Cryopreservation: It's Not Just About Cell Yield

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Improved biological packaging for cells, tissues, and organs is necessary to meet the worldwide shipping demands critical to the success of the regenerative/reparative medicine revolution (**1**). In the first article of our biopreservation series we reviewed the general principles underlying cryopreservation (storage at – 80 to –196 ºC) and hypothermic storage (storage at 4 to 8 ºC) — the two major biological packaging processes by which cells and tissues are stored and shipped for the in vitro toxicology, drug discovery, cell therapy, and organ transplant markets (**2**). That article served, in part, as a tutorial detailing how each of the two processes is used, how they differ in complexity, and how a specific bioprocessing application can be best matched to the appropriate biopreservation process. Noted was a common feature shared by both processes and not generally recognized: Most preservation techniques yield suboptimal preservation at multiple levels of transcriptional and enzymatic biochemical responses that occur many hours after return to normothermic conditions (37 ºC, for example). Those changes can lead to a loss of cellular function critical to applications that range from the hybridoma bioprocessing industry to cell therapy applications such as pancreatic islet transplantation. Expanding on concepts presented in the first article, this second article focuses primarily on cryopreservation. It discusses the cellular changes and molecular alterations that occur as a result of cryopreservation and their influence on cell viability and function.

Product Focus: Cells and tissues (regenerative medicine)

Process Focus: Production and storage

Who Should Read: Process engineers, manufacturing, research and development

Keywords: Hypothermic storage, cryopreservation, regenerative medicine

Level: Intermediate

Suboptimal Cryopreservation Activates Cell Death Pathways

Recently, our research team put forth the *multisolution hypothesis of cell preservation*: a new paradigm in the approach to the development of biopreservation technologies (**1–3**). It indicates that optimized hypothermic and cryopreservation of cells can be achieved only through understanding and subsequently manipulating the cell stress pathways activated by the cold. A key tenet of the paradigm is that some activated cold-induced stress pathways have been demonstrated to be cell-specific. As such, the future of improved hypothermic storage and cryopreservation protocols may demand a portfolio of solutions designed to match and manage those cell-specific stress pathways. The HypoThermosol (HTS) and CryoStor platform solutions (BioLife Solutions, Inc., www.biolifesolutions.com) are built on this principle. This hypothesis originated from a number of experiments analyzing the molecular responses of cells subjected to either a hypothermic or freezing insult.

Photo by K.K. Snyder, BioLife Solutions, Inc.

Normal human dermal fibroblasts (NHDF) were hypothermically preserved for two days in either HTS-FRS (BioLife Solutions) or ViaSpan (University of Wisconsin solution), the latter a solution commonly used for shipping and preserving human organs (Barr Laboratories). The cells were then returned to normothermic temperatures and observed under a phase-contrast microscope. Little difference could be discerned in the morphology between the two sets of cells (data not shown). Yet when the cells were stained with JC-1, a cationic carbocyanine dye that reveals active mitochondria, it was apparent that the cells stored in HTS-FRS had active mitochondria as evidenced by the orange, punctate staining pattern; whereas the cells stored in ViaSpan had compromised mitochondria as revealed by the diffuse green staining (Figure 1). (JC-1 is in the green monomeric form

with mitochondria proton motive force lower than approximately –80–100 mv; whereas it forms orange J aggregates when the proton motive force is greater than –100 mv. The average proton motive force of mitochondria is approximately –160 mv.) The nature behind this difference may be due to JC-1's known ability to discern between open and closed mitochondrial permeability transition pores that can be opened based on either intrinsic or extrinsic (activation of cell death receptors) cell stress (**4**). Although additional experiments need to be accomplished to determine whether the difference between the efficacies of the two solutions does, indeed, rotate around the mitochondrial axis, it is clear that cells that appear to be similar through standard optics may be quite different when stress pathways and selected physiological parameters are analyzed. As detailed below, this is the case with cryopreserved cells as well.

Cryopreservation of blood vessels serves as another relevant example demonstrating that the biochemistry of poorly preserved or frozen cells can be seriously impaired. Often the deleterious molecular cascades activated as a consequence of cold stress may not be manifested for days after preservation. An example of this phenomenon is illustrated with blood vessel preservation. Human blood vessels are often cryopreserved and stored for use in a number of applications for cardio-prosthetic applications (**5, 6**). Although cryopreservation practices have been widely applied in both the academic and commercial processing of blood vessels, improved cryopreservation approaches are needed to facilitate enhanced post-thaw vascular patency. Accordingly, we hypothesized that improved preservation of blood vessels could be achieved if the cold-induced, cell stress responses to the constituent blood vessel cell types were manipulated, which first required analysis of those pathways. Once those cell-specific stress pathways were understood, vessel-specific cryopreservation solutions could be designed to modulate them.

