8 Tissue Preservation

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8.1 INTRODUCTION

The purpose of this chapter is to provide an overview of the "state of the art" for biopreservation of tissues ranging from simple clumps of cells to complex tissues containing multiple cell types. Preservation of specific tissue types including ovaries, corneas, vascular grafts, articular cartilage, heart valves, skin, and pancreatic islets have been the subject of recent reviews.[1] The use of human allogeneic tissues, such as skin, heart valves, and blood vessels in medicine has become normal practice. However, while clinical demand for tissues continues to grow, supply of these valuable human resources has become a limiting factor. As a result, the development of living engineered constructs has become an important new field of biomedical science and biopreservation of donor tissues and manufactured product is generally recognized as an important issue without which many market applications may never be fully achieved.

The differences between simple cell suspensions and structured, multicellular tissues with respect to their responses to cooling, warming, and dehydration clearly impact their requirements

for biopreservation. These differences have previously been described in detail with respect to the response of structured tissues to freezing and thawing.[2,3] We regard biopreservation to be a crucial enabling technology for the progression from preclinical and translational clinical research on cellular tissue products for regenerative medicine and transplantation. Tissue biopreservation is also needed for samples to be used for various research and toxicology test purposes. The need and advantages of tissue biopreservation are widely recognized and well documented.[3,4] There are several approaches to biopreservation, the optimum choice of which is dictated by the nature and complexity of the tissue and the required length of storage. Obviously, tissues such as bone and tendon that are banked successfully without a viable cell component (often referred to as nonliving tissues) are far more robust in withstanding the stresses of preservation than "living" tissues that invariably contain cells that must retain viability for maintenance of tissue functions. Maintenance of structural and functional integrity of living tissues is demanding and will be the principal focus of this chapter.

Short-term preservation of tissues and organs that cannot yet be successfully cryopreserved because they sustain too much injury at deep subzero temperatures can be achieved using either normothermic organ culture, in the case of corneas, or more commonly hypothermic storage at temperatures a few degrees above the freezing point. Normothermic organ culture is usually limited to corneal preservation for periods of up to a month or more.[4,5] In contrast, hypothermic storage is commonly employed for many types of tissue for transport between tissue donation sites, processing laboratories and end users, and short-term storage. These approaches to short-term biopreservation are dealt with in other chapters (see Chapter 2) and will be covered only in outline here for completeness.

With the exception of normothermic organ culture, all approaches to biopreservation aim to stabilize biological tissues by inhibiting metabolism and significantly retarding the chemical and biochemical processes responsible for degradation during *ex vivo* storage. Long-term preservation calls for much lower temperatures than short-term hypothermic storage and requires the tissue to withstand the rigors of heat and mass transfer during protocols designed to optimize cooling and warming in the presence of cryoprotective agents (CPAs). As we will explain below, ice formation in structured tissues during cryopreservation is the single most critical factor that severely restricts the extent to which tissues can survive cryopreservation procedures involving freezing and thawing. In recent years, this major problem has been effectively circumvented for several tissues by using ice-free cryopreservation techniques based upon *vitrification.*[6]

Long-term preservation in the presence of ice is achieved by coupling temperature reduction with cellular dehydration. In principle, stabilization by dehydration without concomitant cooling can be achieved for long-term storage at ambient temperatures.[7,8] However, the application of this approach to mammalian systems is in its infancy and will be addressed at the end of this chapter as a prospective development for the future.

Obtaining adequate and reproducible results for cryopreservation of most tissues requires an understanding of the major variables involved in tissue processing and subsequent preservation. Optimization of these variables must be derived for each tissue by experimentation guided by an understanding of the chemistry, biophysics, and toxicology of cryobiology.[2,3,6,9,10] Before discussing the factors affecting tissue quality in detail, it is necessary to consider the meaning of "viability" with respect to tissue function *in vivo*. Viability may simply be defined as the ability of preserved tissues to perform their normal functions upon return to physiological conditions. Many means of assessing cell viability within tissues have been described including amino acid uptake, protein synthesis, contractility, dye uptake, ribonucleic acid synthesis, and 2-deoxyglucose phosphorylation.[11] The assay(s) used to determine viability should give clear indications that the cells are alive and, preferably, should report on activities important for long-term tissue functions.

Multicellular tissues are considerably more complex than single cells, both structurally and functionally, and this is reflected in their requirements for cryopreservation. Some cell systems,

such as platelets and sperm, may be subject to thermal or cold shock upon cooling without freezing. In general, tissues are not known to be sensitive to cold shock. However, due to concerns that CPAs, such as DMSO, may increase tissue sensitivity to cold shock[12,13] or result in cytotoxicity, CPAs are usually added to tissues after an initial cooling to 4°C prior to further cooling. Frozen tissues have extensive extracellular and interstitial ice formation following use of tissue bank cryopreservation procedures. Such frozen tissues may, in some cases, have excellent cell viability. In other cases, as discussed later, viability may be very poor or cell viability can be good but cells in the tissue may no longer operate as a functional unit. It is usually not possible to detect where the ice was present after thawing by routine histopathology methods. Cryosubstitution techniques, which reveal where ice was present in tissues, have, however, demonstrated significant extracellular tissue matrix distortion and damage.[14–16] The extent of freezing damage depends upon the amount of free water in the system and the ability of that water to crystallize during cooling.

Other factors, in addition to ice formation, have biological consequences during biopreservation: the inhibitory effects of low temperatures on chemical and physical processes and, perhaps most important, the physiochemical effects of rising solute concentrations as the volume of liquid water decreases during crystallization. The latter process results in cell volume decreases, pH changes, and the risk of solute precipitation. There have been several hypotheses on mechanisms of freezing-induced injury based upon such factors.[3,14,17,18,19]

Two main approaches to tissue cryopreservation are currently in use or development. The first involves traditional freezing methods, based upon the cell preservation methods developed shortly after the Second World War, versus approaches involving ice-free vitrification. Both approaches involve the application of cryobiological principles.[3,6,14,17,18,20,21] Cryobiology may be defined as the study of the effects of temperatures lower than normal physiologic ranges upon biologic systems. Simply cooling cells or tissues with spontaneous ice nucleation and crystal growth results in dead, nonfunctional materials. Little advance was made in the field of cryobiology, with respect to significant post-freeze cell survival, until 1949 when Polge et al.[22] reported the cryoprotective properties of glycerol. Shortly thereafter, Lovelock and Bishop[23] discovered that dimethyl sulfoxide (DMSO) was also an effective cryoprotectant. Since the discovery of these CPAs, the field of cryopreservation has flourished and many other cryoprotectants have been identified. Before considering cryopreservation of tissues further, we will review some of the principles of short-term preservation in the absence of freezing.

8.2 SHORT-TERM TISSUE PRESERVATION

This section deals with the issues relating to the selection and design of solutions for hypothermic preservation and tissue transport. Most tissues are transported on ice for short periods of time before being processed for an application or cryopreserved for long-term storage. Reference was made at the end of Chapter 2 to the importance of interventional control of the extracellular environment of cells and tissues to optimize preservation. More specifically, the composition of the buffer medium used to nurture the tissue during the preservation protocol is very important but often overlooked on the assumption that conventional salt buffers such as Ringer's lactate and Krebs' solution, or regular tissue culture medium will be adequate. There are very good reasons (outlined in Ch 2) that these types of solutions may be suboptimal for cell preservation in tissues at reduced, hypothermic temperatures. Since control of the component cell and tissue environments is of fundamental importance for the outcome of preservation, it is worth considering some of the basic principles of solution design in relation to low-temperature storage. In essence, two basic types of solution are considered and often referred to as "extracellular-type" or "intracellular-type" solutions to reflect their basic ionic composition. Over the years we have encountered some confusion and inconsistencies in the use of these terms, hence definitions of these terms are footnoted.*

Some more sophisticated hypothermic blood substitution solutions such as Hypothermosol (Biolife Solutions), Unisol™ (Organ Recovery Systems), and KPS1 (Belzer's UW kidney perfusion solution; Organ Recovery Systems), the formulations of which are tabulated in Chapter 2, also contain an oncotic agent in the form of a high-molecular-weight colloid such as Dextran or hydroxyethyl starch. These solutions are perfused through the vascular bed of an individual organ, or even the whole body, at a pressure sufficient (typically 25–60 mm Hg) to achieve uniform tissue distribution. To balance this applied hydrostatic pressure and prevent interstitial edema, an oncotic agent such as albumin or synthetic macromolecular colloids (e.g., Dextran or hydroxyethyl starch) is incorporated into the perfusate. These perfusates may have an "intracellular" or "extracellular" complement of ions depending upon the temperature of perfusion preservation.[24–28]

Methods of short-term hypothermic preservation are neither standardized nor optimized for various tissues and organs. Currently the formulation of solutions employed differs depending upon the type of tissue or organ and whether the excised organs are stored statically on ice or mechanically perfused (see Chapters 2 and 9). Historically, a variety of preservation solutions have been developed and used for organs, but their application for tissues has been generally neglected. Most tissues are still transported in cell culture media and the fallacy of assuming that physiological culture media is acceptable for low-temperature storage is illustrated below. While there are undisputed industry standards for certain organs and applications, the concept of a "universal" preservation solution for all tissues and organs has still to be realized in practice. In general, the solutions adopted for abdominal visceral organs (kidney, liver, and pancreas) have not proved optimal for thoracic organs (heart and lungs) and vice versa. In contrast, a new approach to "universal" tissue preservation solutions has been developed for bloodless surgery using hypothermic blood substitution (HBS) to protect the whole body during profound hypothermic circulatory arrest (clinical suspended animation, or "*corporoplegia*" — literally meaning body paralysis).[24,26,28,29]

In recent years we have used this approach based upon the "Unisol" concept,[27] in which two new solutions (a "*maintenance"* and a "*purge"*) formulated for separate roles in the procedure have been tested.[30,31] The principal solution is a hyperkalemic, "intracellular-type" solution designed to "maintain" cellular integrity during hypothermic exposure at the nadir temperature (<10°C). The companion solution is an "extracellular-type" purge solution designed to interface between blood and the *maintenance* solution during both cooling and warming. This novel approach to clinical suspended animation has been established in several large animal models[24,26] and more recently explored for resuscitation after traumatic hemorrhagic shock in preclinical models relevant to both civilian and military applications.[30,32–35] Most recently the efficacy of hypothermic blood substitution

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* *DEFINITIONS*

"Intracellular-type" solution: A preservation solution that is typically hypertonic having a composition that is designed to restrict the passive exchange of water and ions during hypothermic exposure when cell membrane pumps are inhibited. This is achieved by raising the concentration of potassium, and reducing sodium, to mimic that of the intracellular space and thereby restrict passive fluxes of these ions. More importantly, an I-type solution usually includes a non-permeating (impermeant) anion to partially replace chloride ions in the extracellular space and thereby provide osmotic support to balance the intracellular oncotic pressure generated by macromolecules and their associated counter-ions locked inside the cell (these molecules do not cross the plasma membrane even passively due to their size and charge). Energy-consuming pumps normally control the water content of cells, but during hypothermia (and/or energy depletion) this control mechanism is compromised and cells imbibe water due to the oncotic pressure of the intracellular milieu. Cell swelling due to this passive hydraulic flux can be inhibited by raising the osmolality of the extracellular medium and by incorporating an impermeant anion such as lactobionate, or gluconate. Hence, these biophysical characteristics are the basis of why such solutions have been termed "intracellular-type" although in truth the solutions do not mimic the intracellular composition of the cytoplasm in many other respects.

