

# Cryopreservation: Evolution of Molecular Based Strategies

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## Abstract

Cryopreservation (CP) is an enabling process providing for on-demand access to biological material (cells and tissues) which serve as a starting, intermediate or even final product. While a critical tool, CP protocols, approaches and technologies have evolved little over the last several decades. A lack of conversion of discoveries from the CP sciences into mainstream utilization has resulted in a bottleneck in technological progression in areas such as stem cell research and cell therapy. While the adoption has been slow, discoveries including molecular control and buffering of cell stress response to CP as well as the development of new devices for improved sample freezing and thawing are providing for improved CP from both the processing and sample quality perspectives. Numerous studies have described the impact, mechanisms and points of control of cryopreservation-induced delayed-onset cell death (CIDOCD). In an effort to limit CIDOCD, efforts have focused on CP agent and freeze

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media formulation to provide a solution path and have yielded improvements in survival over traditional approaches. Importantly, each of these areas, new technologies and cell stress modulation, both individually and in combination, are now providing a new foundation to accelerate new research, technology and product development for which CP serves as an integral component. This chapter provides an overview of the molecular stress responses of cells to cryopreservation, the impact of the hypothermic and cell death continuums and the targeted modulation of common and/or cell specific responses to CP in providing a path to improving cell quality.

### Keywords

Cryopreservation • Apoptosis • Molecular control • Biopreservation • Thawing • Cell Storage • Cryopreservation induced cell death • Improved survival • Necroptosis

## Abbreviations

CCM	Complex cryopreservation media
CIDOC	Cryopreservation induced delayed onset cell death
CP	Cryopreservation
CPA	Cryoprotective agent
DMSO	Dimethyl sulfoxide
ER	Endoplasmic reticulum
RNAi	RNA interference
ROCK	Rho-associated kinase
TAC	Target apoptotic control
Tg	Glass transition temperature
TNF- $\alpha$	Tumor necrosis factor alpha
UPR	Unfolded protein response

as advances in areas such as cell therapy, stem cell research, personalized medicine, cell banking and cancer research drive the need for optimal processing and storage of the unique biologics (cells and tissues) for downstream utilization [3]. Even as advances continue to improve outcomes, there remain significant issues with current preservation practices including: continued cell death, loss of function, use of animal based products in storage media, activation of molecular stress response pathways in ‘surviving’ cells and downstream effects on epigenetic control of gene expression and protein levels [4, 5]. This last point highlights the profound change in our understanding of the consequences of low temperature, as the focus has moved from the physical/chemical changes (i.e. osmolarity, membrane disruption, etc.) to the complex molecular and stress pathway associated alterations over the last few decades.

## 2.1 Introduction

Biopreservation is a multi-disciplinary scientific endeavor which focuses on the development of methods for the storage of cells, tissues and organs and their subsequent return to pre-storage functionality [1]. As a field, biopreservation incorporates a number of areas including cryobiology, bioengineering, computer sciences, molecular biology, and cellular biology among others [2]. Currently, biopreservation is experiencing unprecedented growth and development

Typically, the utilization of low temperatures is the primary method for the preservation of biologics with the goal to extend the utility and deliver them “on demand”. Biopreservation can be divided into two branches, hypothermic storage and cryopreservation. Hypothermic storage is the use of non-frozen temperatures to store biologics, from below normothermic temperatures (32–37 °C) to usually no lower than 0 °C. A temperature range of 4–10 °C is most commonly uti-

lized for this regime. In contrast, cryopreservation is the utilization of ultra-low temperatures, typically at or below  $-80^{\circ}\text{C}$ , and usually below  $-140^{\circ}\text{C}$ , as this is below the reported nominal glass transition temperature ( $T_g$ ) of water (an important point which will be addressed later) to achieve long term storage. While this approach has demonstrated successful outcomes, advancements in fields such as stem cell research, regenerative medicine and tissue engineering are placing increasing demands on current protocols revealing the limitations of current cryopreservation strategies [6].

As such, a molecular-based focus has evolved, which is not only identifying stress pathway responses subsequent to storage (i.e. apoptosis), but is also guiding the development of next generation methodologies focused on stress response mitigation in an effort to improve outcome. This molecular focus has impacted both the area of cryopreservation as well as hypothermic storage. This chapter focuses primarily on the molecular evolution in cryopreservation while touching upon hypothermic storage to provide perspective into discipline overlap.