To this end, human coronary artery endothelial cells (CAEC) and coronary artery smooth muscle cells (CASMC) were subjected to mild freezing temperatures without a cryoprotectant such as DMSO. They were allowed to recover for various intervals at normothermic temperatures, and their constituent DNA was analyzed to follow cell death patterns as a result of cold stress. Data revealed that many cells survived this treatment, as evidenced by intact genomic DNA at the top of the gel that failed to migrate (Figure 2). Analysis of cells that died following freezing revealed that the endothelial cells succumbed primarily by apoptosis within three hours post-thaw (Figure 1A). In contrast, the

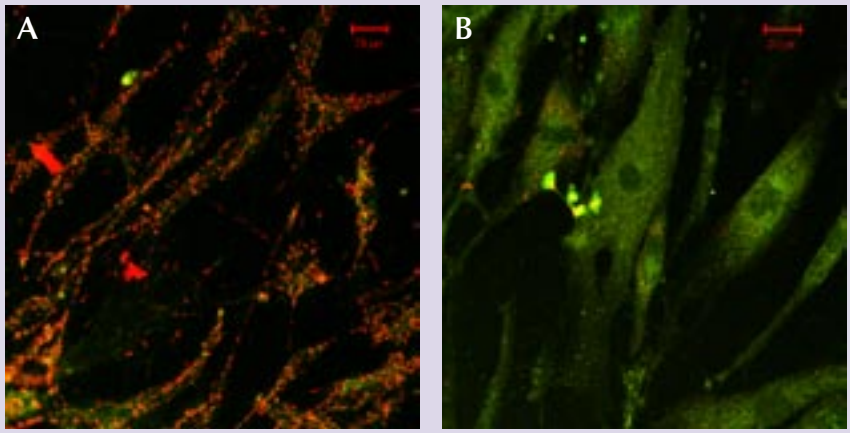

Figure 1: Change in mitochondrial proton motive force subsequent to two days hypothermic preservation in either HTS-FRS (A) or ViaSpan (B). Normal human dermal fibroblasts (NHDF) were stored for two days at 4 °C, returned to normothermic temperature (37 °C), and then stained with JC-1. The orange punctate staining of cells stored in HTS-FRS indicates a robust proton motive force; whereas the diffuse green staining of cells stored in ViaSpan indicates poor proton motive force that may be due to the opening of the mitochondrial permeability transition pore. It should be noted that these two sets of cells looked nearly identical under standard phase optics (data not shown).

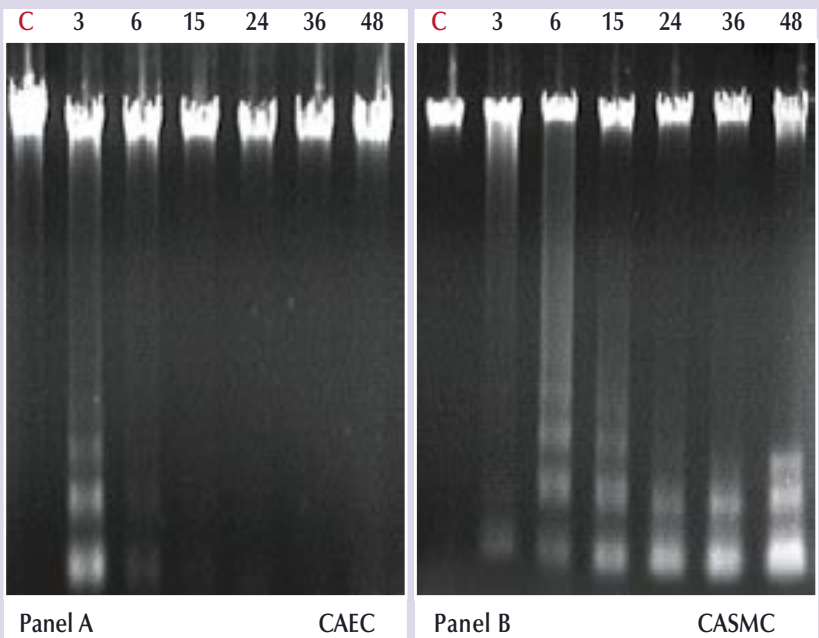

Figure 2: Agarose gel electrophoresis of coronary artery endothelial cells (CAEC) and coronary artery smooth muscle cells (CASMC) genomic DNA after a mild (–5 °C) freezing episode. Each panel depicts the separation of genomic DNA isolated from control cells at 37 °C (lane C), followed by cells harvested at various time points subsequent to freezing over a two-day recovery period (3, 6, 15, 24, 36, and 48 hours postexposure). Note that CASMC exhibited sustained and distinct apoptotic banding, whereas CAEC demonstrated apoptotic banding only at the three-hour period subsequent to freezing.

