Organogenesis 5:3, 90-96; July/August/September 2009; © 2009 Landes Bioscience

Cryopreservation
An emerging paradigm change

John G. Baust,[1,*] Dayong Gao[2] and John M. Baust[1,3]

[1]Department of Biological Sciences and Institute of Biomedical Technology; Binghamton University; State University of New York; Binghamton, NY USA; [2]Departments of Mechanical Engineering and Bioengineering; University of Washington; Seattle, WA USA; [3]Cell Preservation Services Inc.; Owego, NY USA

Key words: cryopreservation, biopreservation, apoptosis, cell death, cell storage, cryobiology

Introduction

In 1949 Polge, Parks and Smith[1] reported on the "chance" discovery of glycerol's cryoprotective function during their efforts to preserve avian spermatozoa in the frozen state. In the following year, Smith[2] extended these observations by successfully cryopreserving human red blood cells (RBCs) in glycerol. These two reports identified key elements that would play a crucial role in the evolution of the field of biopreservation including the need for a cryoprotective agent (CPA), the process by which cells could successfully be exposed to penetrating CPA and the manner of freezing and thawing. In 1959 Lovelock and Bishop[3] first described the use of dimethyl sulfoxide as a CPA with its advantage of enhanced permeability versus glycerol for many cell types. In the following decades incremental advances were made focusing on changes in and study of the carrier media containing the CPAs as well as the mechanisms of cell cryoinjury and cryopreservation. Most notable was the Mazur et al. report[4] in 1972 which put forth the "Two-factor Hypothesis" to describe the interrelationships between cooling rates and survival as influenced by either toxic "solution effects" experienced at sub-optimal slow cooling rates or lethal intracellular ice present at high cooling rates. In effect, these studies established a biophysical foundation upon which cryopreservation experimentation rested for nearly four decades. Other notable developments were discoveries by Fahy et al.[5] and Rall and Fahy[6] in the mid-1980s. This group reported on the novel vitrification strategy of cell preservation in which high concentrations (approaching 8 molar) of a cryoprotectant mixture could be titrated over a concentration gradient to create a medium that when "frozen" was devoid of ice even at liquid nitrogen storage temperatures. Beginning in 1998 a series of studies revealed that perturbations in the cell's proteome and genome during and following the cryopreservation process would significantly impact survival. This effect was observed regardless of the cryopreservation protocol utilized, "optimized" or other.[7-11]

With the continued development of cellular-based technologies (e.g., bioreactors with stem cells, tissue engineering, etc.), there is a need for improved methods of preservation that meet the requirement for rapid return to normophysiological function. These methodologies must protect the genome and proteome thereby avoiding stress-related genetically selectivity,[12] a criterion not met by most preservation processes, even today. In addition, the use of animal-derived products, proteins, serums, etc., raises concern over contamination with non-native components (i.e., prions, etc.,).[13] Efforts to maintain mammalian cells in a *dormant* state capable of "on demand" restoration have centered primarily on either hypothermic (refrigerated) storage or preservation in the frozen state.[14] Hypothermic storage often includes processes that maintain cells at temperatures above 0°C but below a normothermic temperature range (32–37°C). Advances in the hypothermic storage research have depended on improvements in organ preservation media in support of cell and tissue storage and organ transplantation including those factors related to ion balance, buffering capacity, free radical scavenging, oncotic support and nutrients.[15-17] Cryopreservation may be defined as the maintenance of biologics at sub-freezing temperatures, below -80°C and typically below -140°C. As stated above, Mazur's "Two-Factor Hypothesis" established the principles and a priori evidence of lethal conditions that need to be avoided if successful structural preservation is to be accomplished, a supposition influenced by Lovelock's classic demonstration of the relationship between survival and sodium chloride content in human RBCs.[18]

Throughout much of its development, cryopreservation research has focused less on integration of the cellular fundamentals that emerged from organ-based, hypothermic storage research and more so on physical factors, events and engineering principles.[19] Cryopreservation research has had as its central focus on the physical parameters associated with the water-to-ice phase change (i.e., water flux) during the preservation process with less attention focused on the impact of the hypothermic continuum experienced by cells during freezing until a glassy storage state is achieved.[20] Cells that are structurally preserved (avoid intracellular ice formation) remain in a state of deepening hypothermia until attaining the vitrification state of the preservation medium.[11,21] Importantly, during this thermal excursion, solute levels continue to elevate due to freeze concentration.[21] Cell function, while suppressed, does not cease until the intracellular glass transition (Tg) temperature is reached.[22]

A Long Cold Journey

Biopreservation is now recognized as an integrative specialty defined to include processes that suppress biological aging while supporting post-preservation restoration of function. Successful

*Correspondence to: John G. Baust; Email: jgbaust@binghamton.edu
Submitted: 09/04/09; Accepted: 09/08/09
Previously published online:
www.landesbioscience.com/journals/organogenesis/article/10021

cryopreservation incorporates relevant engineering principles with developments in cellular and molecular biology. Biopreservation represents the simultaneous application and management of numerous, often poorly defined (and not fully recognized), lethal conditions with the expectation of normal recovery.[20]

Biopreservation begins with a reduction in temperature typically from 37°C to the 0–10°C range. A hold temperature of 4°C is common for a brief interval (nominally 10 minutes) to allow for cryoprotectant equilibration. The selection of the hold temperature is less related to a cellular rationale and more to the fact that liquid water reaches its maximum density at 4°C. With cooling, a change in the energy balance of the cell occurs due to heat flow from the biologic. This loss of kinetic energy of molecules results in the uncoupling and re-coupling (shunting) of biochemical reactions.[23] The metabolome experiences imbalances that cause the failure of aerobic production of ATP, disruption of membrane-mediated transport (i.e., rapid gains in calcium, the loss of intracellular potassium and gain of sodium) and intracellular acidosis with pH approaching 4.[23-26]