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## 2.2 Hypothermic Preservation

Hypothermic storage is built upon the well documented fact that the use of cold temperature has a protective effect on biologic materials. While the benefits of hypothermia have been known for centuries, our understanding of the molecular level effects on biologics has only recently evolved. This “modern era” of low temperature cell preservation began in the 1950s [5]. This was led by Carrel’s investigations on normothermic perfusion of organs prior to transplantation in which he described the fundamental characteristics of a perfusion medium leading to the advancement of the cold perfusion technique [7–9].

Both hypothermic and cryopreservation techniques were developed during this “modern era”. Each provide a means of effectively storing biologics for various intervals while limiting the negative consequence associated with removal of

cells and tissues from their normal, *in vivo* setting including ischemia and hypoxia [10–13]. The ability of cold temperatures to slow metabolic activity within cells in a reversible manner is the driving principle behind these techniques. This reduction in activity decreases cellular metabolic needs as well as the accumulation of damaging products from the ischemic/hypoxic environment, such as free radicals [14], thereby allowing biologics to be held in a state of “suspended animation” [1, 5]. Further, the use of ultra-low temperatures in cryopreservation can effectively halt molecular motion [3, 15], theoretically allowing for indefinite storage. However, the effectiveness of current cryopreservation techniques is restricted to cell suspensions or simple tissues. Cryopreservation of complex, whole organ systems results in significant cryoinjury as current protocols result in incomplete and uneven cooling and freezing, followed by cell death and in turn, loss of higher order functions [16]. Vitrification, the process of adding high molar concentrations of cryoprotectant in a step-wise fashion during the cooling interval allowing for cryopreservation in an “ice-free” environment, is another approach to tissue and organ cryopreservation. While this technique has demonstrated potential for organ cryopreservation [17, 18] it has limited usefulness and introduces other challenges, such as the cytotoxic ‘solution effects’ resulting from the inherent high cryoprotectant concentration exposure during the transient hypothermic state [19]. As such, hypothermic storage currently serves as the most effective paradigm for organ preservation [20]. While this approach has proven to be successful in increasing the effective storage interval for organs compared to warm perfusion and storage [21], it is essentially a tradeoff of more profound and fast acting stresses for less severe, but nonetheless significant, stresses associated with cold exposure. In an effort to understand the complex changes associated with hypothermic storage, numerous reports have investigated the metabolic, biochemical and physical characteristics of cells in their normothermic environment and how they change in response to exposure to cold [22–27]. Concurrent advancements in cell biology

have furthered our understanding of many of the intricate normal cellular functions, from biochemical processes to cell survival pathway activation. Through a more complete and thorough understanding of how a cell normally functions, it has become possible to establish a basis for comparison when examining cells exposed to cold. Further, this deeper understanding has allowed for a targeted and intelligent approach to improve storage outcome; a fact illustrated by the advances made in storage solution design over the last few decades.

As our understanding of cellular processes has progressed, including how cells regulate the flow of ions to create gradients and electron potential across membranes to drive reactions or how specific biochemical processes such as the citric acid cycle creates energy for the cell, it has allowed for calculated improvements focused on the formulation of the storage solutions. A major evolution was achieved with the development of an ‘intracellular-like’ preservation media, ViaSpan® (University of Wisconsin solution), which was formulated in the 1980s to mitigate many of the known causes of cell death at that time and became the ‘gold standard’ for organ transplantation [20, 28]. In the decades that followed, additional preservation solutions have been developed including Celsior, HTK-Custodial, HypoThermosol, Unisol, etc. [5, 26, 29]. This shift in media design has allowed for more successful short-term storage of cells, tissues and organs at hypothermic temperatures, as well as built the foundation upon which many recent advances in cryopreservation solution design have been based (discussed below) [2]. These advances include the addition of compounds, such as antioxidants and inhibitors, in a targeted molecular-based approach to improve storage outcome [3, 5]. As such, 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, osmotic balance and the supply of energy substitutes [30, 31]. Traditional media fall short in addressing changes in solution pH, free radical production, energy deprivation, among others. Accordingly, the basal properties of these histori-

cal preservation media often do not provide for protection at the cellular level [32]. While this approach has yielded significant improvements, there remains a considerable void that solution design alone cannot bridge. Moving forward, the field of biopreservation will need to continue to elucidate the complex molecular changes associated with preservation stress pathway activation. This will likely result in a process which carefully controls biologic response through all stages of the storage process (before, during and after), to be optimized in a cell/tissue specific manner to achieve protocols better suited for today’s demands.

