Heart Failure Reviews, 8, 277–284, 2003
© 2003 Kluwer Academic Publishers. Manufactured in The Netherlands

Principles of Low Temperature Cell Preservation

Boris Rubinsky
Departments of Bioengineering and Mechanical Engineering, University of California at Berkeley, Berkeley CA 94720, USA

Abstract. **Cell transplantation is becoming an important technique for treatment of heart failure. Preservation is an integral step in any procedure using cells. There are two primary modes of cell preservation at low temperature, hypothermic preservation at temperatures above freezing and cryogenic preservation at temperatures below freezing. Optimal preservation protocols require a fundamental understanding of the principles involved. This review briefly describes the basic mechanisms of damage during hypothermic and cryogenic preservation and the basic principles for developing optimal protocols for preservation of cells.**

Key Words. **hypothermic preservation, cryopreservation, freezing damage**

Introduction

Cell transplantation is becoming an important technique for treatment of heart failure. The logistics of cell transplantation require the ability to preserve cells for extended periods of time, a requirement common to every method of tissue engineering. The principle of cell preservation is ingrained in the observation that life processes are temperature dependent chemical reactions, whose sum is the metabolism. Similar to the kinetics of every chemical reaction, the metabolism can be also reduced by lowering the temperature. Most enzymes of normothermic animals show a 1.5 to 2-fold decrease in metabolic activity for every 10°C decrease in temperature, purely from kinetic predictions. A decrease in temperature from 37 to 0°C will decrease the metabolism by 12–13 folds. Consequently, the preferred method for long term cell preservation is through reduced temperatures. (Another, much less commonly used method is by dehydration; which will not be discussed here.) It is important to recognize that cell preservation techniques are cell type specific, and can vary widely from cell type to cell type and even with the age of the cell and organism. As new cell types are being used, with various properties and from various sources it is important to have the ability to develop optimal preservation protocols for each of these cell types. The goal of this review is to summarize in a concise form the general principles of cell preservation.

Since the reduction of metabolic rates is a strong function of temperature, it is obviously most desirable to preserve cells at close to absolute zero—where the chemical reactions cease and cells can be preserved indefinitely. However, biological materials are mostly water, in a physiological saline solution. At atmospheric pressure, physiological saline freezes at about −0.56°C. The freezing removes water from solution, in the inert form of ice crystals and completely modifies the biophysical environment the cells experience. Therefore preservation protocols are designed differently when ice is present in the solution. As a function of the presence of ice and temperature there are three modes of low temperature preservation. Hypothermic preservation—in which there is no ice, freezing preservation in which there is ice and vitrification in which the fluid is in a vitreous (glassy) form. Freezing preservation and vitrification are known as cryopreservation—because they involve cryogenic temperatures, i.e. temperatures in the range in which gases liquefy at atmospheric pressure.

Hypothermic Preservation

Hypothermic preservation is mostly used for the preservation of whole organs, while cryopreservation is more commonly used with cells. However, during short term transportation it is sometimes desirable to preserve cells by hypothermia rather than freezing. Short term hypothermia is often reversible. The extent of reversibility is time and temperature dependent [1]. The techniques for preserving cells by hypothermia are similar to those used for preserving the whole organ, heart in this case. The information summarized here is therefore also relevant to methods for preserving the whole heart by hypothermia.

Most mammalian cells can withstand low temperatures, for short periods of time, when ice is not

Address for correspondence: Boris Rubinsky, The Barbara and Arnold Silverman, Distinguished Professor of Bioengineering, Department of Bioengineering and Department of Mechanical Engineering, University of California at Berkeley, Berkeley, CA 94720, USA, 6105 Etcheverry Hall. Tel.: (510) - 642-8220; Fax: (510) - 642-1274; E-mail: rubinsky@me.berkeley.edu