"**Extracellular-type" solution:** By contrast, this is an isotonic solution having a plasma-like complement of ions that mimics the normal extracellular environment of cells. Examples of this type of solution can range from simple saline ("extracellular" in terms of the concentration of NaCl and osmolality) to tissue culture media that contain a more complete complement of ions, amino acids, and other metabolites to mimic the extracellular composition of plasma and other body fluids. Such solutions are generally poor preservation solutions at reduced temperatures principally because they do not counteract the passive biophysical processes outlined above.

with the hybrid Unisol solutions for whole-body protection has been demonstrated in a porcine model of uncontrolled lethal hemorrhage (ULH).[30,35,36]

In considering the efficacy of a solution for universal tissue preservation there is no better test than to expose all the tissues of the body to cold ischemia. Moreover, protection of those tissues most exquisitely sensitive to an ischemia/hypoxia insult, namely the heart and brain, provides the greatest challenge. Current interests in the development of hypothermic arrest techniques to facilitate resuscitation of hemorrhagic shock victims in trauma medicine has parenthetically provided an opportunity to examine the efficacy of new hypothermic blood substitution solutions for universal tissue preservation. In accordance with earlier baseline models,[26,37,38] these studies validate the unequivocal benefit of profound hypothermia combined with specially designed blood replacement fluids for the protection of heart, brain, and the other major organs during several hours of cardiac arrest and ensuing ischemia. Moreover, the preservation of higher cognitive functions in these animals subjected to hypothermic arrest further corroborates previous reports of the high level of neuroprotection provided by these hypothermic blood substitute solutions.[39] The general *in vivo* tissue-preservation qualities of this hypothermic blood substitution technique are clearly demonstrated by the consistent resuscitation of exsanguinating animals after traumatic hypovolemic shock. More specifically, biochemical profiles of the surviving animals showed that, apart from a transient elevation in liver enzymes that normalized within the first post-op week, there were no metabolic signs of organ dysfunction.[35] This is consistent with previously published observations in a canine model of clinical suspended animation in which specific markers of heart, brain, and muscle injury (creatine kinase isozymes) all showed transient increases in the immediate post-op period and returned to normal baseline levels within the first post-op week.[24,26]

In conclusion, the demonstrated efficacy of these synthetic, acellular hypothermic blood substitute solutions for protection of all the tissues and organs in the body during clinical suspended animation justifies their consideration for multiple organ harvesting from cadaveric and heart-beating donors. Furthermore, these observations support the findings of parallel studies for longer-term hypothermic storage of a variety of cell types derived from vascular tissues and kidney in which the Unisol-UHK *maintenance* solution has provided excellent cell survival compared with a variety of commonly employed solutions.[27,31] This provides further evidence for the protective properties of hypothermic blood substitutes such as *Hypothermosol* and *Unisol* solutions used for global tissue preservation during whole body perfusion in which the microvasculature of the heart and brain are especially vulnerable to ischemic injury.[24,39] Moreover, the application of solution-design for clinical suspended animation under conditions of ultraprofound hypothermia places the Hypothermosol and Unisol solutions in a unique category as universal preservation media for all tissues in the body. In contrast, all other preservation media, including the most widely used commercial solutions such as UW-ViaSpan are established for specific organs, or groups of organs, e.g., UW for abdominal organs and Celsior or Cardiosol for thoracic organs.

8.2.1 Hypothermic Storage of Tissues: Illustrative Studies Using Blood Vessels

Hypothermic storage of whole organs flushed or perfused with a preservation solution is common practice in clinical transplantation. This procedure leaves the vascular endothelial cells in direct contact with the preservation medium during the cold ischemic period. The effect of storage conditions on the integrity of vascular endothelium is therefore of crucial importance for the quality of preservation of intact organs. Moreover, it has been established in recent years that the long-term patency of vascular grafts used in reconstructive surgery may be significantly affected by cold storage.[40] We have reviewed the effects of hypothermia upon vascular function elsewhere.[31,41] The importance of these effects is illustrated by some experiments we conducted to compare the microscopic changes in tissue morphology when excised blood vessels were immersed and transported in Unisol-UHK hypothermic preservation solution compared with Dulbecco's Minimum

Essential Medium (DMEM), which is a common culture medium used to incubate and transport tissues *ex vivo*. The total cold ischemia time in these experiments was relatively short, 34.5 ± 9.5 minutes for samples transported in DMEM, and 37 ± 4 minutes for samples in Unisol-UHK. Tissue immersed and transported in DMEM on ice exhibited microscopic changes within the tunica intima and tunica media as shown in Figure 8.1A. The intima was intact, but there was extreme vacuolization (indicated by the arrows) of the underlying basal lamina causing, in turn, extrusion of the endothelial cells into the lumen and giving a "rounding-up" appearance. Apart from this vacuolization, the endothelial cells had a near normal appearance. The smooth muscle cells (SM) had a somewhat shrunken appearance with irregular contours. The tunica adventitia was essentially normal.

In contrast, jugular veins transported in Unisol demonstrated little if any histological changes compared with the DMEM group as shown in Figure 8.1B. The tunica intima was intact with little evidence of vacuolization of the underlying basement membrane. The smooth muscle cells (SM) did not appear shrunken and were in a normal, horizontal orientation.

These preliminary observations have been extended recently in a comprehensive study of interactive determinants that impact the preservation of autologous vascular grafts.[31] A multifactorial analysis of variance was used in the design and analysis of a study to evaluate the interaction of solution composition with time and temperature of storage. In summary, these *in vitro* studies that have examined both the function and structure of hypothermically stored blood vessels have clearly shown that optimum preservation of vascular grafts is achieved by selection of the type of storage medium in relation to time and temperature. Synthetic preservation solutions such as Unisol, designed specifically to inhibit detrimental cellular changes that ensue from ischemia and hypoxia, are clearly better than culture medium or blood and saline, which are commonly used clinically.[31]

8.2.2 Basic Rationale for the Design of Hypothermic Solutions

The scientific rationale for the choice of components selected in the design of Unisol is largely the same as that described previously for other hypothermic solutions[24–26] and will be summarized here. Based upon results from decades of organ preservation studies, desirable properties of hypothermic preservation solution have been determined and are listed in Chapter 2. The strategic design of solutions used for organ preservation have differed depending upon their ultimate use, either as a flush solution for static storage of the organ, or as a perfusate for continuous, or intermittent,

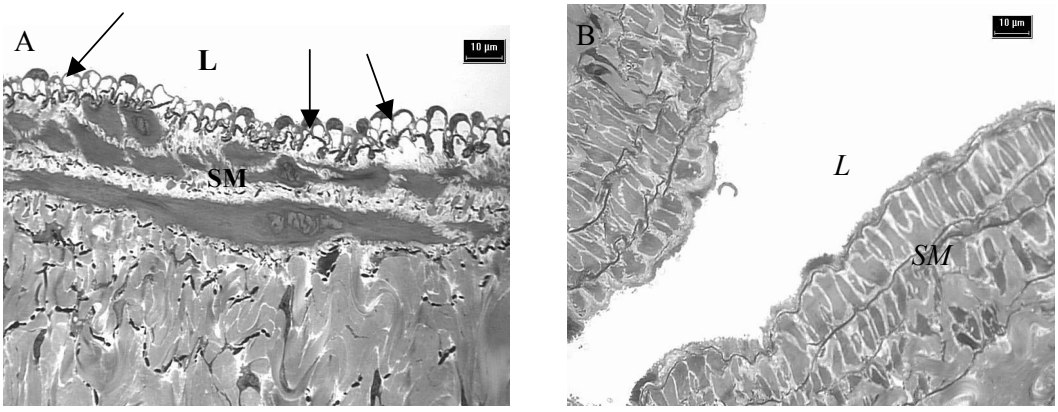

FIGURE 8.1 Light microscope histology of jugular vein segments after a period of cold ischemia in either DMEM culture medium (A) or Unisol-UHK hypothermic preservation solution (B). Note: L = Lumen; SM= smooth muscle cells.

perfusion of the organ. Taking a unique approach, Unisol has been formulated with a view to developing a universal baseline solution that may be used for both hypothermic static storage of tissues and organs, and also for machine perfusion preservation.[27,42] By design Unisol contains components that help to (1) minimize cell and tissue swelling, (2) maintain appropriate ionic balance, (3) prevent a state of acidosis, (4) remove or prevent the formation of free radicals, and (5) provide substrates for the regeneration of high-energy compounds and stimulate recovery upon rewarming and reperfusion. Parenthetically, these are regarded as the minimum essential characteristics for what can be regarded as baseline formulations. Additional classes of compounds can be considered as additives to these baseline solutions to fine-tune the cytoprotective properties. Examples are listed below and in Table 2.2 in Chapter 2. The scientific basis for new strategies to counteract cold ischemic injury by modifying storage solutions and perfusates is still emerging. Most of these strategies focus on combating oxidative stress and cold or hypoxia-induced apoptosis. Some insights into the cellular and molecular mechanisms of cold-storage injury have recently been reviewed by Rauen and DeGroot.[43] While these mechanisms have been elucidated using various experimental models, potential strategies[44,45] to counteract their effects have yet to be demonstrated in clinical practice.

Table 2.2 in Chapter 2 included combining the main characteristics of effective hypothermic solutions with attention toward selection of multifunction components. By carefully selecting components that possess multiple properties, the intrinsic protective properties of these hybrid solutions are maximized.

A fundamental biophysical property of the Unisol design is to provide the optimum concentration of ions and colloids to maintain ionic and osmotic balance within the organ or body tissues during hypothermia. In particular, an effective impermeant anion is included to partially replace chloride in the extracellular space and prevent osmotic cell swelling (i.e., to balance the fixed ions inside cells that are responsible for the oncotic pressure leading to osmotic cell swelling and eventual lysis during ischemia and hypothermia). A number of anions including citrate, glycerophosphate, gluconate, and lactobionate, or the anionic forms of aminosulphonic acids such as HEPES* could be suitable candidates. Lactobionate (FW = 358) was used exclusively as the principal impermeant in many solutions developed in recent years; these include ViaSpan®, Hypothermosol, Celsior, Cardiosol, and Churchill's solution, among others.[46–52] Lactobionate is also known to be a strong chelator of calcium and iron and may, therefore, contribute to minimizing cell injury due to calcium influx and free radical formation.[53] However, for organ perfusion Belzer and Southard recommended against using lactobionate in a perfusion solution, especially for kidneys.[54] Instead, gluconate was selected and shown to be an important component of Belzer's machine perfusion solution (currently marketed as KPS1 by Organ Recovery Systems). As a hybrid solution, Unisol has effectively incorporated both impermeants and uses gluconate (70 mM) as the predominant impermeant plus lactobionate (30 mM) for its additional cytoprotective properties (Table 8.1).

The osmoticum of Unisol is supplemented by the inclusion of sucrose and mannitol, the latter of which also possesses properties as a hydroxyl radical scavenger and reduces vascular resistance by inducing a prostaglandin-mediated vasodilatation that may be of additional benefit.[55,56]

A macromolecular oncotic agent is an important component of a perfusate to help maintain oncotic pressure equivalent to that of blood plasma. Any oncotic agent that is sufficiently large to prevent or restrict escape from the circulation by traversing the fenestration of the capillary bed may be considered. Examples of acceptable colloidal osmotic agents include:

- blood plasma expanders such as human serum albumin
- hetastarch or hydroxyethyl starch (HES) — an artificial colloid derived from a waxy starch and composed almost entirely of amylopectin with hydroxyethyl ether groups introduced into the alpha (1–4) linked glucose units[57]

* N-2(hydroxyethyl-piperazine)N-2-ethanesulfonic acid

TABLE 8.1
Formulation of Cryoprotectant Vehicle Solutions

Components (mM 1–1)	EC	Unisol-CV
Ionic		
Na^+	10.0	60.0
K^+	115.0	70.0
Ca^{++}	–	0.05
Mg^{++}	–	15.0
Cl^-	15.0	30.1
pH Buffers		
$H_2PO_4^-$	15.0	–
HPO_4^{2-}	42.5	–
HCO_3^-	10.0	5.0
HEPES	–	35.0
Impermeants		
Lactobionate$^-$	–	30.0
Sucrose	–	15.0
Mannitol	–	25.0
Glucose	194.0	5.0
Gluconate	–	70.0
Colloids		
Dextran 40	–	6%
Pharmacologics		
Adenosine	–	2.0
Glutathione	–	3.0
Osmolality (mOsm/Kg)	375	350
pH		7.6

EC (EuroCollins Solution); UNISOL-CV (Unisol Cryoprotectant Vehicle — phosphate free Unisol-UHK[31,80])

- Haemaccel (Hoechst) — a gelatin polypeptide[58]
- pluronic F108 (BASF — a nonionic detergent copolymer of polyoxyethylene and polyoxypropylene[59]
- polyethylene glycol[60]
- polysaccharide polymers of D-glucose such as the dextrans[61]

For a variety of reasons, dextran-40 (average mol wt = 40,000 daltons) was selected as the preferred colloid of choice for oncotic support to balance the hydrostatic pressure of perfusion and help prevent interstitial edema. It has long been known that dextran can improve the efficiency of the removal of erythrocytes from the microvasculature of cooled organs by inhibiting red cell clumping and by increasing intravascular osmotic pressure and reducing vascular resistance.[62–65] These attributes of dextran may be particularly important during washout, both *in vivo* and *ex vivo*. Dextran is widely used clinically as a plasma expander and is readily and rapidly excreted by the kidneys.[66] There is ample recent evidence that dextran-40 is also an effective and well tolerated

colloid in modern cold-storage solutions for organ preservation.[67–69] Moreover, in 1996, a Swiss clinical study verified that dextran-40 safely replaces HES in the UW solution for the purpose of human kidney preservation for transplantation.[70]

Retention of the colloid in the vascular space is an important consideration for achieving optimal oncotic support. Any dextran that permeates into the interstitial space during the hypothermic procedure will be readily eluted upon return to physiological conditions. Another advantage of the use of dextran is that the viscosity of the solution will not be as high as with some other colloids such as HES.

