Biobanking: The Future of Cell Preservation Strategies

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Abstract

With established techniques cryopreservation is often viewed as an "old school" discipline yet modern cryopreservation is undergoing another scientific and technology development growth phase. In this regard, today's cryopreservation processes and cryopreserved products are found at the forefront of research in the areas of discovery science, stem cell research, diagnostic development and personalized medicine. As the utilization of cryopreserved cells continues to increase, the demands placed on the biobanking industry are increasing and evolving at an accelerated rate. No longer are samples providing for high immediate post-thaw viability adequate. Researchers are now requiring samples where not only is there high cell recovery but that the product recovered is physiologically and biochemically identical to its pre-freeze state at the genominic, proteomic, structural, functional and reproductive levels. Given this, biobanks are now facing the challenge of adapting strategies and protocols to address

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these needs moving forward. Recent studies have shown that the control and direction of the molecular response of cells to cryopreservation significantly impacts final outcome. This chapter provides an overview of the molecular stress responses of cells to cryopreservation, the impact of the apoptotic and necrotic cell death continuum and how studies focused on the targeted modulation of common and/or cell specific responses to freezing temperatures provide a path to improving sample quality and utility. This line of investigation has provided a new direction and molecular-based foundation guiding new research, technology development and procedures. As the use of and the knowledge base surrounding cryopreservation continues to expand, this path will continue to provide for improvements in overall efficacy and outcome.

Keywords

Cryopreservation • Apoptosis • Molecular control • Biopreservation • Thawing • Cell storage • Cryopreservation induced cell death • Freeze injury • Improved survival • Necroptosis

Abbreviations

CCM	Complex cryopreservation media
CIDOCD	Cryopreservation
CPA	Cryoprotective agent
DMSO	Dimethyl sulfoxide
DOCD	Delayed onset cell death
ISBER	International Society of Biological and Environmental Repositories
LN_2	Liquid nitrogen
NCI	National Cancer
NIH	National Institutes of Health
TAC	Target apoptotic control
Tg	Glass transition temperature
UPR	Unfolded protein response

4.1 Introduction

Whether a biobank exists as an asset of an individual research laboratory or as an "industry/not-for-profit/government" mega-bank archiving an extensive diversity of samples, its purpose is to preserve biological specimens with the expectation of future recovery to support knowledge development relevant to a purpose such as disease control. In view of the diversity of sample types and methodologies available for banking, the concept of "fit for purpose" often serves as the strategic guide for the selection of sample processing methodologies. Implicit in this approach, but often unstated, is the probability that the samples may best serve other (unanticipated) purposes in the decades ahead. Today, biobanking preservation strategies should portend accurate predictions of future needs. In effect, tomorrow's successes will be dependent on the application of current mid-twenty century methodologies of preservation, which, unfortunately, often yield samples of limited utility [1–3]. How then do we reconcile the uncertainty, and therefore assure future utility of many millions of cryopreserved mammalian cell collections?

Today's biopreservation is characterized by a diverse scientific foundation integrating the fields of cryobiology, engineering, computer sciences, structural chemistry and cell/molecular biology [1, 3, 4]. Successful biopreservation requires the effective use of or the development of methodologies that support the preservation of cells, tissues and organs with post-storage return to pre-storage functionality [2, 3, 5]. Biopreservation is characterized by rapid growth as advances in cell therapy, stem-cell research, personalized medicine, cell banking, cancer research, etc. drive the need for optimized storage protocols. However, the field still experiences significant

problems with the current techniques including: sub-optimal survival, loss of cell function post-storage, addition of animal components in storage solutions, and activation of cellular stress pathways which can lead to alterations in gene expression, protein composition, micro RNA's, etc. [4, 6].

The first successes in cryopreservation can be traced to the Polge et al. [7] serendipitous discovery that avian spermatozoa could be successfully preserved in 20 % glycerol when pelleted on to a block of dry ice and a subsequent report on human erythrocytes [8]. In the decades that followed scores of empirical studies investigated process manipulation of two atypical cell types (the human RBC and spermatozoa) from diverse species. These cell types served as the models of choice due clinical/agricultural need, ease of experimental manipulation and availability. In 1959 Lovelock and Bishop [9] reported on the first use of dimethyl sulfoxide (DMSO) as a cryoprotective agent. Successful cryopreservation of spermatozoa is assessed not by normal physiologic function but by artificial insemination outcome which effectively requires only that acrosomal degradative enzymatic activity and DNA integrity be maintained. RBC survival was determined by percent hemolysis based on hemoglobin leakage immediately post-thaw. Long term viability of thawed RBC remains uncertain. Fortunately, and some might counter unfortunately, the application of these "… *penetrating cryoprotectants … enabled empirical cryoprotection to leap frog basic research*" [2].