present. While hypothermia reduces metabolism, energy storage degradation and oxygen demand, thus increasing ischemic tolerance, lowering the temperature has also negative effects. Cells contain a highly specific intracellular chemical content separated from the non-specific extracellular milieu by a selective membrane barrier made of a lipid bilayer. A detrimental aspect of cooling the cell to a temperature lower than normothermic is the lipid phase transition, which affects every membranous structure in the cell, in particular the cell membrane [2]. The lipid membrane bilayer is in a fluid state during physiological temperatures. As the temperature is reduced the lipids undergo phase transition into a gel phase or into other three dimensional crystalline structures with lower Gibbs free energy. In the process membrane proteins become segregated and very often also the various lipids in the membrane segregate. The phase transition and the segregation make the membrane leaky and usually allow ions to enter the cell in uncontrollable ways [3,4]. While passive ion channels are usually not affected by hypothermia, the energy efficiency of ionic pumps ($Na+/K+$ ATPase, $Mg2+/Ca2+$ ATPase) is temperature depended and reduced. The combination of ionic leakage and reduced ionic pump efficiency cause changes in the intracellular composition and osmolality, with significant consequences. These include protein denaturation and edema. The damage is cumulative, a function of time and is particularly expressed when the cells are returned to their normal physiological temperature. In addition to ionic imbalance hypothermia can cause acidosis, free radical production and contracture. Because the cytoskeleton depends on chemical bonds between membrane proteins and the cells scaffold, lowering the temperature weakens these bonds and makes them particularly vulnerable to mechanical damage [5]. Furthermore, increased levels of intracellular calcium during hypothermia could activate calcium dependent proteases and phospholipidases which can damage the cell cytoskeleton. Apoptosis could be also induced by exposure to cold. Because all the mechanisms of damage listed above are related to reaction kinetics, and therefore dependent on time most cells can naturally withstand exposure to cold for short periods of time and recover. Many of the cold induced changes occur at temperatures lower than the lipid phase transition temperature, from about 6 to 18°C, depending on the cell type. For preservation on the order of days cells should be preserved near freezing temperatures. However, for short periods of time, on the order of single hours it may be advisable to keep cells at room temperature rather than on ice. This is particularly true with cells in which the ionic composition plays a key role in their function and which can be irreversible modified by changes in their ionic content. For instance, at temperatures below the lipid phase transition platelets cannot be preserved because they become activated by ionic leakage and uncontrollable calcium influx.

In developing methods for hypothermic preservation, preserving solutions are designed which address the issues listed above [6]. Nonelectrolytes (sucrose, raffinose, saccharoids), citrate and magnesium chelates or high molecular weight anions (lactobionates) are used to prevent intracellular edema. Buffers (phosphate, histidine, citrate), manitol or glutathione and glutamate are used to address the issues of acidosis, free radical production and contracture, respectively. After preservation the cells are washed with solutions that have high energy substrates added for energy regeneration and compounds that reduce apoptosis.

There are several cardioplegic solutions used for hypothermic preservation. They differ in the basic ionic composition, which can be either intracellular (rich in $K+$) or extracellular (rich in $Na+$). Solutions that are based on the intracellular composition include: standard University of Wisconsin solution (UW), Bretschneider solution (HTK), Stanford solution (STF), and Euro-Collins solution (EC). Solutions that are based on the extracellular composition include: Celsior solution, St. Thomas Hospital solutions 1 and 2 (STH-1, STH-2), the modified University of Wisconsin solution (UW-1). These solutions have slightly different indications and effects on myocytes. For instance, the original Collins solution was developed first for kidney preservation, and is extensively used for this application. It tries to mimic the intracellular composition, and is rich in $K+$. There is a strong phosphate buffer and the osmolal load is carried by glucose. Some of the problems with this solution are related with the glucose which when broken down to lactate can increase intracellular osmolality. The University of Wisconsin (UW) solution was developed first for liver preservation. Because of its success it is now used essentially for every organ and type of cell, although not specifically designed for all. The high viscosity solution is also based on an intracellular composition and boasts three improvements. Glutathione, allopurinol and adenosine were added as oxygen radical scavengers. Metabolically inert lactobionates and raffinose are used to attain the desired osmotic load instead of glucose in the Collins solution. Also the colloidal carrier hydroxyethylstarch (HES) was added. While commonly used with every organ, including the heart, there is a potential of problems with myocytes. The high concentration of potassium (125 mmol) can cause calcium sequestration, enzyme dysfunction and myocyte depolarization and decreases membrane stability.

