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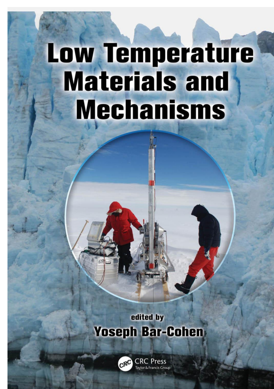

Low Temperature Materials and Mechanisms

Yoseph Bar-Cohen

Medicine and Biology: Technologies Operating at Extremely Low Temperatures

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11

Medicine and Biology: Technologies Operating at Extremely Low Temperatures

Alasdair G. Kay and Lilia L. Kuleshova

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11.1 Introduction

Medical and biological technologies operating at extremely low temperatures have been developed as part of the discipline of low temperature biology (cryobiology), and the wider field of bioengineering [Kuleshova and Hutmacher, 2008]. The direct aim of medical cryobiology is to utilize reduced temperatures to facilitate the long term preservation of cells, tissues, and organs with guaranteed phenotypic and genotypic stability. Cryobiology enables the induction of a state whereby “translational molecular motions are significantly arrested, marking the end of biological time” [Fahy et al., 1984].

Advances in medical technologies, such as organ transplantation, *in vitro* fertilization (IVF), or commercialization of cell therapies, are limited by the availability of consistent and safe viable materials for use. Reliable and reproducible protocols for the preservation of living materials are required to facilitate banking and subsequent supply. In addition to storage of biological material, low temperature therapies contribute effectively to the treatment of debilitating disorders and cancers.

Cryobiology covers a broad range of activities within a diversity of scientific aspects. Reference information specific to these individual areas can be accessed from multiple sources, including but not limited to, the journals of *Cryobiology*; *Cryoletters*; *Cell Preservation Technologies and Cryobiology*; and organizations including the Society for Cryobiology (SfC); the Society for Low Temperature Biology (SLTB); the Japan Society of Low Temperature Medicine (JSLTM); the Japanese Society for Cryobiology and Cryotechnology (JSCC); the European Alliance for Medical and Biomedical Engineering & Science (EAMBES); the International Society of Cryosurgery (ISC); and the American College of Cryosurgery (ACC).

“Low temperature” refers to temperatures below physiological mean (e.g., 34°C–37°C). “Extremely low temperatures” outlined in this chapter may be different from those described in materials science where scientists study and utilize significantly reduced temperatures, as low as –269°C or 4 K (the temperature of liquid helium). As opposed to scientific material presented in other chapters, cryobiology predominantly operates at temperatures of liquid nitrogen (–196°C or 77 K) or above. The reader may ask whether it would not be beneficial to further examine these lower temperatures. Temperatures below –196°C have been assessed in a cryobiological context; however, no significant benefits are found from applying such strategies for cryopreservation of cells or tissues. This chapter also describes the technology used for short-term preservation of organs at subzero or hypothermic temperatures (around 0°C).

11.2 Cryostorage and Mechanisms of Cryopreservation

The goal of cryopreservation technology is to achieve amorphous solidification of cells and preserve this state during long-term storage. Moreover, ice formation within the cells also has to be avoided on cooling and warming. Successful cryopreservation of cells or tissues allows for almost indefinite storage in effective stasis without loss of cell vitality [Mazur, 1984; Fahy et al., 1984; Hoffmann and Bischof, 2002]. At subzero temperatures, energy production reduces to around 10% of that at normothermia [Hoffmann and

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Minor, 2014], and consequently cell degradation due to metabolic processes is reduced. Depending upon the cell type and location, the water content of a cell is 60%–85% of the total volume, some of which is bound to intracellular solutes or molecules [Mazur, 2004]. To achieve long periods of storage without damage to the cells (attributable to storage conditions), there must be no liquid state present. At temperatures approaching liquid nitrogen temperature, chemical reactions cannot occur due to insufficient thermal energy [Mazur, 1984].

Vitrification eliminates ice formation inside and outside cells on cooling, cryostorage, and warming and involves exposure of cells and tissue to aqueous solutions of special supporting chemicals, commonly at room temperature [Fahy et al., 1984; Kuleshova et al. 2004, 2007, 2009a]. Many of these chemicals, termed cryoprotectants (CPAs), form solid "glass" at –130°C [Boutron and Kaufman,1979; Boutron, 1990; Fahy et al., 1984; Luyet and Rasmussen, 1967; Kuleshova et al., 1999a; Kuleshova and Hutmacher, 2008; Macfarlane et al., 1987, 1991; Shaw, 1997]. The challenge in the cryopreservation of cells or tissues comes in careful preparation using the combined action of CPAs to be preserved without damage by vitrification rather than in the long-term survival of cells once cooled and stored [Kuleshova, 2009a]. Alternative ways exist to avoid lethal formation of ice inside the cells, namely, to cool the cells slowly enough in the presence of low-concentration CPA that freezable water is osmotically drawn out of the cell to freeze externally. The initial theory outlined in this section serves as the basis of this and describes the challenges in cryopreservation of cells and tissues faced by cryobiologists pursuing a slow cooling approach [Mazur and Leibo, 1972; Mazur, 1984].

Storage below –130°C of cells preserved in both ways ensures that only solid physical states exist, and can be achieved through transfer of cells or tissue for storage in liquid nitrogen, liquid nitrogen vapor phase (ranging from –145°C to_–156°C), or electrical freezers (–150°C) [Fahy et al., 1984; Pegg, 2009; Pegg et al., 2006a, 2006b, 2006c; Kuleshova and Hutmacher, 2008]. Vapor storage is of great benefit when large tissue samples need to be preserved. Heart valves for transplantation, for example, are widely stored in nitrogen vapor. Storage above the level of liquid nitrogen at the temperature of –145°C makes the samples less brittle and prevents them from cracking. Second, numerous viruses and microorganisms of infected materials previously stored in these liquid nitrogen tanks can survive and become a source of cross-contamination. Fluctuation of temperature during the opening of vapor storage tanks is not significant for large samples [Wood et al., 1999]. Small specimens warm significantly faster than large specimens. As storage temperature is higher in the vapor phase than in liquid nitrogen, the risk of accidental thawing or the effects of greater fluctuations in storage temperature may counteract the benefits of using this storage method for small samples. Some sealing strategies are more effective than others; therefore, extra protection, for example, wrapping straws used for cell vitrification in a plastic film or a double bag, may be needed to further reduce the risk of leakage/contamination of samples [Russell et al., 1997]. Developing protocols to prevent contamination of very rapidly cooled or vitrified biological material may prove more difficult. A strategy to minimize the likelihood of contamination while allowing specimens to be cooled by direct immersion in liquid nitrogen and warmed by direct immersion into a water bath was developed using a double straw arrangement as a simple strategy to prevent cross-contamination [Kuleshova and Shaw, 2000; Kuleshova et al., 2004, 2009a]. The major advantage of electrical freezers is in removing the necessity of refilling the storage container with liquid nitrogen, but the storage volume available is more limited.

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11.3 General Principles of Vitrification

A deep understanding of physical chemistry was a foundation for the development of vitrification as a method of cryopreservation [Luyet and Rasmussen, 1967; MacFarlane et al., 1987, 1991]. A broad class of solutes are able to dissolve uncharged in water. The common factor in alcohols, amides, ketones, and other lower-molecular-weight solutes of interest is an ability to enter into hydrogen-bonding interactions with water. Generally, high-molecular-weight solutes tend to suppress ice nucleation, and therefore promote glass formation, more effectively than low-molecular-weight solutes. First, because of this high molecular weight, larger weight by weight (w/w) percentage solutions have small molar concentrations. Consequently, there is no colligative effect of these solutions on water. Second, the high molecular weight of the solute causes the viscosity of the solutions to rise rapidly, which is beneficial in achieving glass transition.

Chemicals such as propane diols, butane diols, sugars, and polymers are able to undergo vitrification and form a glass [Luyet and Rasmussen, 1967; MacFarlane et al., 1987, 1991; Fahy et al., 1984; Kuleshova and Hutmacher, 2008; Kuleshova, 2009a], yet research aims to find the optimal composition and conditions to achieve a vitreous state of moderately concentrated aqueous solutions [Shaw, 1997; Kuleshova et al., 1999a] and avoid the toxicity of CPAs. The glass-forming ability of the first group has been investigated for decades [Luyet and Rasmussen, 1967; Boutron and Kaufman, 1979; Boutron, 1990]. Low-molecular-weight agents (MW < 100 Da) were characterized for lowest total solute concentration required for vitrification calculated from the diagram of phase and physical transitions of CPAs based on differential scanning calorimetry thermograms and other physical methods [MacFarlane et al., 1991; Shaw et al., 1997; Kuleshova et al., 1999a]: 2,3-butandiol [Boutron, 1990], propylene glycol [Boutron and Kaufman, 1979], ethylene glycol [Rasmussen and Luyet, 1969], dimethyl sulfoxide [Rasmussen and Mackenzie, 1968], and glycerol [Rasmussen and Luyet, 1969]. For example, 1,2-propanediol can form a stable glass at a solute concentration ≥45% (w/w). Another popular CPA, ethylene glycol, is able to form a stable glass at 59% (w/w) solute concentration. If concentration is sufficient at a given cooling rate, crystallization may not occur and the solute remains wholly amorphous. It has been observed that ice crystals do not form during rapid cooling; however, as the cell returns to normothermic temperatures, it passes through the range –100°C to –40°C and devitrification occurs. Cubic ice may form from supercooled liquid, which can transform into hexagonal ice. Furthermore, recrystallization occurs with an increase of temperature, whereby small or isolated ice crystals combine to form large crystals [Luyet and Rasmussen, 1967; Boutron and Kaufman, 1979; Fahy et al., 1984; Boutron, 1990; Kuleshova and Hutmacher, 2008].

In a cryobiology context, vitrification is the cooling of a suspension or tissue exposed to a CPA solution in stepwise manner to below the glass transition point. Following glass transition, biological stasis is achieved such that the tissue does not appear to alter or degrade over time [Fahy et al., 1984]. The challenge with such a technique is to achieve exposure to high levels of CPA while minimizing CPA toxicity. Processes involve the design of a pre-equilibration protocol to allow the cells or tissue to adapt to the final vitrification solution as well as a subsequent dilution procedure. Toxicity can also be avoided through the addition of high-molecular-weight additives to solutions that have not only the advantage to penetrate the cells but additionally are able to dehydrate cells as described earlier. To participate in the vitrification process, two main classes of chemicals were described, namely, penetrating cryoprotectants with molecular weight (MW) less than 100 Da and nonpenetrating CPAs that are in turn divided into two classes, namely, sugars

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(180 Da < MW < 600 Da) and polymers with MW up to hundreds of kDa [Luyet and Rasmussen, 1967; Kuleshova et al., 1999a, 2009a]. Although vitrification is a dynamic process, reduction of total solute concentration is required for vitrification; this is a challenge. It is essential to decrease cooling/warming rates by orders of magnitude to reduce the total solute concentration by 1%–2%. Ice crystals could still be formed on warming from supercooled liquid (devitrification) if the total solute concentration has reduced below the calculated level, as well as through recrystallization of small crystal nuclei, further damaging biological material. The phenomenon of devitrification is a major concern in cells. For this reason, warming has to be achieved faster than cooling.

Insufficient dilution of CPA during warming is an issue for larger tissues, as CPA toxicity increases with rising temperature. Retention of excess CPA in the tissue will have a toxic effect on the cellular component. A narrow range of CPA combinations and concentrations will allow a tissue to be cooled to super-low temperatures while inhibiting ice formation, will not be excessively toxic to cells, and will not be prone to excessive ice formation during devitrification. This range of concentrations, related to the temperature at which exposure occurs, will vary depending upon the tissue type, content, size, and cellularity.

Applications of vitrification to medical cryopreservation date back to the 1940s in the cryopreservation of spermatozoa and protoplasm [Hoagland and Pincus, 1942; Luyet and Gehenio, 1947a; Polge et al., 1949]. The technology has, however, seen an upsurge in use since Greg Fahy and William F. Rall reintroduced the method and applied it to embryo and organ preservation [Fahy and Hirsh, 1982; Rall and Fahy, 1985; Fahy, 1986].

Practical realization of vitrification technology broadly falls into two categories: those that utilize very rapid cooling and warming, usually in stepwise manner with a brief application of high-concentration CPA in the initial stages followed by very rapid reduction in temperature; and those that use controlled slow rate cooling with a gradual increase in CPA, best termed as "liquidus tracking" (LT). Vitrification of tissue-engineered constructs involving preservation of encapsulated clusters of cells, several layers of cells, and substrate or neo-tissue has several specific challenges described in Section 11.8 (Figure 11.1).