In the decades that followed numerous hypotheses were proposed to account for freeze injury. These included: Lovelock's salt concentration, Meryman's "minimal cell volume" [10], Mazur's "two-factor hypothesis" [11, 12] and Steponkus' "membrane deletion concept" [13]. Mazur's two-factor hypothesis remains widely accepted amongst research cryobiologists as one of the "factors," rapid cooling rates fail to permit adequate water efflux from cells resulting in the formation of lethal intracellular ice [12]. The second factor focused on the toxic "solution effect" which predicts that if cells experience long exposures to concentrated solutes such as cryoprotectants, solute toxicity will be evident.

Accordingly, an optimal cooling rate would be required to minimize the destructive consequence of cooling at either too rapid (= intracellular ice) or too slow (= cryoprotectant toxicity) a rate. What followed were thousands of studies on the biophysical aspects of changes in cell volume and calculated water efflux rates with various cryoprotectants, cell types, cooling rates and strategies for cryoprotectant addition and removal. The goal since the 1960s has been to manage cellular water to prevent intracellular ice formation, and as a consequence of solute freeze concentration, to lower preservation temperatures below the nominal glass transition (Tg) range for pure water (Tg = ~ −135 °C) [14]. Below Tg "liquids" (also referred to as amorphous or acrystalline solids) are of high viscosity (10^{12} Pas) and reactions are determined by diffusion kinetics (WLF kinetics) rather than energetically driven Arrhenius kinetics. Since, for example, below Tg the rate of diffusion of a proton (hydrogen ion) has been estimated to take over 200 years to move one molecular diameter, chemical reactions are improbable. To terminate low temperature storage, cells are rapidly thawed to both minimize time above Tg thereby preventing energetically driven reactions, devitrification (ice formation at temperatures just above Tg) and recrystallization (disparities in ice crystal surface energy that causes changes in ice crystal size). Ice-free preservation, a form of vitrification, is designed to eliminate extracellular ice by exposing cells to very high solute concentrations (up to 8 M) in a time specific manner thereby minimizing the "solution effects" above Tg while relying on increased viscosity to suppress ice crystal growth [15, 16].

While the above strategies generally yield survival as measured immediately post-thaw [6], many cells in most samples continue to die over the following 24–48 h (Delayed On-set Cell Death) [17–20]. Surviving cells may lose key functional characteristics that may not be recovered until following generations [21, 22]. As a result studies focused nearly exclusively on structural parameters effectively treat cells as passive participant in the preservation process, as passive osmometers, thereby ignoring the critical

biological responses to the severe oxidative stress of cryopreservation. In effect, every processing step beginning with cell harvest through chilling, cryoprotectant exposure, freezing to Tg and thawing, *the cold chain*, occurs within the "hypothermic continuum" characterized by multiple cellular survival/death responses. Today, and in the future, it will be the management of these biological cascades that will determine not just whether or not a cell survives but whether or not it retains normophysiologic function. Several distinct mechanisms of cell death are now recognized following the application of an "optimized" cryopreservation protocol: physical cell rupture, apoptosis and necrosis [17, 20, 23–25]. Other possible forms of cell death such as autophagy, anoikis and more recently necroptosis may also be associated with cryopreservation but necessitate further study.

4.2 Hypothermic Storage

Hypothermic storage is primarily a metabolic suppression strategy for the maintenance of biological material. While the protective effects of cold have been documented for centuries, our understanding of the biological consequences of cold exposure is relatively recent. The "modern era" of low temperature cell preservation began with Carrel's investigations on the perfusion of organs prior to transplantation which related the characteristics of a perfusion medium [26–28].

As cold preservation entered the modern era, both hypothermic and cryopreservation techniques were developed to increase storage intervals by limiting the negative effects (i.e. ischemia, hypoxia, etc.) associated with cell and tissue harvest and isolation [1, 3, 28]. The central principle underlying the use of cold as a preservation tool is grounded in the reversible depression of cellular functions. Cryopreservation relies on ultralow temperatures to bring a cell's metabolism to a halt in support of an indefinite storage period. However, the current state of cryopreservation is only effective for single cell suspensions and a few simple tissues [1]. The cryopreservation of complex tissues or whole organs results are limited by "cryoinjury," manifest by cell death and the loss of higher order functions [4, 29]. For complex biologics hypothermic storage remains the most effect strategy for the preservation [1, 2, 28, 30].