On the other hand the UW solution may inhibit intracellular calcium levels from rising. Nevertheless, at this stage it seems that the UW solution is ubiquitously used whenever a new preservation protocol is tested. While commonly used with the heart the St. Thomas Hospital solution is not a prolonged storage medium. It was developed for brief periods of heart preservation *in situ* and temperatures that are moderately hypothermic. In principle for applications in which the preservation is at moderately hypothermic temperatures and short, a buffered physiological saline solution may suffice. Celsior was specifically designed for the heart. It contains manitol and lactobionates to reduce cell swelling; histidine, glutathione and manitol to reduce oxygen free radical injury; glutamate to enhance energy production and reduce contracture; high magnesium content and slight acidosis to limit calcium overloading. A recent comparison of several hypothermic preservation solutions can be found in [7]. Various other hypothermic solutions are being continuously developed to address the problems caused by hypothermia. In developing a preservation protocol for various cell types it is recommended that the several solutions be tested, as they could have a different effect on the cell line preserved.

It is interesting to mention that a recent area of major interest to hypothermic preservation is the study of survival of cold tolerant organisms in nature and hibernators. The study of cold tolerant organisms has revealed that two naturally occurring compounds, trehalose and thermal hysteresis proteins, can provide hypothermic protection to cells [8,9]. It is anticipated that major advances in hypothermic preservation will be generated from further studies in the survival mechanisms of cold tolerant organisms and hibernators.

Cryopreservation

Preservation of cells at temperatures lower than the freezing temperature of the solution involves a complex process. In order to develop an optimal cryopreservation protocol it is important to understand the complexity of the process and in particular the mechanisms of damage. Than, on the basis of this understanding the cryopreservation protocol can be designed. In discussing the mechanisms of damage it should be remembered that the basic solution for preservation of cells is either a buffered physiological saline solution, or one of the hypothermic preservation solutions discussed earlier.

a) Damage to Cells during Freezing

During the cryopreservation protocol cells first experience cooling. The process of cooling can be divided into two stages, prior to the onset of freezing and after. Prior to the onset of freezing the cells experience hypothermia and the related mechanisms of damage, discussed earlier. However, because the period of time in which cells are exposed to hypothermic conditions is short, on the order of minutes, the damage from hypothermia is usually minor and can be ignored.

When the temperature becomes lower than the intracellular or extracellular solution freezing point temperature, freezing may occur. However, freezing is a probabilistic event. For ice to form, water molecules must assemble, during their random movement, into an ice like structure (nucleus) with a critical size or assemble around an impurity in a critical nucleus size [10]. Pure water, in the absence of nucleation sites can supercool to temperatures as low as—40°C [11]. Preservation of biological materials at a very low temperature, in a supercooled state, could be an ideal means for significantly lowering the cell metabolism without inducing freezing damage. Unfortunately, supercooling and ice nucleation is a probabilistic event, and in reality solutions of cells usually freeze at temperatures higher than—5°C, in an unpredictable manner.

The probability of ice nucleation is a function of the volume of the solution. Therefore ice nucleation usually occurs first in the much larger extracellular space. The probability for cells to freeze intracellularly is much lower, because of their smaller volume. Furthermore, even when a cell in a solution occasionally freezes the ice will not trigger ice formation in other cells in the solution. Therefore, when cells are frozen in a solution ice will form first in the extracellular space and propagate throughout that space in the direction of the temperature gradient, while most of the cells will remain unfrozen. Freezing is a process in which energy needs to be removed from the system. When a solution begins to freeze the ice front propagates in the direction of the temperature gradients, along which energy is removed from the system. Ice, is a crystalline structure of water molecules assembled very tightly. It can incorporate very few impurities and solutes. Therefore, when a solution begins to freeze the solutes are rejected by the formation of ice and the concentration of solutes on the interface between ice and water increases rapidly. Figure 1 illustrates the rapid growth of solutes on the freezing interface [12]. This accumulation of solutes leads to a phenomenon known as "constitutional supercooling", which causes ice to grow in the form of finger like structures, at the microscale. This phenomenon, which is of importance in understanding the mechanism of damage during freezing of cells, is discussed in detail in many material science texts [13]. Typical finger like ice crystals, known also as