The challenge in vitrifying native tissue, rather than cell suspensions, by either method is in achieving both the correct CPA concentration throughout the tissue to be protective but not toxic, and controlling cooling temperature throughout a tissue. It is a complication

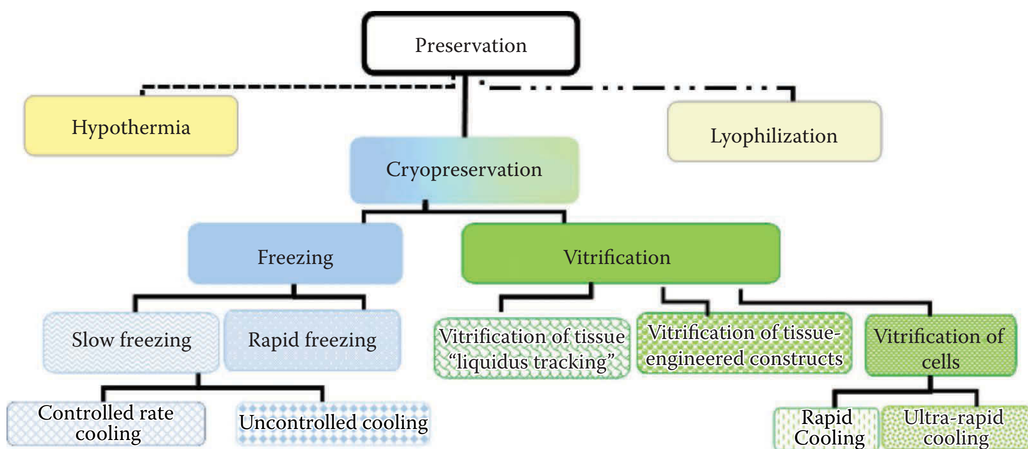

FIGURE 11.1
Schematic representation of medical and biological technologies operating at subzero and low temperatures.

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that difficult-to-permeate deep regions of a tissue will be least responsive to environmental temperature variations. For large-volume solutions or tissues, this often means approximating control of both temperature and CPA concentration at deep levels within the tissue.

Vitrification by rapid and ultra-rapid cooling is a technique used predominantly for single cells like gametes, as well as for clusters of cells or multicellular organisms of small dimensions (embryos). Rapid cooling "freezing" is a method that was tested in the late 1980s for the cryopreservation of embryos using intermediate concentrations of CPA [Shaw et al., 1991]. This technique involves the direct plunging into liquid nitrogen of embryos held in straws. The supporting solutions used are commonly vitrified on cooling and devitrified on warming, which was identified as detrimental. Since this time, cryobiology progressed and the value of vitrification was established in the field of reproductive biology [Mukaida et al., 1998; Kuleshova et al., 1999b]. However, currently the majority of groups working in the field of assisted reproductive technology (ART) in a clinical setting are less focussed on historical ideation, borrowing the terminology "vitrification" due to the attractiveness of the concept while disputing evidence of devitrification. Several studies reported a substantial reduction of total CPA concentration by ~20% from that initially proposed for the vitrification of embryos [Rall and Fahy, 1985; Mukaida et al., 1998]. It is necessary to increase cooling/warming rates to about 10^{6}°C/min for vitrification, which is impractical and rather impossible taking into consideration the size of a sample holder and solution used to carry oocytes or embryos. Unfortunately the suggested current techniques reported by ART groups could not reach strict vitrification criteria, which can lead to nonlethal injury of valuable human gametes and embryos. Thus, these techniques are rapid freezing rather than vitrification.

Availability of transplant tissues and organs is a bottleneck in treating many potentially fatal disorders, such as cancers, renal failure, or liver or heart disease. The cryopreservation of organs or tissues would facilitate the building of biobanks to store healthy organs for transplantation. Organ preservation is discussed in Section 11.8; however, potentially the most appropriate technique for the cryopreservation of whole organs is vitrification (Figure 11.1).

11.4 Mechanisms of Freezing Injury

11.4.1 Mechanisms of Slow-Freezing Injury in Cells Intended for Cryopreservation

A majority of work investigating cryoinjury for therapeutic use has been performed *in vitro* owing to the need to focus on direct cell injury, without cell vitality being affected by immune reaction or vascular damage.

The "two-factor hypothesis" describes the potentially lethal effects of cooling rate of ice formation during temperature reduction and identified from the boundary parameters of lethality between which cell survival is possible. The hypothesis identified two factors of cell injury leading to loss of viability:

- Excessively rapid cooling rates lead to intracellular ice formation (IIF), which is lethal to the cell.
- Excessively slow cooling rates lead to a lethal increase in the concentration of solutes within cells as they dehydrate due to osmotic removal of water from the cell in response to the reduced availability of liquid water after extracellular ice formation, and associated "solution effects."

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11.4.1.1 Formation of Intracellular Ice

When cells or tissues are cooled, supercooling occurs whereby the temperatures reached are below the melting point of the liquid component of the tissue, yet ice nucleation has not occurred. Typically, cells or tissues will supercool down to a temperature of –5°C. Below this temperature, ice will start to form spontaneously or due to crystallization typically in the range –10°C to –15°C [Mazur, 1984]. The cell will remain liquid, with an absence of IIF, to a temperature of around –15°C as ice formation is extracellular while cell contents are supercooled. At these temperatures, intracellular water may osmotically diffuse out of the cell due to increased extracellular solute concentration, which occurs as water "freezes out" of aqueous solutions. The risks of IIF and "solution effects" are the highest at temperatures ranging between –15°C to –40°C [Gao and Critser, 2000]. When cooling below –40°C there is reduced likelihood of ice formation and bound water will not freeze [Mazur, 1984; Walstra, 2002].

The tissue must pass through this potentially lethal temperature range twice, once during cooling and again during warming, and the rate at which the temperature of the tissue must traverse [Gao and Critser, 2000] is considered to be the greatest risk of lethal effects, rather than the ultra-low temperatures used for storage.

Rapid cooling rates lead to IIF as water is unable to diffuse out of the cell sufficiently fast to equilibrate with the surroundings, such that residual water in the cell crystallizes. This enables equilibrium as the osmotic balance is met as the intracellular solute (electrolyte) concentration increases to match the extracellular concentration. IIF causes cell death and increased pressures due to water flux may be sufficient, or analogous to those required to disrupt organelles and membranes [Muldrew and McGann, 1994]. It has been observed that crystals formed during rapid cooling tend to be small; however, as the cell returns to normothermic temperatures, it passes through the range –40°C to –15°C and recrystallization occurs, whereby isolated crystals combine to form large crystals. The effect of recrystallization is the rupture of membranes and damage to cell components [Mazur et al., 1972]. The primary methods of avoiding IIF are either through the use of CPAs of sufficient concentration to fully inhibit ice formation, or through a reduction in the cooling rate sufficient to allow water to osmotically migrate from the cell. However, if the cooling rate is too low, the latter situation may present problems through "solution effects" that are also lethal to the cell.

11.4.1.2 Solution Effects

There are many suggested solution effects that may be lethal or minimally detrimental to the cells within a tissue. Reduction in cooling rate allows supercooled water to diffuse out of the cell; however, excessive loss of water causes harmful cellular dehydration. This may cause binding of membrane proteins that were previously not in contact. On warming, the increased intracellular electrolyte concentration osmotically rehydrates the cell; however, the available volume is reduced and rupture may occur. This is referred to as the maximum cell-surface area hypothesis [Steponkus and Wiest, 1979]. Furthermore, the dehydration of cells can cause damage to membranes, internal architecture, and organelles. Denaturation of lipid protein complexes can also make cell membranes excessively permeable, causing swelling and rupture [Lovelock, 1957]. Similarly, the phase change of water to ice may cause the denaturation of membrane lipids or macromolecules crucial to cell function [Fishbein and Winkert, 1978]. Water loss from the cell may, in extreme cases, remove bound water that is otherwise inaccessible. Many tissues contain a component of nonsolvent, or inaccessible, water that may be either trapped in tissues or covalently bound within the cell and loss decreases cell vitality. [Jaffe et al., 1974; Maroudas and

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Schneiderman, 1987; Muldrew et al., 1996; Elmoazzen et al., 2005; Sun, 1999]. A low cooling rate allows water to exit the cell without IIF; however, ice crystals that form external to the cell accumulate into larger blocks leaving high solute concentration liquid channels separating them. Cells accumulate in these liquid channels where they will be exposed to rising concentrations of solutes as the aqueous components freeze out and cell volume reduces, which, combined with the decreasing size of these channels adding physical pressures to the cell, could theoretically cause irreparable cell damage [Mazur, 1984]. These narrowing channels may also bring cells into direct close contact with other cells or the ice front, or allow interactions that are potentially harmful to the cell. Pegg and coworkers described a "packing effect" whereby the increased proportion of cell density in a system prior to freezing causes increased loss of cell vitality [Pegg and Diaper, 1988; Mazur and Cole, 1985]. Equally, damage caused by extracellular ice formation can also be both lethal to cells and damaging to the biomechanics of tissues through similar processes [Pegg et al., 2006c).

Osmotic stresses due to increases in solute concentration as water freezes out of aqueous solutions are a major cause of cell damage. Metabolic processes are vital in cells in a liquid state [Hoffmann and Minor, 2014]. During slow cooling or hypothermic temperature storage, metabolic damage may occur due to low oxygen (ischemic injury); cell starvation due to decreased availability of nutrients during storage; accumulation of toxic products through metabolism or from storage solutions; loss of necessary substrates during perfusion; and simple cold damage [Pegg et al., 1984]. Ischemia from tissue freezing will prompt tissue necrosis and is a mechanism of action in cryosurgery.

11.4.2 Mechanisms of Freezing Injury *In Vivo*

There exist many mechanisms leading to cryoinjury in tissues. The properties of ice formation may be used beneficially to selectively destroy tissues such as cancers. The mechanism by which these procedures cause cellular damage are now openly discussed and applied to cold-temperature therapies, particularly that of vascular damage within the tissue [Hoffman and Bischof, 2002; Fraser and Gill, 1967].

IIF inflicts extensive damage to cells. Minor freezing does not cause extensive cell damage but does prompt an immune response that aids recovery and reduces symptoms of disorders [Gage et al., 2009]. Slow freezing may cause lethal concentration of the ionic components within the cell or dissociation of the tissue [Han and Bischof, 2004; Edd and Rubinsky, 2006]. The severity of damage from application of a cryoprobe in cryotherapy depends largely on the minimum temperature reached and the rates of cooling and thawing. Repeated cycles of freeze-thawing and slower rates of thawing cause more extensive damage. Typically, malignant tissue may be treated with cycles of freeze thaw, while benign tissues may be treated with a single freeze thaw. The temperatures experienced over time for a tissue can be referred to as the "thermal history" of the tissue. This history can be described whether the tissue has undergone cryopreservation or cryosurgical therapy, and briefly comprises cooling rate, final temperature reached, storage/hold time, and warming rate [Hoffmann and Bischof, 2004; Gao and Critser, 2000; Mazur, 1963]. In contrast, with cryopreservation of a tissue the CPA content of the tissue/cell suspension affects cell vitality, in particular the addition of CPA, final concentration of CPA, length of exposure related to temperature, and the efficiency of clearing the warmed tissue of residual CPA [Hoffmann and Bischof, 2004]. Following treatment for around 24 h, inflammation and immune-mediated responses cause further cell death. When freezing is applied to a region, a cryogenic lesion forms comprising a region of coagulation necrosis that corresponds to the extent of tissue freezing that has occurred [Gage and Baust, 2007].

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Benign tumors can be effectively destroyed using temperatures of –20°C to –30°C, while malignant tumors or neoplastic tissue requires lower temperatures of –40°C to –50°C or less. Prolonged freezing will involve an immune response but also prompt formation of a volume of coagulation necrosis and vascular stasis [Gage and Baust, 2007; Gage et al., 2009]. The destruction of vascular tissue is the primary site of injury induction and is vital for the treatment of cancerous tissues, particularly at the microvascular circulation level [Daum et al., 1987; Rabb et al., 1974]. The location of vascular damage can be extensive, but particular attention is on the vascular bed, or wall, where damage may occur due to mechanical injury to the vessel wall, injury to the cells lining the vessel walls, or injury through reperfusion or immune response post-thaw [Hoffmann and Bischof, 2002].

In cancers, continual rapid replication is necessary as cancerous cells require a steady supply of nutrients from blood. Often angiogenesis causes necrosis by drawing blood supply from surrounding tissues. The applied cryosurgical damage to the vascular system of tumors starves the cells and isolates the tumor, aiding the adjacent tissues and advancing the destruction of the tumor. The principle applied in general is that the freeze aspect of the cycle should be performed as rapidly as possible and the thaw stage performed as slowly as possible to maximize cell damage [Gage and Baust, 1998]. Vascular injury is the predominant surgical means to control difficult-to-access tumors [Fraser and Gill, 1967]. Subsequent to cryosurgery, however, an immune response initiated by the cryosurgical damage will destroy tissues, both healthy and compromised, due to the heightened sensitivity of the host immune system. This is referred to as "freezing-stimulated immunologic injury" [Ablin, 1995]. Equally, evidence suggests that sublethal cryosurgical injury may prompt the cell to initiate apoptotic mechanisms for programmed cell death [Baust et al., 2000].

In broad terms, these three types of damage describe the mechanisms of cryoinjury *in vivo* [Hoffmann and Bischof, 2004]. Knowledge of the mechanisms of cryoinjury is vital to the successful application of thermal therapies such as cryosurgery. The mechanisms described here fit broadly into all of the following chapters as these processes underlie both the successful cryostorage of cells and tissues and also the controlled ablation of diseased tissues.