While select mammalian species have evolved mechanism supportive of whole-body hypothermia (i.e., hibernators)[27,28] or seasonally adaptive heterothermy,[29,30] few human cells in vivo tolerate low temperature exposure beyond a few hours. Hypothermia without manipulative intervention yields progressive cell injury during each of its three phases (cooling, maintenance in the cold and rewarming). In addition to metabolic imbalances measurable changes in cell and organelle membrane lipid domains occur in a cell. These structural characteristics (transitions) result in a change in membrane fluidity from the liquid-crystalline state to the solid gel state[31-34] yielding a "leaky" membranous state. A cascade of damaging events may follow including: activation and leakage of lysosomal and lipoprotein hydrolases,[35,36] activation of calcium-dependent phospholipases[37,38] and the release of free fatty acids,[39] activation of the apoptotic cascade[40-44] and disruption of the cytoskeletal matrix.[45-47] Along with the generation of free radicals, the oxidative stressors attendant to hypothermia may result in the onset of apoptosis or gene regulated cell death.[11,12,20,48-51]

Cryopreservation of Cells

Cryopreservation protocols begin with hypothermic exposures which persist through the period of active extracellular ice growth until equilibrium is reached in the glassy-state (vitrified). This journey of deepening hypothermic stress experienced by a cell has been termed the hypothermic continuum.[52] CPA exposure represents the second step in the preservation process introducing a diversity of penetrating (membrane permeable) and non-penetrating agents contained within a carrier media to the hypothermic cell.[53] Incubation in the cryoprotective cocktail lasts between 10–30 minutes at 4°C followed by cooling at a nominal ("optimal") cooling rate (ranging from 1 to 10°C·min^{-1} is common for many mammalian cells). Seeding (ice nucleation) at a temperature close to the equilibrium freezing point of the cryoprotective medium (-2 to -6°C) supports the gradual growth of extracellular ice and limits "supercooling" of the system. This supports the osmotic efflux of water from the cell and the equilibration of cryoprotective agents across the cell membrane.[54] As extracellular ice formation continues, freezing reduces the availability of freezable water in the cell while freeze concentration of solutes increases the intracellular viscosity. When cooling rates are too rapid, cellular dehydration is inadequate increasing the probability of lethal intracellular ice formation.[55] Non-optimal freezing effects are recognized post thaw by increased cell rupture and early stage necrosis occurring over the first few hours post-thaw.[7,9,11,56] If cooling rates are too slow, prolonged exposure to multimolar levels of the freeze concentrated solutes results in cell toxicity (solution effects).[4,57] An indication of "solution effect" toxicity is the appearance of delayed necrosis peaking 6–12 hours post-thaw as well as apoptosis 12–36 hours post-thaw (cell type dependent)[58-60] followed by a secondary bout of necrosis related to the interplay between the progression of the modes of cell death.[11]

Cooling rate control (CRC) is accomplished by devices which support microprocessor controlled injection of liquid nitrogen to achieve active controlled rate cooling, or by passive methods often using insulated alcohol baths placed in a -80°C freezer. Active CRC devices monitor a representative sample vial, straw or bag and follow a pre-established program to achieve a desired cooling profile. Profiles are typically set to maintain a standard rate of cooling (e.g., -1°C/min for a specific cell type) over a prescribed temperature range and include a "seeding" event, nucleation through a thermal shock administered by a surge in cryogen to "flatten" the temperature rebound resulting from the latent heat of fusion of ice formation. Active CRC devices also provide records of the cooling profile. Passive CRC devices contain the sample surrounded by, but isolated from, an alcohol bath or a thermal-insulation material, and when placed in a freezer (-80°C), a curvilinear rate (e.g., approximately -1°C/min for a given cell type) is achieved in the samples. In passive CRC process seeding is often accomplished via mechanical agitation to create a nucleation event at a prescribed time during the cooling period.

Upon completion of freezing, samples are placed into long-term storage. During storage, the cell surrounded by a thin layer of vitrified cryoprotectant is maintained in a vitreous state encased in the mass of extracellular ice as long as the temperature remains above the glass transition temperature (Tg) of the solution. In addition to controlled rate cooling, high molar concentrations of cryoprotectant mixtures introduced in a stepwise manner can also be used to vitrify samples creating an ice free state both in the cell and extracellular matrix. This approach eliminates most ice crystal structure formation and has been reported to be of benefit when attempting to cryopreserve complex tissues and organs.[5,6,61-63] Retrieving samples from storage requires rapid thawing often accomplished by placing the sample in a 37–40°C stirred water bath until most of the ice melts. Once the ice has dispersed, elution of the cryoprotectant cocktail with cell culture media in a single-step or a step-wise (for high CPA concentrations) dilution process is used. Step-wise elution minimizes the volume excursions of the cell thereby preventing mechanical damage to the cell membrane and rupture. The effect of physicochemical changes that occur during cryopreservation has been extensively reported by Mazur.[21,55]

Despite intensive research focused on improving cell preservation, not all mammalian cells cryopreserve "equally." To highlight this issue, Lane[57] states that "Few scientific problems have proved as intractable as cryopreservation" and "…cryobiology has been straitjacketed by its need to conform to the intractable laws of biophysics. For all its successes, cryobiology has been stuck in a rut." Further, Mazur[21] has stated that "The problem today (with cryopreservation) is that applying basic principles of biophysics simply cannot solve many of the remaining challenges in cryobiology." As traditional approaches to cell storage are applied to non-terminally differentiated mammalian cells, many of these native and engineered cell types prove refractory to cryopreservation. Even in "successfully preserved" cell systems, significant death (30–70%) is often observed within 24–48 hours post-thaw.[11] Structural protection is afforded to these cells, but mitigation of the preservation-induced stress response resulting in biomolecular-based cell death many hours post-thaw remains a critical issue. As such, it is often the case where the cryopreservation sciences have provided effective strategies for structural preservation of most mammalian cell types but, until recently, have lacked to the molecular-based tools necessary to understand and mitigate much of the post-thaw damage. Recent studies have linked numerous stress factors associated with cryopreservation to known initiators of molecular-based apoptotic cell death processes (**Table 1**).