The process of whole organ preservation requires an initial step of cold perfusion upon harvest. The hypothermic preservation solution supports transport prior to implantation at which time a warm reperfusion process flushes the hypothermic solution from the organ and returns it to normothermic temperature prior to implantation. While this method has proven to be far superior to warm perfusion and storage, it supports very limited preservation times (hours to days) [28]. Progress in preservation solution design was dependent on advances in the recognition of cellular responses to stress and a growing knowledge of tolerable limits that challenge normophysiological processes. This focus led to the development of the first intracellular-like cold perfusion solution, University of Wisconsin solution (ViaSpan®) which remains the "gold standard" of preservation solutions for many organ systems [3, 28, 30] since the late 1980s. While a physiological approach to preservation solution design continues [3, 27], there remains a significant limitations in complex tissue/organ preservation [3, 28, 30]. More recently, advancements in our understanding of the molecular response of cells to cold are supporting targeted approaches (cell and tissue specific) to extended preservation intervals and higher quality "product" [1, 3, 25].

4.3 Hypothermic Continuum

The medical literature generally defines hypothermia as mild (32–35 °C), moderate (27–32 °C), deep or profound (10–27 °C) and ultraprofound (0–10 °C) [2, 28]. We suggest that these divisions at above freezing temperatures along with differences in the aims of hypothermia and cryopreservation, have led to "disciplinary isolation" by those focused on these distinct preserva-

tion strategies [3]. As noted, the successes of hypothermic preservation have made significant gains through the understanding of fundamental cellular processes. Cryopreservation, on the other hand, has historically focused on the physical aspects of freezing leaving a disconnect between these related fields of study.

In cryopreservation research a distinction between pre- and post-freeze chilling exists. This distinction provides an artificial boundary at the temperature at which extracellular ice is manifest (nominally −2 °C). For a cryopreservation protocol to be "successful," intracellular freezing must be avoided whether by freeze concentration of the protective solute or the addition of initial high concentrations of solute. Both strategies result in intracellular vitrification. As a prelude to cryopreservation, the biologic transitions from its normothermic state (typically 37 °C) to hypothermic temperatures (~0 to 10 °C). This initial cooling can provide short-term benefit such as decreased metabolism, reduced oxygen consumption, and reduced nutrient demand thereby increasing overall survival. Cold exposure does, however, initiate numerous negative effects correlated the change in the energy state. Lowered temperature yields a decrease in the kinetic energy necessary to support normal physiological reactions resulting in a depletion of ATP [25, 28, 29, 31]. Early targets of hypothermic damage include cell membrane structure changes from liquid-crystalline to solid gel-like state, and functionally, as membrane mediated transport fails ionic imbalances become pronounced [2, 28, 30]. Increase levels in cellular calcium and sodium, losses in potassium and intracellular acidosis (pH approaching 4) occurs [28]. Numerous other disruptive events occur simultaneously within the cell including the leakage of hydrolases, generation of free radicals, disruption of cytoskeletal elements and mitochondrial-linked events leading to the activation of apoptotic machinery [4, 32–34]. With prolonged chilling, many molecular-based responses will activate or be suppressed which must be recognized and possibly be altered through molecular-based strategies to assure post-thaw survival [3, 4, 28, 35, 36].

4.4 Cryopreservation Process

Cryopreservation is a technique for maintaining biologics at cryogenic temperatures (at or below −80 °C, for prolonged periods of time). Cryopreservation processes begins with the exposure (~10 to 30 min) of cells to a cold cryopreservation solution. The process proceeds with further cooling to extend the hypothermic continuum to the storage temperature. Equilibrium is reached as the system reaches a glassy or vitrified state. As discussed, cells experience profound stress during the cooling interval of the cryopreservation process (up to 2 h) before reaching Tg where it is assumed that there are no further deleterious effects. It is often unappreciated that in order for a cell to be successfully cryopreserved the cell itself must avoid freezing, therefore remaining in a state of deepening hypothermia until Tg is reached. In essence, for a cell to be successfully cryopreserved, it must remain in a ultra-cold liquidous state until transitioning to a glassy state. Generally speaking, if ice forms within a cell during any part of the process, survival will be compromised.

Cryoprotective agents (CPAs) function, in part, to lower the probability of intracellular ice formation. Since glycerol was described as an effective CPA for both avian spermatozoa and human erythrocytes, numerous other compounds have been identified as CPAs. Today's, CPAs include a variety of penetrating (membrane permeable) and non-penetrating compounds contained in an appropriate cell culture media [6, 19, 37] with or without serum. An optimal cooling rate, nominally 1 °C per minute, is commonly applied for mammalian cell [12, 29, 31]. This allows for cellular dehydration and reduces the probability of intracellular ice formation which otherwise would cause cells to rupture upon thawing [12]. While generally accepted, studies by Acker et al. [38] have shown that some cells can tolerate a limited amount of intracellular ice during the process. While avoidance of intracellular ice is critical, if cooling rates are too slow, prolonged exposure to high solute concentrations can result in toxic effects (i.e. "solution effects") [3, 12].