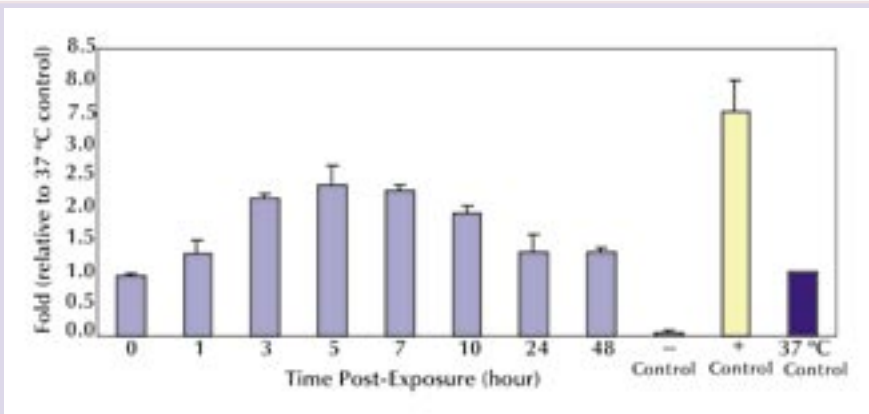

Figure 3: Post-exposure time-course analysis of caspase-3 activity (SEM) in CASMC over a 48-hour recovery period following a mild freeze exposure (–5 °C). Caspase-3 activity was elevated between three and 12 hours post-exposure and peaked at five hours. Activity levels were expressed as fold relative activity in comparison to 37 °C controls. Preincubation of samples with caspase-3 inhibitor served as a negative control. The positive control was provided by caspase-3 induction with the apoptosis-inducer, cisplatin.

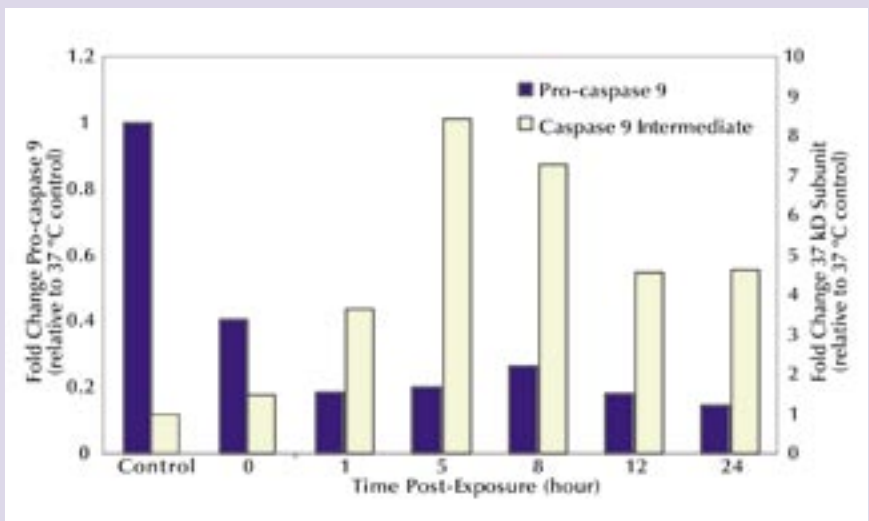

Figure 4: Post-exposure time-course analysis of caspase-9 activity in CASMC over a 24-hour recovery period following a mild freeze exposure (–5 °C). Detection of pro-caspase-9 (47kD) cleavage into its 37-kD subunit was achieved through Western blot analysis. CASMC exhibited a sustained increase in 37-kD band intensity accompanied by a prolonged decrease in procaspase-9 levels. Procaspase-9 never returned to control levels by 24 hours post-exposure.

smooth muscle cells appeared to die initially through necrotic mechanisms over an extended interval of three to 15 hours post-thaw, then from apoptosis 24 to 48 hours post-thaw (Figure 1B). Those data demonstrated that various cell types from a single tissue die in response to freeze stress through different functional pathways over distinct time intervals.

An interesting discovery in these studies was that the freezing response in smooth muscle cells demonstrated that apoptosis/necrosis occurred for up to two days following return to normothermic temperature — a concept previously termed *cryopreservation-induced delayed onset cell death* (**7**, **8**). This delayed onset cell death is especially critical to consider in the cell therapy sciences, where thawed cells may appear viable immediately upon implant but succumb a day or two later in the patient, resulting in transplant failure. Thus, it was incumbent upon us to further explore this phenomenon to improve cell survival.