The combination of partial physical damage to a cell coupled with cell stresses experienced during the freeze-thaw cycle can result in necrosis. Further, "cross talk" between the apoptotic and necrotic cascades may also yield secondary necrosis. This complex series of events and factors demonstrates the critical involvement that a cell's "biology" plays in cryopreservation outcome.

Molecular-Based Cell Death Associated with Cryopreservation

Apoptotic activation in response to low temperature exposure has been documented in a variety of systems including renal cells, fibroblasts, hepatocytes, peripheral blood mononuclear cells, cord blood, spermatozoa, oocytes, ovarian-tissue, vascular tissue, et cetera.[11,48,53,64-68] Comparison of the stressors associated with cryopreservation and those known to activate apoptosis reveals substantial overlap. An analysis of these stresses and the cryopreservation literature demonstrates the well documented involvement of apoptosis in cryopreservation failure and the benefits of cell stress response modulation to improve outcome.[51,69-76] Although apoptosis was described in association with these reports, it was not until 1998 that apoptosis and cryopreservation failure were directly linked.[8] Over the last decade, there has been the emergence of numerous studies focused on understanding the role apoptosis plays in cryopreservation failure.[7,9,11,49,50,65,66,68,70,77-89] The involvement of apoptotic cell death in cryopreservation has now been reported in renal cells,[7,8] fibroblasts,[11] blood cells,[90-92] cornea,[93] stem cells,[94-96] cord blood,[78] lymphocytes, sperm,[97] ovarian tissue[98] and oocytes[66,99] to name a few. One aspect of this molecular involvement is the temporal component of post-cryopreservation cell death.[44] Molecular-based cell death often takes hours to days following thawing to manifest following thawing as various pathways are activated. This results in a delay in necrotic (6 hours) and apoptotic (12-hours) activity and ultimately observable cell death.[9,11,85,87,100] This temporal component continues to elude many investigators attempting to characterize the extent of molecular cell death following preservation in a variety of settings. While much research has been focused on identifying and quantifying apoptosis following cryopreservation, few have detailed the initiating stresses. While this area remains in the early stage, studies have, none-the-less, begun to provide insight into the pathways associated with cryopreservation-induced molecular cell death. These studies have implicated a host of initiation sites including the cell membrane, nucleus and mitochondria. These reports continue to solidify of the universal role molecular-based cell death exerts on cryopreservation.[7,48,51,81,83,86-88,101,102]

Table 1. Stress factors characteristic of cryopreservation

Hypothermia	Known initiators of apoptosis
Metabolic Uncoupling/Shunting	Biochemical Alterations/Inhibitions
Energy Deprivation	Energy Deprivation
Ionic Imbalances	Ionic Imbalances
Cellular Acidosis	Cellular Acidosis
Protease Activation	Protease (caspases) activation
Membrane Phase Transitions	Membrane Alterations
Free Radical Production	Free Radical Accumulation
Cytoskeletal Disassembly	
Freezing	
Water Solidification (Solute Concentration)	
Cell Volume Excursions	Membrane Alterations
Hyperosomolality	Ionic Imbalances
Protein Denaturation	Biochemical Alterations

Major Problems in Cryopreservation of Tissues and Organs

Tissue/organ cryopreservation is much more complicated and difficult than cryopreservation of individual cells. First, in addition to the cell cryoinjury caused by the intracellular ice formation (IIF) and "solution effects" as described above, there are several following major problems associated with organ cryopreservation: (a) vascular damage/rupture caused by ice formation/expansion in blood vessels (water moves into blood vessels from dehydrated cells).[116-118] To minimize this cryo-destructive effect, increased understanding and prediction of the fundamental mechanisms of ice formation and cell dehydration in tissues/organs are required. Although these biophysical events have been extensively studied in single cells with cryomicroscopy techniques,[106-108] experimental data in whole tissues are very limited. (b) Fracture of frozen tissue/organ caused by the thermal stress during the warming process. Thermal stress is one type of mechanical stress caused by non-uniform warming in a solid/frozen body (e.g., the fracture of glass when a surface is rapidly heated or cooled). To reduce the thermal stress, uniform heating is needed. Unfortunately, biological tissues have relatively low thermal conductivity and high specific heat.

Therefore, the conventional heating method (e.g., heating in a stirred water bath) causes a large temperature gradient within the tissue, resulting in high thermal stress and tissue fracture. Pegg et al.[109] and Cui and Gao, et al.[110] have developed a slow-cooling technique for relatively uniformly warming frozen rabbit carotid arteries to prevent the fracture. However, generally speaking, the slow warming may cause intracellular ice re-crystallization killing cells. A rapid and uniform heating technology is desired to prevent both potential lethal ice re-crystallization and thermal-stress-induced fracture. Apparently, rapid and uniform heating cannot be achieved by conventional heating methods. Recently, scientists have been developing single mode microwave resonance technology to achieve very rapid and uniform heating of frozen biomaterials.[111,112] (c) Problems associated with vitrification: a dramatically different approach to cryopreservation is to use either high concentrations of certain CPAs or ultra-rapid cooling rates (>10^6°C/min) to induce the cell cytoplasm to form a glass (i.e., to vitrify cells/tissues) rather than to crystallize. Indeed, vitrification is an ideal approach for organ cryopreservation. However, ultra-rapid cooling rates are technically difficult to achieve for tissues/organs. Several of the CPAs that are effective in ameliorating slow freeze injury also act to promote glass formation, but the required concentrations are so high, e.g., 4–8 M,[113,114] that they can be very toxic to the cells/tissues.[115] In addition, a vitrified organ is very brittle and can be easily fractured by thermal stress if not uniformly heated. (d) The lack of a single optimal cryoperservtion condition for all cell types in a tissue/organ. As we know, the optimal cryopreservation condition (e.g., cooling rate) is cell-type dependent due to the cell-type dependence of membrane permeability to water and CPAs, intracellular ice formation, osmotic tolerance limits, cryo-sensitivity and other physical/biological factors for cell cryoinjury. Because of many different cell types in a tissue or organ, it is difficult to define one single condition which is optimal for cryopreservation of all cell types. Up to now, cryopreservation (in either frozen or vitrified conditions) of large tissues and organs is not successful, in general.