Advancements in cryopreservation over the last several decades have helped to "optimize the process" yet have yielded varying degrees of success [2, 3, 5, 19, 29]. The most commonly practiced process is as follows: (a) cells are incubated in a culture media containing a cryoprotective agent such as DMSO (dimethyl sulfoxide), the most commonly employed CPA. To this end, over the last decade there has been a paradigm shift in solution design to include "intracellular-like" solutions as the CPA carrier media as a substitute for traditional culture media [18, 22, 39, 40]. As discussed later in this chapter, studies have shown that this shift in carrier solution design yield a significant increase in post-thaw cell survival and function [18, 21, 39, 40]. Following (b) a 10–30 min incubation at 4 °C, the cells are cooled (typically) at a uniform rate of 1 °C/min. A uniform cooling rate may be produced in an active or passive manner. Programmable controlled rate coolers provide active cooling. These devices monitor sample temperature and vary cryogen injection to provide a pre-determined cooling rate. Passive cooling methods utilize containers in which samples are surrounded by, but isolated from, alcohol. The container (c) is placed into a −80 °C freezer to achieve an approximate cooling profile of −1 °C/min. Ice nucleation (seeding) is often performed between −2 and −6 °C to prevent damage associated "flash" freezing due to sample supercooling. Seeding in active cooling devices is initiated through a programmed, thermal shock to the samples or through physical agitation of samples passive devices. Cooling (d) continues at a controlled rate to a predetermined temperature (i.e. −40 to −80 °C). Samples (e) are then transferred to ultralow temperature storage (i.e. liquid nitrogen immersion, liquid nitrogen vapor, or mechanical storage of < −135 °C). These ultralow temperatures fall below the reported glass transition temperature (Tg) of pure water [14, 41, 42] which arrests all molecular interactions (i.e. metabolism) and is thought to prevent a free radical generation [3]. In the glassy or vitrified state the viscosity of the solution is high causing the translational motion of molecules to cease. After storage, the cryopreserved sample is rapidly thawed to limit further exposure to negative effects associated with chilled liquid state. Sample thawing while agitated in a 37–40 °C water bath progresses until the last ice crystal is observed. Dilution of the sample is then accomplished by the addition of fresh culture media. New innovations in thawing rely on programmable thaw devices that provide repeatable, uniform and documentable sample thawing are discussed below.

4.5 Vitrification Strategies

Sample vitrification may be attained with an alternate technique. With this alternate technique a step-wise addition of high molar concentrations of cryoprotectant during the cooling process achieves an "ice-free" state [15, 16]. While similar in aim, vitrification procedures are different from that of controlled rate cooling. It has been shown that both extracellular and intracellular ice formed during cooling are damaging. The avoidance of ice formation makes vitrification a potentially viable option for the preservation of more complex tissues [15, 16]. A detailed discussion of vitrification is beyond the scope of this chapter. For additional discussion on vitrification procedures we refer the readers to articles [43–47].

4.6 Sample Thawing

One aspect of the cryopreservation process which received little attention is that of sample thawing. It is well established that rapid thawing of samples provides for improved cell viability post thaw compared to slow rates [11, 48–51]. Rapid warming rates allow for thawing of samples while minimizing recrystallization of ice and cellular exposure time to high osmolality and CPA concentrations. Rapid thawing is most often achieved via removal of samples from storage followed by immediate placement into a warm (37 °C) water bath. The time between removal from cryogenic temperatures to placement into the warm bath ("air time") is critical and should be kept as short as possible (few seconds). Prolonged time (>30 s, nominally) at tempera-