The ionic balance, notably the Na^+/K^+ and Ca^{2+}/Mg^{2+} ratios, of Unisol has been adjusted to restrict passive diffusional exchange at low temperatures when ionic pumps are inactivated. Table 8.1 shows that in the Unisol formulation, the concentration of monovalent cations Na^+ and K^+ are approximately equimolar to restrict their passive transmembrane exchange. Due to documented concerns regarding the very high potassium levels in commercial organ preservation solutions, including ViaSpan and EuroCollins,[50,71,72] the potassium concentration in Unisol is lower by comparison, but sufficiently elevated to fulfill the requirements of an "intracellular-type" preservation medium. In the area of cardioplegia and myocardial preservation there is good evidence for improved survival using elevated concentrations of magnesium and very low, but not zero, calcium to avoid the putative calcium paradox.[73–75] Some glucose is included in these hypothermic solutions as a substrate, but the concentration is low to prevent exogenous overload during hypothermia, which may potentiate lactate production and intracellular acidosis by anaerobic glycolysis.[76]

Acidosis is a particular hazard during hypothermia and attention has been given to the inclusion of a pH buffer that will be effective under nonphysiological conditions that prevail at low temperatures (see Chapter 2 for a detailed discussion of acid-base regulation under hypothermic conditions). HEPES was selected as one of the most widely used biocompatible aminosulphonic acid buffers that have been shown to possess superior buffering capacity at low temperatures,[77–80] and have been included as a major component of other hypothermic tissue preservation media.[77,81,82] Synthetic zwitterionic buffers such as HEPES also contribute to osmotic support in the extracellular compartment by virtue of their molecular size (HEPES = 238 daltons).

Adenosine is a multifaceted molecule and is included in the hypothermic preservation solutions not only as an essential substrate for the regeneration of ATP during rewarming, but also as a vasoactive component to facilitate efficient vascular flushing by vasodilatation.[83,84] Glutathione is included as an important cellular antioxidant and hydroxyl radical scavenger, as well as a cofactor for glutathione peroxidase, which enables metabolism of lipid peroxides and hydrogen peroxide.[46,85,86]

Unisol, by design, is a base vehicle solution to which any combination of pharmacological additives might be added for optimization of the preservation of a particular tissue or organ. Moreover, there is the potential benefit of a wide variety of pharmacological and biochemical agents that may be selected from the following categories:

- Cytoprotective agents and membrane stabilizers
- Energy-producing substrates and nucleotide precursors
- Calcium channel blockers
- Oxygen-derived free radical scavengers/antioxidants
- Apoptosis inhibitors
- Vasoactive agents
- Trophic factors
- Molecules for oxygen delivery

8.3 TISSUE SCREENING, PREPARATION, AND ANTIBIOTIC STERILIZATION

If tissues are destined for transplantation, it is normal practice for them to go through an extensive donor screening, microbial testing before and after processing to check for potential contaminants, and incubation in antibiotic formulations to hopefully kill low levels of microbial contaminants that are below the resolution of the microbial sampling protocols employed. In the USA the United States Food and Drug Administration (FDA) regulates tissue-engineered products, and the allograft tissue community has its own regulatory body, the American Association of Tissue Banks (AATB). The AATB has established standards that provide minimum performance requirements for all aspects of tissue banking activity.[87] These guidelines include requirements for donor suitability as well as the handling of transplantable human tissue. Their intent is to assure allograft tissue recipients disease- and contaminant-free implants, and to help ensure the optimum clinical performance of transplanted cells and tissues.

In contrast, tissue-engineered product regulations are in various stages of development by the FDA. One of the issues from a regulatory perspective has been that many tissue-engineered products combine features of drugs, devices, and/or biological products, so the normal division of work structure at the FDA has had to be modified. The Center for Biologics Evaluation and Research (CBER), the Center for Devices and Radiological Health (CDRH), and the Center for Drug Evaluation and Research (CDER) have established intercenter agreements to clarify product jurisdictional issues within the FDA. The FDA has also established an InterCenter Tissue Engineering Work Group to address scientific and regulatory issues and members of this group have been very active at large. Two other special interest groups are presently involved in the development of standards for tissue-engineered products, the American Society for Testing and Materials (ASTM) and the Tissue Engineering Special Interest Group of the Society for Biomaterials.

Recently the FDA published final rules establishing donor eligibility criteria for donors of human cells, tissues, and cellular- and tissue-based products (HCT/Ps) to help prevent the transmission of communicable disease when these products are transplanted. This new rule is part of the agency's plan to regulate tissues and related products with a comprehensive, risk-based approach. The new rule on donor eligibility pertains to donors of traditional tissues such as musculoskeletal, skin, and eye tissues that have been required to be screened and tested for HIV, hepatitis B virus (HBV), and hepatitis C virus (HCV) since 1993. Under this new rule, reproductive tissue (semen, ova, and embryos), hematopoietic stem cells derived from cord blood and peripheral blood sources (circulating blood sources as opposed to bone marrow), cellular therapies, and other innovative products are also regulated. In addition to including a broader range of tissues and cells, the new rule extends the scope of protection against additional communicable diseases that can be transmitted through transplanted tissues and cells. The new regulation adds requirements to screen for human transmissible spongiform encephalopathies, including Creutzfeldt-Jakob disease (CJD), and to screen and test for syphilis. Screening and testing for still other relevant communicable disease agents [human T-lymphotropic virus (HTLV)] would be required for viable cells and tissue rich in leukocytes such as semen and hematopoietic stem cells. The new rule also provides a framework for identifying emerging diseases that may pose risks to recipients of transplanted HCT/Ps and for which appropriate screening measures or testing are available. Thus, this new regulation gives the FDA the flexibility to rapidly address new disease threats as they appear, providing substantial new protections for patients receiving tissue transplants. Examples of such diseases include West Nile virus and Severe Acute Respiratory Syndrome (SARS). The rule also contains requirements related to record keeping, quarantine, storage, and labeling of the HCT/Ps, all important to the prevention of disease transmission. The final rule became effective on May 25, 2005. FDA documents are available on the FDA's web site at http://www.fda.gov. References for ethical and safety considerations in Europe were recently provided by Wusteman and Hunt.[1]

Regardless of whether the transplantable material is an allograft or an engineered product, recipient safety must be ensured through administration of strict donor screening criteria along with stringent quality control measures that encompass the entire tissue preparation protocol. In contrast to most inanimate medical products, which are sterilized to eliminate bacterial, viral, and fungal contaminants, it is not possible to terminally sterilize products that contain living cells. It is necessary, therefore, to ensure the safety of these products by stringent control of the living component source.

In engineered products, just as with allogeneic tissues and organs, procedures for the screening of donors and handling of materials must be strictly performed. These procedures are presently in the process of being defined and may vary depending upon the type of cell source employed. There is no need for modification from existing clinical practice in the autologous situation, in which cells or tissues are removed from a patient and transplanted back into the same patient in a single surgical procedure. If the autologous cells or tissues are banked, transported, or processed with other donor cells or tissues, however, there then exists opportunities for product adulteration and the introduction of transmissible disease. When this is the case, good manufacturing practices (GMPs) and good tissue practices (GTPs) should be implemented and it becomes necessary to screen for infectious agents (US FDA, 61 CFR 26523, 1996). For example, in the case of the Carticel™ process, in which biopsies of healthy cartilage are used as a source of chondrocytes, the biopsies are minced, washed, and cultured with cell culture medium containing antibiotics. However, years ago Brittberg et al.[88] found that presence of antibiotics (50 µg/ml gentamicin sulphate and 2 µg/ml amphotericin B) in the culture medium may prevent contaminant detection; therefore the culture medium should be changed to an antibiotic-free formulation prior to testing for bacterial contamination and extensive washing of the biological material may be required to remove inhibitory antibiotics to allow their proliferation and subsequent detection.

Utilization of allogeneic donors is associated with greater risk than an autologous donation because of the risk of infectious disease transmission from the donor to the recipient. Donor screening similar to that outlined in the following section on allogeneic tissue grafts should be performed, along with the implementation of product screening and/or quarantine procedures (US FDA, 21 CFR Parts 16 & 1270, 1997). The allogeneic cells may then be (1) used with no modifications after expansion *in vitro*, (2) genetically manipulated, or (3) turned into continuously proliferating cell lines. As an additional safety measure, specimens of each donor tissue should be archived for future pathogen screening.

There is a similar concern about unidentified diseases in the use of xenogeneic cell, tissue, and organ sources. The range of infectious diseases potentially transmissible by xenogeneic cells is unknown because infectious agents that produce little or no effects in animals may have severe consequences in human patients. The FDA has published draft recommendations on infectious disease issues in xenotransplantation (US FDA, 61 CFR 49920, 1996 & 62 CFR 3563, 1997). Donor screening issues again play a significant role in the prevention of infection. With xenogeneic materials, the pedigree and health status of environmentally isolated herds or colonies of animals to be used as donors become critically important.

All tissues should be delivered to patients with the highest possible assurance that they are free of pathogens. The two most effective and common methods for the prevention of infectious agent transmission are thorough donor screening and adherence to sterile techniques during harvesting, transport, and processing. The first step in confirming a potential donor in most parts of the world is to obtain permission for organ and/or tissue donation from the donor's legal next of kin. Regulations in some countries and states may differ. Once permission has been obtained, the donor must be screened to minimize the potential for transfer of infectious or neoplastic disease. In most organ donors, the donor has been declared brain dead and organ functions are supported by extracorporeal life support. This is to ensure maintenance of tissue viability while permission for donation is obtained and donor screening and infectious disease testing is performed. The AATB

standards require donor tissue be tested for blood-borne infectious disease prior to acceptance for transplantation, so tissues are placed in quarantine until the test results are complete.

The most commonly employed viable tissue allografts involving antiseptic treatments are tricuspid heart valves (aortic and pulmonary valves) and blood vessels. Other tissues, such as skin where viability is not considered a major issue and, less frequently, transplanted tissues, are subject to antiseptic treatment methods similar to those used for heart valves. Donor tissues for transplantation are obtained aseptically in an operating room or, alternatively, at autopsy in a clean fashion. The donor is usually prepared in a manner similar to preparing the incision site of a patient for surgery. For example, skin donors are usually shaved and the areas of skin to be removed are scrubbed with an iodine-based wash (for example, Betadine™), rinsed with isopropyl alcohol, and again painted with iodine.[89] Kirklin and Barratt-Boyes[90] have presented surgical techniques for the recovery of hearts for valve procurement in an autopsy setting. In order to provide a sterile allograft for transplantation, identification and elimination of any potential contaminants are required. The antiseptic treatment stage of tissue processing begins once the tissues have been prepared and dissected employing aseptic technique. AATB standards dictate that "processing shall include an antibiotic disinfection period followed by rinsing, packaging, and cryopreservation" and that "disinfection of cardiovascular tissue shall be accomplished via a time-specific antibiotic incubation."[87] Following immersion in the antibiotic solution of choice, the tissues are incubated, while immersed, at either 4°C or 37°C for up to 24 h (temperature and time being variable between tissue banks). Following incubation the tissues are packaged aseptically for tissue storage by cryopreservation.

Many different antibiotic mixtures for treatment of tissues for transplantation have been employed. Heart valve indicators of effectiveness, documented in many studies over the years, include cellular viability, host ingrowth rate, disinfection efficiency, and valve survival rates.[91–98] Various formulae using various combinations of penicillin, gentamicin, kanamycin, axlocillin, metronidazole, flucloxacillin, streptomycin, ticarcillin, methicillin, chloramphenicol, colistimethate, neomycin, erythromycin, and nystatin have been tried for heart valve treatment. Skin is usually treated with gentamicin (personal communication, American Red Cross Tissue Services, 1999) or combinations of penicillin and streptomycin.[99,100] Csonge et al. reported the best results with combinations of ceftazidime, ampicillin, and amphotericin.[101] Currently, a modified version of the antibiotic treatment regimen recommended by Strickett et al., in which cefoxitin, lincomycin, polymyxin B, and vancomycin are added to sterile-filtered RPMI 1640 tissue culture medium, is being commonly used in the USA to disinfect allograft heart valves.[102] Various nutrient media have also been used, including modified Hank's solution, TCM 199, Eagle's MEM, and RPMI 1640 in conjunction with various antibiotic "cocktails."[98,102–105] While many different antibiotics in various tissue culture media have been employed, all are employed at relative low doses with varying incubation times at either 4°C or 37°C. Care should be taken to optimize the antibiotic concentrations and conditions to minimize loss of tissue viability and function while maximizing antimicrobial effectiveness.

The antibiotic solutions developed for the antiseptic treatment of heart valves were originally formulated to ensure sterility after weeks of storage at 4ºC. However, Angell et al. showed that antibiotics are more effective at physiological temperatures (~37ºC) than at refrigerator temperatures.[106] The simple protocol of collection of heart valves within 24 h of death combined with low-dose antibiotic treatment has been reported to be sufficient to produce pathogen-free valves in >95% of cases.[107] In fact, there is little evidence that antibiotics even provide bactericidal action at 4ºC since most antibiotics function through interference with temperature-dependent processes of nucleic acid synthesis or the bacterial cell wall. It is possible that the effectiveness of some of the 4ºC antibiotic treatment protocols can be credited to antibiotic binding during low-temperature incubation, and that upon subsequent rewarming the antibiotic action is actually activated. Nevertheless, and regardless of the mechanism, cardiovascular tissue programs usually advocate the use of antibiotics, and in some cases residual antibiotics in the tissue may actually reduce the risk of subsequent graft infection. These tissue grafts are often preferred for implantation in infected patient sites.