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## 2.3 Hypothermic Continuum

Medical literature references four stages or intensities of hypothermia, including mild (32–35 °C), moderate (27–32 °C), deep or profound (10–27 °C) and ultraprofound (<10 °C) [33]. Classically, there has been a relative isolation between hypothermic storage and cryopreservation research, likely explained by a disparity in conventional aims between these two techniques. While hypothermic storage research has made considerable advancements through a deeper understanding of complex cellular processes, cryopreservation initially focused on the physical facets associated with the phase change and osmotic flux during the freezing process. Considering the significant overlap within these two fields, this divide has delayed potential gains that an integrated approach could have achieved; particularly given the fact that the cryopreservation process involves transitioning biologics through a deepening state of hypothermia ending when the sample transitions through the glass transition temperature ( $T_g$ , nominally –140 °C). This is again repeated during the warming phase.

As stated previously, while the use of hypothermic temperatures increases the time that biologics can remain viable through decreasing metabolism thus reducing oxygen and nutrient demand, there are a number of negative consequences associated with cold exposure. A central problem revolves around the depletion of energy

(ATP) within the cell as the temperature drops resulting in a decrease of kinetic energy necessary to drive the biochemical reactions within the cell [34, 35]. As a result, a number of essential cellular functions become compromised, including membrane mediated transport [36]. The combination of decreased membrane mediated transport along with the structural changes in the membrane as it changes from liquid-crystalline to solid gel-like state in the cold temperature results in pronounced ionic imbalances within the cell. Specifically, a cell experiences increases in intracellular sodium and calcium concentrations coupled with decreased potassium and intracellular acidosis approaching a pH of 4 [23, 26, 37]. Concurrent with these membrane-related events, a number of other deleterious effects occur within a cell including generation of free radicals, disruption of cytoskeletal components, leakage of hydrolases, and mitochondrial-related events leading to apoptotic activation [3, 36, 38–40]. Taken together, these accumulated stresses trigger molecular-based stress and death responses and illustrate the importance of managing these effects as future developments are made in biopreservation.

## 2.4 Evolution of Cryopreservation Strategies

Cryopreservation is the use of ultra-low temperatures, usually at or below  $-80^{\circ}\text{C}$ , to preserve biologics for extended periods of time (days to years). Cryopreservation studies initially focused on efforts to reduce intracellular ice formation through the use of cryoprotective agents (CPAs). In 1949 Polge, Parks and Smith [41] 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 [42] 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 cryopreservation including the need for

a 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 [43] 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 the study of carrier media containing the CPAs as well as the mechanisms of cell cryoinjury and cryopreservation [1, 44]. Most notable was a report by Mazur et al. [45] in 1972 which put forth the “Two-factor Hypothesis” to describe the inter-related relationships 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. Specifically, it was determined that a faster than optimal cooling rate results in an incomplete dehydration of cells and thus increases the likelihood of intracellular ice formation [45–48]; conversely, if cooling is done too slowly, cells become exposed to higher concentration of solutes, a condition known as “solution effects”, for extended periods of time and as a result experience toxicity [19, 45]. From this it was determined that for mammalian cell systems a cooling rate of  $-1^{\circ}\text{C}$  per minute provided the optimal rate for maintaining viability following cryopreservation. Further, a rapid thawing process was also shown to improve outcomes by limiting the time cell systems are exposed to these damaging temperatures and solutes following the storage stage [49]. In effect, these studies established the biophysical foundation upon which cryopreservation research has rested for nearly half a century.

### 2.4.1 Cryopreservation Process

Cryopreservation protocols begin with hypothermic exposure which persist through the period of active extracellular ice growth until equilibrium is reached in the glassy-state (vitrified). As previously discussed, this journey of deepening hypothermic stress experienced by a cell has been

termed the hypothermic continuum [1]. 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 [32]. Incubation in the cryoprotective cocktail lasts between 10 and 30 min at 4 °C followed by cooling at a nominal (“optimal”) cooling rate (ranging from  $-1$  to  $-10$  °C · min<sup>-1</sup> 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 controlled growth of extracellular ice and prevents “supercooling” and resultant “flash freezing” of the system. Seeding supports the osmotic efflux of water from the cell and the equilibration of cryoprotective agents across the cell membrane [50]. As extracellular ice formation continues, the availability of freezable water in the cell decreases, 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 [47]. Non-optimal freezing effects are recognized by increased cell rupture and early stage necrosis occurring over the first few hours post-thaw [48, 51, 52]. If cooling rates are too slow, prolonged exposure to multimolar levels of the freeze concentrated solutes results in cell toxicity (solution effects) [45, 53]. An indication of “solution effect” toxicity is the appearance of delayed necrosis peaking 6–12 h post-thaw as well as apoptosis 12–36 h post-thaw (timing is cell type dependent) [54–56].