Improving Cryopreservation Outcome

Differences in the sensitivity of various cell types to cryopreservation processes are well known. In an article by Van Buskirk et al.[20] it was suggested that the basis for differing cellular survival is linked to individual cell stress response and the resultant differential activation of cell death processes. The discovery of molecular responses in cells to the preservation process has therefore resulted in a variety of attempts to control these events in an effort to improve outcome. These attempts have included alteration in solution design (cryoprotectant carrier media), addition of cryoprotective agent cocktails, and the incorporation of select compounds for the Targeted Control of Apoptosis (TAC) during the cryopreservation process.

The mitigation of the molecular-based stress responses to low temperature exposure and storage has been shown to be attainable with cryopreservation solution formulation that addresses both physical and cellular related events.[44] The concept of specialty preservation media has evolved out of the organ preservation specialties. The Belzer and Southard team[15,16] first developed ViaSpan® (the University of Wisconsin solution) to support the transport of organs (pancreas, kidney and liver). ViaSpan®, formulated for hypothermic storage, was the first solution designed to manage select putative stress factors (**Table 1**) and became the first "intracellular-like" preservation medium. In the decade that followed additional preservation solutions were developed (i.e., Celsior, HTK—Custodiol, HypoThermosol, Unisol and others). More recently, cryopreservation solution formulation has moved beyond the addition of a penetrating cryoprotective agent such as DMSO (5–15%) to cell culture media, buffered saline or these media plus serum or a protein component.[53] Now recognized as essential to optimization of the cryopreservation process is the maintenance of proper cold-dependent ion ratios, control of pH at lowered temperature, prevention of the formation of free radicals, oncontic balance, the supply of energy substitutes, etc.[17,44] Traditional media fall short in addressing changes in solution pH, free radical production, energy deprivation, etc. Accordingly, the basal properties of these historical preservation media often do not provide for protection at the cellular level.[53] In attempt to address this issue, the cryopreservation sciences have taken lead from the organ preservation and molecular biology arenas combining these knowledge bases to increasing cell survival. Media including Viaspan, CryoStor, Unisol, Adesta, Celsior and others, to name a few, when combined with CPAs for have been reported to improve cell survival to varying degrees. Improvements have been observed in systems including hepatocytes,[58,103] cord blood stem cells,[104] PBMC's,[67,105] fibroblasts,[11] keratinocytes,[69] blood vessels[101] and engineered tissues.[56] In these studies, evaluation of the cryopreservation media was conducted and correlated with improvements in cell survival, function and growth. The improvement was not noted immediately post-thaw but not until following manifestation of the molecular-based events was the effect observed. It is now recognized that the integration of an intracellular-type solution with a penetrating cryoprotectant along with an understanding of the molecular responses of the cell at low temperature, provides for improved cryopreservation outcome.[44,53] The success of these solutions is linked to an in depth knowledge and understanding of the cell death pathways activated as a result of cryopreservation-induced cell stresses. To this end, studies have suggested that the improvement in cell survival and function was due to a reduction of both apoptosis and necrosis during post-thaw recovery although the mechanism of which remains unknown.[11,44,48,77]

Closing Thoughts

Cell-based applications in cell therapy, regenerative and reparative medicine, biobanking and tissue engineering are now focusing on normal, predictable and timely return to function of the cells after cryopreservation. This is often not achieved with today's technologies and approaches. In order to address this issue, continued improvement in cryopreservation outcome will rely on the integration of cellular biology, molecular biology, biophysics, engineering and cryobiology. Furthermore, with the growing body of evidence suggesting that CPAs, such as DMSO, affect the cellular,

proteome, genome and structures such as the mitochondria, the cell membrane and nucleus, it is obvious that successful preservation requires new strategies for the new definitions of success.

Traditionally, cryopreservation developments focused on structural preservation of cells through the inclusion of penetrating cryoprotectants and the management of ice and chemo-osmotic perturbations. New strategies improved preservation outcome through alteration of preservation solution to mitigate some of the detrimental effects of stress that contribute to the post-thaw launch of apoptotic and necrotic cell death cascades. The literature base utilizing the integrated approach to understanding and developing new approaches for preservation grows slowly. Current studies are now focused on linking the "management" of gene regulated stress dependent effects on a cell with the traditional cryopreservation approaches. In combination cell cryopreservation outcome will doubtlessly improve to meet the increased needs in biomedical applications.