Because DNA gel electrophoresis experiments indicated that apoptosis appeared to contribute to freeze-activated cell death, we expanded our analysis to include the apoptotic proteolytic enzymes — caspase-3 and caspase-9, respectively — in CASMC. Through that, we found activation of both caspase-3 and caspase-9 following freezing (Figures 3 and 4). Activation of caspase-3 peaked at five to seven hours, whereas activation of caspase-9, the cleavage of pro-caspase 9 to its intermediate form, was primarily noted five to 12 hours post-thaw. Considering that DNA cleavage occurs in the later stages of apoptosis and necrosis, the caspase profiles corroborated the gel-electrophoresis data (Figure 2), supporting freeze-stress initiation of delayed-onset cell death pathways. But could other cell stress pathways be activated during freezing as well?

We previously reported that freezing can increase the Bcl-2/Bax ratio in cells (**9**). Clarke et al. noted that the ratio of anti-apoptotic Bcl-2 to pro-apoptotic Bax increased over a 48 hour period, peaking at a ratio of 3× control at 24 hours following return to normothermic temperatures. Thus, these data suggest that the PC-3 cells were responding to freezing by increasing Bcl-2 — a survival response to the stress. Yet in all cells studied to date, there appears to be an increase in freezing-induced caspase activity (Figures 5 and 6; references **9**–**11**). The increase in the anti-apoptotic protein Bcl-2 suggested that cells were activating a survival pathway due to freezing; whereas activation of the caspases suggested that cells may also be activating cell death pathways during the same period. In both cases, contrasting, cold-induced stress pathways were not manifested until several hours after return to normothermic temperatures.

Although a complete investigation of all death pathways activated as a consequence of cold stress has yet to be accomplished using primary human cells, Odani et al. note that DNA microarrays have shown that cryopreservation of yeast cells results in changes in gene activity that relate to cell rescue, defense and virulence, energy and metabolism (**12**). Many of those genes encoded for heat shock proteins, oxidative stress scavengers, and enzymes related to glucose metabolism. This notion of the activation of competing biochemical pathways as a result of complex stressors (such as freezing associated with

cryopreservation) has recently evolved into what we call the *cell-preservation competitive stress pathway hypothesis*. Thus, not only may cells activate different stress pathways as a consequence of cryopreservation, but our data suggest that a possible "tug-of-war" between death and survival pathways may have to be understood to develop improved cryopreservation solutions that serve the emerging regenerative/reparative medicine marketplace.

Understanding Cell Stress Pathways

The previous data examined the response of cells to freezing both with and without incorporation of cryoprotective agents such as DMSO. As we described earlier, adding DMSO does not necessarily ameliorate the delayed onset cell death. Over the past five years numerous studies by our group and other groups have illustrated the involvement of delayed onset cell death in cryopreservation failures. In attempts to further understand these events, investigation including RT-PCR (reverse transcription-polymerase chain reaction), Western blots, protease activity, and fluorescence staining have been undertaken.

In 2002, Baust et al. reported for the first time an up-regulation of transcriptional activity of several caspases following cryopreservation in a human fibroblast model (**8**). This study reported an increase in caspase 3, 8, and 9 on RNA transcripts following cryopreservation. They further went on to compare the extent of caspase up-regulation between standard cryopreservation solutions such as media +5% DMSO to newly developed cryopreservation solution technologies such as CryoStor CS5: a protein-free, serum-free DMSO containing (5%) cryopreservation solution being used in many cell therapy applications. This study found a substantial up-regulation in caspase-9 activity following cryopreservation in both the standard media and in the CryoStor approach (Figure 5). The data in Figure 5 illustrate that the delayed caspase expression was still apparent even when DMSO was present in the preservation solution — a delayed expression phenomenon