Controlled rate cooling (CRC) is accomplished by devices which support the 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) 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. 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. After the nucleation point, sample cooling continues at the controlled rate until a user-defined temperature is achieved, usually  $-40$  to  $-80$  °C. Once this target temperature is reached, samples are typically transferred to an ultra-low temperature environment, such as liquid nitrogen immersion, liquid nitrogen vapor phase, or mechanical storage of  $<-135$  °C. While not all cryopreservation storage is conducted at ultra-low temperatures, with some instead choosing to maintain samples at  $-80$  °C, ultra-low temperatures are critical to reach given that they fall below the reported glass transition temperature ( $T_g$ ) for water [56]. This threshold is important as once crossed all molecular translational movement ceases and thus theoretically stops all biochemical activity, including the accumulation of damaging products, such as free radicals [15]. The transition through  $T_g$  also signifies an end to the hypothermic continuum as cells enter a vitrified (glassy) state surrounded by extracellular ice. It is at these temperatures that an indefinite storage period is theoretically possible given this state of ‘suspended animation’ in which cells are held. Retrieving samples from storage requires rapid thawing often accomplished by placing the sample in a  $37$ – $40$  °C stirred water bath or dry thawing system (SmartThaw, ThawStar or other device) until most of the ice melts. The rapid thaw limits the time biologics spend in the damaging post-storage hypothermic conditions (between  $T_g$  and  $0$  °C, nominally). 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.

Overall the cryopreservation process has advanced from these initial findings and others relating to physical parameters of the freezing/thawing process to become an “optimized” technique which is used today with varying degrees of success on more complex cell systems [24, 57]. However, even as these techniques have improved, there continues to be limitations in effectiveness when applied to more complex and sensitive cell systems.

### 2.4.2 Cryopreservation Induced Delayed Onset Cell Death

Despite the improvements and an increased understanding of cryopreservation, there remain significant challenges which need to be overcome. As “successful” methods of cryopreservation are applied to more sensitive and complex cell systems, less than ideal results are obtained. Often cells appear healthy and viable when examined immediately after thawing; however when examined 24–48 h later, a significant portion (30–70%) of these cells are lost due to cryopreservation-induced delayed-onset cell death (CIDOC) [30, 48]. This phenomenon has been reported in many cell systems and demonstrates that while the cryopreservation process may be optimized to protect cells structurally, it fails to adequately manage the other stresses associated with this process. In sensitive biologics, such as stem cells, CIDOC can account for a significant loss (>50%) of the total population [58–60] and result in high system failure, compromise in functionality, spontaneous differentiation and alterations in gene expression following what was initially deemed a ‘successful’ cryopreservation event [3]. As researchers began investigating this phenomenon, it became apparent that cell stress response and the activation of apoptosis played a critical role in CIDOC. Given all that is now known about both apoptotic induction and cryopreservation related stresses, a connection is now clear. Following apoptosis

identification, subsequent research has revealed how complex the cell death process is and the myriad of ways that molecular pathways within a cell can be activated following thawing. It is precisely this shift towards a deeper understanding of the molecular stress responses of a cell that continue to drive the field of cryopreservation to meet the ever-changing demands on it, such as the preservation of stem cells.

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## 2.5 Modes of Cell Death

It is well understood that multiple modes of cell death contribute to cryopreservation failure. The two main molecular paths of cell death associated with cryopreservation failure include necrosis and apoptosis. Cellular rupture due to intracellular ice formation is a critical third mode, however, this is primarily controlled through CPA incorporation. While ice related cell rupture has been extensively researched [61] resulting in significant improvements since cryopreservation’s inception, there remains significant issues to address with the other modes of cell death that contribute to preservation failure, particularly in complex, sensitive systems.