Besides the issues of microbial effectiveness, there is also the issue of cytotoxic effects of antibiotics upon cells and tissues. Cram et al. provided evidence that reduction in antibiotic concentrations improves viability of refrigerated stored skin.[100] There have been many reports of antibiotic effects on heart valve cell viability,[106,108–111] alterations in cell morphology,[108] and inhibition of cell ingrowth.[92,112] In particular, amphotericin B may destabilize mammalian cell membranes during cryopreservation by altering the mobility of the cholesterol in the lipid phase of the plasma membrane or alterations in osmoregulation.[111] Alternatively, amphotericin B is supplied as a colloidal suspension that has been dispersed by the detergent deoxycholate. This detergent may directly alter the membrane permeability properties of mammalian cells and such changes may render the cells less resistant to the osmotic stresses of freezing and thawing. There have been few reports on alternative antifungal agents; however, Schmehl et al. assessed the water-soluble fungicide flucytosine as an alternative to amphotericin B in combination with imipenem,[113] a wide spectrum β-lactam, but this fungicide is not being used clinically for tissue processing. Elimination of amphotericin B from the antibiotic regimen used to sterilize grafts emphasizes the fact that postprocurement treatment cannot be relied upon to guarantee recipient safety. It further highlights the importance of thorough donor screening. Permission for autopsy and obtaining pertinent medical history, including detection of symptoms associated with systemic mycoses or infective endocarditis, are paramount to exclusion of fungal contaminants originating from the donor graft. Strict sterile technique during recovery, transport at 4°C, and cold, sterile processing are additional measures to prevent fungal proliferation. A sterile specimen is preferred because evidence of fungus may be masked by an overgrowth of competitive bacteria. Coprocessed specimens are usually tested before and after antibiotic treatment, just prior to packaging for cryopreservation. Antibiotics cannot be expected to unfailingly disinfect every allograft.[95,114,115] The issue of how to assure sterility of tissue allografts while maintaining cell viability and tissue functions has no effective solution in sight.

8.4 CRYOPRESERVATION

The principles that govern the cryopreservation of mammalian systems at the cellular level are covered in other chapters in this book and other recent reviews.[18,19] Our objective here is to give an overview of tissue preservation by relating these principles to the practical demands of developing techniques for the preservation of living tissues. It is important to emphasize at the outset that successful cryopreservation of tissues is not a simple matter of extrapolating the well-established principles of cell cryopreservation to more complex tissues. The reason is that tissues are much more than the aggregate sum of their component cells. They invariably comprise a variety of cell types intimately associated with basement membranes, an extracellular matrix, and often a vascular supply such that the structure of the integrated tissue demands special considerations in its response to cryopreservation conditions for its successful preservation. These differences are manifest in additional mechanisms of injury, identified some years ago,[2] that must be circumvented for successful preservation. Ultimately these differences in their response to freezing between individual cells and tissues are principally due to extracellular ice formation.

A variety of factors are known to influence cell survival during cryopreservation (Table 8.2), but the role of the vehicle solution for the CPAs is often overlooked. It is generally assumed that simple salt buffers or conventional culture media used to nurture cells at physiological temperatures will also provide a suitable medium for exposure at low temperatures. In a manner similar to our earlier discussion of optimum control of the cells' environment during hypothermic short-term storage, cryopreservation also demands consideration of the chemical composition of the buffer medium used as a vehicle for the CPAs as well as the temperature to which the cells are exposed. It has been a common practice in tissue banking to use tissue culture media as the base solution for preservation media. However, for the reasons outlined above, tissue culture media, which are designed to maintain cellular function at normal physiological temperatures, are inappropriate for

TABLE 8.2
Major Cryopreservation Variables

Freezing-compatible pH buffers
Vehicle solution selection (may vary with cryoprotectant selection)
Apoptosis inhibitors (may be required to get long-term post-thaw cell survival for some cells)
Cryoprotectant selection (optima may vary with vehicle solution selected)
Cooling rate
Storage temperature
Warming rate
Cryoprotectant addition/elution conditions (number of steps, temperature)

optimum preservation at reduced temperatures and we have long advocated the use of intracellular-type solutions as more appropriate vehicle solutions for CPAs.[77,81,116–118] Maintaining the ionic and hydraulic balance within tissues during cold exposure can be better controlled in media designed to physically restrict these temperature-induced imbalances and can be applied equally to the choice of vehicle solution for adding and removing CPAs in a cryopreservation protocol.[119] Moreover, the nature of the vehicle solution used to expose cells and tissues to cryoprotectants at low temperatures has been shown to impact the outcome of cryopreservation,[77,117,120,121] and has recently become the focus of additional research aimed at optimization and attenuation of the so-called cryopreservation cap.[80,119,122–124]

Figure 8.2 illustrates the marked effect that an intracellular-type vehicle solution can have on the outcome of cryopreservation. In a study of factors that influence the survival of vascular smooth muscle and endothelial cells it was discovered that the choice of carrier solution significantly impacted the optimum survival of the cells. Moreover, the survival varied with the nature of the CPA and the cell type suggesting that nature of the vehicle solution should be included as one of the variables that must be optimized for a given system. Our aim is to use the approach of a hybrid universal formulation in an attempt to nullify the wide differences in available solution choices. Baust et al. have corroborated this approach and shown that an intracellular-type solution, *Hypothermosol,* provides a significantly better vehicle solution for CPAs than a range of extracellular-type media in other cell systems.[123–125]

Another common practice in tissue banking is to employ serum of animal origin in the cryopreservation formulation. Serum-free procedures have been reported for a variety of tissues[126] and mammalian sources of serum can be removed providing that cryopreservation conditions are subsequently reoptimized.[127]

8.4.1 Synthetic Cryoprotectants

Historically, serendipity has been largely responsible for most discoveries of cryoprotectants. Cryoprotectant selection for cryopreservation in general is usually restricted to those that have conferred cryoprotection in a variety of biological systems (dextrans, DMSO, ethylene glycol, glycerol, hydroxyethyl starch, polyvinylpyrrolidone, sucrose, and trehalose).[10] Combinations of two cryoprotectants may result in additive or synergistic enhancement of cell survival.[128,129] Comparison of chemicals with cryoprotectant properties has revealed no common structural features. These chemicals are usually divided into two classes: (1) intracellular cryoprotectants with low molecular weights that penetrate and permeate cells and (2) extracellular cryoprotectants with relatively high molecular weights (greater than or equal to sucrose [342 daltons]) that neither penetrate nor permeate cells. A variety of biologic chemicals with cryoprotective activity for one or more biological systems have been reported (Table 8.3).

Intracellular cryoprotectants, such as glycerol and DMSO at concentrations from 0.5 to 3.0 molar, are effective in minimizing cell damage in slowly frozen biological systems. Extracellular

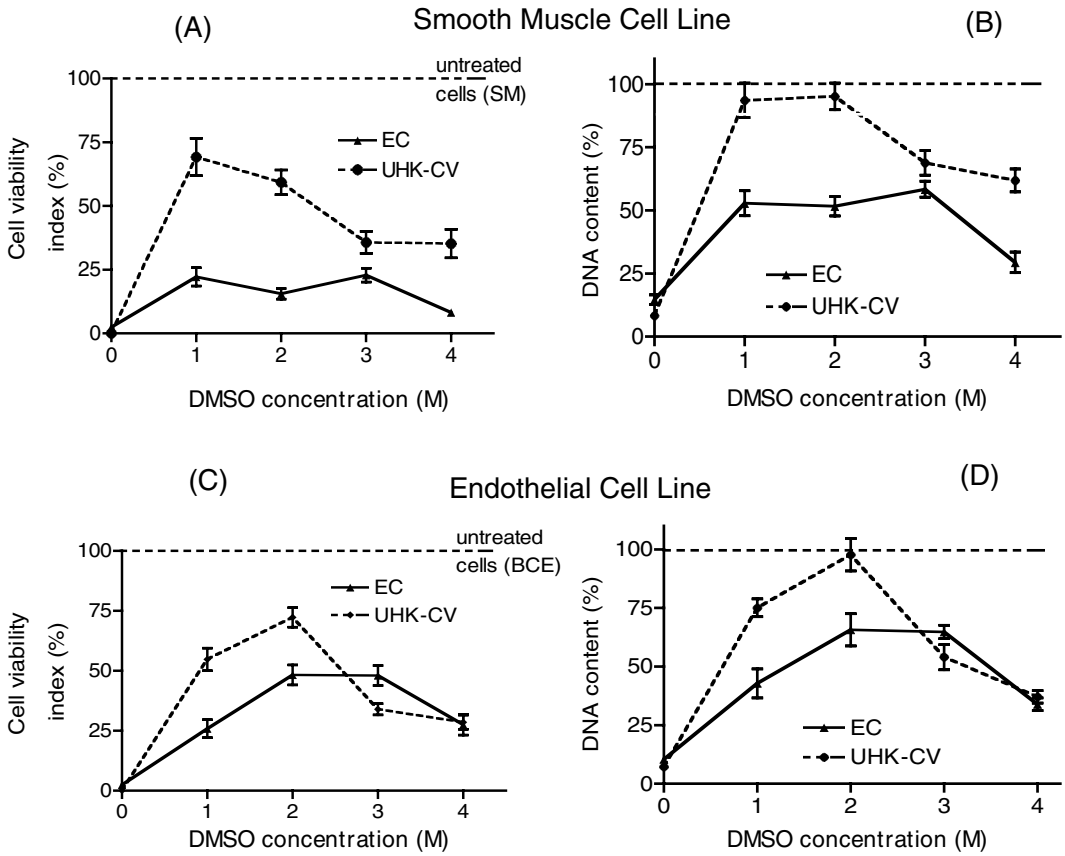

FIGURE 8.2 Cell survival (viability: A and C and DNA content; B and D) after freezing and thawing with varying concentrations of DMSO in either Unisol-CV (UHK-CV) or EuroCollins. Cells were frozen and thawed as adherent populations in microtiter plates. Data was normalized to untreated cells and is the mean (±SEM) of 12 replicates. (From Taylor, M.J., Campbell, L.H., Rutledge, R.N., and Brockbank, K.G.M. (2001): Comparison of Unisol with EuroCollins solution as a vehicle solution for cryoprotectants. Transpl. Proc., 33: 677–679. With permission.)

cryoprotective agents such as polyvinylpyrrolidone or hydroxyethyl starch are often more effective at protecting biological systems cooled at higher rates. Such agents are often large macromolecules that affect the properties of the solution to a greater extent than would be expected from their osmotic pressure. These nonpermeating cryoprotective agents are thought to have direct protective effects on the cell membrane. This protection may be due to the oncotic forces (colloidal osmotic pressure) exerted by large molecules and alterations in the activity of the unfrozen water caused by hydrogen bonding to water molecules. Although cryoprotective agents also reduce the amount of extracellular ice at each subzero temperature with a resultant increase in the volume of the unfrozen fraction, it is not known if fewer ice crystals are responsible for any of the reduction in cell damage.[130] The latter function of cryoprotective agents may also relate to their role in reducing membrane fusion during cryopreservation.[131] The pharmacologic effects of cryoprotective agents such as DMSO and glycerol were reviewed by Shlafer.[132] According to Mazur, cryoprotectants protect slowly frozen cells by one or more of the following mechanisms: suppression of salt concentrations, reduction of cell shrinkage at a given temperature, and reduction in the fraction of the solution frozen at a given temperature.[10] Cryoprotectants and their mechanisms of action have been the subject of a number of useful reviews.[14,17,20,133]

TABLE 8.3
Chemicals with Demonstrated Cryoprotective Activity

Acetamide	Ethylene glycol*	Mannitol	Pyridine N-oxide
Agarose	Ethylene glycol	Mannose	Ribose
Alginate	Monomethyl ether	Methanol	Serine
Alanine	Formamide	Methoxy propanediol	Sodium bromide
Albumin	Glucose	Methyl acetamide	Sodium chloride
Ammonium acetate	Glycerol*	Methyl formamide	Sodium iodide
Butanediol	Glycerophosphate	Methyl ureas	Sodium nitrate
Chondroitin sulfate	Glyceryl monoacetate	Methyl glucose	Sodium nitrite
Chloroform	Glycine	Methyl glycerol	Sodium sulfate
Choline	Hydroxyethyl starch*	Phenol	Sorbitol
Cyclohexanediols**	Inositol	Pluronic polyols	Sucrose*
Dextrans*	Lactose	Polyethylene glycol	Trehalose
Diethylene glycol	Magnesium chloride	Polyvinylpyrrolidone*	Triethylene glycol
Dimethyl acetamide	Magnesium sulfate	Proline	Trimethylamine acetate
Dimethyl formamide	Maltose	Propylene glycol*	Urea
Dimethyl sulfoxide*			Valine
Erythritol			Xylose
Ethanol			

* Chemicals that have conferred substantial cryoprotection in a wide variety of biological systems, modified from Shlafer.[132]

** Synthetic ice blockers.[6,225]

8.4.2 Natural Cryoprotectants

Through millions of years of evolution, nature has produced several families of proteins that may have benefits during cryopreservation, which help animals and plants survive in cold climates. 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 antifreeze molecules are diverse in structure and, to date, four main types have been characterized. The first to be discovered and best characterized are the antifreeze peptides and glycoproteins (AFPs) found in Antarctic fish and northern cod species. The natural AFPs found in polar fish and certain terrestrial insects are believed to adsorb to ice by lattice-matching[134] or by dipolar interactions along certain axes[135] of forming 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.[136] Regardless, these molecules confer a survival advantage upon certain animals. These observations led to the hypothesis that naturally occurring antifreeze molecules might be improved upon by synthesis of molecules that will either bind to other ice nuclei domains or upon stable ice crystals.