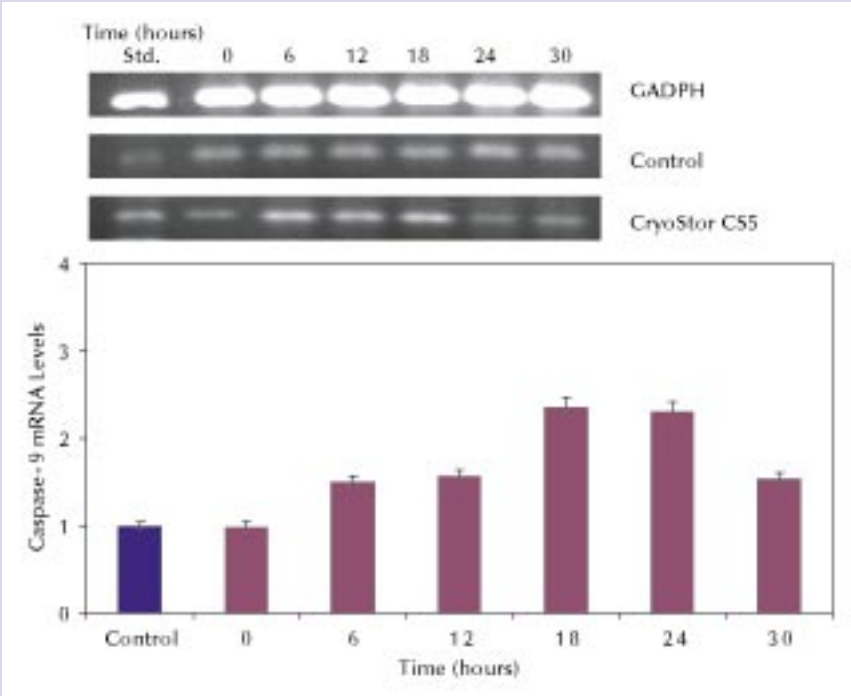

Figure 5: Post-thaw time course RT-PCR analysis of caspase-9 expression in normal human dermal fibroblasts following cryopreservation in CryoStor CS 5. Caspase-9 expression increases slightly following thawing, peaking at 18 hours post-thaw and returning to that of controls by 30 hours. Caspase-9 expression levels were normalized internally to a matched GADPH standard and then compared directly to matched time point nonfrozen controls (37 °C) and converted to graphical format for comparative purposes.

consistent with the freezing-induced cell stress pathways depicted in the previous section. Use of cryoprotectants did not appear to alter the timing of this delayed activation of cell death pathways. Yet it occurred to us that there might be a correlation between the level of cell survival and the extent to which these cell-death pathways are activated. In other words, do improved cryopreservation solutions result in any reduction in the delayed activation of the cell death pathways?

Following the above referenced studies, post-thaw caspase-3 expression was analyzed in a system cryopreserved in media + 5% DMSO or CryoStor CS5 to determine whether improved survival in the CryoStor samples correlated with a reduction in caspase-3 levels.Caspase 3 expression was found to be greater in normal human dermal fibroblasts cryopreserved in cell culture media (FBM + 5% DMSO) than in cells cryopreserved in CryoStor CS5 (Figure 6). If there is a correlation between viability and caspase activation, then the viability of normal human dermal fibroblasts cryopreserved in CryoStor CS5 would be approximately twice that of cells cryopreserved in FBM + 5% DMSO. In fact, it has been reported elsewhere (**2, 6**) that twice the number of cells survive following cryopreservation in CryoStor CS5 compared with cells stored in FBM + 5% DMSO (Figure 7). Viability, however, is only one measure of cell survival. A recent shift in thought is bringing cell function after cryopreservation to the forefront. Thus, our next question was to determine whether a connection can be made between cell function and cell yield. In other words, if more cells survive, is their function (on an equal viable cell basis) improved as well? Or is cell survival, per se, sufficient to result in full restoration of cell function following cryopreservation?