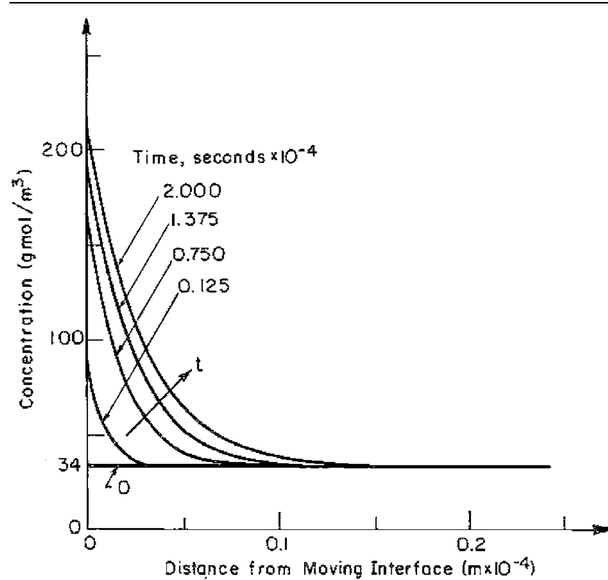

Fig. 1. *Concentration distribution in the aqueous solution adjacent to the freezing interface as a function of the time after freezing begins and as a function of distance from the freezing interface.*

dendrites, grown in physiological saline are shown in Figure 2. The figure was obtained with a directional solidification cryomicroscope, [14]. (Cryomicroscopy is an important technique, commonly used for studying the fundamental aspects of the process of freezing in cells [15]. During the freezing of cells in a solution, the cells usually are unfrozen and entrapped in the unfrozen channels between the ice fingers like structures. These channels also contain the solutes rejected by ice. The solution surrounding the unfrozen cells becomes

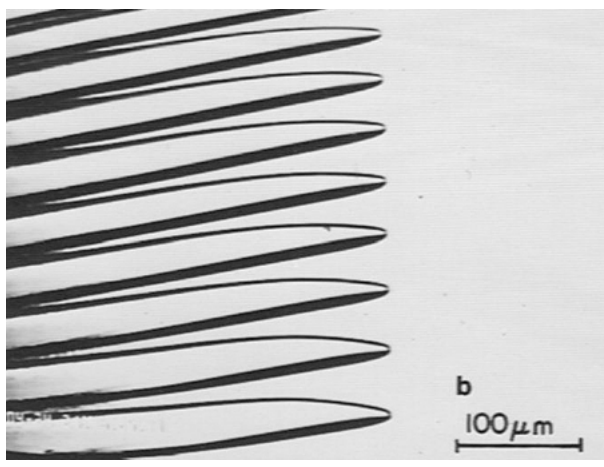

Fig. 2. *Microscale ice crystal, finger like, morphology during the freezing of a physiological saline solution. The freezing interface propagates from left to right. Scale bar 100 micron.*

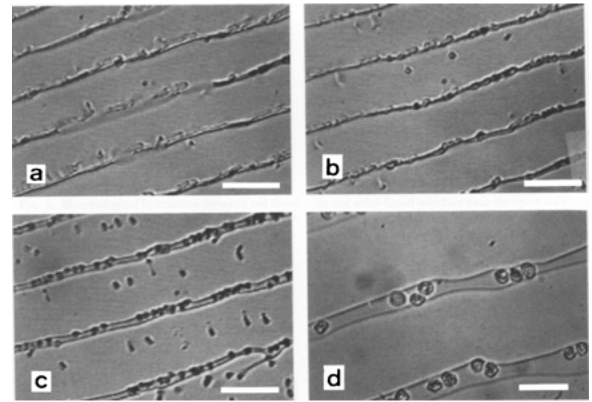

Fig. 3. *Red blood cells in the unfrozen solution between ice crystals. The temperatures for which these figures were taken increase from (a) to (d). It is possible to observe how at lower temperatures and high consequently higher extracellular concentrations the cells have shrunk more. Scale bar 50 micron.*

increasingly hypertonic as the temperature of the solution is reduced. The exposure of cells to the hypertonic extracellular solution between the ice crystals is one of the major mechanisms of damage during freezing. Figure 3 illustrates this mechanism [16]. When unfrozen cells are exposed the hyperosmotic solution, to equilibrate the difference in chemical potential between the intracellular solution that is supercooled and the extracellular solution that is hypertonic and in equilibrium with ice, water will leave the cell through its membrane. As water leaves the cell, it shrinks and the intracellular solution becomes also hypertonic. Figure 3 shows how red blood cells shrink as the temperature of the freezing solution is reduced to lower values. It was originally proposed by Lovelock [17] and later incorporated in his comprehensive theory by Mazur [18] that increased hypertonic intracellular solution damages the cells. It is not entire clear what is the mechanism; chemical denaturation of proteins and other molecules or changes in the cell structure or both. Nevertheless, it appears that the damage to cells increases with increasing extracellular concentration and with time of exposure, which is typical of a chemical related damage.