Conflicting results have been obtained by scientists following up on the proposal of Knight and Duman that many of the problems associated with ice formation during cryopreservation might be limited by the addition of naturally occurring AFP.[135] However, the studies of Hansen et al. demonstrated that AFP Type I inhibited ice recrystallization in the extracellular milieu of cells, but increased ice crystal growth associated with the cells, and resulted in AFP concentration-dependent cell losses compared to untreated control cultures.[137] A major focus of our research for the past six years has involved the identification of synthetic ice blockers (SIBs) that may combine with certain naturally occurring antifreeze compounds and cryoprotectants to minimize ice damage during

freezing or risk of ice formation during vitrification. The best SIBs we have identified to date are 1,3-cyclohexanediol and 1,4-cyclohexanediol (patent pending).[6]

In recent years, some of the challenges for cryopreservation of living tissues have been more fully characterized and new approaches are under development for circumventing the problems that have thus far limited the extrapolation of established principles and techniques for cells to more complex tissues and organs. A synopsis of the state of the art of biopreservation of living tissues utilizing cryopreservation methods follows.

8.4.3 Cryopreservation by Controlled Freezing

Successful biopreservation by freezing is dependent on the optimization of several major factors. Advances in the field of cryopreservation had been modest until Polge et al. discovered the cryoprotective properties of glycerol.[22] Subsequent research by Lovelock and Bishop showed that dimethyl sulfoxide was also a cryoprotectant (CPA).[138] The use of cryoprotectants during freezing and thawing of biological materials has become established and many other cryoprotectants have been identified. When cryoprotectants are used in extremely high concentrations, ice formation can be eliminated during cooling to and warming from cryogenic temperatures. Under these conditions the solution and tissue become vitrified; this is discussed further in a later section. In addition to cryoprotectant selection several major variables must be considered in development of cryopreservation methods (both freezing and vitrification approaches; Table 8.2).

A wide variety of isolated cells in suspension can be preserved using conventional cryopreservation methods involving freezing. In such methods the cells are concentrated and vitrify in ice-free channels between regions of extracellular ice. In general terms, each cell type has a freezing "window" in which the change in temperature with time provides for optimal cell survival. This proposed "window" is narrow at high temperatures and becomes increasingly wider as the temperature decreases, suggesting that deviation from a given cooling rate at high temperatures may be more critical to cell survival than deviations at low temperatures. Studies on the survival of various mammalian cell types, frozen in glycerol or DMSO, both as single cell suspensions and in tissues, frozen at a variety of rates suggest that optimal survival occurs at a cooling rate somewhere between 0.3°C and 10°C per minute.

The cell viability of cardiovascular tissues including veins, arteries, and heart valves can be preserved by a number of cryopreservation freezing techniques. However, smooth muscle and endothelial functions are usually impaired to varying degrees depending upon species, tissue type, and preservation methods employed.[1,141] Clinically the fundamental issue with cryopreserved cardiovascular tissues, regardless of whether they are cryopreserved by freezing or vitrification, is that they are allogeneic and there is an inevitable immune response unless immunosuppressive therapy is employed. We have compared cryopreservation techniques in syngeneic and allogeneic rat models and concluded that the changes observed in allogeneic heart valves are primarily due to immunological incompatibility of the graft and recipient, as previously suggested in rats[142] and human infants,[143,144] not cryopreservation method.[145] The immune response is not clinically significant for the majority of cryopreserved allogeneic heart valves; however, cryopreserved small-diameter vascular allografts typically have only short-term patency and this is probably a consequence of immunogenicity. There has been considerable discussion of whether or not cell viability is needed for allograft heart valves. The conclusion appears to be that viability correlates with "minimally traumatized" tissue with the result that most allograft heart valves employed today retain viable cells at the moment of implantation.

In marked contrast to cardiovascular tissue, studies using a variety of animal articular cartilage models[146–149]and human cartilage biopsies[150] have revealed no more than 20% chondrocyte viability following conventional cryopreservation procedures employing either DMSO or glycerol as cryoprotectants. Ohlendorf et al. used a bovine articular cartilage, osteochondral plug model to develop a clinical cryopreservation protocol.[147] This protocol employed slow-rate cooling and 8% DMSO

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. previously investigated chondrocyte survival in a similar sheep model.[148] These researchers observed cells surviving post-cryopreservation 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. 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 mid-portion of the graft.[151] The reason for lack of cell survival deeper than the superficial layers of articular cartilage is most likely multifactorial and related principally to heat and mass transfer considerations.[9] 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 DMSO (8–20%) may not penetrate adequately to limit intracellular ice formation. Recent data from Jomha et al. demonstrated that increasing DMSO concentrations to 6 M can result in higher overall cell survival (40%) after cryopreservation.[152] These observations suggest that use of higher DMSO concentrations results in better penetration of the DMSO into the cartilage.

We are aware that other factors, in addition to ice formation, may have biological consequences during freezing procedures. Two of these factors are the inhibitory effects of low temperatures on chemical and physical processes, and, perhaps more importantly, the physiochemical effects of rising solute concentrations as the volume of liquid water decreases during crystallization. This latter process results in a decrease in cell volume and the risk of solute precipitation. Several hypotheses have been published on mechanisms of freezing-induced injury based upon such factors,[9,17] but our own experiences with mammalian tissues concur with others that the principal disadvantage of conventional cryopreservation revolve primarily around ice formation.[3,15,16,153–156]

8.4.4 Cryopreservation by Avoidance of Ice Formation — Vitrification

It is now generally accepted that extracellular ice formation presents a major hazard for biopreservation by freezing of multicellular tissues. This has led to a major focus during the last decade 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 previously reported.[2,3,6,15,155,157,158]

Prevention of freezing by vitrification means that the water in a tissue remains unfrozen in a noncrystalline state during cooling. Vitrification is the solidification of a liquid without crystallization. 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. It is this conversion of a liquid into a glass that is called vitrification (derived from *vitri*, the Greek word for glass). 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 has been shown to provide effective preservation for a number of cells, including monocytes, ova, and early embryos and pancreatic islets.[159–162] Vitrification is potentially applicable to all biological systems.

Vitrification preservation procedures are very similar to those employed for freezing tissues. Generally speaking, the cryoprotectants are added in stepwise or gradient manner on ice. In some cases, due to risks of toxicity, lower temperatures may be employed for the final higher CPA concentrations. The cooling rates employed are typically as fast as can be achieved for the tissue in question to temperatures around –100°C and then more slowly to the final vapor phase nitrogen storage temperature between –135°C and –160°C. Warming is performed in a similar manner, slowly to –100°C and then rapidly to 0°C. Rapid cooling and warming in our laboratory is usually performed by immersion in either chilled alcohol (–100°C) or warm water baths (4°C or 37°C).

Microwave warming has been attempted but has never been successful using conventional devices due to the uneven warming of specimens and problems with thermal runaway, which results in heat-denatured tissues. In 1990, Ruggera and Fahy reported success in warming test solutions at rates of up to about 200°C/min using a novel technology based on electromagnetic techniques (essentially microwave heating).[163] Unfortunately, unpublished results indicate that this method is also problematic due to the uneven warming of specimens and problems associated with thermal runaway. Others have taken a systematic approach to develop a dielectric heating device to achieve uniform and high rates of temperature change.[164,165] This has been achieved in some preliminary model systems but application of this warming technology with survival of cells has yet to be reported.

Vitrification and freezing (water crystallization) are not mutually exclusive processes; the crystalline phase and vitreous phase often coexist within a system. In fact part of the system vitrifies during conventional cryopreservation involving controlled freezing of cells. This occurs because during freezing the concentration of solutes in the unfrozen phase increases progressively until the point is reached when the residual solution is sufficiently concentrated to vitrify in the presence of ice. Conventional cryopreservation techniques by freezing are optimized by designing protocols that avoid intracellular freezing. Under these cooling conditions the cell contents actually vitrify due to the combined processes of dehydration, cooling, and the promotion of vitrification by intracellular macromolecules. Phase diagrams have proved to be a useful tool in understanding the physicochemical relationship between temperature, concentration, and change of phase. For detailed discussion of the role and interpretation of solid-liquid state diagrams in relation to low-temperature biology, please refer to a previous review.[21] 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. [6,21]

The term vitrification is generally used to refer to a process in which the objective is to vitrify the whole system from the outset such that any ice formation (intracellular and extracellular) is avoided.[166–171] In cryopreservation by vitrification dehydration occurs by chemical substitution alone, while in cryopreservation by freezing dehydration occurs by both osmotic dehydration and chemical substitution. In the former case the cells appear normal, while in the latter case the cells appear shrunken.

Stability of the vitreous state is critical for the retention of vitrified tissue integrity and viability. Comprehensive studies of vitreous stability for a variety of potentially important cryoprotective mixtures have been made.[172] Glass stability of vitrified blood vessel samples stored in vapor phase liquid nitrogen storage with retention of smooth muscle function has been demonstrated up to 4 months of storage.[173] The stability of glasses formed from aqueous solutions of 1,2-propanediol are much greater, for the same water contents, than for all other solutions of commonly used cryoprotectants including glycerol, dimethyl sulfoxide (DMSO), and ethylene glycol. Unfortunately, solutions of polyalcoholic cryoprotectants (CPAs) such as propanediol and butanediol that show the most promise in terms of cooling rates and concentrations necessary for vitrification, also required unrealistically high heating rates to avoid devitrification during rewarming. Moreover, due principally to isomeric impurities that form a hydrate at reduced temperatures, 2,3-butanediol has proved to have an unanticipated biological toxicity at concentrations below that necessary for vitrification.[174–177]

Advances in biostabilization require process development for optimization of chemical and thermal treatments to achieve maximal survival and stability. At this time the consensus opinion is that viable tissues such as blood vessels, corneas, and cartilage that have proven refractory to cryopreservation by conventional freezing methods, despite decades of intense research by many investigators, can only be successfully preserved if steps are taken to prevent or control the ice that forms during cooling and warming. In contrast, other tissues in which the cells do not function in an organized manner or in which the extracellular matrix water is not highly organized are well preserved by traditional cryopreservation by freezing methods (i.e., heart valves and skin). Our

laboratory has developed a cryopreservation approach using vitrification, which thus far has demonstrated >80% preservation of smooth muscle cell viability and function in cardiovascular grafts[16,153] and similar levels of chondrocyte survival in articular cartilage.[6,178–181] In addition to *in vitro* studies of cardiovascular tissues, transplant studies have been performed that demonstrate normal *in vivo* behavior of vitrified cardiovascular and cartilaginous tissues. Most recently, this technology has been successfully applied to tissue-engineered blood vessels[182] and encapsulated cells described below.[183,184]

Avoidance of ice by vitrification has been achieved by cooling highly concentrated solutions (typically >50% w/w) that become sufficiently viscous at low temperatures to suppress crystallization rates. The original formulation and method was licensed from the American Red Cross, where it was developed for organ preservation.[154,167] However, even though rabbit kidneys were successfully vitrified, they could not be rewarmed with significant retention of function. Viability was lost due to ice formation during the rewarming process. The rewarming of vitrified materials requires careful selection of heating rates sufficient to prevent significant thermal cracking, devitrification, and recrystallization during heating. The use of carefully designed warming protocols is necessary to maximize product viability and structural integrity. Vitrified materials, which may contain appreciable thermal stresses developed during cooling, may require an initial slow warming step to relieve residual thermal stresses. Dwell times in heating profiles above the glass transition should be brief to minimize the potential for devitrification and recrystallization phenomena. Rapid warming through these temperature regimes generally minimizes prominent effects of any ice crystal damage. It is presently not possible to rewarm organs rapidly enough due to their high volume relative to the volume of tissues. Development of optimum vitrification solutions requires selection of compounds with glass-forming tendencies and tolerable levels of cytotoxicity at the concentrations required to achieve vitrification. Due to the high total solute concentration within the solution, stepwise protocols are commonly employed at low temperatures for both the addition and removal of cryoprotectants to limit excessive cell volume excursions and lower the risk of cytotoxicity. For a current comprehensive review of vitrification see Taylor et al.[6]

Despite developments to devise solutions that would vitrify at practically attainable cooling rates for sizeable biological tissues, the corresponding critical warming rate necessary to avoid devitrification remains a critical challenge. Conceptually, elevated pressures,[185] electromagnetic heating,[163–165] the use of naturally occurring antifreeze molecules,[186] and synthetic ice blockers[6] have been proposed as means to tackle the problem.