tures above −80 °C results in slow sample warming which can compromise cell viability and function. To this end, protocols often call for the transfer and transport of frozen samples in dry ice (−79 °C) or in LN_2 (LN_2 baths or dry shippers) to maintain the ultra-cold temperature of the sample until immediately before thawing. As described, the most common thaw procedure is to place samples into a warm (37 °C) bath. Gentle mixing or agitation is recommended to reduce the formation of steep thermal gradients within a sample throughout the thawing process. This prevents the formation of a microenvironment within a given sample where a portion of the sample is exposed to elevated temperatures (approx. >10 °C) where CPA's can be toxic. Samples are held in the warm bath until the last bit of visible ice has dissipated at which point samples should be removed and placed into a cool rack or on ice until dilution in culture media and plating or use. While thawing using a warm water bath has been practiced for over 50 years, this process is being reexamined as the "art of thawing" is not compatible with today's regulated and documentation intensive research and clinical environments. To address this need, a number of devices are being developed to provide for rapid, controllable, repeatable and documented sample thawing. These devices fall into the classification of "dry thawers" wherein samples are warmed in a dry heated chamber. While concerns over the reduced heat transfer efficiency of dry thawers compared to wet water baths have been expressed, reported thaw rates are comparable between the approaches. Further, dry thawers offer a number of advantages over water baths including improved processing and reduced risk of contamination and user error among others. To this end, BioCision recently introduced the *ThawSTAR* system designed to rapidly thaw a single cryovial while reportedly providing for a recorded thermal history of the sample [52]. In the blood banking arena, several dry thaw systems are available (*Plasmatherm*, *Sahara III*, *CytoTherm*) which utilize heated metal plates to thaw frozen blood product bag samples. Most recently, the *SmartThaw* system has been introduced as a next generation dry thawing device supporting the thawing of multiple container configurations (vials, bags, ampules, syringes, etc.) [53]. The *SmartThaw* system achieves rapid thawing of sample vials (1–4 vials), 25 ml cell therapy bags, 250 ml blood bags among others via a soft compliant thaw surface interface between which samples are placed. This compliant interface results in a sandwiching of a sample and provides for 360° of uniform warming. The system also provides for gentle agitation of the sample during the thaw interval. Like the *ThawSTAR* cryovial thawing system, the *SmartThaw* device provides a downloadable sample thermal history allowing for documentation of the thaw process. While ultimately these systems provide a similar outcome to that of water bath approaches, these dry thawers provide for more consistent and repeatable sample thawing which is documentable and can be performed in a clean or even sterile manner which is not possible with water bath approaches. The shift to dry thawing systems will enable end users to recover the highest quality sample possible while reducing the risk of sample loss, contamination, user error, thereby eliminating the "art" necessary for sample thawing.

4.7 Post-storage Outcome

Over the past half century, the improvements in cell preservation technologies have been modest with significant challenges remaining to be overcome. Cells post-thaw often appear viable in the hour or two after thawing. However, when examined 24–48 h later, a significant portion (30–70 %) of these cells succumb to delayed-onset cell death (DOCD) [6, 20, 25]. In effect "optimized" cryopreserved processes do protect cellular structure but fail to adequately manage the biological stresses associated with cryopreservation. An inability to manage the oxidative stresses attendant to cryopreservation results in the delayed initiation of complex cell death cascades leading to a loss of viability [4, 20, 25].

Studies have shown that the delayed molecular effects following thawing extend beyond that of cell survival or death, but impact function as well. Overall function of cellular systems following

cryopreservation has been an issue often overlooked due to the immediate challenges presented by working to improve "survival". The literature contains numerous reports citing high post-thaw cell viability and function [4]. Further examination of these studies, however, reveals that in many cases there are significant compromises in function post-thaw in cell systems such as hepatocytes [22, 54, 55], pancreatic islets [56], cardiac cells [57], blood cells [58], and stem cells [59]. Abrahamsen et al. [60] used flow cytometry to assess sample quality levels (apoptosis and necrosis) following cryopreservation as a means of establishing dosing parameters for cancer patients the cryopreservation process significantly affected the level of $CD34^+$ expressing cells in PBMC samples. de Boer et al. [61] have also reported the impairment of function in $CD34^+$ cells which resulted in a reduction in the effectiveness of stem cell graft procedures. Reports detailing similar reduction/losses in post-thaw functionality in gametes have also been described [62, 63]. Studies on the cryopreservation of spermatozoa have now linked molecular based stress responses and the loss of acrosomal and motility functions. Other studies have now associated negative effects of cryopreservation on the impairment of biochemical functionality in hepatocytes [22, 64] and cardiomyocytes [57]. These studies have helped to further our understanding and increase our recognition of the downstream effects cryopreservation may have on cellular function.

4.8 Cryopreservation Induced Cell Death

Biobanks experience a difficult, if not intractable, situation when faced with changes in cryopreservation protocols. While a number of organizational best practices exist (i.e. NIH NCI, ISBER, etc.), most focus on biobank management. Few effectively address process changes necessary improve cell functionality [2, 3]. Further, despite intensive research focused on improving cell preservation, not all mammalian cells cryopreserve "equally." To highlight this issue, Lane [5] stated 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 [65] 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. As described, even in "successfully preserved" cell systems, significant death (30–70 %) is often observed within 24–48 h post-thaw [20]. Structural protection is afforded to these cells, but mitigation of the preservation-induced stress response resulting in cell death many hours post-thaw remains a critical issue. As such, it is often the case that today's cryopreservation protocols provide effective strategies for structural preservation of most mammalian cell types but lacked to the molecular-based tools necessary to understand and mitigate much of the post-thaw damage. Multiple modes of cell death are recognized as contributors to cryopreservation failure.