It's Not Just About Cell Yield

Many investigators are now focusing their attention on cell yield following

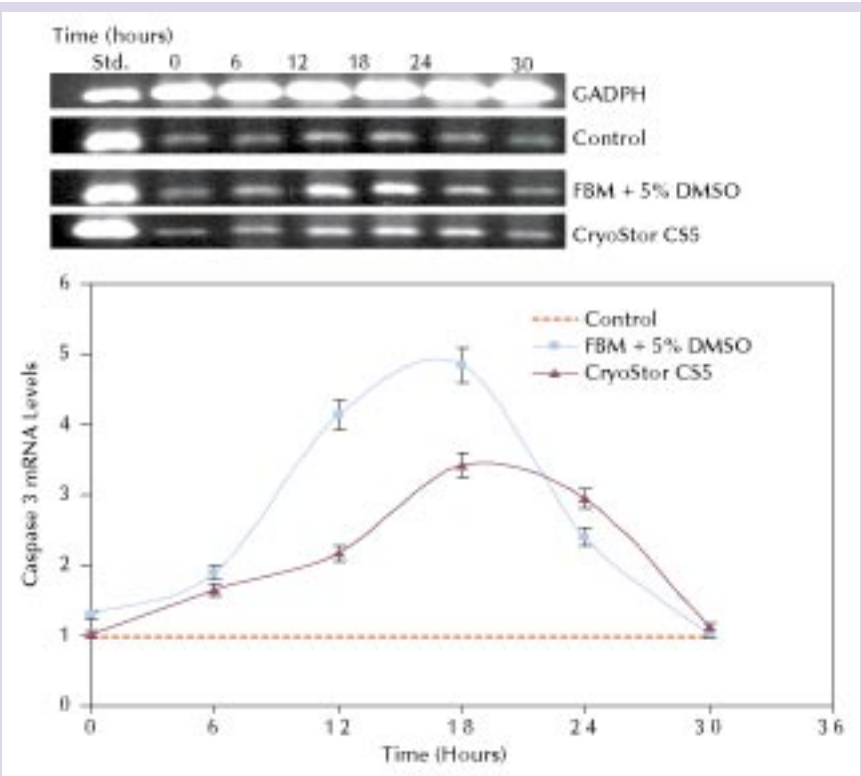

Figure 6: Post-thaw time course RT-PCR analysis of caspase-3 expression in normal human dermal fibroblasts following cryopreservation in either media + 5% DMSO or CryoStor CS 5. Caspase-3 expression increases significantly following thawing, peaking at 18 hours postthaw, and returning to that of controls by 30 hours. Preservation of fibroblasts in CryoStor CS 5 resulted in a reduction in the extent of caspase-3 up-regulation in comparison with media + DMSO samples. Caspase-3 expression levels were normalized internally to a matched GADPH standard and then compared directly to matched time point nonfrozen controls (37 °C) and converted to graphical format for comparative purposes.

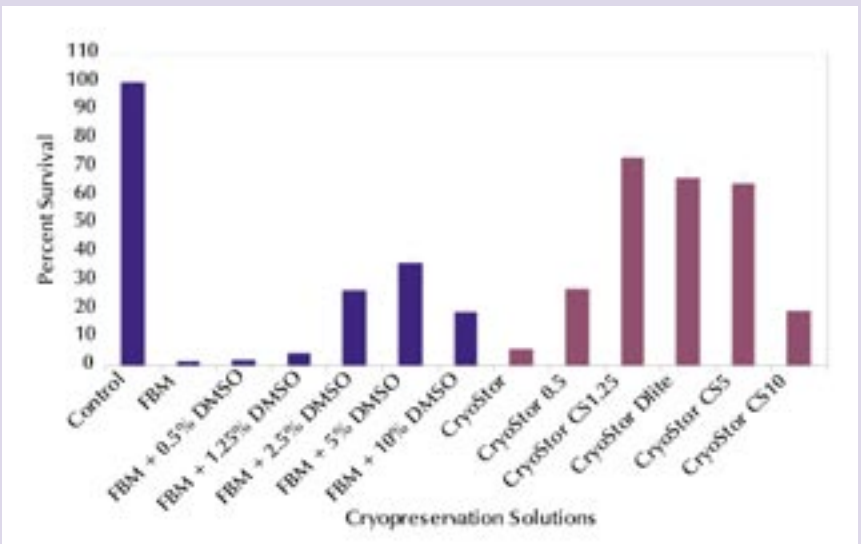

Figure 7: Normal human dermal fibroblasts following cryopreservation in fibroblast cell culture media (FBM), FBM supplemented with different levels of DMSO, CryoStor, or CryoStor supplemented with different levels of DMSO. Cells were cryopreserved using a standard stepwise process to –196 °C, thawed rapidly, and assayed 24 hours subsequent to return to normothermic temperatures for cell survival using the metabolic probe, alamarBlue. Note that improved preservation was noted with CryoStor, twice the number of cells survived in CryoStor compared to cells stored in cell culture media that contained two to four times the concentration of DMSO, and increasing concentrations of DMSO did not reduce the "cryopreservation cap" beyond a certain threshold that was solution-specific.

cryopreservation to determine how it may affect the function of the cryopreserved cells. Indeed, if a cryopreservation process yields only 50% recovery after manifestation of delayed onset cell death, then typically a three- to five-day cell culture period after return to normothermic temperatures will allow the population to replicate back to the same number originally cryopreserved. So at the expense of time and materials (which can be substantial when factoring in person-hours and product delay), the system appears to return to 100% yield. Thus, little attention has been paid to cell yield for cells that can replicate and make up the difference in the loss due to cryopreservation. Yet typical cryopreservation procedures/solutions of this type, such as media + DMSO, may artificially select for cells that are more robust in their ability to withstand the cryopreservation process — an often overlooked fact that can contribute to genetic drift.

Many cells currently being used for cell therapy applications, however, are terminally differentiated and as such are postmitotic. For instance, human pancreatic islets (**13**) and hepatocytes (**14, 16**) are currently being used or considered for cell therapies. Even replicating cells such as skeletal myoblasts used for cellular cardiomyoplasty (reviewed in the first article in this series) must be fully functional when injected directly into diseased myocardial tissue (**1, 2, 15**). Given these applications, it is even more important to consider the issue of cell function in addition to cell viability.