11.5 Cell Cryopreservation in Mammals by Conventional Slow Freezing

Observation of natural survival through freeze thaw cycles dates back to 1670 in vinegar eels reported by Henry Power and subsequently in 1683 in frogs and fish reported by Robert Boyle [Thomson, 1964]. The targeted cryopreservation and successful revival of living cells developed from significant advances in 1949 with applications for the freezing of sperm using glycerol as a CPA [Polge et al., 1949] applied in cattle farming and livestock breeding, with human IVF adaptations following. The cryopreservation of gametes is discussed in more detail in Section 11.6. Improvements in cell survival came with the identification of dimethyl sulfoxide (Me_2SO) as an improved preservative with enhanced permeation in cells, in particular red blood cells [Lovelock and Bishop, 1959]. Me_2SO offers advantages and disadvantages as a CPA. Unlike polyvinylpyrrolidone (PVP), Me_2SO penetrates the cell, enabling reduced IIF, however making it more difficult to ensure complete removal of CPA post-thaw, although Me_2SO is not excessively toxic to cells at low concentrations [Pegg et al., 2006b, 2006c].

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Many different cell types are cryopreserved for a range of applications [Fuller et al., 2004]. Cells may be stored for clinical use or reuse by research teams, banked for commercial sale, or archived as part of a cell library. Fertility treatments, discussed later, and commercial applications of freezing sperm have fueled early development of cell cryopreservation options.

The preservation of cells *in situ* is complicated by the need for CPA to permeate matrix and cell layers, potentially of varied types and sensitivities. In cell suspensions, there is rapid and even exposure to CPA, allowing accurate control of concentrations experienced by the cell, which may not be possible in all whole or partial tissues.

Cell preservation protocols aim to restrict the formation of intracellular ice crystals, using CPA to remove water from the cell and the surrounding extracellular matrix to inhibit ice crystallization [Fuller et al., 2004]. Mechanisms of damage to cells due to this process are described in Section 11.4. Clinical cell banking is used to preserve cells as characterized cell lines for response testing or as cell suspensions for pharmaceutical use in the production of biological or biotechnological products from cell substrates such as vaccines [Petricciani and Sheets, 2008; Knezevic et al., 2010]. Cell substrates are cell lines that produce target biologicals either naturally or through genetic modification, or have been inoculated with vaccine viral proteins. Viral replication utilizing cell architecture is used to produce large quantities of vaccine. Cell substrates are preferably rapidly replicating cells of animal origin or microbial cells such as fungi or yeasts [Knezevic et al., 2009]. Characterized diploid cells or cell lines capable of continuous culture without senescence or terminal differentiation can be effectively stored long term using cryopreservation. This facilitates consistently high-quality products derived from these cells that remain closely associated to a single original culture. Once a cell line has been isolated, characterized, and stored, the culture can be tested and confirmed negative for contaminations. This results in reduced post-thaw contaminations [Schiff, 2005].

The results from testing within cell lines can be variable; however, this variability can be counteracted by using confirmed cell lines from trusted sources. WHO retains stocks of useful cell lines, such as the Vero cell line (Vero 10-87). This cell line can be used as a seed to culture a master cell bank, particularly for use in the development of new vaccines [WHO website].

The first stem cell bank was set up in 2002 in the UK [MRC UK Stem Cell Bank], and is currently supplying 25 human embryonic stem cell (hESC) cell lines for research, fully characterized for differentiation capacity and surface markers representing cell "stemness" [NIBSC]. The cryopreservation of mammal cells in small scales is now a standardized process that is largely unchanged with the increased demand for cell therapies, and the loss of a significant proportion of cells is accepted as inevitable even though improvements to the process may be possible.

Cell lines are commonly preserved using Me_2SO as CPA. Cooling may be performed using an automated controlled rate freezer (Figure 11.2), giving monitored and accurately reproducible cryopreservation protocols that can be tailored to the individual sensitivities of the cell line being preserved, or a simple device to control heat transfer, such as the commonly used "Mr. Frosty." This technology uses a buffer of 100% isopropyl alcohol surrounding cryovials of cells, with the entire unit placed into a mechanical freezer at –80°C overnight prior to transfer to liquid nitrogen storage. This method gives approximate control and regulation of heat transfer to provide a cooling rate of ~1°C/min. Certain sensitive cells benefit greatly from more specifically defined cooling protocols, such as hESC and other stem cell types, which are preferentially cryopreserved using vitrification (Figure 11.1) [Hunt, 2011]. Freeze-drying technologies are only used for preservation of

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FIGURE 11.2
Automated cooling for cells and tissue using a controlled rate freezer (Kryo-560, Planer Plc). (Courtesy of Geoffrey Planer, CEO, Planer Plc. Middlesex, UK.)

nonsensitive cells. These are two-step processes, that is, vitrification, followed by freeze-drying; however, this is complex as many of the CPAs used to prepare cells are toxic, so lyophilized storage at temperatures above 0°C with these CPAs is challenging.

A freezing medium, often 10% Me2SO CPA in fetal bovine serum, is used to suspend 1.0×10^6 to 4.0×10^6 cells in specially designed cryovials. These are then cooled slowly using either a controlled rate freezer, or an approximation such as the Mr. Frosty. Following 24-h storage, cells cooled in this manner are transferred to liquid nitrogen at –196°C for long-term storage.

With the exception of advances in vitrification technologies, the basic protocols for cell cryopreservation remain largely unchanged. The establishment of cell banks has led to the creation of standardized protocols for the cryopreservation of mammalian cells such as defined by the European Collection of Cell Cultures.

Warming of cell suspensions is performed in a two-step process. Frozen cell suspensions are rapidly thawed by immersing in water from 37°C up to 42°C, and once liquid, the suspension is diluted using a dropwise process into the appropriate growth medium for cell expansion [Geraghty et al., 2014]. Diluted cell suspensions are seeded into adherent plastic tissue culture flasks and left for several hours prior to a complete medium change to remove residual CPA. The temperature in blood warmers is currently limited to a maximum of 42°C by the American Association of Blood Banks; however, no significant changes in blood cells have been found after exposure to 47°C for 1 h [Nienaber, 2003].

The demand for cell cryopreservation in research and clinical use has been driven by the need for consistent, characterized, early-stage cell lines to give results comparative

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for multiple studies or reliable clinical results. The clinical application of cell preservation facilitates cell screening, banking for storage, quality control, and maintenance of adequate stocks of cells for use and distribution of cells in a cryopreserved state [Karlsson and Toner, 2000]. In research laboratories, it is standard practice in regenerative medicine, when using cell lines from either commercial or primary sources, to routinely cryopreserve a stock (one to two ampoules) of early passage (early stage) cells for later use. The ability to store and supply cells has been integral to long-term or longitudinal studies of cell therapy. With the more recent classification and description of stem cells, particularly embryonic, cord blood, and iPS cells, the need to sensitively preserve cells at an early stage is evident.

11.6 Cryobiology as a Cornerstone of Assisted Reproductive Technology

Cryobiology is a cornerstone of assisted reproductive technology. There are several reasons for such a statement. A significant fall in female fertility occurs after 34 years in humans. Oocytes (female eggs) are the largest single cells in the human body. They age much faster than women plan to have children, which creates health concerns in modern society [Jolly et al., 2000]. A decline in sperm concentration and morphology attributed to socio-psycho-behavioral factors has also been documented over the past few decades. Reproductive cryobiology originated from the preservation of sperm more than 50 years ago and has gradually developed into a mature science over the past six decades [Polge et al., 1949; Holt et al., 1996, 1999; Leibo, 2002; Pickard and Holt, 2004]. Sperm cryobiology has been explored in three major directions—agriculture, laboratory animal medicine, and human clinical assisted reproduction. Cryopreserved human spermatozoa were first used in the 1950s [Sherman, 1964]. Complete description of the biophysical fundamentals of sperm cryobiology that may be of particular interest to readers with engineering physics backgrounds can be found in the review published by Critser's group [Benson et al., 2012]. There is a vast body of knowledge about the cryopreservation of sperm [Watson, 1995; Holt et al., 1996, 1999; Leibo, 2002; Benson et al., 2012; Morris et al., 2012]; thus, the main focus of this subchapter will be on the cryopreservation of oocytes, a subject of enormous interest and complexity.

Since 1999, when the birth of the first baby from an embryo derived from vitrified-warmed oocytes was reported by Kuleshova and coworkers in the journal of *Human Reproduction* [Kuleshova et al., 1999b], this concept has been recognized and implemented worldwide [Chian and Chao, 2009; Wood, 2012]. This first breakthrough in achieving ice-free low temperature preservation of human oocytes made an important advance in our knowledge and practice of the IVF process, as it removed the ethical problems associated with embryo cryopreservation. Over 500 births have been reported after the application of this strategy worldwide (Figure 11.3). This invention removed the problem of chromosome aberration associated with oocytes freezing and proved critical for oncology patients [Chian and Chao, 2009; Kuleshova, 2009a; Wood, 2012].

The first birth using vitrified oocytes is one of the main research achievements of the cryobiology community and is closely related to the ethical and social needs of society. Social implications of this invention involve 13% of the couples around the world. Couples with embryos frozen and stored in cryobanks face serious legal, ethical, and moral dilemmas. Just in the last two decades in the United Kingdom alone, 1.7 million embryos have been discarded as they are no longer needed by couples trying for children under IVF programs. In sharp contrast to embryo donation, vitrified surplus oocytes are not discarded, but usually donated by couples who have successfully had their babies from IVF and who

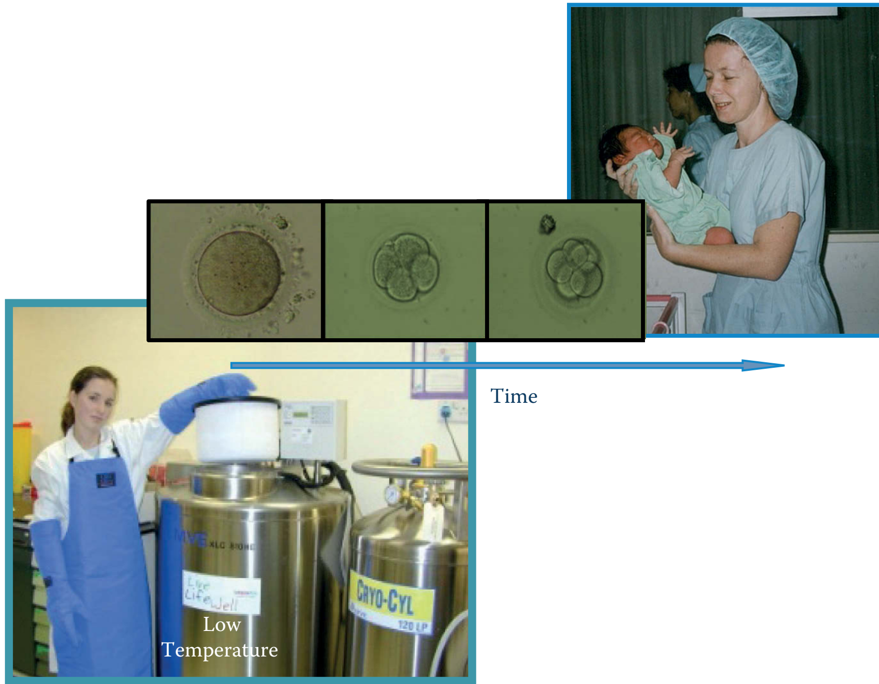

FIGURE 11.3
Embryo development and child birth following oocyte vitrification-warming and intracytoplasmic sperm injection.

want to help other couples. The main reason for donation lies in the fundamental difference between oocytes and embryos. Similar to red blood cells and bone marrow, oocytes are a person's cells, not an embryo developing into a new individual.

Since the concept was proven and quickly widespread, application of a vitrification strategy has led to conclusive, statistically confirmed results in recent years. The incidence of birth anomalies after vitrification of human oocytes does not differ from that observed in natural conception. The rates of ongoing pregnancy, embryo cleavage, and fertilization do not differ between vitrified and fresh oocytes. The difference in average survival rates after vitrification and after slow cooling shows that vitrification is superior to slow cooling [Kuleshova, 2009a]. Vitrification also resulted in significantly higher fertilization and embryo cleavage rates compared with slow cooling. The birth rate for slow cooling studies is approximately half of that observed in vitrification studies [Kuleshova, 2009a].

In clinical practice, the Practice Committee of the American Society for Reproductive Medicine estimates the live birth rate per vitrified-warmed oocytes at 4% in their early report. With time in embryo culture, embryo transferred efficacy is further improved [Patrizio and Sakkas, 2009]; equally the studies on human oocytes vitrification and comprehensive meta-analysis [Cobo and Meseguer, 2010; Cobo and Diaz, 2011] showed that complete outcomes are significantly higher. Finally, identical outcomes for vitrified and fresh oocytes have been demonstrated in an oocyte donation program [Nagy et al., 2009]. The Practice Committee is continuously updating their guidelines to assist the community and physicians with their decision-making process [Practice Committee of the American Society for Reproductive Medicine, 2013]. Recent clinical evaluation of the efficiency of donation programs demonstrated that pregnancy rates are similar with either fresh or

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vitrified oocytes and there is good evidence that the same is applicable in young patients. Different groups give the name of the method by the type of containers/carriers that has been used during exposure of oocytes to low temperature. The evaluation of holders employed for human oocyte vitrification revealed that they are not greatly different in their efficacy. More fundamental research regarding the vitrification concept is needed in the quest for a proper scientific approach to understanding the mechanism of success and injury to make any real progress in the field.

