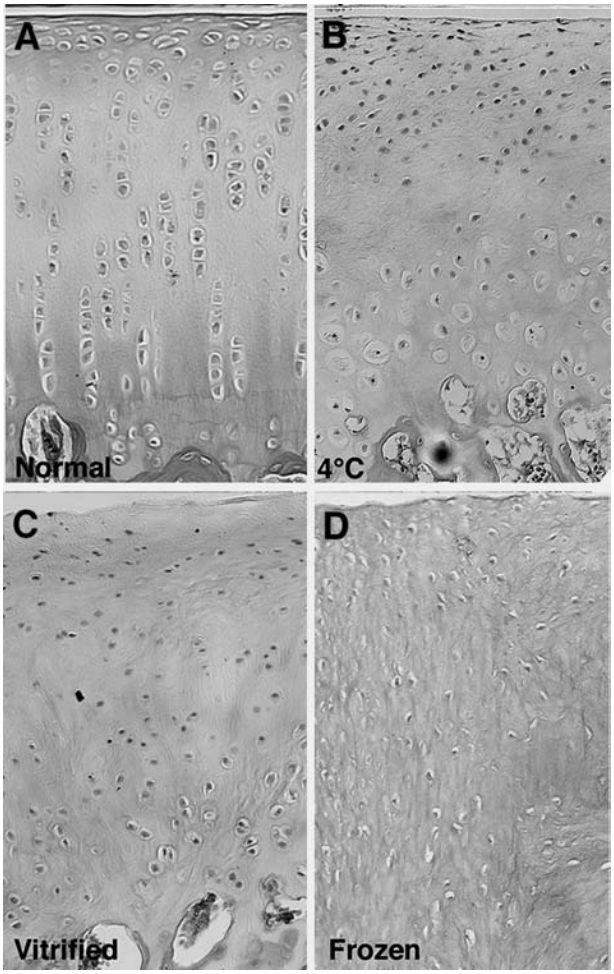

FIGURE 22.10 Histological comparisons of representative 12-week explants from three preservation procedures and control cartilage. (A) *Normal nonoperated rabbit articular cartilage:* In the deeper zone, large cubical chondrocytes lie in vertical columns. In the superficial perichondrial zone, cells are smaller, flat and lie parallel to the joint surface. (B) *4°C preservation:* Composed of hyaline cartilage-like tissue in the deeper zone and fibrous cartilage-like tissue in the superficial perichondrial zone. (C) *Cryopreserved by vitrification:* Similar to B in composition. (D) *Cryopreserved by Freezing:* The tissue is devoid of chondrocytes and most cells are representative of fibrous connective tissue.

elevates the preservation technique from one that yields inferior survival using freezing to one that provides respectable recovery using vitrification, it nonetheless represents a threshold that provides room for further improvement. The ultimate goal is 100% survival, and this might be approached by further optimization of ice-free technologies or by attention to the "molecular paradigm" that has recently been purported to be a way forward to remove the so-called preservation cap (Baust, 2002; Baust et al., 2001, 2002).

Moreover, as mentioned in section 22.4.1, the nature of the vehicle solution used to expose cells and tissues to cryoprotectants at low temperatures has been shown to affect the outcome of cryopreservation (Elford and Walter, 1972a, 1972b; Taylor, 1982; Taylor et al., 1978) and has recently become the focus of additional research aimed at optimization and attenuation of the cryopreservation cap (Baicu and Taylor, 2002; Baust, 2002; Baust et al., 2001, 2002; Taylor, 2002; Taylor et al., 2001).

TABLE 22.3
Contrasting Survival of Tissues after Cryopreservation in Either the Frozen or Ice-Free State

Model Tissue	Assay	Survival Outcome (%)		Reference
		Frozen	Ice-Free	
Taenia coli smooth muscle (Guinea pig)	Histamine-induced contractility	21	78	Taylor and Pegg, 1983
Jugular vein (Rabbit)	Various contractile agonists	6–22	84–87	Taylor et al., 1999 Song et al., 2000a
Carotid artery (Rabbit)	Various contractile agonists	<30	>80	Song et al., 2001b
Articular cartilage (Rabbit)	Esterase activity (Calcein AM)	13	80	Song et al., 2001a Brockbank et al., 2002
	Metabolic activity (alamarBlue)	11	85	Song et al., 2004a, b