An example of how important both cell viability and function are to successful cell therapy applications is provided by the cryopreservation of hepatocytes in two different solutions with differing preservation qualities (**16, 17**). In a recent study by Sosef et al., primary rat hepatocytes were cryopreserved in CryoStor CS10 or hepatocyte cell culture media supplemented with 10% DMSO (**17**). In that study, albumin secretion, urea synthesis, and cytochrome activity were measured in cryopreserved samples post-thaw and compared with unfrozen matched controls (Figure 8). This study

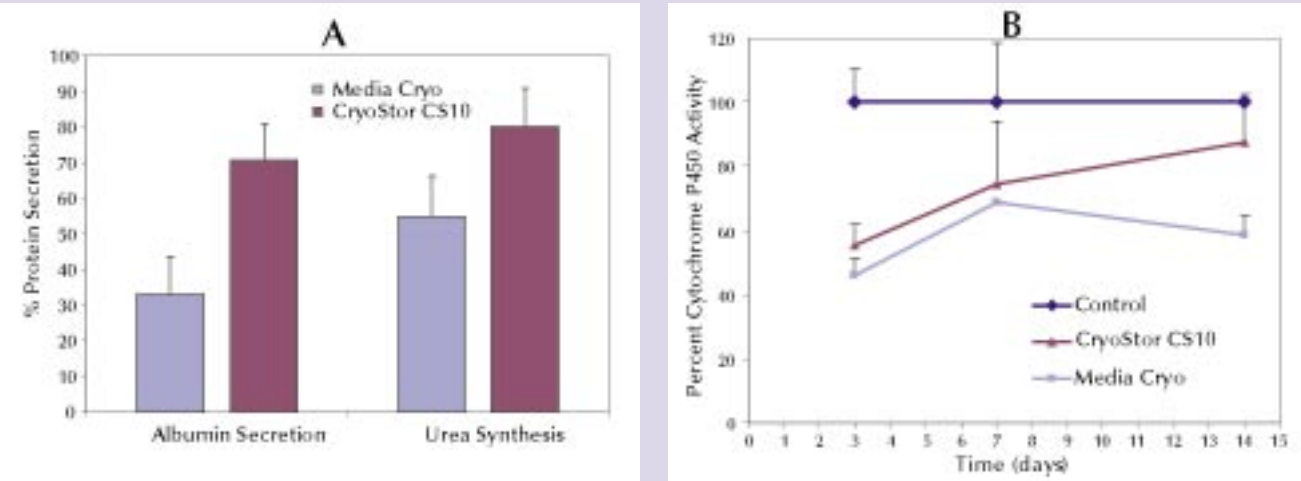

Figure 8: Analysis of cellular function following cryopreservation. Primary hepatocytes were cryopreserved in either media + 10% DMSO or CryoStor CS10 and placed into long-term culture following thawing. Analysis of (A) albumin secretion, urea synthesis, and (B) cytochrome P450 activity was assessed for a period of 7–14 days postthaw. The data show that cryopreservation of hepatocytes using specialized preservation solutions results in a greater protection/retention of cellular function as well as increased cell viability as demonstrated in previous figures. (Data reproduced from Sosef, et al.)

reported that hepatocyte cell survival and function were substantially improved in samples cryopreserved in CryoStor CS10 in comparison with the media + DMSO solution. Examination of cell function per cell revealed that the three cell-function parameters measured demonstrated a substantial improvement in the samples stored in CryoStor compared with DMSO-supplemented cell culture media (Figures 8A and B). Thus, these data support the notion that improved cryopreservation technologies can protect both cell viability and function — two criteria critical for cryopreservation protocols now serving the global cell therapy markets.

Cryopreservation and the Proteome/Genome

Developing cryopreservation protocols and solutions to satisfy the demands of the regenerative/reparative medicine market necessitates a multifaceted approach. Traditionally, formulation of cryopreservation cocktails focused on ice formation and its concomitant osmotic and physical stress on the cells. Recently, it has been demonstrated that it is critical that the cell-specific stress pathways be understood so that approaches and technologies applied for cryopreservation of cell products can be developed to target those stress pathways. One approach is to move beyond the level of the detail of the apoptotic pathways (caspase and Bcl-2/Bax) by using a proteomic and genomic approach. Initial studies accomplished in our laboratory using SELDI-TOF (surface-enhanced laser desorption and ionization time-of-flight) have shown a substantial difference in protein expression levels in cryopreserved cells. Cells were cryopreserved, total cytosolic protein isolated, and proteins were coated on a ProteinChip with selective charges. SELDI-TOF was then used to analyze the lower molecular weight proteins in cells stored in both media + DMSO and CryoStor CS5. A panel of representative SELDI-TOF protein profiles is illustrated in Figure 9 (top). These profiles were then analyzed and scored for protein abundancy in both samples (Figure 9, bottom). Note that many proteins demonstrated a several-fold change (+) in the cryopreserved samples when compared with controls. Thus, protein and DNA microarrays analysis may provide an avenue to uncover further clues to the cell stress pathways that are activated during cryopreservation and, as such, serve as a map to direct development of future cryopreservation solutions for the bioprocessing markets.