8.4.5 Vitrification Versus Freezing

We are often asked how we make the decision to use a vitrification approach rather than a freezing method for a particular tissue. The answer is usually a combination of method efficacy, based upon our experience and the literature with respect to the specific tissue, and ease of use or cost.

An excellent example of efficacy is conventional cryopreservation of articular cartilage by means of freezing, which typically results in death of 80–100% of the chondrocytes plus extracellular matrix damage due to ice formation. These detrimental effects are major obstacles preventing successful clinical use of osteochondral allografts[147,150,187] and commercial success of tissue-engineered cartilage constructs. Cryosubstitution studies of frozen and vitrified articular cartilage plugs revealed negligible ice in vitrified specimens and extensive ice formation throughout frozen specimens.[180] Transplantation studies in rabbits demonstrated that vitrified cartilage performance was not significantly different to fresh untreated cartilage. In contrast, frozen cartilage performance was significantly different when compared to either fresh or vitrified cartilage.[179] These studies combine to demonstrate that the vitrification process results in ice-free preservation of rabbit articular cartilage plugs and that about 85% of cellular metabolic activity is retained following rewarming. In contrast, frozen tissues contained ice within the cells and the matrix, with the exception of the articular surface, where some viable cells were observed. In our experience

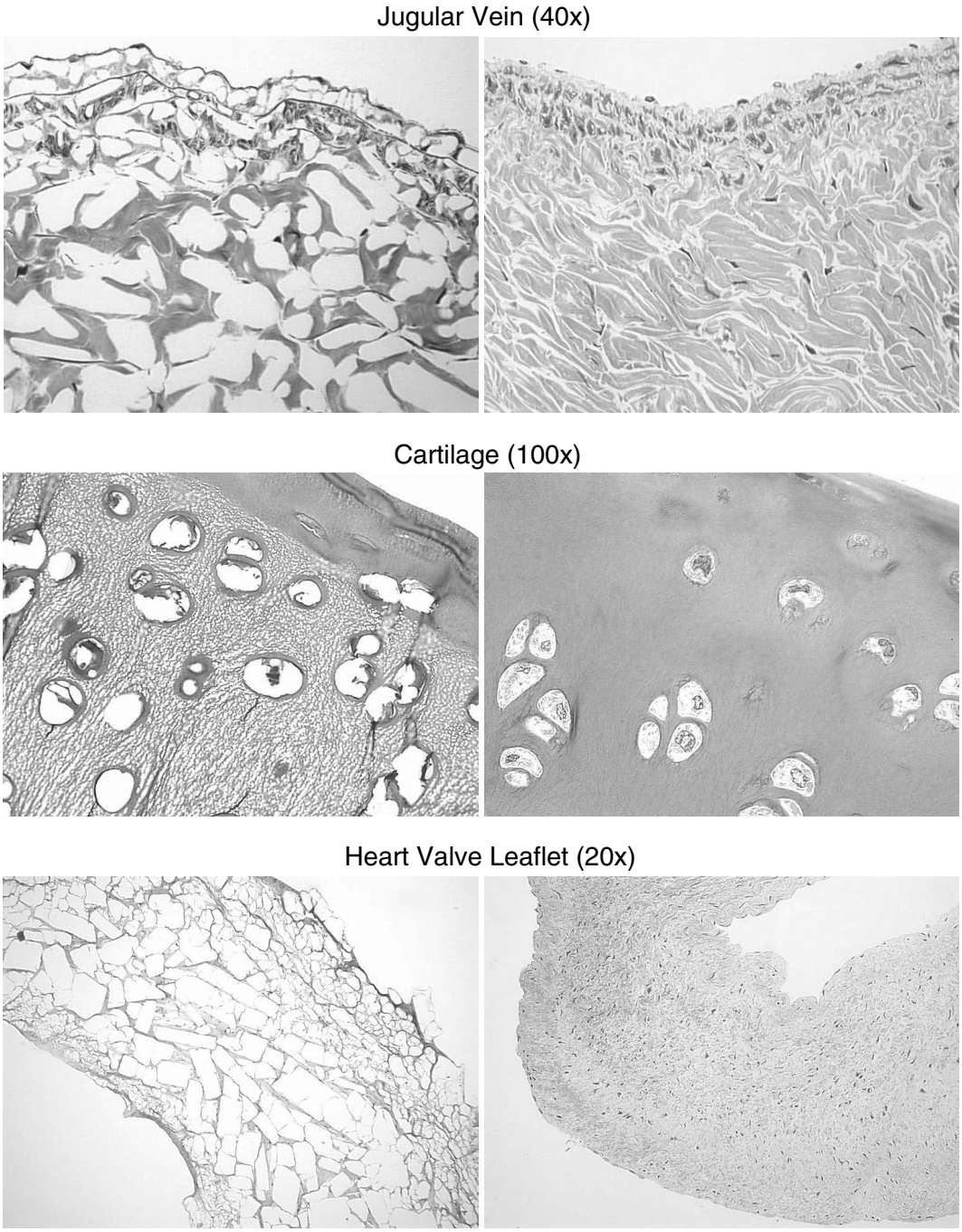

FIGURE 8.3 Light microscopy of cryosubstituted frozen (A, C, E) or vitrified (B, D, F) specimens from a variety of natural tissues: Jugular vein (A, B); articular cartilage (C, D) and heart valve leaflet (E, F). Cryosubstitution is a process whereby the location and size of the domains occupied by ice in the cryopreserved tissue are revealed and appear as white spaces in the tissue section (see Brockbank, K.G.M., Lightfoot, F.G., Song, Y C., and Taylor, M.J. (2000): Interstitial ice formation in cryopreserved homografts: A possible cause of tissue deterioration and calcification in vivo. J. Heart Valve Dis., 9:(2) 200–206). Extensive ice formation is present in the frozen veins (A), cartilage (C) and heart valve leaflet (E). In contrast, the respective vitrified specimens (B, D and F) appear to be free of ice and retain a more normal, undistorted morphology.

vitrification has been superior to freezing for rabbit cartilage plugs, porcine cartilage plugs, and human biopsy specimens (unpublished data). That does not mean that effective freezing methods can't be developed, just that no one has come close to date.

In cardiovascular tissues the decision to use a vitrification method over freezing is not as clear cut. Both approaches to cryopreservation result in high cell viability, but in our experience smooth muscle and endothelial functions are better preserved by vitrification than freezing. At this time we hypothesize that this is due to prevention of extracellular ice damage to tissue matrix. We fully agree with criticism that we have compared vitrification with freezing methods that may be improved upon; however, we would also point out that both the vitrification and the freezing methods that we have employed may be improved by further research. We have primarily employed freezing protocols derived by extensive research and in clinical practice for allografts in the United States. In the case of cardiovascular allografts the use of vitrification techniques may have cost benefits because there is no need for control-rate freezers. However, regardless of whether vitrification or freezing is employed, the tissue is still allogeneic with respect to the potential recipient and there is no reason to anticipate that vitrification will reduce immunogenicity. The research that we have performed on vitrification of cardiovascular tissues was intended for tissue-engineered cellular constructs; however, when the work was initiated there were no constructs available so we employed autologous and allogeneic tissue models. There are still no tissue-engineered cellular constructs approved for human use, but there are several well-established experimental models. We have compared the published vitrification and freezing procedures for two experimental vascular graft models based upon collagen or polyglycolic acid matrixes combined with smooth muscle cells. The results reflect our earlier results with rabbit jugular vein segments: cell viability was well preserved by both freezing and vitrification methods. However, in the graft model capable of developing detectable contractile forces in response to various drugs, the polyglycolic acid construct, smooth muscle function was significantly better preserved by vitrification than freezing (see Figure 8.4).[182]

In many small tissue structures (such as tissue organoids, cell aggregates, or encapsulated cells) it is anticipated that optimized freezing and vitrification procedures will provide similar levels of cell viability and tissue functions. We are finding that cryopreservation by freezing and vitrification methods may be equally effective in preservation of small pieces of tissue. The question is often which method is easiest and most consistent. We recently performed cryopreservation studies on rat embryo metanephroi (embryonic kidneys).[188] One potential solution to xenotransplantation immunological complications is the transplantation of embryonic kidneys whose blood supply is not yet fully developed. The metanephroi (MN) may be less immunogenic in comparison to their adult counterparts, at least in part due to the fact that post transplantation their vascular supply is derived from the host.[189] It has been shown that MN from E15 Lewis rat embryos that are transplanted into the omentum of adult C57BI/6J mice receiving costimulatory blockade undergo growth and differentiation.[189] Also, the E28 pig MN growth and development occurs post-transplantation across an allogeneic or highly disparate xenogeneic barrier with costimulatory blockage.[190] For such a therapy to be commercially viable, long-term storage of embryonic kidneys is crucial. In these studies the effects of controlled-rate freezing and ice-free vitrification on MN viability were investigated. Metanephroi were isolated from 15-day (E15) timed pregnant Lewis rats and either (1) control-rate frozen at –0.3°C/min in a DMSO formulation or (2) vitrified in VS55. The MN were then stored at –135ºC for 48 h. After storage the MN were rewarmed, placed in culture media, and their viability was assessed using the alamar Blue assay and histology (light microscopy, TEM, and cryosubstitution). There were no statistical differences in embryonic kidney metabolic activity of either of the cryopreserved MN groups relative to the untreated control group. Cryosubstitution demonstrated the presence of significant ice formation during controlled-rate freezing (see Figure 8.5). This was confirmed by TEM, where vacuolation of the cytoplasm of control-rate frozen MN was observed. In contrast, no ice was observed in vitrified MN and there was very little cytoplasmic disruption. However, vitrified MN showed mitochondrial and nuclear injury suggestive of CPA cytotoxicity. This injury was not observed in frozen MN, nor

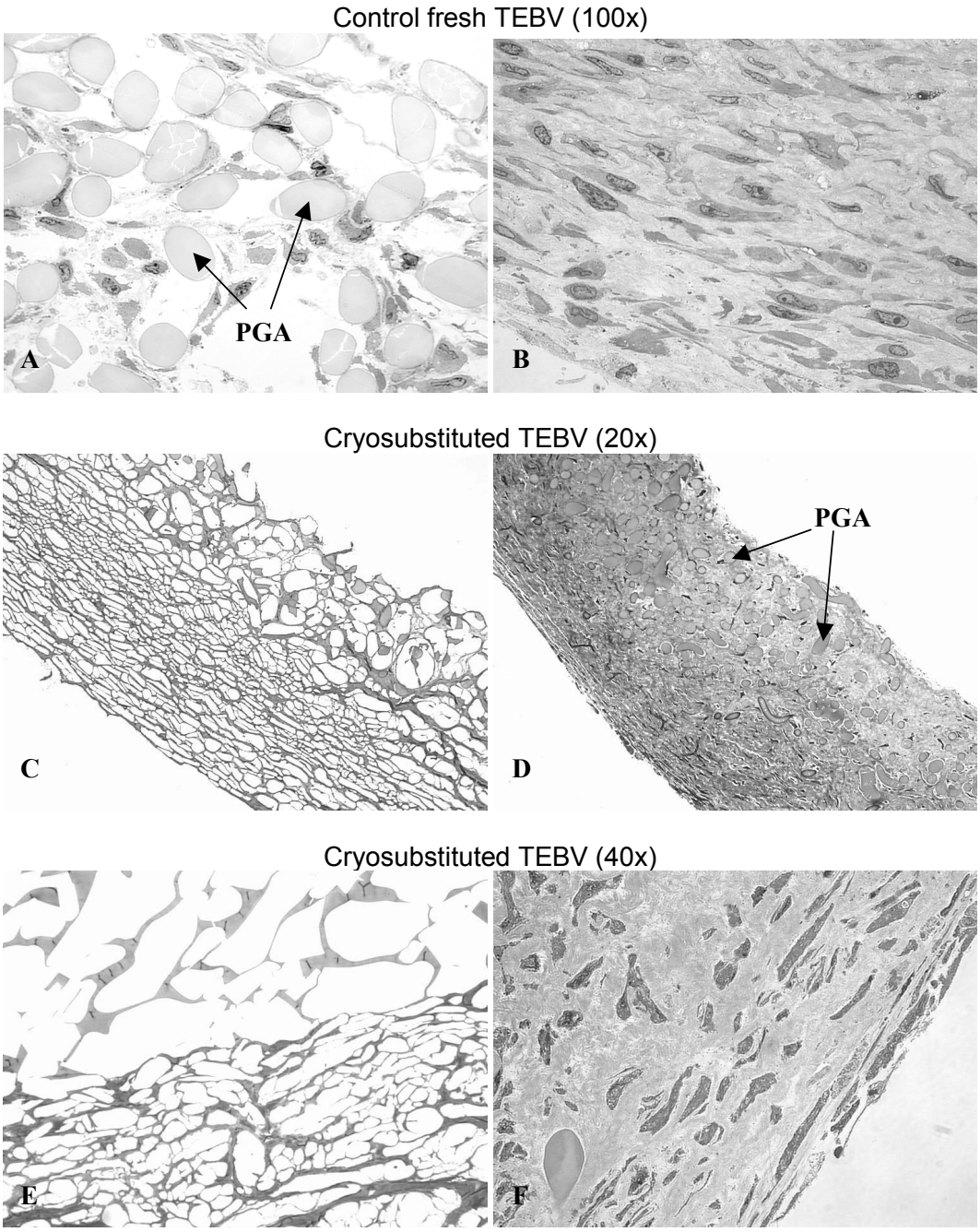

FIGURE 8.4 Cryosubstitution of tissue-engineered blood vessels (TEBV) at –90°C. Low-power micrographs of frozen TEBV (left panels C, E) reveal the noticeable distortion of the tissue structure by the prevalent ice domains (white spaces) of variable size scattered throughout the extracellular matrix of the vessel wall. The tissue matrix appears shrunken and sandwiched between the ice crystals. In contrast, the vitrified specimens (D,F) appear to be ice-free with a morphology that resembles the normal structure of these engineered constructs (A, B are control non-cryopreserved specimens). These constructs comprise vascular smooth muscle and endothelial cells on highly porous, degradable polyglycolic acid (PGA; arrows) scaffolds.