One of the first elegant methods that proved to be effective derived from the utilization of electron microscopy grids as miniature carriers. Current methods are numerous variations on the same strategy, mainly employing immersion of biological material directly into LN_2 with a minimum amount of vitrification solution (VS), while VSs currently in use suffer from some limitations. A major concern is the total solute concentration of proposed VSs. Ice crystal formation may take place inside and outside of cells and not be visible as solutions have insufficiently low concentration to support a stable amorphous state. Overall, it can be seen from the analysis of all reports on vitrification of human oocytes that lead to child birth that there is no distinct link between the success rate and the type of container used. The basis of success is a correspondence between equilibration and dilution steps that allow sufficient oocyte dehydration in combination with penetration and subsequent removal of CPAs post-warming. The composition of VS is also important. Ethylene glycol (EG)-based vitrification solutions are shown to be effective for human oocyte and embryo vitrification [Kuleshova et al., 1999b, 2009a; Yoon et al., 2003; Cha et al., 2011; Mukaida et al., 1998, 2001]. This idea was found to be valuable by others during application to porcine and bovine oocytes. Overall, despite some breaches of the protocols designed, many oocytes survived cryopreservation and produced good-quality embryos after fertilization and resulted in many healthy live births.

In a cryobiology context, human oocytes are unique cells. In contrast to other mammalian oocytes, some parameters of human oocytes at the same stage of maturation vary for different infertility patients. This may be a vital factor due to which vitrification of mammalian oocytes, except possibly for swine species, has been relatively successful for a number of years. It should be also clarified that success rates for cryopreserved embryos continued to be higher than for cryopreserved oocytes in most mammals [Fuller, 2009]. The challenges of human oocytes cryopreservation, development of protocols, and equipment for control rate cooling protocols have been comprehensively described by Fuller [2009].

Oncology patients are benefitting significantly from the invention of an effective strategy for oocyte vitrification [Kuleshova et al., 1999b; Chian and Chao, 2009]. The gonadotoxicity of chemotherapy is a well-established fact. The main strategy for preservation of fertility in young female oncology patients or those without partners is vitrification of human oocytes rather than embryos [Chian and Chao, 2009]. It is known that hormone stimulation is required for generation and collection of several mature oocytes simultaneously. The procedure covers a substantial period while the concern for cancer patients is the time constraints. Shorter protocols take only 3–4 days and result in a few immature oocytes ready for collection and *in vitro* maturation. Even with the rise in popularity of the vitrification concept, child birth following cryopreservation of immature oocytes is found to be difficult to achieve for reasons related to the substructure of immature oocytes, and it is an inappropriate practice for patients with a poor state of health or those undergoing severe operations. Therefore, human oocyte maturation followed by a preservation approach was adopted as a working hypothesis, giving the first fruitful outcomes in recent years. In general, there are two strategies of preservation for fertility in female oncology patients, both involving low temperature preservation by vitrification of either ovarian

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tissue or oocytes. Although ovarian tissue can also be preserved effectively, the overall procedure can have complications for patient health. Collection of tissue requires major surgery, which is suboptimal and may also cause inflammation in some cases. Thus, while vitrification of *in vivo* matured oocytes still remains more effective than vitrification of *in vitro* human matured oocytes, it is believed and it is our view that it is the most promising approach in the preservation of fertility of female cancer patients. In our time, embryos at all stages of development have been preserved by vitrification [Mukaida et al., 1998, 2001; Kuleshova and Lopata, 2002]. However, freezing of embryos is still common practice. Preimplantation genetic diagnosis and/or screening allow the assessment of the genetic health of an embryo before transferring it into the uterus. These techniques require the removal of cellular material in order to perform genetic analysis. Freezing of human embryos after biopsy is less effective than vitrification, which is in turn under development. An important issue to mention here is cryobiology research in the context of the preservation of fertility in male oncology patients. All methods of interest are based on cryopreservation and cryostorage at deep cryogenic temperatures (–196°C), irrespective of approaches explored for fertility preservation in prepubertal male oncology patients or spermatozoa preservation for adult male oncology patients.

Reproductive cryobiology related to mammals demonstrates that humans and wildlife share common environmental and genetic challenges. Comprehensive overviews published by Bill Holt and Paul Watson over the years provide understanding about the status of international conservation efforts for wildlife and the preservation of genetic resources of mammals in captivity as well as other breeding programs [Watson and Holt, 2001; Holt et al., 1996, 1999, 2008]. Frozen-thawed spermatozoa, similarly to human sperm banking, have become an integral component of animal agriculture and laboratory animal genome banking. The protocols, particularly for cryopreservation of oocytes, and testicular and ovarian tissue, are still in a stage of optimization and cannot readily be extrapolated to other species' reproductive tissue and cells [Leibo and Songsasen, 2002]. Human reproductive cryobiology is only part of this fascinating science.

From a background in physics and chemistry, an issue unsolved for decades has been resolved, that is, effective preservation of human oocytes has been achieved [Kuleshova et al., 1999b]. Subsequent years of successful vitrification of human oocytes have confirmed the validity of this idea. This research resulted in immediate improvements in the quality of life revealing the inevitability of bioethics in modern society, taking into account the ethical aspects of cryobiology research along with discussions on AIDS and the genome project, discarding embryos and surrogacy and dilemmas faced by scientists in today's highly challenging global environment.

11.7 Vitrification of Tissue: The LT Method

It has been shown that controlled rate cooling of large tissue volumes results in a temperature gradient within the tissue or solution, whereby the temperature experienced on the outer surface is lower than that experienced within inner tissue during cooling and higher during warming, with compressive tensions due to freezing causing extensive damage [Rubinsky et al., 1980]. This temperature gradient encourages ice formation in predominantly liquid regions of tissue where there is less tissue organization, such as blood vessels [Hunt, 1984] and articular cartilage [Pegg et al., 2006a]. There may also be a concentration gradient in CPA between the outer surface and the deep tissue due to

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diffusion characteristics and kinetics of CPA uptake. Permeation of CPA is particularly an issue in larger tissues where surface tissue absorbance of CPA may equilibrate with environmental CPA, while deep tissue equilibration is incomplete at the stage where freezing temperatures are reached. This leads to increased propensity for ice formation in deep tissue. Attempts to increase deep tissue permeation of CPA by increasing surface concentrations risk CPA toxicity causing fatal damage to surface layers of tissue and are ineffective in raising final equilibration coefficients [Carsi et al., 1985]. Attaining a balance between surface CPA toxicity and deep tissue freeze damage is a complex issue.

LT is a vitrification method that uses balanced control of CPA concentration against temperature during cooling and warming [Pegg et al., 2006c]. The goal of LT is to promote cell survival at low temperatures experienced by tissue, using exposure to the minimal amount of CPA necessary to inhibit ice formation at the temperature being experienced only as that temperature is reached. This is facilitated due to the diminution in toxicity of high-concentration CPA at low temperatures [Matheny et al., 1969]. For vitrification of bulky tissue, as previously described, a very high concentration of CPA is required, levels toxic to most tissues at even a brief-duration exposure in a previtrified state. If high levels of CPA are reached only at very low temperatures, toxicity is reduced and vitrification can occur with minimal cellular damage, potentially as little as 5% [Wang et al., 2007]. By gradually increasing CPA content during the cooling process, rather than prior to cooling, the solution composition matches tracks defined by the "liquidus line," which marks the transition between complete solution and ice crystallization/melting point, to beyond the glass transition temperature, achieving vitrification [Pegg, 1986]. Ice crystallization is fully inhibited if the degree of tracking is sufficiently precise. This enables ultra low temperature cooling with the level of CPA exposure experienced by the cell not exceeding that experienced during conventional preservation methods [Pegg et al., 2006c].

Vehicle solutions for CPA during cooling contain a complex electrolyte component to provide nutrients to cells and tissues, protect from damage during cooling, vitrify, and balance osmolarity and pH across a wide range of temperatures. The LT method also controls for changes in electrolyte concentration as temperature decreases [Farrant, 1965].

The concept of LT was first proposed by Farrant modeling on a NaCl-only electrolyte system. The first successful application of the LT method was in 1972 with smooth muscle [Elford and Walter, 1972]. The LT method was not substantially revisited until the research group of Professor David Pegg applied the technique to the vitrification of cartilage [Pegg et al., 2006c]. This group demonstrated the possibility of a technique that would allow clinical-grade living articular cartilage grafts to be stored for off-the-shelf use by orthopedic surgeons, potentially as an alternative to prosthetic total knee replacement surgeries. This is discussed further in Section 11.10. Cartilage is a particularly appropriate tissue to apply the LT technique to because it is uniform in structure with broadly speaking only one tissue composition involved. Standard cryopreservation techniques are particularly ineffective due to the high water content in cartilage and the increased susceptibility of chondrocytes to ice nucleation, preferentially in the lacunae, causing irreparable damage to the cells [Pegg et al., 2006b].

In LT, the variables to be optimized are as follows:

- Kinetics of permeation for the chosen CPA
- The duration of exposure to CPA, determined by cooling rate
- The final concentration of CPA, determined by the eutectic temperature of the system applied.

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LT will typically use CPA that exhibits good uptake by cells and tissue, such as Me_2SO. Penetration rates are partially determined by the extraction of water as Me_2SO experiences less rapid osmotic diffusion. It is vital, therefore, to maintain osmotic balance to avoid a large change in cell volume or rapid dehydration of the cell.

LT may be achieved through the transfer of tissue between CPA solutions of increasing concentrations, or the increase in CPA concentration for a single solution bath in which the tissue remains static. Both methods require preparation of accurate solution concentrations precooled to the correct temperatures, and both require good mixing techniques. The timing (and duration) of tissue transfer or CPA increase must be handled precisely and at the correct temperature, so the risk of operator error is large. For these reasons, an effective automated process is preferred and one strand of current investigations is looking at an automated system for LT [Wang et al., 2007]. At present, the only commercially available automated LT system is produced by Planer Plc (Sudbury-on-Thames, London). This system provides a programmable approach to LT where the operator is able to control the rate of cooling/warming aligned with the exposure concentration of CPA. This is achieved through a calculated application of high concentration CPA to a constant-volume solution in the sample holder, such that target CPA is achieved accurately at the temperature instructed. A Planer 560 controlled rate freezer (Planer Products, London, UK) is used with standard Delta-T software to control temperature from built-in resistance thermometers, while a purpose-built control unit (CfgPid) designed by Planer Plc controls pumps that adjust the inflow of high-concentration CPA to achieve a temperature-matched target in the medium surrounding the tissue.

The issue of surface temperature to inner temperature gradient is important as it is likely that the deep tissue will remain vitrified, and therefore not available for CPA reduction, to higher temperatures than surface tissue. For this reason, most LT protocols for warming will contain a "devitrification" step that holds the tissue at a low temperature but above the melting point of the CPA solution, to allow free movement of CPA during warming.

LT presents a promising technique for the vitrification of living organs. CPA can be delivered through perfusion to complex organ tissues using intact vascular systems that run throughout the body of the tissue. While the toxicity and rate of diffusion remain significant issues, particularly in complex tissues, the promise of LT as a vitrification method for long-term storage of organs is an enticing one. The current state of organ preservation is discussed fully in Section 11.9.

11.8 Cryobiology as an Integral Aspect of Tissue Engineering

Developing effective cryopreservation strategies to enable off-the-shelf availability of complex cell-containing constructs and neo-tissues is necessary to realize their clinical potential. It is vital to provide an effective link between tissue engineering and biomaterial science and its biomedical application. The promises of tissue engineering rely upon the ability to physically distribute ready-to-use products of regenerative medicine without compromising their quality. The current research and practical interest is to establish how complex tissue-engineering principles can be sustained during cryopreservation to facilitate biomedical application of cell-containing constructs for applications in tissue repair and regeneration, drug development, and diagnostics. To date this emerging subject has received little consideration outside the work of a few groups, one of them being Kuleshova's laboratory [Kuleshova et al., 2004, 2007, 2009b, 2013; Kuleshova and Hutmacher,

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2008; Gouk et al., 2011; Magalhaes et al., 2008, 2009; Magalhães et al., 2012; Wu et al., 2007, 2015; Wen et al., 2009; Bhakta et al., 2009].

There are several challenges in the cryopreservation of tissue-engineered constructs (TECs):

- Preservation of the integrity of constructs
- Maintaining high cell viability after cryopreservation
- Maintaining cell function
- Preserving cell proliferation and differentiation potential (particularly if stem cells are involved)
- Preserving cell–cell interactions

This leads to several specific challenges:

- The hydrogel matrix that allowed migration of cells should retain its original properties after cryopreservation.
- Maintaining the cell attachment ability to substrate is essential for cell-scaffold systems.