References

1. Polge C, Smith AU, Parkes AS. Revival of spermatozoa after vitrification and dehydration at low temperatures. Nature 1949; 164:666.
2. Smith AU. Prevention of haemolysis during freezing and thawing of red blood cells. Lancet 1950; 2:910-1.
3. Lovelock JE, Bishop MW. Prevention of freezing damage to living cells by dimethyl sulphoxide. Nature 1959; 183:1394-5.
4. Mazur P, Leibo SP, Chu EH. A two-factor hypothesis of freezing injury. Evidence from Chinese hamster tissue-culture cells. Exp Cell Res 1972; 71:345-55.
5. Fahy GM, MacFarlane DR, Angell CA, Meryman HT. Vitrification as an approach to cryopreservation. Cryobiology 1984; 21:407-26.
6. Rall WF, Fahy GM. Ice-free cryopreservation of mouse embryos at -196°C by vitrification. Nature 1985; 313:573-5.
7. Baust JM, Van Buskirk, Baust JG. Cell viability improves following inhibition of cryopreservation-induced apoptosis. In Vitro Cell Dev Biol Anim 2000; 36:262-70.
8. Baust JM, Van Buskirk R, Baust JG. Cryopreservation outcome is enhanced by intracellular-type medium and inhibition of apoptosis. Cryobiology 1998; 37:410-1.
9. Baust JM, Van Buskirk RG, Baust JG. Gene activation of the apoptotic caspase cascade following cryogenic storage. Cell Preserv Technol 2002; 1:63-80.
10. Baust JM, Van Buskirk R, Baust JG. Modulation of the cryopreservation cap: elevated survival with reduced dimethyl sulfoxide concentration. Cryobiology 2002; 45:97-108.
11. Baust JM, Vogel MJ, Van Buskirk R, Baust JG. A molecular basis of cryopreservation failure and its modulation to improve cell survival. Cell Transplant 2001; 10:561-71.
12. Snyder KK, Van Buskirk RG, Baust JM, Mathew AJ, Baust JG. Biological packaging for the global cell and tissue therapy markets. BioProcessing 2004; 3:34-45.
13. Dimond PF. Prion disease mechanisms and activation. Genet Eng News 2005; 2516-22.
14. Gao D, Mazur P, Critser KK. Fundamentals of mammalian spermatozoa. Karow AM, Critser JK; Reproductive Tissue Banking: Scientific Principles. San Diego: Academic Press 1997; 263-328.
15. Southard JH, van Gulik TM, Ametani MS, Vreugdenhil PK, Lindell SL, Pienaar BL, et al. Important components of the UW solution. Transplantation 1990; 49:251-7.
16. Southard JH, Belzer FO. Organ preservation. Annu Rev Med 1995; 46:235-47.
17. Taylor MJ, Campbell LH, Rutledge RN, Brockbank KG. Comparison of Unisol with Euro-Collins solution as a vehicle solution for cryoprotectants. Transplant Proc 2001; 33:677-9.
18. Lovelock JE. The haemolysis of human red blood-cells by freezing and thawing. Biochim Biophys Acta 1953; 10:414-26.
19. Coger R, Toner M. Preservation techniques for biomaterials. Brunzion JD, Ed; The Biomedical Engineering Handbook. Boca Raton: CRC Press 1995; 1567-79.
20. Van Buskirk RG, Snyder KK, Baust JM, Mathew AJ, Baust JG. Hypothermic storage and cryopreservation—The issues of successful short-term and long term preservation of cells and tissues. BioProcess International 2004; 2:42-9.
21. Mazur P. Principles of cryobiology. Fuller BJ, Lane N and Benson EE, Eds., Boca Raton: Academic Press 2004; 3-65.
22. Taylor MJ, Song YC, Brockbank KGM. Vitrification in tissue preservation. Fuller BJ, Lane N, Benson EE, Eds; Life in the Frozen State. Boca Raton: Academic Press 2004; 603-92.
23. Tani M, Neely JR. Role of intracellular Na^+ in Ca^{2+} overload and depressed recovery of ventricular function of reperfused ischemic rat hearts. Possible involvement of H^+-Na^+ and Na^+-Ca^{2+} exchange. Circ Res 1989; 65:1045-56.
24. Renlund DG, Gerstenblith G, Lakatta EG, Jacobus WE, Kallman CH, Weisfeldt ML. Perfusate sodium during ischemia modifies post-ischemic functional and metabolic recovery in the rabbit heart. J Mol Cell Cardiol 1984; 16:795-801.
25. Neely JR, Grotyohann LW. Role of glycolytic products in damage to ischemic myocardium. Dissociation of adenosine triphosphate levels and recovery of function of reperfused ischemic hearts. Circ Res 1984; 55:816-24.
26. Southard JH, Van der Laan NC, Lutz M, Pavlock GS, Belzer JP, Belzer FO. Comparison of the effect of temperature on kidney cortex mitochondria from rabbit, dog, pig and human: Arrhenius plots of ADP-stimulated respiration. Cryobiology 1983; 20:395-400.
27. Galletti G. Hypothermia and hibernation. Karow AM Jr, Pegg D, Eds; Organ Preservation for Transplantation. New York: Marcel Dekker, Inc 1984; 101-11.
28. Nedergaard J, Cannon B. Mammalian hibernation. Philos Trans R Soc Lond B Biol Sci 1990; 326:669-85.
29. Brown RT, Baust JG. Time course of peripheral heterothermy in a homeotherm. Am J Physiol 1980; 239:126-9.
30. Baust JG, Brown R. Adaptive heterothermy in the arctic ground squirrel. Comp Biochem Physiol 1980; 67:447-52.
31. Wilson JM, McMurdo AC. Chilling injury in plants. Morris GJ, Clarke A, Eds; Effects of Low Temperature on Biological Membranes. London: Academic Press 1973; 4:285-309.
32. Lyons JM, Raison JK. A temperature-induced transition in mitochondrial oxidation: Contrast between cold and warm blooded animals. Comp Biochem Physiol 1970; 37:405-11.
33. Raison JK. The influence of temperature-induced phase changes on the kinetics of respiratory and other membrane-associated enzyme systems. J Bioenerg 1973; 4:285-309.
34. Ricciutti MA. Lysosomes and myocardial cellular injury. Am J Cardiol 1972; 30:498-502.
35. Weglicki WB, Owens K, Ruth RC, Sonnenblick EH. Activity of endogenous myocardial lipases during incubation at acid pH. Cardiovasc Res 1974; 8:237-42.
36. Liu X, Engelman RM, Rousou JA, Das DK. Myocardial reperfusion injury during adult cardiac surgery. Das DK, Ed; Pathophysiology of Reperfusion Injury. Boca Raton: CRC Press 1993; 263-93.
37. de Jong JW. Cardioplegia and calcium antagonists: a review. Ann Thorac Surg 1986; 42:593-8.
38. Schwertz DW, Halverson J, Isaacson T, Feinberg H, Palmer JW. Alterations in phospholipid metabolism in the globally ischemic rat heart: emphasis on phosphoinositide specific phospholipase C activity. J Mol Cell Cardiol 1987; 19:685-97.
39. White BC, Wiegenstein JG, Winegar CD. Brain ischemic anoxia. Mechanisms of injury. JAMA 1984; 251:1586-90.
40. Jennings RB, Ganote CE. Mitochondrial structure and function in acute myocardial ischemic injury. Circ Res 1976; 38:80-91.
41. Jennings RB, Ganote CE. Structural changes in myocardium during acute ischemia. Circ Res 1974; 35:3156-72.
42. Clarke DM, Baust JM, Van Buskirk RG, Baust JG. Addition of anticancer agents enhances freezing-induced prostate cancer cell death: implications of mitochondrial involvement. Cryobiology 2004; 49:45-61.
43. Baust JG, Gage AA. The molecular basis of cryosurgery. BJU Int 2005; 95:1187-91.
44. Baust JM. Molecular Mechanisms of Cellular Demise Associated with Cryopreservation Failure. Cell Preserv Technol 2002; 1:17-31.
45. Russotti G, Brieva TA, Toner M, Yarmush ML. Induction of tolerance to hypothermia by previous heat shock using human fibroblasts in culture. Cryobiology 1996; 33:567-80.
46. Brinkley BR, Cartwright J Jr. Cold-labile and cold-stable microtubules in the mitotic spindle of mammalian cells. Ann N Y Acad Sci 1975; 253:428-39.
47. Stefanovich P, Ezzell RM, Sheehan SJ, Tompkins RG, Yarmush ML, Toner M. Effects of hypothermia on the function, membrane integrity and cytoskeletal structure of hepatocytes. Cryobiology 1995; 32:389-403.
48. Fu T, Guo D, Huang X, O'Gorman MR, Huang L, Crawford SE, Soriano HE. Apoptosis occurs in isolated and banked primary mouse hepatocytes. Cell Transplant 2001; 10:59-66.
49. Jaeschke H, Lemasters JJ. Apoptosis versus oncotic necrosis in hepatic ischemia/reperfusion injury. Gastroenterology 2003; 125:1246-57.
50. Fowke KR, Behnke J, Hanson C, Shea K, Cosentino LM. Apoptosis: a method for evaluating the cryopreservation of whole blood and peripheral blood mononuclear cells. J Immunol Methods 2000; 244:139-44.
51. Mathew AJ, Hollister WR, Addona T, Baust JG, Van Buskirk RG. Vitamin E and EDTA improve the efficacy of hypothermosol-implication of apoptosis. In Vitr Mol Toxicol 1999; 12:163-72.
52. Baust JG. Concepts in Biopreservation. Baust JG, Baust JM, Eds.; Advances in Biopreservation. Boca Raton: CRC Press 2007; 1-14.
53. Baust JM. Advances in media for cryopreservation and hypothermic storage. Bioprocess Int 2005; 3:46-56.