### 2.5.1 Ice Rupture

As ice forms outside the cell during the freezing process, solutes are excluded resulting in their concentration. Freeze concentration of solutes results in an increase in sample osmolality from approximately 350 mosmol to upwards of 10,000 mosmol [45, 61]. Cells exposed to these conditions shrink severely, but do not necessarily experience a lethal event. During thawing, many cells will be subject to significant cell membrane damage resulting in rupture while other cells may experience membrane damage that is repairable. The majority of membrane rupture occurs within minutes after thawing. Those cells rupturing 1 or more hours post-thaw experience non-repairable membrane damage and typically die through necrosis.

### 2.5.2 Necrosis

Necrotic cell death has been observed in numerous cases of cryopreservation failure [51, 62, 63]. 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 [64–67]. 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* [64, 65, 67]. The progression of necrosis often occurs rapidly in a matter of minutes to hours. Induction is typically seen in 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 and toxic agents. Interestingly, many of these necrotic activating stressors are involved in or associated with cryopreservation.

### 2.5.3 Apoptosis

Apoptosis is a form of gene regulated cell death often referred to as programmed cell death. 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” [64–69]. Unlike necrosis, apoptosis does not elicit an immune response *in vivo* but instead the cell blebs which are then recycled through phagocytosis. Apoptosis is induced by a number of different stressors which can activate responses in the mitochondria, the plasma membrane or the nucleus [68–70]. Apoptosis can be induced by starvation (nutrient deprivation), temperature changes, viral infection, hypoxia, radiation, toxic compounds, osmotic stress and many other stresses. The two canonical “branches” of apoptosis which have been identified in cryopreservation failure are the extrinsic (membrane-mediated) and the intrinsic (mitochondrial-mediated) path-

ways. Additionally, studies show that cross-talk, feedback and amplification pathways exist [71–73]. The identification of a third, nuclear-mediated apoptotic pathway further complicates a complete delineation of the cell death process following cryopreservation.

### 2.5.4 Necroptosis

As ongoing cell death research continues to elucidate the specific biochemical mechanisms that trigger and propagate programmed cell death, an alternative form of cell death has been identified [74]. 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, distinguishing it from conventional necrosis. As such it has been termed necroptosis. 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 [75]. The binding of the respective ligand (TNF- $\alpha$ ) to the death receptor, similar to membrane-mediated apoptosis, results in the recruitment of intracellular signaling proteins and 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 [76, 77]. Continued efforts are necessary to elucidate the specific signaling cascade of necroptosis and how the apoptotic/necroptotic balance is controlled during programmed death. However, the role of necroptosis in cryopreservation related stress conditions such as ischemia/reperfusion injury is becoming more evident [78, 79]. As such, efforts to understand the complex cell death interplay at the molecular level will be paramount for improving future cryopreservation approaches.



### 2.5.5 Transitional Cell Death Continuum

The original perception of molecular-based cell death was that there were two clear and distinct modes: apoptosis and necrosis [64]. Apoptosis is characterized by a particular set of molecular events which are highly organized and represent a true molecular response while necrosis was viewed as an uncontrolled process resulting from extreme stresses that involve random molecular changes [67, 80]. As continued research has further elucidated our understanding of the cell death process, it has become clear that necrosis and apoptosis do not exist as separate entities but comprise opposite ends of a ‘cell death continuum’ [81].

Reports have describe instances in which a multitude of cell types have demonstrated a cell death that did not follow a classical route of demise, but instead switches between necrosis and apoptosis and in some case display characteristics of both in a single cell [82–84]. To this end, Leist et al. [73] demonstrated that Jurkat cells can be shunted to a necrotic cell death following apoptotic induction through the removal of energy (ATP) from the system. Further, they demonstrated that returning ATP resulted in a reversion to more apoptotic-like death and that there is a point in the process when reversal can no longer be achieved. More recent reports have detailed two distinct forms of cell death that involve this shifting from apoptosis to necrosis, namely necroptosis and secondary necrosis [83, 84]. Secondary necrosis describes circumstances for when a cell has committed to cell death through apoptosis but experiences a depletion of ATP before completing the process and therefore shunts to necrosis to complete cellular demise. In contrast, necroptosis is the term given to a distinct, regulated form of necrosis which results in a cell death with necrotic hallmarks such as cell and organelle swelling, membrane rupture and the absence of chromatic condensation. To further complicate the picture, the literature also further divides ‘programmed cell death’ into distinct categories with apoptosis as merely one of a host of different programmed pathways a cell can uti-

lize to achieve cell death. Other pathways such as autophagy, caspase-independent apoptosis, necroptosis, anoikis, cornification and exocytotoxicity demonstrate the breadth of programmed cell death.