4.8.1 Physical Cell Rupture

During the freezing process, solute is concentrated from approximately 350 mosmol to upwards of 10,000 mosmol [12, 29]. Cells exposed to these conditions will shrink severely but not necessarily experience a lethal event. During the post-freeze thaw, many cells will be subject to significant cell membrane damage resulting in rupture while other cells may experience membrane damage that is repairable. Not all cells respond the same as cell rupture may occur over many hours. The majority of membrane rupture occurs within minutes after thawing. Those cells rupturing one or more hours post-thaw experience non-repairable membrane damage and typically die through necrosis.

4.8.2 Necrosis

While ice-related rupture has been the primary focus of cryopreservation, necrotic cell death has also been observed in numerous cases of cryopreservation failure [17, 23, 66]. Necrosis is an energy independent form of cell death characterized by the swelling of a cell and its constituent organelles, loss of membrane integrity, lysosomal rupture, random DNA fragmentation by endonucleases and ultimately cell lysis [67–70]. As a result of cell rupture and the associated release of cytokines, there is typically an activation of an immune and inflammatory response *in vivo* [67, 68, 70]. The progression of necrosis often occurs rapidly in a matter of minutes to hours. Induction is typically seen in a response to severe cellular stress and results in the activation of detrimental intracellular signaling cascades. Necrotic cell death has been reported to be activated by stressors such as ischemia, osmotic shock, severe thermal stress, ionic dysregulation, toxic agents, etc. Interestingly, many of these necrotic activating stressors are also involved in or associated with hypothermic storage and cryopreservation.

4.8.3 Apoptosis

Apoptosis is a form of gene regulated cell death often referred to as programmed cell death. It differs from necrosis in that it is an energy-dependent process characterized by cell shrinkage, chromatin condensation, intact membranes but with phosphatidyl serine inversion, non-random DNA cleavage, and the formation of organelle containing "blebs" [67–72]. Unlike necrosis, apoptosis does not elicit an immune response *in vivo* but instead cells shed the apoptotic blebs which recycle cellular materials through phagocytosis. Apoptosis is induced by a number of different stressors that can specifically initiate the apoptotic response in the mitochondria, the plasma membrane or the nucleus [71–73]. Apoptosis can be induced by starvation (nutrient deprivation), temperature changes, viral infection, hypoxia, radiation, toxic compounds, osmotic stress and many other stresses. There are two canonical "branches" of apoptosis which have been identified in cryopreservation failure: the extrinsic or membrane-mediated and the intrinsic or mitochondrial-mediated pathways. Additionally, studies show that cross-talk, feedback and amplification pathways exist [33, 34, 74]. The identification of a third, nuclear-mediated apoptotic pathway further complicates a complete delineation of the cryopreservation process.

4.8.4 Necroptosis

As ongoing cell death research has continued to elucidate the specific biochemical mechanisms that trigger and propagate programmed cell death pathways, an alternative form of cell death has been identified [75]. Given the name necroptosis, this recently identified type of cell death has been shown to result in a necrotic-like execution with classical hallmarks such as cell swelling and membrane lysis while remaining highly regulated which distinguishes it from the conventional definition of necrosis. Research has now begun to reveal the distinct mechanism of action responsible for the activation of this pathway. Specifically, it has been shown that this mode of programmed necrosis is triggered through the signaling of death receptors, such as tumor necrosis factor receptor 1 [76]. The binding of the respective ligand (TNF-α) to the death receptor, similar to membrane-mediated apoptosis, results in the recruitment of intracellular signaling proteins and in turn the formation of an active complex responsible for downstream effects. Central to this necrotic complex is the kinase activity of receptor interacting proteins 1 and 3 (RIP1 and RIP3, respectively) and their substrate, the pseudokinase mixed lineage kinase domain-like protein (MLKL) as the core machinery for execution [77, 78]. Continued efforts will be necessary to further clarify the specific signaling cascade of necroptosis and how exactly the apoptotic/necroptotic balance is controlled by the cell during programmed death. However, the role of necroptosis in particular disease states and other biopreservation related stress conditions such as ischemia/reperfusion injury is becoming more

evident [79, 80]. As such, efforts to understand the complex cell death interplay at the molecular level will be paramount for improving future biopreservation endeavors.

4.8.5 Transitional Cell Death

Molecular-based cell death is typically thought to proceed through either an apoptotic or necrotic pathway. Apoptosis has been viewed as a "true organized molecular response" with necrosis considered "to involve random molecular events" at the intracellular signaling level. While accurate, the cell death landscape has evolved substantially over the last decade to suggest that classical apoptosis and necrosis represent more extremes on a continuum of molecular-based cell death [4]. Apoptosis is now considered to be a mode of cell death that can present in several forms including (a) Type I, the conventional view of apoptosis, not involving lysosomes but relying on caspase activation, (b) Type II, by contrast, is characterized by lysosomal-linked autophagocytosis, and (c) Type III, lysosomal-independent, necrosis-like apoptosis characterized by swelling of intracellular organelles [73]. It is now known that a cell's commitment to death causes an apoptotic activation and progression to cellular execution (type I classical apoptosis) or to a point where the stress becomes too great or energy reserves (ATP levels) too low resulting in a shunting from apoptosis to necrosis (secondary necrosis) [25, 30, 74, 79, 80].