54. Mazur P, Cole KW. Influence of cell concentration on the contribution of unfrozen fraction and salt concentration to the survival of slowly frozen human erythrocytes. Cryobiology 1985; 22:509-36.
55. Mazur P. The role of intracellular freezing in the death of cells cooled at supraoptimal rates. Cryobiology 1977; 14:251-72.
56. Baust JM, Van Buskirk R, Baust JG. Cryopreservation of an engineered human skin equivalent: The apoptosis paradigm. J Am Soc Mech Eng (Adv Heat Mass Trans Biotechnol) 1999; 36:371-6.
57. Lane N. The future of cryobiology. Fuller BJ, Lane N, Benson EE, Eds; Life in the Frozen State. Boca Raton: Academic Press 2004; 645-57.
58. Sugimachi K, Sosef MN, Baust JM, Fowler A, Tompkins RG, Toner M. Long-term function of cryopreserved rat hepatocytes in a coculture system. Cell Transplant 2004; 13:187-95.
59. Fisher RL, Hasal SJ, Sanuik JT, Hasal KS, Gandolfi AJ, Brendel K. Cold- and cryopreservation of dog liver and kidney slices. Cryobiology 1996; 33:163-71.
60. Corominola H, Mendola J, Esmatjes E, Saenz A, Fernandez-Cruz L, Gomis R. Cryopreservation of pancreatic islets prior to transplantation: a comparison between UW solution and RPMI culture medium. Cryobiology 1998; 37:110-8.
61. Huang JS, Downes GL, Childress GL, Felts JM, Belzer FO. Oxidation of ^{14}C-labeled substrates by dog kidney cortex at 10 and 38°C. Cryobiology 1974; 11:387-94.
62. Song YC, Khirabadi BS, Lightfoot F, Brockbank KG, Taylor MJ. Vitreous cryopreservation maintains the function of vascular grafts. Nat Biotechnol 2000; 18:296-9.
63. Brockbank KGM, Taylor MJ. Tissue Preservation. Baust JG, Baust JM, Eds; Advances in Biopreservation. NY: Taylor & Francis 2007; 157-96.
64. Xiao M, Dooley DC. Assessment of cell viability and apoptosis in human umbilical cord blood following storage. J Hematother Stem Cell Res 2003; 12:115-22.
65. Paasch U, Grunewald S, Agarwal A, Glandera HJ. Activation pattern of caspases in human spermatozoa. Fertil Steril 2004; 81:802-9.
66. Men H, Monson RL, Parrish JJ, Rutledge JJ. Degeneration of cryopreserved bovine oocytes via apoptosis during subsequent culture. Cryobiology 2003; 47:73-81.
67. Baust JM, Cosentino M, Meeks E, Baer J, Van Buskirk RG, Baust JG. Apoptotic cell death contributes significantly to peripheral blood mononuclear cells cryopreservation failure. Cryobiology 2005; 51:354-5.
68. Schuurhuis GJ, Muijen MM, Oberink JW, de Boer F, Ossenkoppele GJ, Broxterman HJ. Large populations of non-clonogenic early apoptotic CD34-positive cells are present in frozen-thawed peripheral blood stem cell transplants. Bone Marrow Transplant 2001; 27:487-98.
69. Borderie VM, Lopez M, Lombet A, Carvajal-Gonzalez S, Cywiner C, Laroche L. Cryopreservation and culture of human corneal keratocytes. Invest Ophthalmol Vis Sci 1998; 39:1511-9.
70. Jurisicova A, Varmuza S, Casper RF. Involvement of programmed cell death in preimplantation embryo demise. Hum Reprod Update 1995; 1:558-66.
71. Van Buskirk RG, Taylor MJ, Shen R, Tang W, Mathew A, Baust JG. Optimization of HypoThermosol using cultured cardiomyocytes and the fluorescent multiple endpoint assay. Cryobiology 1995; 32590.
72. Mathew A, Baust JG, Van Buskirk RG. Optimization of HypoThermosol® for the hypothermic storage of cardiomyocytes—Addition of EDTA. In Vitro Toxicology 1997; 10:407-15.
73. O'Flaherty C, Beconi M, Beorlegui N. Effect of natural antioxidants, superoxide dismutase and hydrogen peroxide on capacitation of frozen-thawed bull spermatozoa. Andrologia 1997; 29:269-75.
74. Hadj-Aissa A, Ramella-Virieux SG, Steghens JP, Barbieux A, Pozet N. Calcium antagonists improve kidney function in the rat after cold storage in high-Na UW but not in high-K UW solution. Transplant Proc 1997; 29:2439-41.
75. Nagasaki H, Nakano H, Boudjema K, Jaeck D, Alexandre E, Baek Y, et al. Efficacy of preconditioning with N-acetylcysteine against reperfusion injury after prolonged cold ischaemia in rats liver in which glutathione had been reduced by buthionine sulphoximine. Eur J Surg 1998; 164:139-46.
76. Roberts RF, Nishanian GP, Carey JN, Darbinian SH, Kim JD, Sakamaki Y, et al. Addition of aprotinin to organ preservation solutions decreases lung reperfusion injury. Ann Thorac Surg 1998; 66:225-30.
77. Yagi T, Hardin JA, Valenzuela YM, Miyoshi H, Gores GJ, Nyberg SL. Caspase inhibition reduces apoptotic death of cryopreserved porcine hepatocytes. Hepatology 2001; 33:1432-40.
78. Abrahamsen JF, Bakken AM, Bruserud O, Gjertsen BT. Flow cytometric measurement of apoptosis and necrosis in cryopreserved PBPC concentrates from patients with malignant diseases. Bone Marrow Transplant 2002; 29:165-71.
79. Van Buskirk RG, Baust JM, Snyder KK, Hollister WR, Clarke DM, Mathew AJ, et al. Cryopreservation—It's not just about cell yield. BioProcess Int 2005; 3:64-74.
80. Anzar M, He L, Buhr MM, Kroetsch TG, Pauls KP. Sperm apoptosis in fresh and cryopreserved bull semen detected by flow cytometry and its relationship with fertility. Biol Reprod 2002; 66:354-60.
81. Mathew AJ, Van Buskirk RG, Baust JG. Improved hypothermic preservation of human renal cells through suppression of both apoptosis and necrosis. Cell Preserv Technol 2002; 1:239-53.
82. Chavez-Reyes A, Parant JM, Amelse LL, de Oca Luna RM, Korsmeyer SJ, Lozano G. Switching mechanisms of cell death in mdm2- and mdm4-null mice by deletion of p53 downstream targets. Cancer Res 2003; 63:8664-9.