During freezing of solutions, since the extracellular solution is in thermodynamic equilibrium with ice, that solution will become increasingly hyperosmotic as the temperature of freezing decreases. The damage to cells during freezing appears to be related to chemical reactions and rates of chemical reactions increase with concentration, time and temperature. If the intracellular composition would have been exactly equal to the extracellular composition than the chemical damage to

cells should continuously increase with a decrease in temperature. Experiments show that indeed, the damage to cells during freezing increases with a decrease in temperature—which validates the concept of chemical damage, e.g. [19].

Cellular damage increases with a decrease in the temperature to which the cells are frozen. However, experiments have also shown that it is not only the temperature which affects the survival of cryopreserved cells. In fact, the rate with which the cells are brought to cryogenic temperatures is probably the most important thermal parameter of cryopreservation. This parameter is known as the cooling rate and is usually given in C/min. The effect of cooling rates is related to the process of mass transfer (primarily water transport) across the cell membrane during cooling in the freezing zone of temperatures. The intracellular composition does not become instantaneously equal to the extracellular composition. The mass transfer across the cell membrane and the cell shrinkage are a rate dependent function of time, cell membrane permeability and driving force, i.e. extracellular solution osmolality. Since the chemical damage is a function of temperature and since the mass transfer and cell dehydration are a function of time, it should be anticipated that if cells are frozen rapidly to cryogenic temperatures the chemical mode of damage during freezing could be eliminated. Indeed, it has been observed that increasing the cooling rates during freezing improves cell survival [18,20].

In developing optimal cryopreservation protocols for cells, it is always observed that increasing cooling rates improves cryopreserved cells survival. However, experiments have shown that this increase in viability suddenly reverses when the cooling rates exceed a certain optimal value, and cell survival decreases precipitously with a further increase in cooling rates. When cells survival is plotted as a function of cooling rates, the survival signature has an inverse U shape, with survival gradually increasing with an increase in cooling rates to a certain so-called optimal cooling rate and than decreasing with a further increase in rate. While, this is an ubiquitous behavior of every cell the values of each so-called "inverse U shaped survival curve" is cell type specific and the optimal cooling rate can vary even by orders of magnitude from cell type to cell type. Experiments have correlated the decrease in cell survival with the sudden formation of intracellular ice [18,20–22]. The sudden formation of intracellular ice is also related to the dynamics of mass transfer across the cell membrane during freezing. When cells are cooled too rapidly for the intracellular solution to equilibrate osmotically with the extracellular solution than the intracellular solution is thermodynamically supercooled. The probability for intracellular ice nucleation increases with thermodynamic supercooling. It is not clear if the nucleation site for intracellular ice formation are intracellular, extracellular or on the membrane [23]. However, whatever the cause of intracellular ice may be, it is almost always lethal to the cells, and should be avoided in designing cryopreservation protocols.

In addition to the modes of damage by freezing listed above there are others, there are several others which are less explored. One of them relates to the possible mechanical interaction between ice crystals and the cells entrapped between them, which has been originally observed by Nei [24], and referred to by Mazur as the "unfrozen fraction hypothesis". Recent experimental evidence shows that the spacing between ice crystals can indeed cause cell destruction [16]. Another possible mode of damage is the contact and interaction between ice and the lipid bilayer, which by itself may be damaging [25].

b) Damage to Cells during Storage, Thawing and Warming

At any temperature above absolute zero chemical reactions continue to take place, with a temperature dependent rate. Therefore damaging chemical reactions can continue to take place during both storage and thawing. However, in general when the storage temperature is in the cryogenic range, liquefied gases temperature, cells can be preserved for decades. Nevertheless, experiments show that at temperatures higher than that of dry ice cells become noticeable damaged during the storage and these higher temperatures should be avoided.