There are important differences between the two main methods of cryopreservation in a tissue-engineered context. Vitrification eliminates ice crystal formation during the course of a cooling and warming process not only inside the cells but also inside biomaterials [Kuleshova et al., 2004, 2007, 2009b; Kuleshova and Hutmacher, 2008; Magalhães et al., 2008, 2009, 2012; Wu et al., 2007, 2009]. The freezing process permits ice outside cells in the medium and in the biomaterial, because the concentration of supporting CPA is usually from 1 M to 3 M. This is an advantage over freezing, as vitrification provides amorphous solidification, resulting in less mechanical stress to the cells and TECs.

Several areas of regenerative medicine can benefit from the development of new integrated vitrification strategies through the efforts of groups such as Kuleshova's: (i) hepatic tissue engineering, (ii) tissue engineering of bone and cartilage, and (iii) neural tissue engineering. Cryopreservation of tissue-engineered blood vessels has been developed by of Song and coworkers [Dahl et al., 2006].

It is a challenge to design biomaterials with properties that will not be transformed during interaction with chemicals (e.g., CPA), cooling to cryogenic temperatures, and subsequent warming. Research on development of cryopreservation principles in tissue engineering allied with the exploration of biodegradable biomaterials, serving as permissive substrates for cell growth, differentiation, and biological function, aims to complete this vital step [Kuleshova et al., 2004, 2007; Kuleshova and Hutmacher, 2008; Wu et al., 2007; Wen et al., 2009]. It is believed that an integrated approach such as this would facilitate the medical applications of tissue engineering, stem cells, and biomaterials.

The development of biologically inspired nanoscale materials, including hydrogels that mimic the *in vivo* environment, is a promising strategy and an integral part of future developments. In the biomaterial context, the structure–property relationships of polymeric materials must be explored to ultimately allow the design of a new process for preservation by vitrification [Wen et al., 2009; Kuleshova et al., 2004; Wu et al., 2007]. Research into the modification of collagens for enhancing their properties, cell assembly encapsulation, and cryopreservation has been undertaken for the benefit of hepatic tissue engineering [Wu et al., 2007]. Methylated and, in turn, galactosylated collagen-based matrices with

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superior properties have been synthesized. Although galactosylated collagen results in a less fibrous structure in the shell of capsules with conjugation of a synthetic terpolymer, the work of Kuleshova's group was able to develop a vitrification process that did not affect the integrity of fragile constructs or the structure and chemical properties of collagen. This allowed interactions with receptors of hepatocytes post-cryopreservation. Taking into consideration the acidity of Me_2SO at the high concentrations required for vitrification, it is hypothesized that high acidity may impair the chemical property of collagens and subsequently affect cell migration of hepatocytes and/or viability and proliferation rate in stem cells [Heng et al., 2006]. The results of our studies proved that strategies based on this hypothesis are valid. Freezing allows ice formation, and thus results in rupture of the structure of construct itself, which leads to nearly half of tissue-engineered collagen-based capsules becoming broken [Heng et al., 2004].

Through the exploration of high-molecular-weight polymers as CPA, a strategy for the vitrification of large hepatocyte-containing constructs has been effectively established. A tissue-like structure with high levels of liver-specific functions was formed post-cryopreservation on collagen-coated polyethylene terephthalate fabricated into a thin film. It has been illustrated that the loss in cell viability of post-frozen thawed hepatocytes is due to apoptosis, which follows cytoskeletal disruption. Therefore, the question of why the cryopreservation by vitrification succeeded where freezing protocols generally fail has been answered. Through this comparative approach, effective preservation by vitrification of a bioartificial liver device was developed [Magalhaes et al., 2009, 2012].

It was demonstrated that a combination of mesenchymal stem cells (MSCs) and a hydrogel scaffold can be used successfully for the purpose of formation of new cartilaginous tissue or for regeneration of bone. A stable amorphous state of MSCs growing in alginate–fibrin beads used for bone regeneration has also been maintained during the vitrification-warming cycle [Bhakta et al., 2009]. The alginate–fibrin is a hydrogel, and so has a very high water content and brittleness at low temperatures (–196°C). This result is encouraging in a biomaterial context.

Nanofibrous TECs have been developed for biomedical applications such as cartilage, bone, arterial blood vessels, heart, and nervous system. Fabrication of polymeric nanofibrous poly(caprolactone) (PCL)-gelatin nanofibrous materials involving electrospinning is an area of growing interest. In earlier research, the success of tissue-engineered strategies combined with preservation of MSC-seeded nanofibrous scaffolds fabricated from PCL-gelatin has been demonstrated by Wen and coworkers [Wen et al., 2009]. The excellent results of all assessments after vitrification showed that a vitrification approach is effective in cryopreserving these PCL-gelatin fibrous TECs, retaining high cell viability and the capability to proliferate and differentiate while maintaining structural integrity. It has been demonstrated that cartilage defects can be restored through transplantation of TECs containing MSCs. A reliable strategy for the preservation of hydrogel substrates, serving as a base for MSC growth, is an important current task [Kuleshova et al., 2013]. Cryopreservation of stem cell cultures with their 3D culture support system adds to the flexibility of clinical scheduling and facilitates continuous cell expansion, permitting their effective utilization in the field of regenerative medicine.

Consequently, structural integrity is a high priority in the preservation of TECs at low temperatures. Cell populations can only function effectively as a tissue when the constructs they reside in remain intact; cracks in the constructs can severely affect the functionality of TECs. Numerous reports have demonstrated that the vitrification protocol, which includes a brief three-step exposure to a VS composed of EG and sucrose (40% v/v EG, 0.6 M sucrose), immersion into LN_2, warming, and gradual removal of the VS

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at room temperature, effectively preserves the integrity of dissimilar scaffolds and TECs as a whole. The promising results may be attributed to the regular shape of the constructs. For example, the more uniform heat distribution in thin disc-shaped TECs prevents crack formation and propagation [Wen et al., 2009]. It has been demonstrated that synthetic biodegradable polymers support the growth of neo-tissue and mitigate any thermal expansion constraints of the CPA in the nanofibrous microstructure. It is also believed that the texture of nanofibrous scaffold, where the pore size of the scaffold is larger than the size of CPA molecules employed, facilitates the penetration of the VS [Wen et al., 2009]. A physical transition in the VS enclosing the construct, opposed to a phase transition that occurs during "freezing," apparently plays an important role in preserving the integrity of the TECs.

The duration of exposure to CPA plays a significant role in the success of vitreous cryopreservation. This, together with the appropriate conditions employed, effectively induces vitrification in both the supporting scaffold as well as formed neo-tissue. A "layer-by-layer" approach that constructs a tissue by sandwiching cell layers supported by scaffold layers may be adopted to generate bulky TECs. Vitrification of bulky TECs can be advocated while the length of exposure has to be extended correspondingly. This recommendation is based on (i) the results obtained on 2D, 3D, thin, and thick TECs during decades of our research, in addition to (ii) understanding gained in the area of bulky native tissue cryopreservation through the consistent efforts of Pegg's group, including an original "LT" method [Pegg et al., 2006c] discussed earlier.

Prior to incorporating neuronal stem cells into biomaterials, a series of comparative studies on known approaches to cryopreservation have been undertaken using neurospheres as model systems [Tan et al., 2007; Kuleshova et al., 2009b]. Vitrification is the opposite of conventional freezing and completely maintains the neuronal specific function. The process allowed the maintenance of the structural integrity of 3D neurospheres, suggesting that it may be applicable to other structural cultures such as nerve bridges and matrixes for filling lesions. It was confirmed that cryopreservation of NSCs as 3D clusters is beneficial, drawing a parallel with other 3D tissue-engineered cell cultures.

Cryopreservation of tissue-engineered blood vessels has been successfully attempted by Song et al. [2000a, 2000b; Dahl et al., 2006]. Tissue-engineered blood vessels have been developed as an alternative source of vascular tissue for use in bypass surgery. Cells grown onto a thick polyglycolic acid (PGA) mesh were preserved by freezing and vitrification methods. The contractility results for vitrified TECs were more than 80% of fresh controls and, in contrast, the results for frozen samples were only 11% of fresh controls. Vitrification has been explored with great success in the application to native vascular grafts and other tissues for years as described in previous subchapters [Pegg et al., 2006c; Song et al., 2000a, 2000b; Pichugin et al., 2006]. Yet significantly, Song and coworkers have revealed that it is possible to design a stepwise vitrification procedure for the cryopreservation of a tissue-engineered implant.

In the last decade we have provided evidence that a vitrification strategy is indispensable for the successful preservation of cell-containing TECs [Kuleshova et al., 2004, 2007, 2009b; Kuleshova and Hutmacher, 2008; Magalhaes et al., 2008, 2009, 2012; Tan et al., 2007; Wu et al., 2007, 2015; Wen et al., 2009; Bhakta et al., 2009], since it is able to mantain the attachment ability of cells to the substrate [Wen et al., 2009; Magalhaes et al., 2009, 2012], intactness of the cell membrane [Magalhaes et al., 2009], and interactions of cells in clusters [Tan et al., 2007]. Also demonstrated is the superiority of vitrification in the retention of viability and metabolic function in preserved cells, and proliferation and differentiation potential of 3D cultures of adult stem cells derived from a variety of sources [Kuleshova et al., 2004, 2007, 2009b, 2013; Kuleshova and Hutmacher, 2008; Tan et al., 2007; Bhakta et al., 2009; Wen et al., 2009; Wu et al., 2007, 2015]. Properly evaluated composition and concentration of

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VSs and the cooling/warming rates do not impair the integrity or quality of the hydrogels involved, permitting free migration, aggregation, or proliferation of cells post-vitrification [Kuleshova et al., 2004; Wu et al., 2007; Bhakta et al., 2009]. The subsequent goal is to develop methods of cryopreservation for TECs made of different biodegradable biomaterials with varied geometry.

In summary, an encouraging start strongly supports the view that vitrification concepts for cryopreserving TECs have great potential for tissue engineering and will have a high impact on the application of tissue-engineered implants and novel stem cell–containing devices.

11.9 Organ Preservation

Organ cryopreservation, and tissue banking (Section 11.11), has developed in response to increased demands for tissue. The cryopreservation of organs is a major challenge due to the sensitivity of living organs and the complexity of these tissues. A study of naturally occurring freeze coping strategies has informed researchers about the techniques that may be applied to tissues and organs to achieve vitality after freezing. Kidneys were the first organ to survive cooling and rewarming with cooling to room temperature (1955), and then in 1958, cooling to refrigerated temperatures of 2°C–8°C (1958) [Owens et al., 1955; Stueber et al., 1958]. From here the field has progressed through rigorous research and trial from simple hypothermic storage in specialized solutions through to continuous machine perfusion techniques (Gallinat et al., 2013).

11.9.1 Solutions Used for Short-Term Preservation

Metabolic processes, particularly mitochondrial metabolism and energy production, are vital in maintaining homeostasis in cells [Hoffmann and Minor, 2014]. Metabolic damage can occur through ischemic injury due to low oxygen concentration, cell starvation due to decreased availability of nutrients during storage, accumulation of toxic products through metabolism or from storage solutions, loss of necessary substrates during perfusion, and simple cold damage [Pegg et al., 1981, 1984].

Preservation solutions are used for cold static storage or pulsatile perfusion. Early perfusates were used to flush kidneys for static cold storage. These included Sacks II [Sacks et al., 1973; Beck, 1979], Collins C3 [Beck, 1979], TP-II [Toledo-Pereyra, 1983], and hypertonic citrate [Marshall et al., 1977]. For successful cryopreservation of living organs, preservation solutions include sugars, osmotic agents, and/or buffers to minimize the damage caused by swelling or contraction of the cells. Solubility of oxygen (O_2) during cooling reduces O_2 availability. Ischemia or low O_2 leads to the production of free radicals, which prompt an increased immune response on transplantation and re-establishment of the blood supply in the host, termed reperfusion. Cell membranes may fail due to lipid peroxidation leading to failure of the graft [Suong-Hyu Hyon, 2011]. The inclusion of antioxidants will soak up free radicals [Pegg et al., 1984; Rolles et al., 1984, 1989]. Colloids must also be added to support the vascular bed [Pegg et al., 1984]. Immune reactions are controlled through the use of immunosuppressant treatments. The first solution to be effectively applied to organ (kidney) storage was Eurocollins [Collins et al., 1969; Eurotransplant Foundation Annual Report, 1976]. Eurocollins enables matching of extracellular and intracellular electrolytes, both having high potassium and phosphate, to ensure isotonicity and iso-osmolality [Mühlbacher et al., 1999]. Marshall's solution improved on this with the inclusion of citrate as a buffer to reduce swelling and enhance energy availability [Marshall, 1997].

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In 1986, Jim Southard et al. developed University of Wisconsin (UW) solution, commercially marketed now as "Viaspan." Included were raffinose and lactobionate to maintain osmotic balance and reduce hypothermic cell swelling; a colloid carrier (hydroxyethylstarch [HES]) to reduce vascular damage; and antioxidants (glutathione, allopurinol, and adenosine). UW solution was developed for pancreatic cryopreservation but has since been widely applied, most prominently for the liver and kidney [Southard and Belzer, 1993, 1995].