83. Borderie VM, Laroche L. Ultrastructure of cultured and cryopreserved human corneal keratocytes. Cornea 1999; 18:589-94.
84. Hilbert SL, Luna RE, Zhang J, Wang Y, Hopkins RA, Yu ZX, Ferrans VJ. Allograft heart valves: the role of apoptosis-mediated cell loss. J Thorac Cardiovasc Surg 1999; 117:454-62.
85. Schmidt-Mende J, Hellstrom-Lindberg E, Joseph B, Zhivotovsky B. Freezing induces artificial cleavage of apoptosis-related proteins in human bone marrow cells. J Immunol Methods 2000; 245:91-4.
86. Villalba R, Pena J, Luque E, Villalba JM, Gomez-Villagran JL. Keratocyte injury in human corneas cryopreserved under standard conditions. Cell Tissue Bank 2004; 5:201-4.
87. Paasch U, Sharma RK, Gupta AK, Grunewald S, Mascha EJ, Thomas AJ Jr, et al. Cryopreservation and thawing is associated with varying extent of activation of apoptotic machinery in subsets of ejaculated human spermatozoa. Biol Reprod 2004; 71:1828-37.
88. Sarkar S, Kalia V, Montelaro RC. Caspase-mediated apoptosis and cell death of rhesus macaque CD4$^+$ T-cells due to cryopreservation of peripheral blood mononuclear cells can be rescued by cytokine treatment after thawing. Cryobiology 2003; 47:44-58.
89. Lund PK, Westvik AB, Joo GB, Ovstebo R, Haug KB, Kierulf P. Flow cytometric evaluation of apoptosis, necrosis and recovery when culturing monocytes. J Immunol Methods 2001; 252:45-55.
90. Bontadini A, Tazzari PL, Manfroi S, Tassi C, Conte R. Apoptosis in leucodepleted packed red blood cells. Vox Sang 2002; 83:35-41.
91. Greco NJ, Seetharaman S, Kurtz J, Lee WR, Moroff G. Evaluation of the reactivity of apoptosis markers before and after cryopreservation in cord blood CD34(+) cells. Stem Cells Dev 2006; 15:124-35.
92. Bakken AM. Cryopreserving human peripheral blood progenitor cells. Curr Stem Cell Res Ther 2006; 1:47-54.
93. Ohno K, Nelson LR, Mitooka K, Bourne WM. Transplantation of cryopreserved human corneas in a xenograft model. Cryobiology 2002; 44:142-9.
94. de Boer F, Drager AM, Pinedo HM, Kessler FL, van der Wall E, Jonkhoff AR, et al. Extensive early apoptosis in frozen-thawed CD34-positive stem cells decreases threshold doses for haematological recovery after autologous peripheral blood progenitor cell transplantation. Bone Marrow Transplant 2002; 29:249-55.
95. Heng BC, Ye CP, Liu H, Toh WS, Rufaihah AJ, Yang Z, et al. Loss of viability during freeze-thaw of intact and adherent human embryonic stem cells with conventional slow-cooling protocols is predominantly due to apoptosis rather than cellular necrosis. J Biomed Sci 2006; 13:433-45.
96. de Boer F, Drager AM, Pinedo HM, Kessler FL, Monnee-van Muijen M, Weijers G, et al. Early apoptosis largely accounts for functional impairment of CD34$^+$ cells in frozen-thawed stem cell grafts. J Hematother Stem Cell Res 2002; 11:951-63.
97. Moran JM, Madejon L, Ortega Ferrusola C, Pena FJ. Nitric oxide induces caspase activity in boar spermatozoa. Theriogenology 2008; 70:91-6.
98. Hussein MR, Bedaiwy MA, Falcone T. Analysis of apoptotic cell death, Bcl-2 and p53 protein expression in freshly fixed and cryopreserved ovarian tissue after exposure to warm ischemia. Fertil Steril 2006; 85:1082-92.
99. Parks JE. Hypothermia and mammalian gametes. Karow AM, Critser JK; Reproductive Tissue Banking: Scientific Principles. San Diego: Academic Press 1997; 229-62.
100. Vogel MJ, Baust JM, Van Buskirk RG, Baust JG. Apoptotic cascades are activated following cryopreservation. Cryobiology 2000; 41390.
101. Snyder KK, Baust JM, Van Buskirk RG, Baust JG. Improved cryopreservation of vascular tissue. Cryopreservation 2005; 51:357-8.
102. Riccio EK, Neves I, Banic DM, Corte-Real S, Alecrim MG, Morgado M, et al. Cryopreservation of peripheral blood mononuclear cells does not significantly affect the levels of spontaneous apoptosis after 24-h culture. Cryobiology 2002; 45:127-34.
103. Sosef MN, Baust JM, Sugimachi K, Fowler A, Tompkins RG, Toner M. Cryopreservation of isolated primary rat hepatocytes: enhanced survival and long-term hepatospecific function. Ann Surg 2005; 241:125-33.
104. Stylianou J, Vowels M, Hadfield K. CryoStor Significantly Improves Cryopreservation of Haematopoetic Stem Cells (HSC). Cryotherapy 2005; 7:117.
105. Cosentino LM, Corwin W, Baust JM, Diaz-Mayoral N, Cooley H, Shao W, et al. Preliminary report: Evaluation of storage conditions and cryococktails during peripheral blood mononuclear cell cryopreservation. Cell Preservation Technology 2007; 5:189-204.
106. Diller KR, Cravalho EG. A cryomicroscope for the study of freezing and thawing process in biological systems. Cryobiology 1970; 7:191-9.
107. McGrath JJ. Membrane transport properties. In "Low temperature biotechnology: Emerging applications and engineering contributions" (Mcgrath JJ, Diller KR, Eds.), A.S.M.E. BED-Vol. 10, HTD 1988; 98:273-330.
108. Toner M, Cravalho EG, Karel M, Thermodynamics and kinetics of intracellular ice formation during freezing of biological cells. J Appl Phys 1990; 67:1582-93.
109. Pegg DE, Wusteman MC, Boylan S. Fractures in cryopreserved elastic arteries. Cryobiology 1997; 34:183-92.
110. Cui XD, Gao DY, et al. Cryopreservation and microsurgical implantation of rabit carotid arteries, Cell Preserv Technol 2002; 1:121-8.