During thawing, cells obviously experience elevated temperatures as they reach the melting temperature. In this range the chemical damage continues to occur. In addition, recrystalization, the minimization of Gibbs free energy by formation of large ice crystal through the annealing of ice grain boundaries, is another mechanism of damage during storage and thawing [26]. Recrystalization will cause further disruption to cells entrapped between ice crystals. It is enhanced with an increase in temperature and time of exposure to the elevated temperature, during thawing. Because of the way cellular solutions freeze, the frozen domain is made up of regions with various solute compositions, with different melting temperatures. Therefore, occasionally circumstances may occur in which, as ice melts, cells will be exposed to solutions that are less concentrated than the cells themselves. This can cause water to enter the cell, expand them, and rupture the cell membrane. Experimental studies suggest that usually slow rates of warming are more detrimental to cryopreserved cells than higher rates. Furthermore,

the volume of the container in which the cells are preserved may also be a factor in the ability to rapidly thaw cells—with containers that have a larger outer surface area preferable [27].

c) Damage to Cells by Apoptosis
Most of the studies on the process of cell death during freezing have employed viability tests that evaluate survival of cells immediately after thawing. However, recent studies suggest that the less lethal modes of damage may eventually result in gene regulated cell death (apoptosis) [28]. This area is only beginning to be explored and the use of anti-apoptotic compounds and protocols may benefit cryopreservation protocols.

Cryopreservation of Cells

In 1941, Luyet, reported the survival of frozen vinegar eels when immersed in ethylene glycol [29]. However, the breakthrough in the field was made in 1948 when Polge Smith and Parkes discovered that a 10% solution of glycerol could preserve cattle sperm at −79°C, and that this protective effect is general and applicable to other cells [30]. This ushered in the era of modern cryopreservation. The work of Lovelock, who found that dymethyl sulfoxide can also protect cryopreserved cells, began the quest for chemical additives that protect cells from freezing damage [31]. These chemical are collectively known as cryoprotectants. Many of them are polyalcohols, such as glycerol, ethylene glycol, 2,3 butanediol, and most, with a few exceptions, are chemicals that penetrate the cell membrane and function by replacing part of the water in the cell. It should be emphasized that various cryoprotectants have different effectiveness for various cells, and their optimal use requires designing optimal cryopreservation protocols. Figure 4 illustrates the effect of a commonly used cryoprotectant, glycerol, on the inverse U shaped survival curve of mouse stem cells. It is important to recognize that survival of the cryopreserved cells is a function of cooling rates also in the presence of cryoprotectants. Furthermore the optimal cooling rate usually occurs at lower cooling rate values with an increase in cryoprotectant concentration.

Cryoprotectants usually afford protection during freezing with cooling rates in the ascending part of the inverse-U shaped survival curve. Their effect is therefore obviously chemical and relates to their presence inside the cells. Inside the cell they serve an osmotic function and decrease the concentration of salts at any subfreezing temperature. Because of the presence of cryoprotectants inside the cells, the dehydration is reduced during freezing. While generally beneficial, the larger intracellular volume can induce intracellular ice formation at lower cooling rates, as illustrated by Figure 4. This suggests that the arbitrary use of cryoprotectants without seeking the optimal cooling rate for the particular cell type preserved and the particular cryoprotectant may not lead to optimal preservation.

From the nature of the protecting effect of cryoprotectants it may appear that replacing the

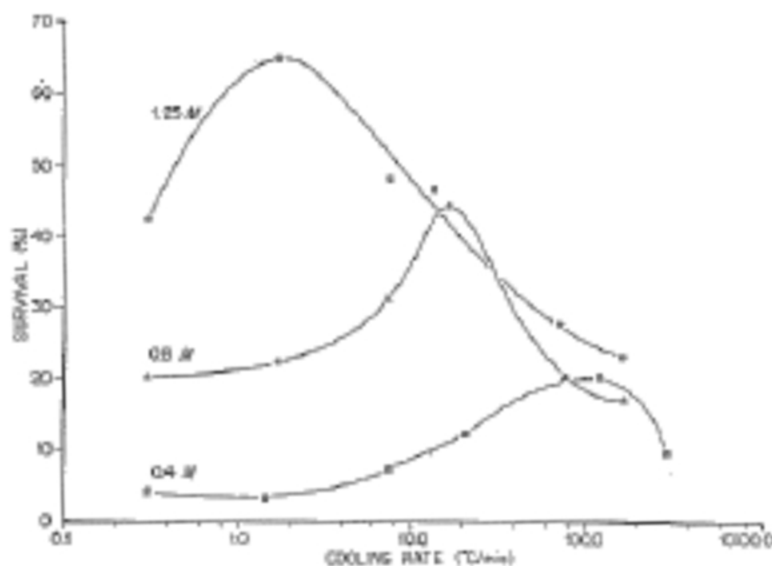

Fig. 4. *Inverse U shaped survival curve for mouse stem cells preserved in concentrations of 0.4, 0.8, and 1.25 M glycerol. The curves depict percentage cell survival as a function of cooling rates, during freezing to cryogenic temperatures.*