More recently, "Unisol-CV," a proprietary CPA medium similar in composition to CPTES, has been shown to be superior to Eurocollins solution as a CPA vehicle solution [Taylor et al., 2001]. "Custodiol-N" builds on increased knowledge of human physiology to include a wider range of amino acids and iron chelators to combat cold-induced injury to cells and hypoxic ischemia. Early results suggest that Custodiol-N improves graft quality through injury reduction [Rauen et al., 2008; Bahde et al., 2008; Stegemann et al., 2010].

11.9.2 Organ Perfusion

In a cold-tolerant organism, a pulse rate is maintained even at very low temperatures. Observation of continued circulation at low temperatures in the animal world led to the exploration of organ perfusion techniques. Perfusion may be used to flush the existing blood or fluids from an organ, to remove wash solutions and introduce preservation solutions, or for continuous perfusion such as used during brief transport [Pegg, 1981]. Organ perfusion must replace blood flow with storage solutions to deliver solutes and oxygen while maintaining osmotic balance. Perfusion solutions must be pure and uncontaminated prior to use [Pegg, 1981]. UW solution is currently popular for continuous organ perfusion [Kosieradzki et al., 1999]. In early research, the primary choice for perfusate was cryoprecipitated plasma [Sterling et al., 1971; Beck, 1979; Veller et al., 1994; Matsuno et al., 1994; Marshall, 1997]. Later studies used plasma protein fraction [Toledo-Pereyra, 1983; Alijani et al., 1985], solutions containing 5% albumin [Halloran and Aprile, 1987], silica-gel plasma perfusate [Mozes et al., 1985; Merion et al., 1990], and plasmanate [Halloran and Aprile, 1987; Jaffers and Banowsky, 1989; van der Vliet et al., 2001]. There are various issues that must be addressed in choice of perfusate, such as cation and anion content; osmolarity; pH; pressure; temperature; filtration; and substrate inclusions, such as oxygen, to support any continual metabolism in process during perfusion [Pegg, 1981].

Perfusion may be single-pass or continuous/pulsatile using a variety of pumps that may be peristaltic, reciprocating, or continuous [Pegg, 1981]. Pulsatile perfusion allows increased storage times and an estimated 20% reduction in the incidence of delayed graft function, giving an opportunity to perform viability testing for the organ and ensure medical clearance, thus expanding the pool of potential donors to include clinically marginal donors [Wight et al., 2003]. Delayed graft function is costly due to increased hospital stays and the need for continued dialysis and is linked with poorer long-term outcomes [Cecka and Terasaki, 1995]. Belzer, a leading pioneer in organ preservation science, was the first to develop a machine for the perfusion of kidneys at low temperatures during transport. However, in the late 1970s to mid-1980s several studies showed no improvement in machine perfusion over static storage, and consequently, largely for financial reasons, static storage dominated [Clark et al., 1974; Opelz and Terasaki, 1976, 1982; van der Vliet et al., 1983]. The use of pulsatile perfusion machines is reported at only 27% in the United States and 7% in the UK [Wight et al., 2003]. The most frequently used machine for kidney perfusion is the Waters MOX 100 [Wight et al., 2003], although also in clinical use are the Belzer LI 400 [Sterling et al., 1971; Beck, 1979], the Gambro [Halloran and Aprile, 1987; Marshall et al., 1977; van der Vliet et al., 2001], and the Nikiso APS-02 [Matsuno et al., 1994; Wight et al., 2003].

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Hoffmann and Minor estimates that 33%–50% of organs received for clinical use are from marginal donors. Consequently, many organs retrieved do not function fully, with estimates of only 20%–40% of kidneys functioning adequately without requiring dialysis support [Hoffmann and Minor, 2014]. While clinical use remains focused on static storage, most current research is on continuous pulsatile perfusion, so strong is the evidence that this will ultimately provide the best solution for longer-term storage and transport of organs for transplantation. The overall progress of hypothermic machine perfusion preservation technologies and multiple organ perfusion *in situ* for clinical use have been comprehensively described by Taylor and colleagues [Taylor and Baicu, 2010].

Currently, an increased interest in matching physiological conditions for storage of tissue grafts has led to heightened interest in normothermic storage of tissues. As discussed in Section 11.10.4, corneas are currently banked at normothermic temperatures and articular cartilage may also benefit from natural temperatures for storage. No hypothermic damage is incurred in the tissue and liver storage has shown 83% success after 20-h storage where cold storage gave no positive outcomes [Brockmann et al., 2009; Jamieson et al., 2011; op den Dries et al., 2013]. There is a good deal of encouraging optimism in the field of organ transplantation, and increased funding is helping support the progress toward an effective solution.

11.10 Contribution of Cryobiology to *In Vitro* Toxicology and Drug Pharmaceutical Development

Exploitation of *in vitro* cell culture systems has proven to be essential in a number of studies of cell biological and physiological processes for over a century. Cryobiology is a vital part in *in vitro* toxicology and pharmaceutical drug development, since the use of cryopreserved primary cells with later reintroduction into culture is a valuable approach to study the problems of clinical relevance, especially those related to studies of cell toxicity and disease screening. However, as with any tool, it is subject to some limitations. For instance, the isolated hepatocyte is particularly convenient for studying the kinetics of hepatic drug uptake and excretion because the hepatocytes can be rapidly taken from the culture in an incubator. Since they show various features of the intact liver, isolated liver cells have also proved valuable for investigating drug metabolism. Yet, they also show important differences such as loss of membrane specialization and some degree of cell polarity. Surplus hepatocytes in small quantities are relatively easy to obtain. Therefore, tissue-engineered 2D or 3D constructs involving primary hepatocytes are widely appreciated as they sustain liver properties for an extended time, up to 2 weeks. Furthermore, a vitrification strategy has been developed by us that ensures long-term performance of cryopreserved units [Kuleshova et al., 2004; Wu et al., 2007; Magalhaes et al., 2008, 2012], paving the way for the use of hepatocyte- and other primary cell-based tissue-engineered constructs for *in vitro* drugs testing or as an integral part of *in vitro* models containing interconnected cultures for metabolic studies.

Cerebral organoids or mini brains are more advanced tissue-engineered systems that have the potential to model development and neurodegenerative conditions [Lancaster et al., 2013]. Alternatively, cerebral organoids can be used to grow specialized regions of brain tissue intended for their transplantation into areas of neurodegeneration as therapeutic treatment. The goal of cryobiology is to develop strategies of preservation for both applications.

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In context of native tissue intended for physiological testing, metabolism studies, and drug screening, cryopreservation of cerebral and liver tissues has been of long-term interest. A vitrification strategy involving an elegant cassette design for liver slices in a number of species was suggested in the mid-1990s [Ekins et al., 1996]. Microscopic examination of rodent brain slices showed generally good to excellent ultrastructural and histological preservation after vitrification. Severe damage in frozen-thawed central villous explants has been reported as an approach to explore drugs in pregnancy disorders [Huppertz et al., 2011]. The study focused on traditional CPA, specifically Me_2SO, and found that a concentration of 3 M is best for maintaining explant viability, morphological integrity, and protein release during cryopreservation. The tested parameters were similar between controls and samples that were cryopreserved, placed on cryostorage, and transported to other locations, demonstrating the possibility of cryostoring explants and the logistics for functional studies.

Higher species require much more consideration for ethical approval. Even though it is possible to assess drugs in rodents *in vivo*, it is considered to be less applicable information. Consequently, cryopreserved rabbit, dog, swine, and canine vascular tissues are often used for drug testing. Further advances in the vitrification strategy involving vascular tissue have been undertaken. Improved tissue functions in vascular tissues cryopreserved using a vitrification approach were observed compared to a standard freezing method. Functional recovery of veins and arteries are usually evaluated by contractile responses and endothelium-independent relaxant responses post-thawing or post-vitrification. The maximum contractions achieved in vitrified vessels were >80% of fresh matched controls with similar drug sensitivities, whereas frozen blood vessels exhibited maximal contractions below 30% of controls and decreases in drug sensitivity [Song et al., 2000a, 2000b]. To date, there is difficulty finding an appropriate *in vitro* model to study human adult cardiac cell biology. A simple model to study the changes at the cellular level is in the culture of cardiomyocytes. Isolation and expansion of human cardiomyocyte progenitor cells from cardiac surgical waste or, alternatively, from fetal heart tissue is one option. Preservation is carried out based on outdated cryopreservation protocols utilized in hospital practice. Therefore, to overcome various issues related to progression of their cryopreservation is challenging. A couple of decades are a typical gap between clinical setting and developing the science of cryobiology. This is one reason why effective cryopreservation of cardiomyocytes has not been developed yet. Another option is emerging, that is, the vitrification of embryonic stem cells and induced pluripotent stem cells that are able to differentiate into cardiomyocytes *in vitro*. As yet, the whole process is still experimental. To conclude, the vitrification strategy is playing an increasingly important role in drug screening and *in vitro* toxicology studies.

11.11 Tissue Banking

In order to supply human tissue for medical treatments, it is necessary to establish tissue stocks. Biobanks exist as discrete units operating from acquisition, processing, and storage to on-demand supply of tissue grafts for surgical treatment and/or research purposes. Initial tissue banks were orthopedic banks supplying bone for surgical grafting [Strong, 2000]. Bone and skin grafts represent the most commonly used cryopreserved allografts in clinical use [Tomford and Mankin, 1999]. The science of transplantation has seen a rapid increase in application since the 1950s leading to an increased demand for tissues for transplantation. Although early reports exist of xenograft (1968) [Filipović-Zore, 2000] and autograft (1820)

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[Chase and Herndon, 1955; Walther, 1821], the first peer-reviewed report of allografting was published by Sir William MacEwen on the reconstruction of the infected humerus in a 4-year-old child using the tibia of a child with rickets [MacEwen, 1881]. During World War II, a book by Inclan and articles by Wilson and Bush & Garber highlighted the demand for bone banks to supply stored bone to treat victims of war injuries [Inclan, 1942; Wilson, 1947; Bush and Garber, 1948]. The availability of tissue through the establishment of tissue banks has facilitated increased tissue transplantation, leading to a demand for rapid tissue supply.

Tissue and cell banking in its current form is tailored more closely to surgical requirements. Tissues routinely banked include bone, articular cartilage and osteochondral allografts, cornea, skin, tendon, heart valves, and vascular grafts utilizing hypothermic or ultra-low temperatures to maintain tissue stability.

11.11.1 Bone

Bone preserves as a natural scaffold that is biocompatible with the human body. It can be frozen in the absence of CPA without controlled rate cooling as the need to maintain cell viability is not a priority. A bone graft is selected for mechanical, osteoconductive, osteoinductive, or osteogenic properties. Osteoconductive materials function as a scaffold for the development of bone, while osteoinductive materials promote the recruitment of immature or progenitor cells and develop these down the osteoblast lineage to initiate new bone growth, termed osteogenesis [Albrektsson and Johansson, 2001].

Femoral head and shaft are commonly cryopreserved. Femoral heads are retrieved from living donors during hip replacement surgery or from deceased donors. Living donors are screened before surgery and again 4–6 months post-retrieval to ensure medical clearance, during which time the graft is stored fresh frozen in quarantine [Delloye et al., 2007]. Processing is undertaken for depletion of soft tissue, cellular content, and bone marrow, to remove donor-specific tissue that may prompt increased immune response or transmit infection. Irradiation of grafts from deceased donors is therefore routinely performed [Lavernia et al., 2004]. Removal of marrow and soft tissue components increases the osteoconductive capacity of the bone [Aspenberg, 1993]. Bone that has been freeze-dried and gamma-irradiated can be stored at room temperature. The primary use of cryopreserved femoral head allografts is cut or ground to aid osteogenic healing in knee or hip revision surgery. Grafts that have been stored fresh frozen, regardless of irradiation status, and grafts freeze-dried without gamma irradiation, are seen to be a reliable replacement of the fresh bone. However, freeze-dried, irradiated bone has reduced mechanical stability due to breakdown of collagen chains in response to ionization [Cornu et al., 2001; Dziedzic-Goclawska et al., 2005]. Freeze-drying has the effect of making bone more brittle unless rehydrated before use while fresh-frozen bone retains the mechanical properties of fresh bone [Delloye et al., 1991]. As result, freeze-dried bone tends to be applied to small defects, while frozen bone is used for larger repairs. Frozen bone allografts may typically be stored for up to 5 years at <–79°C and still be viable for use.

11.11.2 Articular Cartilage and Osteochondral Allografts

Articular cartilage varies from most other transplant tissues in that it is avascular, naturally hypoxic, has no lymphatic system, and relies on diffusion of nutrients and oxygen to nourish the cellular component. Autografts or allografts can be used with postoperative success at 5–10 years [Shasha et al., 2002]. Cartilage has a low cellular component, around 5%, and high water content, around 80%, making ice crystallization a major risk

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during cold preservation [Pegg et al., 2006c]. Cartilage is immunoprivileged with negligible immune response likely post-transplant; the main source of immune rejection of osteochondral grafts is directed toward subchondral bone.

The major limiting factors in the use of osteochondral grafts are the prohibitive costs of allografts and the availability of suitable tissue [Pegg, 2009]. Osteochondral grafts must be size-matched to the recipient, and retrospective studies show that graft contouring must match surrounding tissue for complete integration [Koh et al., 2006; Patil et al., 2008]. These factors and the need for undamaged replacement tissue increase the requirement for a long-term storage methodology for cartilage allografts.