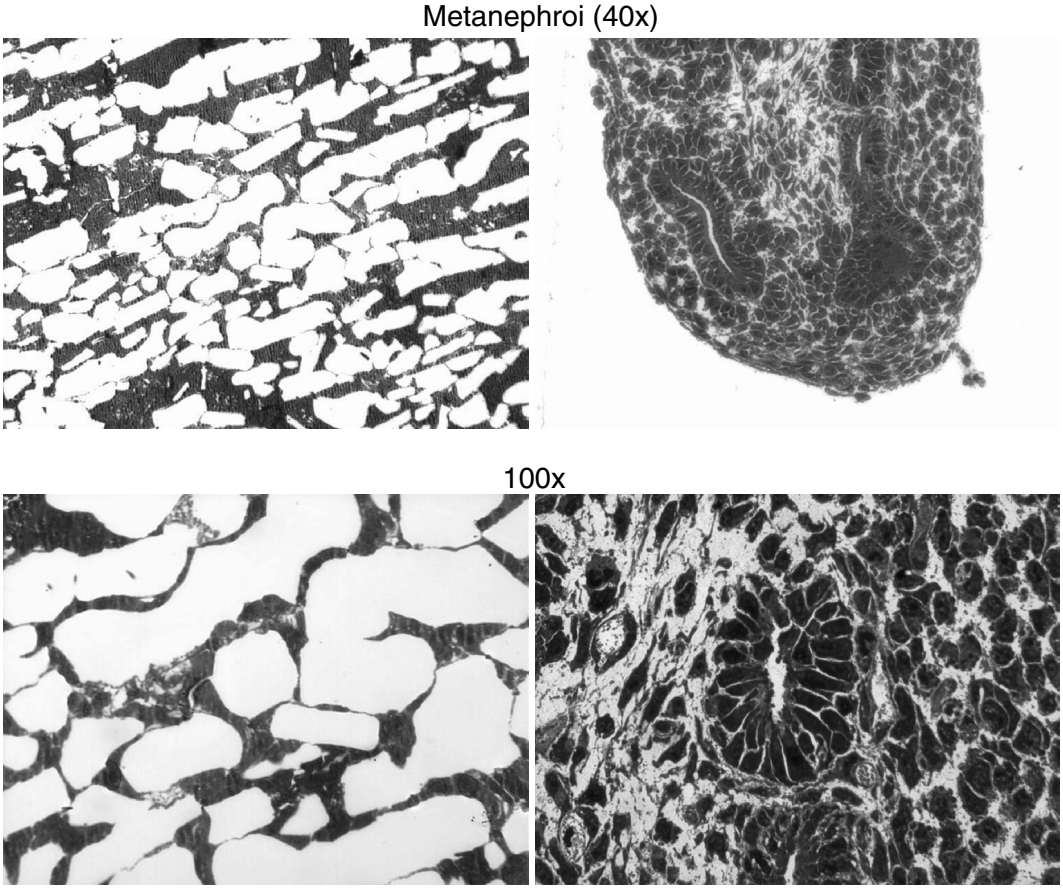

FIGURE 8.5 Light microscopy images of cryosubstituted frozen and vitrified 15-day rat metanephroi (magnification 40X and 100X). The controlled-rate frozen metanephroi show the presence of ice crystals (white spaces) in the extracellular matrix as a result of the cryopreservation method. By contrast, the cryosubstituted vitrified specimen is devoid of large ice domains and illustrate normal tissue morphology. Toluidine Blue was used for resin-embedded tissue section staining.

have we observed similar changes in other vitrified tissues. The effects of vitrification solution formulation, concentration, exposure time, and loading steps on embryonic kidney viability need to be evaluated in future studies.

8.4.6 Microencapsulated Cells as Pseudo-Tissues

Another example is in the development of cryopreservation protocols for microencapsulated cells. Microcapsules are particularly prone to cryodamage using freezing by cryopreservation methods. Since 1991 studies of microcapsule cryopreservation by freezing employing a variety of cell types have accumulated.[184,191–201] Excellent cell viability was obtained in many cases, but capsule integrity is another issue (Table 8.4).

Viability is excellent following preservation in alginate microcapsules. Algae-derived polysaccharides, such as agarose and alginate, are a novel class of nonpermeating (with respect to the cell) cryoprotectants.[191] These polysaccharides had no cryoprotective abilities when used alone, but resulted in enhanced viability when mixed with known penetrating cryoprotectants (such as DMSO). Ice formation in slow-rate, DMSO-protected frozen microcapsules containing insulin-secreting βTC3 cells was demonstrated using cryosubstitution at –90°C and fixation, a method that

TABLE 8.4
Capsule or Cell Cryodamage Induced with Cryopreservation by Freezing

Cell Type	Capsule Composition	Outcome	Reference
Hepatocytes	Collagen matrix enveloped by sodium alginate-poly L-lysine-sodium alginate membrane	Some cryo-samples broke down *in vivo* resulting in inflammatory reaction, poor long-term storage stability.	198
Hepatocytes	Sodium alginate	Fraction (>10%) of capsules broken.	197
Pancreatic islets	Sodium alginate with a poly L-lysine membrane and a further treatment with sodium alginate	No mention of capsule damage; however, the cell viability was low and the authors concluded that further cryopreservation method development was needed.	192
Hepatocytes	Sodium alginate, cellulose sulphate, and poly (methylene-co-guanidine) hydrochloride	A small percentage of capsules (number not given) were broken, good long-term storage stability.	195
Hepatocytes	Sodium alginate, cellulose sulphate, and poly (methylene-co-guanidine) hydrochloride	A small percentage of capsules (number not given) were broken.	196
Adipocytes	Sodium alginate	Capsules were deformed.	196
Kidney cells	Anionic Ter-polymer (composed of methylacrylic acid, 2-hydroxyethyl methylmethacrylate, and methyl methacrylate) with cationic collagen	~40% loss of capsule integrity with best viability retention.	194

permits visualization of ice.[184] In this same study vitrification resulted in freedom from ice (see Figure 8.6).[184] Vitrified insulin-secreting βTC3 cells had significantly better viability (metabolic activity) and function (insulin release) than frozen insulin-secreting βTC3 cells.[184]

Very little investigation of cryopreservation variables has been performed for microencapsulated cells. Most studies have used low-rate cooling with 5–20% DMSO and storage in liquid nitrogen. Single studies have compared cryopreservation with and without nucleation control,[192] duration of DMSO incubation prior to cryopreservation,[193] and cooling rates with several cryoprotectant formulations.[194] The outcome of the DMSO incubation study indicated that a 5-h incubation was required for optimum cell survival. This was curious since we have found that DMSO equilibrates in alginate capsules in 2–3 min (unpublished data). Nucleation was required for optimum cell survival as we would anticipate for an effective freezing cryopreservation method for either isolated cells or tissues. The most in-depth study was performed by Heng et al.[194] In this study rapid cooling cryopreservation protocols with high DMSO concentrations (3.5 M, 25% v/v) resulted in low post-thaw cell viability (<10%), which did not improve with higher concentrations (4.5 M, 32% v/v) and longer exposure to DMSO, even though the majority of microcapsules (60–80%) remained intact. Subsequent investigations of slow cooling with a range of DMSO and EG concentrations resulted in a much higher post-thaw cell viability (80–85%), with ~60% of the microcapsules remaining intact when DMSO was used at a concentration of 2.8 M (20% v/v) and EG at a concentration of 2.7 M (15% v/v). The presence of 0.25 M sucrose significantly improved post-thaw cell viability upon slow cooling with 2.8 M (20% v/v) DMSO, although it had no effect on microcapsule integrity. Multistep exposure and removal of sucrose did not significantly improve either post-thaw cell viability or microcapsule integrity, compared to a single-step protocol. Ficoll 20% (w/v) also did not significantly improve post-thaw cell viability and microcapsule integrity.[194] There have been two reports on vitrification of microencapsulated cells, including our paper on microencapsulated insulin producing TC3 cells.[184] Vitrified encapsulated cells demonstrated no

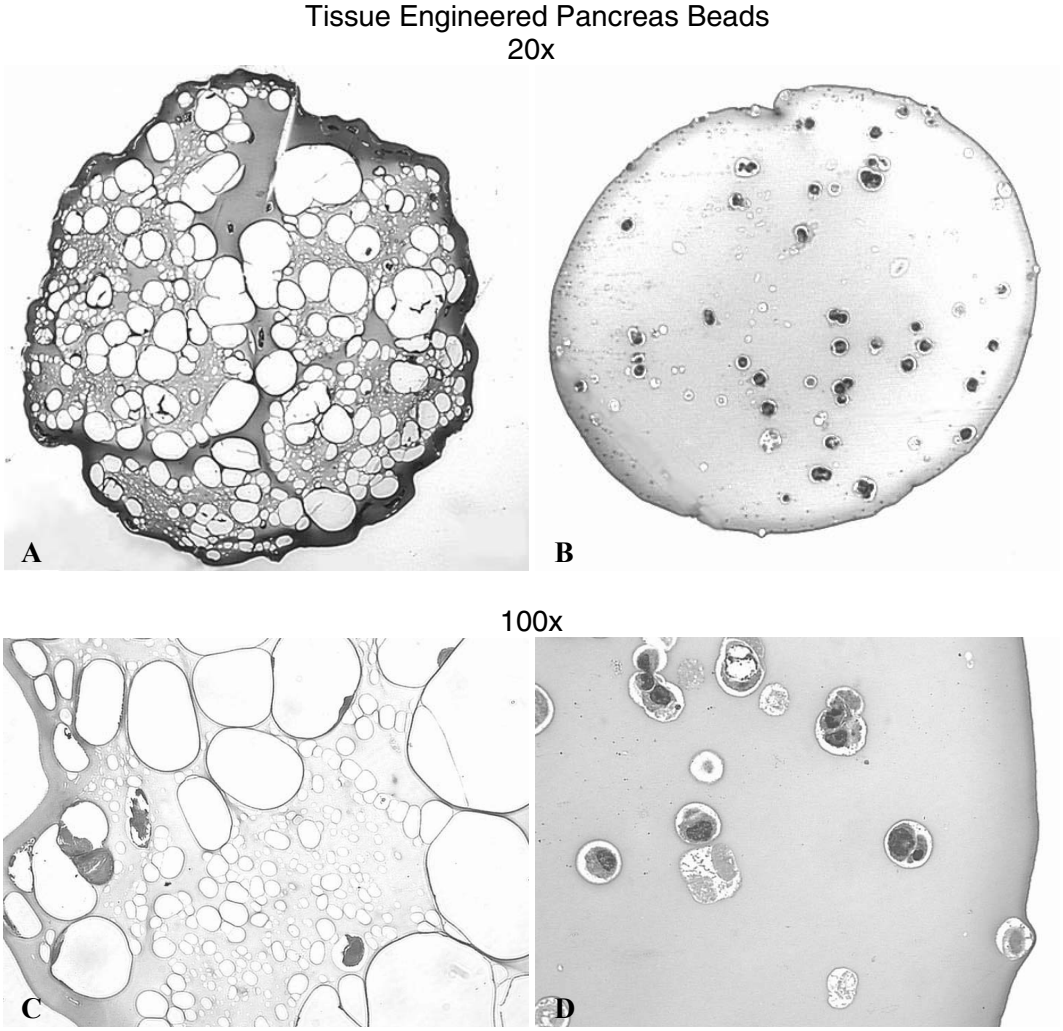

FIGURE 8.6 Morphology of frozen and vitrified pancreatic substitute beads. The beads comprise insulin-secreting _TC3 cells encapsulated in calcium alginate/poly-L-lysine/alginate. Beads frozen using a conventional controlled-rate (1°C/min) protocol with 1 M dimethyl sulfoxide show considerable ice formation throughout the construct (white spaces A, C). In contrast, beads vitrified with VS55 appear to be ice-free (B, D). At higher magnification it is seen that the encapsulated cells are shrunken and compressed within the frozen matrix (arrows) compared with the more normal morphology of the cells embedded in the vitrified matrix (D).

significant difference between fresh and vitrified specimens and no ice was observed in the vitrified specimens. Kuleshova et al. investigated vitrification of encapsulated hepatocytes employing 40% ethylene glycol and 0.6 M sucrose.[202] They employed a modification of vitrification approaches employed for embryos and oocytes that employs straws for handling and storage combined with a capsule made from Ter-polymer and collagen as previously described.[194] These studies combine to demonstrate that both freezing and vitrification procedures for cryopreservation of microencapsulated cells are feasible.