Transitional cell death has been demonstrated in a number of studies, including some in cryopreservation, and has provided a basis for the cell death continuum concept emphasized here. Common stressors such as nutrient deprivation, DNA damage, cytokine exposure, cytotoxic agents, oxygen deprivation, ionic imbalance, etc. have been shown to result in the activation of both apoptosis and necrosis in a multiplicity of cell systems. The determination of apoptotic or necrotic activation is believed to be based on the relative degree of the stress experienced by the cell. The transitional nature of the cell death pathways in response to similar stressors creates a difficult environment to characterize. This is especially true as it applies to situations where multiple stressors are involved, such as cryopreservation.

4.9 Re-optimization of Cryopreservation

4.9.1 Initiation of Cryopreservation-Induced Molecular Death

It is now clear that much of the cell death associated with cryopreservation is linked to the execution of molecular-based cell death cascades [25]. However, limited detailed investigations into the initiating stresses have been reported. As described, the cryopreservation process exposes cells to stressors, many of which can initiate a molecular death response [3, 25, 37]. These factors include metabolic uncoupling, production of free radicals, alterations in cell membrane structure and fluidity, dysregulation of cellular ionic balances, release of calcium from intracellular stores, osmotic fluxes, and cryoprotective agent toxicity. This listing of stresses is by no means complete, but serves as a guide to the complexity of the stress response and multiplicity of potential cellular initiation sites. In an effort to provide insight into the effect of the various stressors associated with cryopreservation, studies have begun to focus on potential initiation sites of apoptosis within a cell. These studies remain in limited but nonetheless shed light onto the role of various pathways of molecular cell death, including the cell membrane, nucleus, and mitochondria associated with low temperature exposure.

4.9.2 Management of Cell Death

Decades of cryobiological research have yielded numerous cell preservation protocols based almost exclusively on one facet of the cold chain – osmometric parameters determinant of water flux as interpreted by changes in cell volume. This information is crucial to successful preservation but only part of the story. The physi-

ological responses of cell stress may direct many cells, especially those undergoing mitosis toward cell death. These molecular responses launch early stage apoptosis during pre-freeze incubation with cryoprotective media. During this period metabolic dysregulation results in free radical production [35, 81], cellular acidosis [28], protein unfolding [82–85] and ion imbalances. These stressors continue to strengthen during the ice growth phase and into the glass transition temperature range. This set of events is also manifest if an extracellular vitrification strategy is employed. Extracellular ice, while participatory in exacerbating stress buildup, is not a defining factor in cell death if "optimal" levels of cryoprotectants are uses. Cell then enter as dormant period but with various cell death pathways activated and primed for execution upon thawing [17, 18, 20, 25].

Differences in the sensitivity of various cell types to cryopreservation processes are well known. In an article by Van Buskirk et al., [30] 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.

4.9.3 Carrier Media

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 [4]. The concept of specialty preservation media has evolved out of the organ preservation specialties. The Belzer and Southard team [86, 87] 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 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) [28]. 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 [37]. 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. [25, 39] 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 [37]. 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. Complex cryopreservation media (CCM) 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 [21, 22], cord blood stem cells [40], PBMC's [88, 89], fibroblasts [20], keratinocytes [90], blood vessels [91] and engineered tissues [92]. 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 [4, 37, 39]. 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 [20, 25, 93, 94].

4.9.4 Target Control of Apoptosis

In an effort to mitigate the pro-death cascades activated as a result of both the pre-storage processing and subsequent preservation steps, a number of different targeted approaches have been taken. Initial strategies for targeted control utilized broad acting stress reduction agents such as free radical scavengers, antioxidants, protease inhibitors and ion chelators as additives to cryopreservation and hypothermic storage media as a means of inhibiting cell death [4, 81, 88, 92, 93, 95–98]. Continued efforts in this approach began to use more specific molecular-based agents to precisely target proteins and cell stress pathways. Specifically, with the use of caspase inhibitors to target pro-apoptotic signaling, studies demonstrated improved biopreservation of numerous different cell systems [17, 18, 88, 92, 93, 98]. Interestingly these improvements were observed as a decrease in both apoptosis and necrosis, again demonstrating the complex interplay of the cell death continuum [25, 36, 84, 88]. More recently, the pro-apoptotic protein Rho-associated kinase (ROCK) has been successfully targeted through the use of a ROCK inhibitor. This finding has been critical for the field of stem cell biology as the use of ROCK inhibitor, both during and post-thaw, has allowed for the successful dissociation and cryopreservation of embryonic stem cells as it has increased post-freeze survival and decreased spontaneous differentiation that resulted from preservation related stress [99, 100]. Additional research has begun to demonstrate improved biopreservation outcomes through the control of cell stress sensing and response elements that lie upstream of the caspase execution pathway. One such pathway that has garnered interest in this respect in the role of endoplasmic reticulum stress and the subsequent triggering of the Unfolded Protein Response (UPR) as a component preservation-induced cell death. Reports have detailed the important and differential role that the UPR pathway has in biopreservation outcome [83–85] and that a more complete understanding and control of this complex signaling will be necessary for next level preservation advances.