Clinical cartilage storage protocols currently specify refrigeration at temperatures ~4°C. After 28 days of hypothermic storage, cell survival is detrimentally affected [Allen et al., 2005]. These grafts show sustained clinical success [Shasha et al., 2002]; however, cell viability and functionality are variable and it still remains preferential to implant fresh stored grafts within 14 days of removal from the donor [Allen et al., 2005]. Normothermic storage (~34°C) promotes increased functionality and cell viability in comparison to hypothermic storage [Pallante et al., 2009; Stoker et al., 2012]; however, the risk of bacterial infection increases. At hypothermic temperatures, enzyme function may be inhibited and the cellular sodium pump inactivated. While beneficial for short periods, long-term effects may include swelling due to water osmotically entering the cell through the membrane. An attempt to mimic the graft physiological environment allows the tissue to continue metabolic activity and cell function; however, metabolic activity results in the production of waste products such that it becomes necessary to refresh medium under normothermic conditions. Storage at normothermic conditions maintains chondrocyte vitality preferentially to bone cells, which may be beneficial in reducing immune responses [Bastian et al., 2011].

Standard preservation techniques at <–2°C cause the formation of both extracellular and intracellular ice crystals in cartilage chondrons [Pegg et al., 2006b]. Crystallization of ice is both directly and indirectly causal in the fatal damage sustained by cells during cryopreservation and consequently must be inhibited to ensure cell survival post-cryopreservation [Mazur, 1984; Pegg et al., 2006a]. Most studies in this field are examining the use of vitrification when applied to cartilage to facilitate a consistent supply of appropriate allografts.

The lack of vascularity and density of the extracellular matrix (ECM) network of collagen fibrils in cartilage makes CPA penetration and inhibition of ice formation a particular challenge [Pegg et al., 2006c]. CPAs inhibit ice crystallization; however, the toxicity of CPAs may severely limit successful cryopreservation [Pegg et al., 2006c]. As ice crystals accumulate, fissures, cracks, and holes form in the tissue, reducing biomechanical function. The vitrification method for storage of tissues is discussed in Sections 11.3 and 11.4. Chondrocytes serve to maintain matrix formation by synthesizing components of matrix structure, such as collagen and proteoglycans. It is therefore important to measure the functionality of cartilage, commonly through uptake of radiolabeled sulfate, in addition to cell viability through membrane integrity and measurement of metabolic activity [Pegg et al., 2006c].

Osteochondral plugs and cartilage slices have been successfully vitrified with post-vitrification cell survival quoted above 80% [Brockbank et al., 2010]. Cartilage is exposed to high-concentration CPA in a preconditioning step to allow diffusion of CPA into the tissue, which is then rapidly cooled (e.g., –43°C/min) to below the glass transition point to avoid prolonged exposure to toxic CPA. Post-vitrification, following a slow warming step to –100°C, tissue is rapidly warmed by placing specimens in a water bath at room temperature until tissue and solution begin to soften, to avoid devitrification and recrystallization of ice [Jomha et al., 2012]. The standard 5-mL vial used for tissue provides an estimated average warming rate of ~–100°C/min. High cooling rates as well as warming rates (typically up to 250°C/min)

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are achievable in small samples of cartilage; however, accurate control of temperature in a large allograft is difficult to achieve. Cartilage without subchondral bone has little clinical application as grafting is not possible. In the repair of large lesions, the orthopedic surgeon measures the contouring of the graft and cuts an osteochondral graft that approximates the size and curvature of the lesion site. Whole or hemi-condyles are necessary to achieve this, while osteochondral dowels have limited clinical application. LT targets the challenge of producing a GMP-compliant, clinically applicable method for preservation of cartilage utilizing a procedure shown to maintain cell function, based on a radioactive sulfate uptake assay demonstrating incorporation of sulfate in gag synthesis, at levels of 75%–95% in ovine articular cartilage and >70% in human tissue slices [Wang et al., 2007].

11.11.3 Tendon and Meniscus

Tendon or ligament grafts are generally from an autologous source using tissue harvested at the time of repair. A revision surgery where damaged tissue occurs in commonly harvested regions, or to avoid donor site morbidity, promotes the use of frozen or cryopreserved allograft tendons [Suhodolčan et al., 2013]. Around 20% of tendon repairs in the United States utilize stored allografts [Buchmann et al., 2008; Cohen and Sekiya, 2007; Mascarenhas et al., 2010] with the most commonly used allografts being Achilles, patellar, and semitendinosus tendons. Commercial sources of fascia lata, rotator cuff, tibialis posterior, tibialis anterior, and gracilis grafts are available [Robertson et al., 2006].

Tendon and meniscus allografts are typically deep frozen in gauze or in saline with antibiotics [Ochi et al., 1995] without prior chemical treatment or CPA. Storage is initially at –80°C, and tendons may undergo freeze-thaw cycles for transit, inventory, and short- and long-term storage [Sterling et al., 1995]. For long-term storage, grafts can be adequately stored long term at –80°C [Robertson et al., 2006; Wascher et al., 1999]. Cryopreservation and freeze-drying are alternate storage methods for tendons and meniscus [Arnoczky et al., 1998; Jackson and Simon, 1992; Robertson et al., 2006]. Cryopreservation of meniscus usually uses glycerol or Me_2SO as a cryoprotective agent and ultimately stores in liquid nitrogen at –196°C [Milton et al., 1990; Mickiewicz et al., 2013]. Cryopreserved meniscus retains between 4% and 54% viability in cells [Gelber et al., 2009; Milton et al., 1990].

11.11.4 Cornea

Corneal transplant is common for repair of many disorders associated with corneal blindness such as keratoconus and corneal scarring. Corneal disease is the most common cause of blindness [Wilson, 1980] but can be treated with restoration of sight using corneal graft transplantation. As with cartilage allografts, corneal transplantation provokes little immune response and therefore makes an ideal tissue for allografting. In the United States, there were 46,196 corneal transplants in 2011 with over 95% successful at restoring vision, and in the United Kingdom in 2013–2014, 5440 cornea were issued and 3313 graft procedures completed, with 91%–94% survival of the graft 1 year post-transplantation. At 4°C, corneas are stored in a hydroxyethyl piperazineethanesulfonic acid (HEPES)–buffered tissue culture medium supplemented with chondroitin sulfate, dextran, and standard antibiotics [Lass et al., 1992], allowing cornea to be stored for 14 days to facilitate testing and organization of the surgical procedure [Chu, 2000; Naor et al., 2002]. Storage at 4°C has been shown to disrupt the F-actin cytoskeleton and tight junctions, which could have the knock-on effect of increasing corneal swelling [Hsu et al., 1999], while storage at organ culture temperatures does not present this issue [Crewe and Armitage, 2001]. Corneal grafts in the UK are stored

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at 34°C for a period of up to 28 days [Armitage and Easty, 1997; Ehlers et al., 1999]. The availability of corneal grafts and increased storage has allowed grafting surgery to become elective and scheduled instead of emergency transplantation [Chu, 2000]. Initial tests using cryopreserved cornea to extend the storage period has prompted loss of viable endothelial cells and reduced success in grafting [Van Horn et al., 1970; Bourne, 1978].

11.11.5 Skin and Amniotic Membrane

Human dermis forms the underlying matrix of skin comprising collagen, elastin, and glycosaminoglycans (GAGs) synthesized by fibroblasts. The upper stratified epidermis has a rapidly regenerating avascular epithelium barrier surface populated predominantly by keratinocytes. Skin can be retrieved from deceased donors and cryopreserved for allografting. The primary uses of banked skin are for treating burns, injuries, and skin conditions such as ulcers. Open or full-thickness wounds, where both the dermis and the epithelium are lost, benefit from treatment with skin allografts. Without the barrier protection of the epithelium, wounds are prone to infection and dehydration leading to necrosis. Equally, without the dermis full-thickness skin cannot easily regenerate. Burn victims experience a great deal of pain and potentially hypothermia, symptoms ameliorated by the application of skin allografts. Skin grafts may come from an autologous or allogeneic source. A full-thickness skin graft will include epidermal and dermal layers with subcutaneous base tissue present, while partial-thickness, or "split-thickness," grafts contain epidermis and a limited thickness of dermal base. Modern grafts may also comprise bioengineered cell scaffolds populated with keratinocytes retrieved from the host or an allogeneic donor, bioengineered artificial skin, or decellularized dermis [Badiavas et al., 2002]. In severe burn victims, a combination approach will be taken with surviving autologous donation sites harvested for autologous grafts that will not be rejected, while allografts are used to protect wound regions for short-term recovery of damaged and undamaged host skin for further graft harvesting [Hermans, 2011].

Skin grafts are commonly stored for 10 days at hypothermic temperatures in saline solutions, prompting reductions in metabolic activity and oxygen consumption while maintaining graft integrity [Knapik et al., 2013]. Skin cryopreservation is performed using controlled rate freezing, with variable pattern cooling in Me_2SO. Storage options using glycerol or lyophilization do not maintain cell viability. Reviews of clinical studies suggest that there is no requirement for living cells for a skin graft to function and allow epithelial repair [Hermans, 2011; Kagan, 1998; Bravo et al., 2000] and decellularized skin is in clinical use, although it may be that growth factors and signalling proteins secreted by living cells in the graft aid healing [Mansbridge, 2008].

Studies remain divided on the effects of cryopreservation on skin. Biomechanical properties may be altered by cryopreservation with Me_2SO, while Young's modulus may remain unchanged [Wood et al., 2014], suggesting that cryopreservation may produce a suboptimal graft. However, clinical results with cryopreserved grafts have been positive, and the benefit of maintaining stocks of allografts without resort to fresh-only grafts may be sufficient to offset the damage caused by cryopreservation.

11.11.6 Heart Valves

Valves for use in replacement surgery are broadly divided into mechanical heart valves (MHV) and bioprosthetic heart valves (BHV). The trend in the United States and Europe has been toward greater use of tissue rather than mechanical valves [Singhal et al., 2013]. Today, the most commonly used BHVs are those from human cadavers (homograft),

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porcine aortic valves, and calf pericardium [Siddiqui et al., 2009]. Human heart valves have been stored and utilized as allografts since 1962 [O'Brien et al., 2001]. The success of heart valve allografts has previously involved the presence of a population of viable cells within the grafts at the time of transplantation, requiring sensitive cryopreservation protocols to address this need. Consequently, relatively elaborate preservation protocols were developed to deliver final post-freeze temperatures of around –150°C or storage in LN_2 while retaining cell viability. More recently, however, acellular heart valves have been successfully applied in heart valve replacements, demonstrating that allograft scaffolds created from decellularized valves with no living cell component or DNA content function extremely well as transplanted allografts with host cells repopulating the acellular graft and returning to normal function and maintenance [da Costa et al., 2010; Konuma et al., 2009; Iop et al., 2014]. Decellularization is generally undertaken using detergent washes (e.g., SDS/SDC), enzyme digestion with detergent wash (e.g., Triton with EDTA and enzymes RNase and DNase), or purely enzymatic washing (e.g., trypsin, RNase, DNase) [Khorramirouz et al., 2014].

The introduction of acellular valve allografts greatly simplifies the cryopreservation procedures. The traditional method of cryopreservation involved dissection of the heart followed by immersion in nutrient medium with CPA. This was then cooled in a stepwise procedure at fixed rates with the protocol in use dependent upon the tissue bank [Birtsas and Armitage, 2005; Armitage et al., 2005]. Specific protocols are developed by individual banks and patented for commercial protection. For example, Cryolife, Inc., patented protocol from warming using –0.01°C/min to 4°C; –1.5°C/min to –3°C; –95°C/min to –140°C; holding at –140°C for 1 min; 20°C/min to 100°C; holding at 100°C for 6 min; 10°C/min to –70°C; 20°C/min to –26°C; holding at –26°C for 2 min; –1°C/min to –80°C, then transfer to LN_2 for long-term storage, with subsequent warming protocol comprising a substantial thaw step in a sterile saline water bath between 37 and 42°C, then removal of CPA through dilutions of CPA at 7.5% and 5% for 1 min each prior to transfer to nutrient medium. This example protocol estimates post-thaw retained viability at >70% of fresh control tissue [McNally et al. 1987, US Patent 4,890,457, 1990].

A consistent study of freeze-drying bovine pericardium suggests that treatment of tissue with chemical substances appears to prevent harmful calcification of the matrix [Aimoli et al., 2007]. Recent work of this group reviewed the reduction of the inflammatory effect post-implantation while no anticipated reduction of calcification after lyophilization was found [Maizato et al., 2013]. Without the requirement for a retained living cell component, decellularized heart valves can be refrigerated in glycerol or sucrose, cryopreserved using vitrification techniques as discussed earlier, or freeze-dried [Aimoli et al., 2007; Maizato et al., 2013]. Yet, cryopreserved and stored homografts at vapor phase of liquid nitrogen have been and still remain the gold standard.