water inside the cells entirely with cryoprotectants could completely eliminate freezing damage. However, cryoprotectants are toxic themselves and their toxicity is both concentration and cell type dependent. Hobbs and Higgins have studied the toxic effect of several cryoprotective compounds on the heart [32]. They have found that dymethyl sulfoxide and glycerol have great chemical toxicity on the heart than ethylene glycol, which could be used with concentrations as high as 3 mol. It should be noted that the toxicity of various cryoprotective compounds may be manufacturing process related. The following example is one of many. For instance, since ethylene glycol is commercially prepared from the sterilization used ethylene oxide gas, any traces remaining in the solution can kill cells. This toxic effect can be removed by allowing the ethylene glycol to stay for 48 h at 37°C [32].

In summary, optimal preservation of cells by freezing can be accomplished through the design of protocols that involve the optimal choice of type and concentration of cryoprotectants and the appropriate optimal cooling rate. The design requires experimentation, as it is cell type specific, and can vary significantly. In addition to experiments, mathematical models exist which can be also used to guide in the developing of the protocol, e.g. [33]. In designing optimal cryopreservation protocols, attempts should be made to determine the effect of warming rates—although rapid warming is usually beneficial. In addition considerations may be given to removing recrystalization damage—which could be done by adding trace concentrations of antifreeze proteins (0.001 mg/ml). Anti apoptosis treatment has also been proposed, such as the addition of Caspase I inhibitorV [28].

Vitrification

Luyet was the first to report in 1938 cryogenic preservation of frog sperm after dehydration in sucrose [29]. This may have been the first report of cryopreservation by vitrification. In cryopreservation, the goal is to reach cryogenic temperatures without chemical damage and without the formation of intracellular ice. This can be ideally accomplished by vitrification. Thermodynamics show that if a liquid is cooled sufficiently quickly to cryogenic temperatures it can avoid freezing and form a highly viscous and amorphous glassy state, known as a glass or vitreous solid. A glass in amorphous (unlike a crystal it has no long range order) and is essentially a fluid with the mechanical properties of a solid. A material is said to be a glass if its viscosity reaches 10 E-14, Pa [11]. If a cell could be brought to a glassy state at cryogenic temperatures, while still technically a liquid the high viscosity halts molecular diffusion and the probability for ice nuclei formation becomes negligible—allowing essentially preservation for millennia. The necessary conditions for vitrification of pure water are cooling rates of millions of degrees per second. However, conventional cryoprotectants which naturally increase the viscosity of the intracellular solution require much lower cooling rates, on the order 10 to 1000°C/min, depending on the concentration of the cryoprotectants. While highly desirable, developing cryopreservation protocols by vitrification require: (a) the use of high concentrations of cryoprotectants—which may be toxic when introduced into cells, (b) the use of high cooling rates which may be difficult to achieve in large volumes and (c) high warming rates—which may be also difficult to achieve in high volumes. Nevertheless reports on successful vitrification of cells are available, e.g. [34].

Conclusion

Low temperature preservation of cells is an important aspect of cell transplantation for treatment of heart failure. There are two primary modes of cell preservation at low temperature, hypothermic preservation at temperatures above freezing and cryogenic preservation at temperatures below freezing. Optimal preservation cannot be achieved by indiscriminant cooling. This review briefly describes the basic mechanisms of damage during hypothermic and cryogenic preservation and the basic principles for developing optimal methods of preservation.

References

1. Zieger MAJ, et al. Factors influencing survival of mammalian cells exposed to hypothermia. *Cryobiology* 1990;27:452–464.
2. Morris GJ, Clarke A eds. *The Effects of Low Temperature on Biological Membranes*. London, Academic Press, 1981.
3. Quinn PJ. A lipid phase separation model of low temperature damage to biological membranes. *Cryobiology* 1985;22:128–147.
4. Wolfe J, Bryant G. Drying and/or vitrification of membrane-solute-water system. *Cryobiology* 1999;39:103–130.
5. Grout BWW, Morris GJ eds. *The Effect of Low Temperature on Biological Systems.* London, Edward Arnold Ltd., 1987.
6. Belzer FO, Southard JH. Principles of solid organ preservation by cold storage. *Transplantation* 1988;45:673–676.
7. Michel P, et al. A comparative study of the most widely used solutions for cardiac graft preservation during hypothermia. *Journal of Heart & Lung Transplantation* 2002;21(9):1030–1039.
8. Singer MA, Lindquist S. Thermotolerance in Saccharomyces cerevisiae: The yin and yang trehalose. *Trends in Biotechnology* 1998;16:460–468.