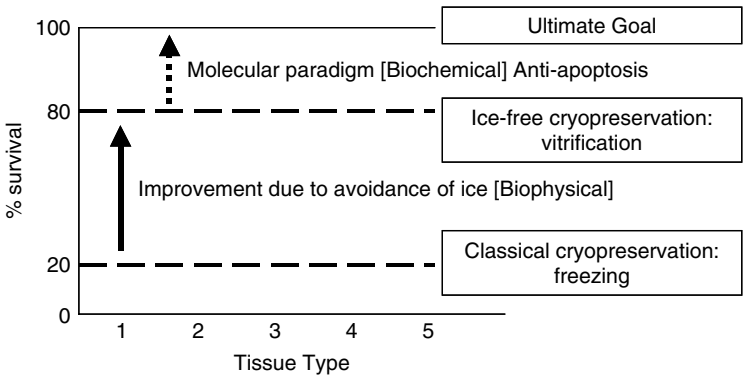

FIGURE 22.11 Schematic diagram to illustrate the paradigms that are considered to influence the outcome of the cryopreservation of multicellular tissues.

22.7 FUTURE DEVELOPMENTS IN RELATION TO TISSUE BANKING AND TISSUE ENGINEERING FOR TRANSPLANTATION

22.7.1 The Commercial Opportunity for Cryopreserved Tissues

Traditional tissue and cell storage cryopreservation methods have well-recognized technical problems that include tissue cracking, matrix disruption, and posttransplantation apoptosis and calcification. Freeze-drying of tissues is still nowhere near a commercial process. The potential for U.S. economic benefits of widely available tissue products was made very clear in the request for proposals from the National Institute of Standards and Technology, Advanced Technology Program, in 1997 which specifically identified "defining and designing conditions for long-term tissue and cell storage that will make products globally available in varying environmental conditions" (http://www.atp.nist.gov). The bottom line is that it was estimated that tissue engineering may address diseases and disorders that account for about one half of the existing U.S. health care costs, which in 1995 dollars had exceeded $1 trillion. We anticipate that this technology will first be applied to research products followed by orthopedic clinical products, such as tissue-engineered cartilage and bone constructs. Cardiovascular products using this technology, including allogeneic human tissues, will follow close behind the orthopedic products. The potential markets are enormous. In 1996, a Frost and Sullivan report indicated that the heart valve replacement and skin

TABLE 22.4
Potential U.S. Organ and Tissue Markets

Structure	Procedures/Year
Skin	4,750,000
Cartilage	1,132,100
Blood Vessels	1,100,000
Pancreas	728,000
Kidney	600,000
Breast	261,000
Liver	155,000
Tendon and Ligament	123,000
Intestine	100,000
Ureter and Urethra	81,900
Heart Valves	65,000
Bladder	57,200

Sources: American Heart Association, 1996; Langer and Vacanti, 1993.

repair product markets alone have maximum potential market sizes of $225 and $5945 million, respectively. Revenues in the total market are anticipated to continue growth at double-digit rates (Frost and Sullivan, 1996). The ultimate potential annual U.S. market size for organ and tissue transplants projected by Drs. Vacanti and Langer (Langer and Vacanti, 1993), assuming unlimited supply of transplants for all potential applications, is indicated in Table 22.4. Many scientific advances in the fields of tissue engineering and xenotransplantation, however, are required for this potential to materialize. The numbers are also predicated on transplantation being such a safe procedure that it would be considered appropriate therapy for a wide range of organ and tissue diseases. Tissue engineering may eventually address diseases and disorders that account for approximately half of all existing health care costs (Langer and Vacanti, 1993; Nerem and Sambanis, 1995; Wilkerson Group, 1992). This market is expected to be affected by major technological changes, and most products will contain highly specialized cell components that will require effective transport solutions and devices to enable product distribution and increase shelf-life.

Research efforts in bioengineering of tissues and organs are driven by the shortfall in allogeneic tissues and organs. It is anticipated by both the scientific and business communities that with new discoveries in tissue engineering, xenotransplantation, and the development of new immunosuppressive therapies, many more diseases will be treated by replacement of defective components (Table 22.4). Hence, the need for reliable methods of storage and shipping will become increasingly important as these developments emerge.