111. Robinson MP, Wusteman MC, Wang L, Pegg DE. Electromagnetic re-warming of cryopreserved tissues: effect of choice of cryoprotectant and sample shape on uniformity of heating. Phys Med Biol 2002; 47:2311-25.
112. Dawei Luo, Chun Yu, Liqun He, Caicheng Lu, Gao DY. Development of a single mode electromagnetic resonant cavity for rewarming of cryopreserved biomaterials. Cryobiology 2006; 53:288-93.
113. Fahy GM, MacFarlane DR, Angell CA, Meryman HT. Vitrification as an approach to cryopreservation Cryobiology 1984; 21:407-26.
114. Fahy GM. The relevance of cryoprotectant toxicity to cryobiology. Cryobiology 1986; 23:1.
115. Fahy GM, Biological effects of vitrification and devitrification. In Biophysics of Organ Preservation (Pegg DE, Karow AM, Eds.), Plenum Press, New York 1987; 265-97.
116. Rubinsky B, Lee CY, Bastacky J, Onik G. The process of freezing and the mechanism of damage during hepatic cryosurgery. Cryobiology 1990; 27:85-97.
117. Rubinsky B, Onik G. Cryosurgery: advances in the application of cold to Qdicine. Int J Refrig 1991; 14:190-9.
118. Bischof LC, Rubinsky B. Microscale heat and mass transfer of vascular and intracellular freezing in the liver. ASME J Heat Transfer 1993; 115:1029-35.