4.10 Summary

The cryopreservation of biologics such as cells and organs, has relied on low temperature to provide “on demand” access. While today’s standard of practice for cryopreservation still focuses primarily on the control of osmotic flux, ice formation and associated stressed, numerous reports have emerged over the last decade demonstrating the critical role of molecular-based stress response pathways and their control plays in cryopreservation outcome. The impact of this molecular aspect extends well beyond influencing cell death but also has a long term impact on biochemical pathways and cellular functionality post-thaw. As such, the ability for today’s biobanks to provide the highest quality samples to enable future discovery will depend on a paradigm shift in cryopreservation strategy. This shift must recognizes that (a) structural methodologies are reasonably effective (“optimized”) in preventing ice-related damage and (b) there remains a compelling need to decipher the cell’s responsiveness to the sever oxidative stressors attendant to a freeze-thaw excursion is required to overcome the significant cell death after thawing [4, 20].

Beginning with the initial step of cell processing (i.e. lifting cells in culture or surgical tissue excision), oxidative stresses parameters begin to compromise the normal physiology the cell. An initial element of any cold chain optimization strategy is the standardization of the isolation

steps (time, temperature and immersion media) and the addition anti-stress agents (i.e. free radical scavengers, molecular-based cell death blockers, buffers operative at low temperatures and oncotic agents to mitigate cell swelling). Cell death cascades that "play out" post-thaw are triggered (sensitized) during pre-freeze manipulation. The suppression of cryopreservation-induced molecular-based cell death can further be accomplished by a number of other strategies either individually or in combination. These include the utilization of (a) complex cryopreservation media (with an intracellular-like ion distribution, appropriate organic buffer and impermeants to protect against osmotic extremes), (b) various free radical scavengers, (c) Targeted Apoptotic Control strategies (apoptotic inhibitors). The timing of the addition of a complex cryopreservation media (CCM) may be cell type specific [4, 30, 37, 39]. While the majority of these efforts focus on the pre-freeze and freezing portion of the cryopreservation process, a similar TAC-based strategy can be employed following a rapid thaw to samples currently banked utilizing today's standard of practice protocols. While not as effective as front end intervention in the process, post-thaw manipulation strategies offer the potential to salvage cell populations enhancing downstream utilization through improving overall survival and/or cell function.

As the literature clearly demonstrates, a shift to molecular-based cryopreservation strategies can provide for improved outcome, it is important to recognize that each of the "optimized" cryopreservation protocols established over the past five decades are not likely to remain optimal with the incorporation of a CCM or other biochemical stress control strategy during or following freezing [17, 18, 20, 22, 39, 88]. This suggests that many if not all of the stages of the cold chain associated with cryopreservation may warrant re-investigation in the future. For example, nominal cooling rates of 1 °C/min are commonly applied despite reports indicating that higher cooling rates are beneficial with varying CPA concentration and types are used [51, 101–103]. In this regard, the use of a CCM with traditional levels of CPAs ought to support the use of higher cooling rates. Further, some cells survive well at cooling rates up to hundreds of degrees per minute. Similarly, thawing rates and the manner in which thawing is applied warrants continued study. Thawing should be as rapid as possible but, as previously discussed, with a methodological approach that supports a uniform thaw and is reproducible.

It is without doubt that the discovery of a complex molecular response of cells and the influence of cryopreservation-induced cell death on overall cell survival and function has had tremendous impact on cryopreservation research over the last 10–15 years. While recognized, these new principles and practices have yet to be implemented into biobanking strategies or even into mainstream discussion in the biobanking community. As the demand for the highest quality frozen cell products continue in support of growth in areas such as discovery science, diagnostics, stem cell biology and personalized medicine, the biobanking industry is now faced with the immediate challenge to embrace and incorporate improved strategies and protocols, extending beyond those focused on management and best practice protocol standardization, which address the molecular biological control/preservation aspect of cells during the cryopreservation process.

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