22.7.2 Remaining Hurdles for Commercialization of Vitrification Procedures

Vitrification is a relatively well-understood physical process. There are, however, a number of significant challenges for commercial deployment of this new technology. Vitrification approaches to preservation have some of the limitations associated with conventional freezing approaches. First, both approaches require low-temperature storage and transportation conditions. Neither can be stored above their T_g for long without significant risk of product damage caused by inherent instabilities leading to ice formation and growth (see Chapters 20 and 21). Both approaches also use CPAs with their attendant problems, discussed below, and require competent technical support during rewarming and CPA elution phases before product use. Therefore, it is possible to employ

vitrified products in highly controlled environments, such as a commercial manufacturing facility or an operating theatre, but not in a doctor's outpatient office, or in third-world environments.

22.7.2.1 Challenges Relating to the Use of Cryoprotectants

The high concentrations of CPAs necessary to facilitate vitrification are potentially toxic because the cells may be exposed to these high concentrations at higher temperatures than in freezing methods of cryopreservation. (This point is discussed more fully in Section 22.5.) Cryoprotectants can kill cells by direct chemical toxicity, or indirectly by osmotically induced stresses during suboptimal addition or removal. On completion of warming, the cells should not be exposed to temperatures above 0ºC for more than a few minutes before the glass-forming cryoprotectants are removed. Chemical toxicity of CPAs is invariably temperature dependent, but in some cases even subzero exposure can be detrimental. (Chilling injury is a thermal event and not related to CPAs, which may actually protect against thermal shock.) There may be issues concerning safety of some CPAs in conjunction with medical products. Formamide, one of the components of VS55, is a known mutagen. Alternatives to formamide with fewer safety risks and potentially easier clinically acceptance are being tested. In addition, a better understanding of molecular signaling pathways involved in cell injury caused by vitrification is required. It is likely that both inhibition of apoptotic cell death (Baust, 2002; Baust et al., 2000, 2001) and stabilization of cell membranes during vitrification may have significant benefits.

Since 1986, computer-operated organ perfusion devices have been employed to introduce and remove cryoprotectants from animal organs (Fahy, 1994). The primary organs studied to date have been the rabbit kidney and rat liver. At the present time, it is possible to introduce into these organs CPA solutions that are capable of vitrification at ambient pressure, by employing subzero perfusion temperatures for both addition and removal. Use of subzero perfusion temperatures helps to overcome cryoprotectant cytotoxicity. Kidneys, which have been perfused with vitrification solution, cooled to subzero temperatures (but not vitrified), and then perfused to remove the vitrification chemicals, have been reimplanted. These kidneys were shown to function *in vivo* and in some cases supported life for several months (Fahy et al., 1995). This, and our own demonstration of the tolerance of vascular grafts and articular cartilage to vitrifiable concentrations of CPA cocktails, shows that the constraints of CPA toxicity and fluxes can be overcome by selection and optimization of appropriate conditions. This leaves heat transfer issues as a primary hurdle for scaling up the successes in model tissue samples to larger specimens of clinical dimensions.

22.7.2.2 Challenges Relating to Thermomechanical Stresses

As outlined in this chapter, techniques have recently been developed to preserve native tissues such as blood vessels and articular cartilage in an essentially ice-free condition. However, although vitreous cryopreservation has been demonstrated to provide superior preservation compared with conventional freezing methods in these small model systems, cryopreservation of large tissue samples continues to be hampered by thermomechanical constraints. These include problems arising from the limits of heat and mass transfer in bulky systems and damage induced by mechanical stresses including fractures. Such fractures are attributed to stresses that can arise because of the nonuniform cooling of larger tissues. In fact, the higher cooling rates that facilitate vitrification will typically lead to higher mechanical stresses. The competing needs of vitrification and minimization of mechanical damage demand a greater understanding of both vitrification and stress development. Although mechanical stress has long been recognized as an important mechanism of tissue destruction, it has received very little attention in the context of cryobiology. It is our opinion that in some circumstances, even a single major fracture may prevent the tissue from recovery, or effective use after cryogenic storage. Reduction of mechanical stress, and thereby prevention of fracture formation, is a necessary integral condition for successful cryopreservation of large specimens.