Cryopreservation most likely triggers an array of stress factors and cell death mechanisms. As research on cell death continues to expand our understanding of the complexities involved in cellular demise, it further complicates the effective management in cryopreservation regimes. This needs to be more fully understood so that more effective modulation strategies can be developed to improve outcome.

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### 2.6 Preservation-Induced Stress-Dependent Cell Death

The identification of programmed cell death involvement following cryopreservation, while a relatively recent discovery, has led to a number of improvements and preservation techniques in an effort to meet the demands of newer and more complex biologics. Given what is now known about both programmed cell death and low temperature induces cell stresses, it seems logical that a multitude of cell death processes, including apoptosis, are triggered in cells during cryopreservation. Since initial reports on the observation of apoptosis following cryopreservation, numerous reports have documented this phenomenon occurring post-thaw in a wide variety of cell types [25, 85–92]. Further, a myriad of reports also demonstrated its involvement in response to hypothermic storage and other related stresses, such as ischemia and hypoxia [11, 32, 79, 93–96]. As such, it is now recognized that molecular-based cell death has a significant role and effect on storage outcomes that is seemingly independent of cell type.

The temporal aspect is one of the most important components to understand the impact of molecular-based cell death on a cell system. While many assume that once a cell emerges from the cryopreservation process *intact*, it has “survived”, remaining a viable cell. However, studies have shown that accumulated stresses

during the preservation event can trigger molecular-based cell death pathways within the cell hours to days following thawing resulting in delayed cell death. Cryopreservation-Induced, Delayed-Onset Cell Death (CIDOCD), a term coined by Baust et al. [48], describes the phenomenon of a delayed peak in both necrosis and apoptosis many hours following cryopreservation. It is this delayed manifestation which demonstrates why timing is vital when assessing cell survival as well as investigating CIDOCD. If the assessment of cell death is conducted too early, while there may be an observable apoptotic and necrotic population, a significant portion may be missed as death continues to occur over the 24–48 h post-thaw, thus resulting in inflated cell survival estimates. On the other hand, if assessment is too late, the observed cell survival may be closer to the actual survival, but the contributions of apoptosis or necrosis may be missed analytically, leading to the incorrect conclusion that they did not play a role. Continued research has further proven this occurrence through more in-depth analyses showing the up-regulation of pro-apoptotic genes and proteins and activation (cleavage) of caspases and other pro-apoptotic signaling molecules [4, 30, 88].

While assessing viability is an important indicator of preservation efficacy, particularly when performed at the proper time post-thaw, functionality is an equally important post-preservation consideration that is often overlooked. While a cell may emerge from the cryopreservation process “viable” and have avoided CIDOCD, its functionality may have been compromised by the process. As improved techniques continue to enhance our ability to maintain viability in more complex and sensitive biologics, it has become clear that “cell in does not equal cell out”. Studies have demonstrated changes to cells following the preservation process such as diminished functions, loss of surface markers, and molecular differences such as changes in gene expression [54, 56, 97–103]. A potential concern is that the preservation process will select for cells with a molecular constitution with increased resistance to preservation stresses. Whether differences existed prior to preservation or as a result of pres-

ervation based selection, if the technique is selective for cells which have increased thermal or stress tolerance, it can be a cause for concern. The alteration of cell survival signaling pathways is a hallmark of cancer formation and progression. Changes in cell signaling related proteins, such as an over-expression of Bcl-2 or modification of P53, have been shown experimentally to be involved with cancer formation [104–107]. In this respect, preservation techniques must be designed to prevent changes in a cell’s molecular constitution that would otherwise limit downstream utilization.

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## 2.7 Targeted Control of Molecular-Based Death

The identification of a molecular-based cell death component to cryopreservation failure has served to complicate our understanding of the complex post-storage response. Despite the complications revealed by our furthered understanding of the entire post-storage cellular response, this also identifies a wide array of targets whose modulation may improve preservation outcome. Recent advances have revolved around changes to preservation solutions as well as the addition of chemical modulators to help control the molecular response following storage.