9. Rubinsky B, Arav A, Fletcher GL. Hypothermic protection—A fundamental property of antifreeze proteins. *Biochem Biophys Res Comm* 1991;180:566–571.
10. Franks F, et al. Ice nucleation and freezing in undercooled cells. *Cryobiology* 1983;20:44–63.
11. Franks F ed. *Water: A Comprehensive Treatise.* New York: Plenum Press, 1982.
12. Rubinsky B. Solidification Processes in Saline Solutions. *J of Crystal Growth* 1983;62:513–522.
13. Kurtz W, Fisher D, J. *Fundamentals of Solidification*. Switzerland: Trans Tech SA, 1984;242.
14. Rubinsky B, Ikeda M. A cryomicroscope using directional solidification for the controlled freezing of biological material. *Cryobiology* 1985;22:56–68.
15. Diller KR. Cryomicroscopy. In: McGrath J, Diller KR, eds. *Low Temperature Biotechnology: Emerging Applications and Engineering Contributions.* New York: ASME: 1988:347–363.
16. Ishiguro H, Rubinsky B. Mechanical interactions between ice crystals and red blood cells during directional solidification. *Cryobiology* 1994;31:483–500.
17. Lovelock JE. The haemolysis of human red blood cells by freezing and thawing. *Biochem Biophys Acta* 1953;10:412–426.
18. Mazur P. Cryobiology: The freezing of biological systems. *Science* 1970;68:939–949.
19. Tatsutani K, et al. The effect of thermal variables on frozen human prostatic adenocarcinoma cells. *Urology* 1996;48(3):441–447.
20. Merryman HT. *Cryobiology*. New York: Academic Press, 1966:966.
21. Tatsutani K, Rubinsky B. A method to study intracellular ice nucleation. *J of Biomech Eng ASME Trans* 1998;120(1):27–31.
22. Diller KR, Cravalho E, G. A cryomicroscope for the study of freezing and thawing processes in biological cells. *Cryobiology* 1970:7:191–199.
23. Toner M, Cravalho E, G, Karel M. Thermodynamics and kinetics of intracellular ice formation during freezing of biological cells. *J Appl Phys* 1990:69:1582–1593.
24. Nei T. Mechanism of hemolysis by freezing at near zero temperatures. II investigation of factors affecting hemolysis by freezing. *Cryobiology* 1967;4:303–308.
25. Wolfe J, Bryant G. Cellular cryobiology—Thermodynamic and mechanical effects. *Int J Refrig* 2001;24:438–450.
26. Forsyth M, MacFarlane D, R. Recrystalization revisited. *Cryo-Letters* 1986;7:367–378.
27. Rubinsky B, Cravalho E, G. Analysis for the temperature Distribution During the Thawing of a Frozen Biological Organ. *AIChE Symposium Series* 1979;75:81–88.
28. Baust JM, Van Buskirk R, Baust J, G. Cell viability improves following inhibition of cryopreservation-induced apoptosis. *In Vitro Cellular & Developmental Biology Animal* 2000;36(4):262–270.
29. Prehoda RW. *Suspended Animation*. Philadelphia, New York, London: Chilton Book Co., 1969:211.
30. Polge S, A Smith V, Parkes A. Revival of spermatozoa after vitrification and dehydration at low temperature. *Nature* 1949;164:666.
31. Lovelock JE, Bishop M, WH. Prevention of freezing damage to living cells by dimethyl sulfoxide. *Nature* 1959;183:1394–1395.
32. Hobbs KEF, Huggins C, E. Investigation of the effect of cryophylactic agents on the isolated rat heart at 37°C. *Cryobiology* 1969:6(3):239–245.
33. Rubinsky B, Pegg D, E. A mathematical model for the freezing process in biological tissue. *Proc of the Royal Society* 1988;234:343–358.
34. Rall W, Fahy G. Ice free cryopreservation of mouse embryos at minus 196 Celsius by vitrification. *Nature* 1985;313(6003):573–575.