11.11.7 Tissue Banking: Summary

The notable feature of most banked tissues is the ability to graft the tissue without the inclusion of viable cells, or with simple tissues where cell viability is easy to maintain through cryopreservation protocols. Each tissue type requires individual, distinct cryopreservation protocols with various options for cooling rates, CPA choice and concentration, manual or automated cryopreservation, and storage temperatures and durations [Wusteman and Hunt, 2004]. Equally, if not more importantly, protocols have to be defined for the warming of the tissues that will not allow further damage to be inflicted, and again these protocols will be specific to the tissue undergoing cryopreservation. The cryopreservation of whole, complex organs remains an elusive challenge discussed separately.

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11.12 Cryosurgery

Low temperatures may be applied medically to achieve the destruction of cells, notably tumors. This is termed cryoablation, cryotherapy, or cryosurgery. Cryosurgery is an effective method for the removal of damaging or diseased tissue without incurring the injury that invasive resectional surgery would cause as cryosurgery is a noninvasive procedure [Zhao and Chua, 2013]. Cryotherapy procedures use freezing to destroy tissue, incidentally providing anesthetic effect and avoiding bleeding, but tissue remains *in situ* to be resorbed by the body rather than being excised [Onik, 1996]. Cryotherapy may target partial or complete ablation of tissue, depending upon the nature and likelihood of spread, particularly in cancers. An ablative procedure that removes an entire tissue, organ, or gland is defined by the critical temperature (critical isotherm protocol, CIP) commonly addressed as reliable at approximately –40°C or 233 K [Mazur, 1984; Bischof et al., 1997; Hoffmann and Bischof, 2002; Desai and Gill, 2002; Zhao and Chua, 2013], however also cited as higher for some tissues (e.g., renal tissue at –19.4°C [Desai and Gill, 2002]); and the duration or cycles of application, and monitoring of the "freeze front" as this CIP temperature progresses through tissue is key to successful surgery [Rewcastle et al., 1998; Rubinsky, 2000; Otten and Rubinsky, 2000; Edd and Rubinsky 2006].

11.12.1 Development and Applications

"Low temperature" cold compresses were used in Egypt (circa 2500 BC) to reduce infection, inflammation, and bleeding and to treat acute injuries such as skull fractures. Ice as a treatment is also recorded by Hippocrates (circa 460–370 BC) [Bleakley, 2013] and later by Dominique-Jean Larrey in local anesthetic prior to limb amputation [Larrey, 1832; Skandalakis et al., 2006]. Dr. James Arnott applied low temperature treatment to cancers to reduce inflammation and potentially damage cancer cells applying ice, crushed with saline solutions at a temperature of –18°C or lower, as treatment for breast, cervical, and skin tumors [Arnott, 1850, 1851]. Liquid air, temperature –190°C, was used clinically for the treatment of skin cancers in 1889 by Dr. Campbell White in New York [White, 1899] and later in 1907 by Whitehouse, who published the positive clinical outcomes from 15 skin cancers treated with spray or swabbing with liquid air [Whitehouse, 1907]. Pusey used solid carbon dioxide in the form of "carbon dioxide snow" to treat epithelial cancers [Pusey, 1907]. In the same year, Bowen and Towle reported the use of liquid air for the treatment of vascular lesions [Bowen and Towle, 1907]. Major developments in cryotherapy followed on from technological improvements, notably in the ability to produce liquid gases at extremely low temperatures (air, oxygen, and nitrogen) and the development of the dewar flask for transport of cryogens.

In 1910, solid carbon dioxide (~–78.5°C) became popular for cryotherapy on multiple conditions mainly at the skin surface, but also tumors of the bladder [Bracco, 1990], overtaking the use of liquid air and outlasting the use of liquid oxygen (–182.9°C). Solid carbon dioxide was in clinical use for the treatment of multiple conditions mainly on the skin surface, but also for tumors of the bladder [Bracco, 1990]. Liquid nitrogen (LN_2) was first applied in clinical treatments in 1950 by Dr. Ray Allington for the treatment of noncancerous skin disorders [Allington, 1950] and is currently the predominant means for delivering low temperatures for cryopreservation, storage, and cryotherapy. The ability to accurately apply LN_2 to a localized region enabled the treatment of small surface defects. In 1938, hollow surgical instruments to deliver ice and saline for the treatment of tumors

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were designed and built by Temple Fay and George Henny [Fay and Henny, 1938]. Temple Fay reported the observation that cancers preferentially developed in regions of higher body temperature and less at lower temperatures such as in limbs, reinforcing the efficacy of cryosurgery [Alzaga, 2009; Henderson and Fay, 1963; Smith and Fay, 1939].

Early methods for the application of liquid nitrogen to the skin focused on the use of cotton buds as a cryogen carrier. In 1961, Irving Cooper and Arnold Lee designed a cryosurgical probe circulating LN_2 under pressure through the center of the cannula to the tip for cooling. The central conduit is surrounded by a space that returns nitrogen vapor for removal while the inner layers are encased in a vacuum insulation layer for cold retention [Cooper and Lee, 1961]. This system was converted into a sealed circuit, or closed system, by Douglas Torre in 1965, enabling safer, aseptic use of the system on skin disorders, including carcinomas [Torre, 1968]. These developments allowed the eventual introduction of a commercial, handheld cryosurgery probe developed by Setrag Zacarian and Michael Byrne [Zacarian, 1973]. Current cryoprobes use either liquid nitrogen or argon gas to cool the tip. Cryosurgery probes apply a single freeze or repeated cycles of freeze-thaw to tissue to promote the intracellular crystallization of ice to inflict extensive damage to cells, discussed earlier.

Cryosprays apply aerosol liquid nitrogen to a treatment site such as brain tumors or the eye condition trichiasis [Hamlin, 1969, 1971; Fraunfelder and Petursson, 1979]. The application of cryosprays has developed, most recently with the potential for the treatment of esophageal cancers, discussed later [Cash et al., 2007; Dumot et al., 2009]. Cryosprays used for skin disorders were shown to be as effective as earlier cotton bud methods [Ahmed et al., 2001].

The major limitations in the use of cryosurgery had been the inability to fully monitor the extent or severity of freezing and the lack of precise control of the freeze region [Rubinsky, 2000]. A solution to the problem of monitoring treatment and damage was described in the 1980s with the development of an ultrasound system that can be operated during surgery to positively locate the precise location of the tumor or lesion, monitor the insertion of the probe, and then track the progress of the ice front as the tissue freezes. This enables the surgeon to accurately freeze tissue that is being targeted for removal. This system was initially demonstrated in liver and prostate cancers [Onik et al., 1988]. Ultrasound was a cheap and convenient method for tracking cryosurgery [Onik et al., 1991]. The moving front of ice formed during cryosurgery can be monitored due to the differing velocities of sound waves as they travel through either ice or water in tissues [Rubinsky, 2000]. Ultrasound is limited in penetration depth and the clarity of the image produced, particularly through frozen regions where resolution is impossible. For cryosurgical treatment of potentially recurrent or metastatic disorders such as in cancer, it is important to fully remove the malignant tissue without residual remnants [Edd and Rubinsky, 2006]. With variable size and shape lesions, accurate monitoring and control are vital [Rubinsky, 2000]. The development of a vacuum-insulated probe of small diameter that could be accurately placed into a small region increased the versatility of cryosurgical techniques for treating unusual shapes [Baust and Chang, 1995]. Various imaging systems can be applied to monitoring the movement of a freeze front within tissue. These systems include magnetic resonance imaging (MRI) and nuclear magnetic resonance (NMR), computerized tomography scanning (CT), and electrical impedance tomography (EIT) that all examine the topography of an imaged region to give accurate representations of a treatment area. MRI or NMR produces a 3D image of the region by applying alternating magnetic force that exerts upon protons in the tissue causing deflection and relaxation. The deflection occurs under the influence of magnetic forces, while

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the relaxation follows administration and varies greatly between frozen and unfrozen regions. There are multiple forms of MRI related to the rate of imaging and the orientation at which images are taken, but all forms are applicable to monitoring the movement of an ice front during cryosurgery [Rubinsky, 2000]. The CT scan is a form of x-ray scan with the benefit of developing a 3D image that includes details of soft tissues within the body. EIT measures the impedance of electrical signals through tissue, with impedance significantly increasing in frozen tissue [Otten and Rubinsky, 2000; Edd and Rubinsky, 2006]. EIT allows the surgeon to evaluate pre- and post-surgery the volume of tissue affected by the cryotherapy, and therefore assess whether sufficient tissue has been treated to ensure complete eradication of malignant tissue without excess damage to surrounding tissues [Edd and Rubinsky, 2006]. EIT has the benefit that, as cell death occurs and membrane permeability increases, impedance drops, therefore enabling this method to measure directly the loss of cell viability in a region undergoing cryotherapy [Edd and Rubinsky, 2006]. To further control the loss of cell viability due to over-extension of the freeze front, heat may be applied to healthy tissues to counteract freezing by impeding the cooling function of the cryoprobe in the region of targeted healthy tissue. One example of this would be insulation or proactive rectal warming or urethral warming during cryoablation of the prostate, where cell vitality is adversely affected in the anterior wall of the rectum or sloughing experienced in the urethra owing to cryoablation of the prostate. Warming of the rectal tissue protects against the progression of the ice front and prevents damage to healthy tissue during ablation of the cancerous gland, while a urethral warming catheter prevents lethal effects on cells by maintaining temperatures above the freezing point [Chen and Pu, 2014; Favazza et al., 2014; Bischof et al., 1997]. This type of tissue warming technique may be applied elsewhere to elicit a similar defense.

Examples of external conditions that could be treated with cryotherapy include UV-related disorders such as skin cancers and solar keratosis; interdigital, or Morton's, neuroma; and warts, moles, freckles, and skin tags. Internal conditions treated using cryosurgery are extensive, but include multiple cancers such as prostate, liver, cervical, lung, retinal, and oral cancers; fibroma; and plantar fasciitis, although only in severe cases and less commonly now as alternative treatments predominate. In both external and internal treatments, the biggest impact in terms of patient benefit is on the treatment of tumors.

11.12.2 Cryosurgery in Cancer Treatment

Treatment of cancerous tumors using cryosurgery was minimally used prior to the 1990s and introduction of ultrasound monitoring systems. Tumors of the skin may be treated distinctly from tumors in internal locations, as skin cancers may be treated through topical application of liquid nitrogen or probes, whereas accessing and freezing internal lesions must be more precise to avoid detrimental damage to surrounding tissues. Cryosprays may be used for skin cancers and have recently been investigated for esophageal cancer [Cash et al., 2007; Dumot et al., 2009]. Associated with cancer therapies, cryosprays have also been useful in treating chronic radiation proctitis, which occurs as a side effect following radiation therapy for colorectal, prostate, or gynecological cancers. Standard treatments to remove the affected mucosa typically cause tissue damage, whereas cryoablation is able to remove the mucosa without deeper tissue damage [Hou et al., 2011]. Mechanisms for targeting cancer tissue with cryotherapy are described in Section 11.4.

Cryosurgical probes are less useful for metastatic tumors. Cryosurgery can be applied as a treatment for large solid tumors or localized disorders. Initially it was believed that

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–20°C was sufficiently low to prompt cryolysis in any cell [Cooper, 1964] based mainly on frostbite investigations and animal experimentation, not human *in vivo* testing [Gage and Baust, 1998].

11.13 Conclusion

In this chapter, we have attempted a brief overview of the medical applications of low-temperature biology. Medical cryobiology has been practiced across four millennia and across the span of scientific applications; an entire book could be dedicated to the subject without covering every aspect. What we have presented here reflects upon the current and previous state of the art for low temperature applied to medicine. What remains, and perhaps presents the most exciting and optimistic viewpoint, is what is yet to come in the field.

Cryobiology is a developing science, like so many disciplines in a broader field of biomedical engineering. The potential for low temperature biology to solve some of the key issues in medicine and effective therapies for hard-to-treat conditions, most notably, the ability to store living organs for transplantation, could revolutionize medicine, and cryobiology, in particular vitrification, offers a solution to this problem. Similarly, advances in complex tissue-engineered solutions for medical treatments will ultimately rely upon cryobiology to enable a consistent and reliable supply. Developments in biomaterials science will complement this process. Advances in cell therapies also require stocks of clinically suitable cells to be maintained, and biobanking is becoming an increasingly vital commodity to national health services. Pharmaceutical options require testing prior to clinical introduction, and at these trial phases, the application of cryobiology to sample storage is again instrumental in facilitating clinical introduction of novel therapeutic options.

In summary, the advancement of a discipline in science requires three developments. These are (i) the development of innovative technologies to facilitate existing concepts; (ii) the development of new concepts; and (iii) application of the fundamental sciences to existing problems. Many historical developments in cryobiology have been achieved by observation of the natural world and the lateral application of those observations to alternative problems. In the last seven decades, significant advances have been made in knowledge and technology. A deeper understanding has been achieved to give greater insight into physiological function, interactions, and mechanisms through research in fields related to, or dependent upon, cryobiology. This presents us with a wealth of new knowledge regarding how to treat complex medical issues. Many challenges remain to be overcome in low temperature biology, but with continuing dedication and interest from a new generation of researchers, and the wealth of knowledge already achieved in the field, these challenges will be met and overcome so that the discipline will fully achieve its potential.

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