In cryopreservation, typical cryopreservation solutions are prepared by supplementing a cell’s basal culture media with serum proteins and DMSO. While this formulation provides adequate physical protection and control of intracellular ice formation, it provides only low level protection against many of the other stresses a cell encounters during the freeze/thaw process. To address this, the development and utilization of more “intracellular-like” solutions was undertaken in an effort to further buffer against changes that an “extracellular-like” solution (high Na<sup>+</sup>, low K<sup>+</sup>), such as basal culture media, has trouble managing. Studies have shown improved outcomes for cell systems cryopreserved using “intracellular-like” solutions supplemented with CPA’s as compared to standard culture media based cryopreservation solution [31, 60, 103,

108–110]. These advanced cryopreservation media are often referred to as Complex Cryopreservation Media (CCM) as they are designed to balance ionic needs of cells at low temperatures, buffer pH, provide energy substrates, osmotic stabilization, protection against free radical damage, among others. Typically, the use of CCM's results in improved sample quality post-thaw including increased viability, functionality, and regrowth which are usually not evident until hours after thawing. These improvements by CCM's have been shown to be a result of decreases in molecular based cell death during the recovery period [32].

More recently, advances in cryopreservation have been achieved through the incorporation of compounds into the cryopreservation media with the aim to control cell death through the targeting of molecular stress response pathways. This strategy is often referred to as Targeted Apoptotic Control (TAC). In this regard, the use of chemical modulators such as free radical scavengers, protease inhibitors and ion chelators have demonstrated the ability to improve cryopreservation and hypothermic storage outcomes [39, 111–113]. For instance, Mathew et al. [40], demonstrated the beneficial effect of the addition of vitamin E, a potent antioxidant, during hypothermic preservation. Other reports from this group described the beneficial effect of EDTA addition and demonstrated implications of apoptotic involvement [113]. In the area of cryopreservation, early reports by Baust et al. [51], Yagi et al. [114], and others [115] demonstrated the benefit of caspase inhibition in improving cryopreservation outcome. These initial reports were important first steps in demonstrating the ability to control cell death and ultimately improve post-preservation outcomes in a number of different cell systems.

More recent attempts at targeted molecular control of cell death have focused on targeting apoptosis. Through the use of caspase inhibitors during the cryopreservation, reports have demonstrated significant improvements in outcome through the blocking of apoptosis activated by the preservation process [51, 86, 92, 114, 115]. Interestingly, studies have shown that the utilization

of caspase inhibitors has a beneficial effect in multiple cell types through a decrease in both apoptosis and necrosis, further strengthening the linkage of the cell death continuum [30, 90, 95]. Another central finding of this research was the demonstration of the temporal aspect of the molecular-based cell death following cryopreservation. Continued research in this area has suggested that the post-storage addition of agents such as caspase inhibitors, also has a beneficial effect on outcome [92, 115]. To this end, a combinatorial approach of targeting stress pathways both during and after cryopreservation may result in improved outcomes for biologics.

As research continues to reveal the multitude of different pathways and molecular agents that play a role in the balance of a cell's fate following cryopreservation, a number of new targets and strategies have been developed to modulate cellular control. One such area of focus has centered on apoptotic control through the management of Rho-associated kinase (ROCK) through the use of ROCK inhibitor [116, 117]. ROCK has an important role in the morphological changes associated with apoptotic activation as a direct target of active caspase 3. Specifically, research has shown beneficial effects of ROCK inhibitor on stem-cell cryopreservation when incorporated during and post-preservation, including increased survival and decreased spontaneous differentiation [118]. Interestingly, studies have shown that utilization of ROCK inhibitor prior to single-cell dissociation of pluripotent stem cells, a necessary step for cryopreservation and a host of other applications, blocks the dissociation based apoptotic activation in these cells and thus improves preservation outcomes. This important finding again illustrates the impact that the entire preservation process, before, during and after, has on the overall success.