However, as the scale of the tissue increases, vitrification procedures excel and levels of tissue functions and/or cell survival previously not achieved with freezing procedures are achieved (discussed in earlier sections). Conceptually, cryopreservation of tissues by vitrification offers several

important advantages compared with procedures that allow or require ice formation. Complete vitrification eliminates concerns for the known damaging effects of intra- and extracellular ice crystallization. Furthermore, tissues cryopreserved by vitrification are exposed to less concentrated solutions of cryoprotectants for shorter time periods. For example, Rall has calculated that for embryos, during a typical cryopreservation protocol involving slow freezing to –40ºC or –70ºC, the cells are exposed to cryoprotectant concentrations of 21.5 and 37.6 osmolal respectively.[169] In contrast, cells dehydrated in vitrification solutions are exposed for much shorter periods to <18 osmolal solution, although the temperature of exposure is higher. Finally, unlike conventional cryopreservation procedures that employ freezing, vitrification does not require controlled cooling and warming at optimum rates. A principal benefit of vitrification is the elimination of requisite studies to determine optimal cooling rates for tissues with multiple cell types. Successful vitrification requires that the thermal processing be rapid enough to transition regions of maximal ice crystal nucleation and growth that occur above the glass transition temperature of the solution. Thus, it is only necessary to cool solutions at rates in which a negligible fraction of the solution forms ice (typically < 0.2%).[172] Vitrified materials have a similar rate requirement during heating, when samples are rewarmed for subsequent use, to limit ice formation to negligible levels (typically <0.5%).[203]

8.4.7 Applications to Tissue-Engineered Products

There are significant challenges for deployment of both preservation methods for tissue-engineered medical products. 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 glass transition temperature for long without significant risk of product damage due to inherent instabilities leading to ice formation and growth. Both approaches employ cryoprotectants with their attendant problems and require competent technical support during rewarming and cryoprotectant elution phases. The high concentrations of cryoprotectants 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. Cryoprotectants can kill cells by direct chemical toxicity, or indirectly by osmotically induced stresses during suboptimal addition or removal. Upon completion of warming, the cells should not be exposed to temperatures above 0°C for more than a few minutes before the glass-forming cryoprotectants are removed. It is possible to employ vitrified products in highly controlled environments, such as a commercial manufacturing facility or an operating theater, but not in a doctor's outpatient office or in third-world environments. The cryoprotectants employed for vitrification, in contrast to DMSO or glycerol for freezing, are less well known for preservation applications outside low-temperature biology circles. In particular, formamide, one of the components of the 55% (v/v) vitrification solution consisting of 3.10 M DMSO, 3.10 M formamide and 2.21 M 1,2-propanediol in EuroCollins solution at 4°C[16,153] (known as VS55), is a known mutagen. Alternatives to formamide with fewer safety risks and potentially easier clinical acceptance are being sought. However, the cytotoxicity of complex cryoprotectant formulations containing formamide is surprisingly much less than the cytotoxicity of single component formulations at the same concentrations.[204]

Storage and shipping temperatures also have a major impact on maintenance of product quality and can result in cell death mediated by ice formation. Degradative processes occur at temperatures warmer than the freezing solution's glass transition temperature (approximately –125°C). Even cells in heart valve leaflets that are frozen slowly can be negatively affected by storage at temperatures warmer than –100°C.[205] It is anticipated that synthetic ice blocker molecules, such as the cyclohexanediols, will be effective in prevention of recrystallization and allow storage of frozen biological materials for longer periods at warmer subzero temperatures. Ice blockers will also allow vitrification at lower, less cytotoxic CPA concentrations. This improved storage capability

will facilitate longer shipping times, less expensive shipping methods, and larger cryopreserved specimens.

It is well established that storage and shipping temperatures have a major impact on maintenance of product quality and can result in cell death via ice formation. If storage temperature is sufficiently low (below the glass transition point of the freezing solution [approximately –135°C to –95°C]), little, if any, change occurs in biological materials.[14,17] Human heart valve leaflets demonstrate retention of protein synthetic capabilities for at least two years of storage below –135°C.[205] Degradative processes may occur at and above the solution's glass transition temperature. For example, it has been shown that cells in cryopreserved human heart valve leaflets are negatively affected by storage at temperatures warmer than –100°C.[205]

One of the major issues for both frozen and vitrified storage of product relates to mechanical forces generated by cooling and warming conditions. Immersion of frozen human valves directly into liquid nitrogen for as little as 5 min may result in tissue fractures.[206] This problem came to light when a hospital-based frozen valve storage system overfilled during an automatic refill cycle. Valves from this accident were discovered to have numerous full-thickness fractures of the valve conduit following normal thawing procedures in the operating room.[207] Adam et al. reproduced this phenomenon experimentally.[206] The rationale for development of fractures appears to relate to abrupt changes in the physical properties of the solidified tissue matrix. Kroener and Luyet described abrupt temperature-dependent changes in aqueous glycerol solutions.[208] Subsequently they reported[209] that the formation and the disappearance of cracks depended on the interaction of several factors, in particular the mechanical properties of the material, the concentration of solute, the temperature gradients, the overall temperature, and the rate of temperature change. Studies of frozen biological materials have also supported the presence of mechanical forces in cryopreserved tissues.[210,211]

Heat transfer issues are the primary hurdle for scaling up the successes in tissues to larger organs. The limits of heat and mass transfer in bulky systems result in nonuniform cooling and contribute to stresses that may initiate cracking. In fact, the higher cooling rates that facilitate vitrification may lead to higher mechanical stresses. Very little information on the material properties of vitreous aqueous solutions exists. Material properties such as thermal conductivity and fracture strength of vitreous aqueous solutions have many similarities with their inorganic analogues that exist at normal temperatures, e.g., window glass and ceramics. Any material that is unrestrained will undergo a change in size (thermal strain) when subjected to a change in temperature. Calculations of stress in frozen biological tissues have shown that thermal stress can easily reach the yield strength of the frozen tissue resulting in plastic deformations or fractures.[212–214] We need a much better understanding of mechanical stresses during vitrification and freezing if we are to effectively proceed from long-term biopreservation of simple tissue structures to complex organs in the future (see Chapter 13).

8.5 ISSUES FOR THE FUTURE

In this overview of biopreservation we have indicated several areas where further research is urgently required, including sterilization methods for allogeneic tissues that permit retention of cell viability, less toxic CPA formulations, better warming methods for large cryopreserved specimens, and the need for a better understanding of the mechanical forces generated by cryopreservation. There are two other topics that we believe should be mentioned in closing that could have a major impact on biopreservation in the future. The first is the development of methods for the intracellular delivery of disaccharide cryoprotectants that are too large to permeate mammalian cell membranes. Success in this area promises new relatively noncytotoxic methods of cell and tissue cryopreservation and leads directly to the second topic of new biopreservation technologies based upon desiccation and freeze drying strategies.[7,8,215,216]

Both conventional freezing and vitrification approaches to preservation have limitations. First, both of these technologies require low-temperature storage and transportation conditions. Neither

can be stored above their glass transition for long without significant risk of product damage due to ice formation and growth. Both technologies require competent technical support during the rewarming and CPA elution phase prior to product utilization. This is possible in a high-technology surgical operating theater but not in a doctor's outpatient office or in third-world environments. In contrast, theoretically, a dry product would have none of these issues because it should be stable at room temperature and rehydration should be feasible in a sterile packaging system.

Drying and vitrification have previously been combined for matrix preservation of cardiovascular and skin tissues but not for live cell preservation in tissues or engineered products. However, nature has developed a wide variety of organisms and animals that tolerate dehydration stress by a spectrum of physiological and genetic adaptation mechanisms. Among these adaptive processes, the accumulation of large amounts of disaccharides, especially trehalose and sucrose, are especially noteworthy in almost all anhydrobiotic organisms including plant seeds, bacteria, insects, yeast, brine shrimp, fungi and their spores, cysts of certain crustaceans, and some soil-dwelling animals.[216,–218] The protective effects of trehalose and sucrose may be classified under two general mechanisms: (1) "the water replacement hypothesis" or stabilization of biological membranes and proteins by direct interaction of sugars with polar residues through hydrogen bonding, and (2) stable glass formation (vitrification) by sugars in the dry state.

The stabilizing effect of these sugars has also been shown in a number of model systems, including liposomes, membranes, viral particles, and proteins during dry storage at ambient temperatures.[215,219,220] On the other hand, the use of these sugars in mammalian cells has been somewhat limited, mainly because mammalian cell membranes are impermeable to disaccharides or larger sugars. Recently, a novel genetically modified pore former has been used to reversibly permeabilize mammalian cells to sugars with significant postcryopreservation and, to a lesser extent, drying cell survival.[221] Such permeation technologies, which may also include use of pressure or electroporation, may provide some of the most likely opportunities for preservation of tissues in the five- to ten-year vision, either by permitting cryopreservation with nontoxic cryoprotectants or drying. Several methods have been developed for loading of sugars in living cells.[7] Introduction of trehalose into human pancreatic islet cells during a cell membrane thermotropic lipid-phase transition, prior to freezing in the presence of a mixture of 2 M DMSO and trehalose, has resulted in good cell survival rates.[222] We have found that prolonged cell culture in the presence of trehalose results in significant increases in postcryopreservation cell survival (patent pending).[223] Human fibroblast transfection with *E. coli* genes expressing trehalose resulted in retention of viability after drying for up to five days.[224] However, it should be noted that most organisms that reach a dried state during dormancy and drought, do so by air drying (not freeze drying), which suggests this may be innocuous to cells under certain conditions. Further, studies of anhydrobiotic organisms may also suggest methods for conditioning mammalian cells for storage by either cryopreservation or drying in the tissue-engineered products of the future.

8.6 CONCLUDING COMMENTS

The emerging fields of tissue engineering and regenerative medicine for living cell-based therapies embody a wide variety of enabling technologies that include the need for effective methods of preservation. Despite significant advances in many of these technologies, it is generally regarded that the basic knowledge and practical know-how needed for the storage of living tissues and complex tissue constructs lags significantly behind. This is reflected by the four key areas of research identified by the US National Institute of Standards and Technology (NIST) in its request for research proposals (1997). These four research areas are automation and scale up, sterilization, product storage, and transportation of product in which substantial technical innovation is required for the development of manufacturing processes (NIST Advanced Technologies Program Request for Proposals — 1997). Concerns for the issues relating to the transition from the laboratory to the market include the major problem of preservation and storage of living biomaterials. Manufacturers

and/or distributors recognize the need for maintaining stocks of their products to ensure a steady supply, while the unpredictable clinical demand for specific tissues will necessitate the creation of tissue banks at medical centers. Methods of preservation are crucial for both the source of cells and the final tissue constructs or implantation devices. Tissue preservation technology involves both hypothermic (above freezing) methods for short-term storage, and cryopreservation for long-term banking. Both approaches call for consideration of the cell in relation to its environment and as interventionalists we can control or manipulate that environment to effect an optimized protocol for a given cell or tissue.

In conclusion, tissue preservation as it exists today has been developed empirically and basic research on fundamentals of biopreservation has had restricted impact on the field to this point. In the near future vitrification methods will get a lot more attention, particularly for tissue-engineered products in which immunogenicity is not an issue. In contrast, for allografts, unless the tissue is immuno-privileged, it is unlikely that vitrification will result in significant differences in clinical outcomes. There are, however, two basic research areas that we believe may have a significant impact on tissue preservation in the future. Both are based on lessons we are still learning from nature, namely strategies by which living organisms deal with the environmental temperature extremes to which they are exposed. Through evolution, nature has produced several families of proteins that help animals (e.g., fish and insects) and plants survive cold climates. These observations led to the hypothesis that naturally occurring antifreeze molecules might be improved upon by synthesis of molecules that will either bind to other ice nuclei domains or upon stable ice crystals. Other organisms naturally accumulate antifreeze compounds such as sucrose and trehalose. Creative methods are required for placement of these compounds within mammalian cells,[7] followed by the development of effective preservation strategies.

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