We have already indicated that heating constraints impose severe limitations on the ability to rewarm vitrified specimens without danger of devitrification. Hence a major hurdle for deployment of vitrification methods is the development of effective rapid-warming techniques for larger specimens (>10 mL volume) to prevent devitrification and ice growth by recrystallization; devitrification is the freezing of a formerly vitrified solution. To prevent devitrification with current technology, the vitrified material must be warmed uniformly at up to 300°C/min so that ice does not have the opportunity to form in significant quantities. To achieve this warming rate, materials must be warmed in 20 sec or less. Many simple structures, such as single-cell suspensions or cell aggregates (with which vitrification has been successful) are small enough that rapid-warming rates can be achieved using immersion in warm fluids.

The same is true for the small model tissues for which vitrification has been successful. Unfortunately, tissue that has been engineered and allogeneic organs and tissues are generally of a much larger volume; thus, more sophisticated techniques of warming are required. Microwave warming has been attempted but has never been successful because of the uneven warming of specimens and problems with thermal runaway, which results in heat-denatured tissues. Ruggera and Fahy (1990) reported success in warming test solutions at rates of up to about 200°C/min using a novel technology based on electromagnetic techniques (wire length resonance radio frequency warming) developed and owned jointly by the American Red Cross and the U.S. Food and Drug Administration. Unfortunately, unpublished results indicate that this method is also problematic because of the uneven warming of specimens and problems associated with thermal runaway. Pegg et al. are credited with the most comprehensive studies of this approach to rapid heating of cryopreserved specimens. During a period spanning more than a decade, they have systematically developed a new device for dielectric heating to achieve uniform and high rates of temperature change (Marsland et al., 1987; Robinson and Pegg, 1999). At this point in time, the focus has been on the electromechanical developments of the technique, which has been reported to yield maximum warming rates of more than 10°C/sec (600°C/min). Using Pegg et al.'s device, frozen samples may be warmed from –65°C to room temperature in less than 30 sec, with final spatial differences of less than 20°C. Application of this technology to the survival of cells and tissues has not yet been reported, so it is still not possible to say whether this approach will provide an answer to the problem of rapid heating of cryopreserved tissues with adequate retention of cell viability and tissue function.

As we have mentioned already, an alternative, or even adjunctive, approach involves the use of molecular ice-control techniques to prevent the damaging growth of ice crystals during cooling or warming. The objective is to identify molecules that specifically interact with ice nuclei, resulting in either prevention of ice nucleus development or modification of ice crystal phenotype. These natural or synthetic molecules promise to have benefits in both freezing and vitrification preservation protocols either by rendering ice crystals less damaging or by permitting reduction of CPA concentrations, respectively.

There is now unequivocal evidence that ice formation within the extracellular matrix of multicellular tissues is the principal event that limits the survival of cryopreserved tissues using conventional freezing techniques. This mode of injury can be circumvented using vitreous cryopreservation, which in the case of relatively small tissue specimens has recently been shown to markedly improve functional outcome. However, this ice-free method of cryopreservation is not easily applied to larger bulk samples of clinically relevant dimensions because of the effects of nonuniform cooling and rewarming, which are the source of thermomechanical stresses in the vitrified tissue samples. Moreover, these additional stresses in cryopreservation have not heretofore been studied in sufficient detail to affect the rational design of improved methods of preservation. It is our opinion that effective methods of cryopreservation of tissues and engineered tissue constructs will only be realized using methods of preservation that not only minimize the effects of ice formation (via vitrification) but also avoid thermomechanical stresses in the cryopreserved samples during cooling, storage, and rewarming.

Finally, it should be understood that vitrification is a technology that must be deployed with extreme care and diligence. Errors in technique are common and can lead to failure to obtain satisfactory results. A large part of our current research program is definition of the limits for each step in the vitrification process including reduction of CPAs to the lowest possible concentrations, long-term storage conditions and duration, and simplification of the CPA addition and removal steps. Such efforts will produce vitrification procedures that can be deployed with less risk of tissue quality being compromised.

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