As advances in molecular biology techniques continue to improve their efficacy, and in turn increase their utilization, new strategies for improving storage outcomes become more feasible. Specifically, the ability to modulate post-thaw cell stress response at the level of transcriptional and translational events may hold the key for future improvements. Typically,

improved outcomes resultant from targeted approaches act through a change to cellular function at the proteomic level to achieve their effect. However, newer molecular tools such as RNAi, gene manipulation and epigenetic control are demonstrating the potential for alterations at the level of DNA and RNA as novel approaches for improved cryopreservation. RNA interference (RNAi) in particular has become an important and heavily researched cellular system since its initial discovery in 1998 and may offer potential in improving cryopreservation outcome [119]. RNAi works through innate cellular mechanisms that degrade double-stranded RNA in both an effort to protect the cell from foreign genetic material (i.e. retro-virus) and as a form of post-transcriptional gene silencing to regulate protein synthesis. Through the use of microRNA and small interfering RNA, it is possible to insert a complimentary RNA sequence to mRNA transcribed by the cell thus forming a double-stranded RNA segment, which is in-turn, degraded by the cell. The implication for cryopreservation then would be to utilize this technology to insert complimentary sequences to the mRNA of pro-death signaling molecules, such as caspases and pro-apoptotic Bcl-2 family (e.g. Bax, Bid, Bcl-XL, etc.), in order to transiently “knock-down” their expression, thus effectively increasing pro-survival signaling.

Similarly, the ability to manipulate genes at the level of DNA transcription through gene insertion or deletion has the potential to improve cryopreservation outcome. Through these techniques it may be possible to control levels of both pro-death and pro-survival proteins in an effort to increase survival through a targeted approach. However, there still remain concerns when manipulating genes, especially in a cell therapy application, because of the potential for unintended consequences such as cancer development. To the end, research is continuing to develop safer methods of gene manipulation as well as elucidate and expand on our understanding of the related cellular events of epigenetic control. Reports have begun to show a connection between thermal tolerance and epigenetic changes [120], demonstrating the potential for

epigenetic manipulations as a modulator to improve cryopreservation outcome. Epigenetics could potentially allow for the targeted control of gene expression in a reversible manner that may not alter a cell’s DNA sequence, limiting the latent negative consequences of gene manipulation.

Lastly, while our understanding of the roles that organelles such as the cell membrane, nucleus and mitochondria play in the cell death cascade continue to evolve, research has revealed other organelles may contribute to a cell’s fate as well. Specifically, the lysosomes, Golgi apparatus and endoplasmic reticulum (ER) have been shown to play a role in cellular stress monitoring with the ability to trigger a molecular-based response if irregularities are detected [121]. The ER in particular has become a major area of research in recent years as reports have revealed its role in cellular homeostasis and its subsequent ability to trigger an apoptotic response in the event of profound cellular distress [122–124]. Specifically, the mechanism through which the ER conducts these functions is known as the Unfolded Protein Response (UPR) [125–129]. As our understanding of the role and impact these areas have on cryopreservation outcome continues to evolve, they too may provide additional targets for modulation to improve post-thaw outcome (survival, function and genomic stability).

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## 2.8 Summary

As cell based research and development continues to advance at an exponential rate, it has become clear that cryopreservation protocols have become outdated and not kept pace with advances in other related fields. While today’s standard of practice for cryopreservation still focuses primarily on the control of osmotic flux, ice formation and associated stressors, numerous reports have emerged demonstrating that the combined targeting of physical, biochemical and molecular responses to the freezing/thaw process can greatly improve outcome. The impact of this combined approach extends well beyond influencing cell death but also has a long term impact

on biochemical pathways and cellular functionality. The results of these practices are, of course, only realized upon sample thawing and utilization. Given the complexity of events which occur during the seemingly simple and routine process of freezing, it is important to recognize and understand the impact of sample preparation, cooling, storage temperature, storage time, and warming have on overall sample quality. As such, the ability to successfully cryopreserve samples depends on the integration of the molecular strategy to obtain the highest quality cell product post-thaw. This strategic shift includes a) structural methodologies which are reasonably effective (“optimized”) in preventing ice-related damage, b) the use of CCM based cryopreservation media and c) integration of modulation strategies to reduce CIDOCD. While this new approach is well supported, there remains a continuing need to decipher the cell’s responsiveness to the stress events associated with cryopreservation to overcome the significant cell death after thawing [3, 48]. As such, investigations into the complex molecular changes associated with cold exposure are necessary to elucidate additional targets for improving storage outcomes. Particularly important is the integrated study of cell response to hypothermic and sub-freezing temperatures as they are experienced by cells during both freezing and thawing processes associated with cryopreservation. Through a more complete understanding of low temperature stress response and how associated changes alter a cell’s typical, normothermic baseline of function, significant improvements in cryopreservation strategies can be achieved in turn yielding improved biologics for downstream utilization.

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