Future Directions: Modern cryopreservation sciences now have a multiplicity of challenges. One goal is to overcome the *cryopreservation cap* (**18**): the level of cell death that occurs following cryopreservation and cannot be currently overcome with increasing concentrations of cryoprotective agent. Second, whatever new formulations that may be constructed in the future to reduce that "cap" must be less toxic than current formulations that use DMSO as the primary cryoprotective agent. Third, regulatory groups are now pushing for cryopreservation solutions to be free of animal or human products.

One path to solving those three issues is to use a battery of molecular tools including enzymatic essays, RT-PCR, proteomic analysis, and DNA microarray systems to understand the cell-specific stress pathways that are activated as a consequence of cryopreservation. With such data in hand, cryopreservation solutions are being formulated and matched to the cells and tissues that will be shipped worldwide to serve the regenerative/reparative medicine, pharmacological, and bioprocessing markets.

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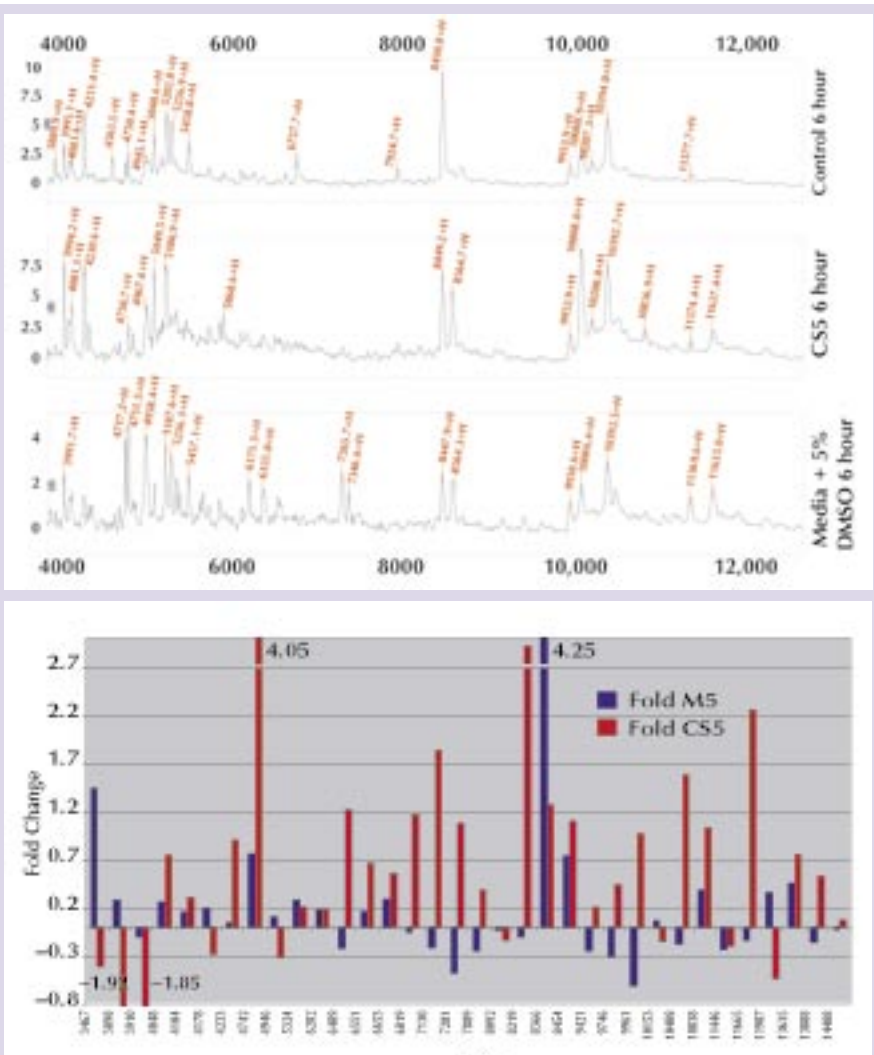

Figure 9: SELDI-TOF analysis of cytoplasmic proteins from Normal Human Dermal Fibroblasts cryopreserved in either CS5, media + 5% DMSO or not cryopreserved (Control). Note that there is a difference in the SELDI-TOF profile between all three groups (top). When comparative differences are represented (bar graph, below), it is noted that several proteins increase/decrease in abundance in cells cryopreserved in media (blue bars) compared with the same proteins identified in control cells; whereas the red bars represent the fold change of proteins in cells cryopreserved in CS5 compared with controls.

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