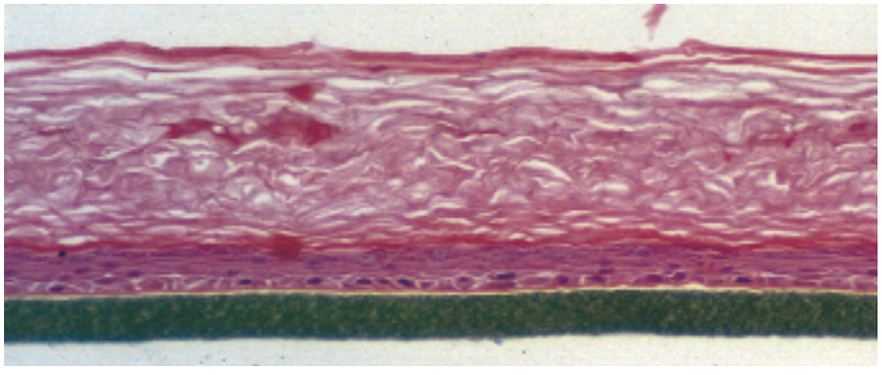
Hypothermic Storage and Cryopreservation

Successful Short- and Long-Term Preservation of Cells and Tissues

Robert G. Van Buskirk, John M. Baust, Kristi K. Snyder, Aby J. Mathew, and John G. Baust



Tissue-engineered human epidermis stored in HypoThermosol (HTS)-BASE (BIOLIFE SOLUTIONS, INC.)

Early successes of cell therapy in applications such as repair of diseased hearts (1) and treatment of Type 1 diabetes (2) support the notion that the use of cells and tissues to treat disease states is one of the most promising avenues in contemporary medical treatment. In part, many cell therapy applications evolved indirectly from the launch of tissue engineering ventures that led to commercial development of engineered products such as skin and cartilage. Cell and tissue therapy has showcased its potential in medicine through recent clinical trials and is maturing under the new rubric of regenerative/reparative medicine.

The emergence of cell- and tissue-based technologies and the move toward a global marketplace are creating a demand for new technologies that allow worldwide shipment of such products while maintaining their viability or function — a concept referred to as biological packaging (3). Our research team has previously reported on how BioHeart Inc. used improved biological packaging methods to launch its successful cellular cardiomyoplasty clinical trials with European patients while using cell production laboratories in the United States (3). Briefly, a patient biopsy is shipped hypothermically (~4 °C) from Europe to the United States. Myoblasts from the sample are isolated and expanded, and the final product of myoblasts is shipped back in hypothermic suspension to a surgical suite in Europe, where they are injected as a cell slurry directly into damaged heart tissue. This transcontinental shipment required hypothermic preservation solutions that maintain viability and function of the cells following sequential storage intervals for transport that approach a week in duration.

In the field of tissue engineering, limited success was recently reported on the development of engineered corneas (4). Ten million people worldwide suffer from corneal blindness; many reside in the Himalayas and other countries at high altitude, where ultraviolet light can be the catalyst for opacities. If optically clear, transplantable, engineered corneas were to be developed, how might such tissues be transported to these remote regions where several weeks or more of hypothermic storage may be necessary? This example also demonstrates the future importance of biological packaging and preservation in the global marketplace.

The principles governing the development of effective biological packaging necessitate an understanding of state-of-the-art hypothermic storage and cryopreservation: the two standard approaches currently used for preserving cells and tissues for

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extended periods. Comparing and contrasting these two processes can initiate a dialog to define the challenges that each system must overcome to serve the future needs of the regenerative medicine market and the bioprocessing community at large. These methodologies are complex, and a complete discussion warrants individual, focused treatises to be presented in two subsequent articles scheduled for upcoming issues of *BioProcess International*.

This first article provides a foundation to begin to explore the nuances of both processes. For example, the development of cryopreservation methods that result in increased cell yield and viability, as well as cellular function — a critical requirement for any bioprocessing program — requires a discussion of the physical, chemical, molecular, and environmental components of the system. Although our review of the elements of biological packaging provides insight into the challenges of hypothermic storage, the identification and modulation of preservation-induced molecular stress pathways and the subsequent development of improved preservation technologies based on these discoveries should be pursued at greater depth. The information provided here introduces several contemporary issues facing preservation science.

Comparing Hypothermic Storage and Cryopreservation

Hypothermic storage can be defined as the preservation of cells and tissues at chilled temperatures that often range between 4 and 10 °C; whereas cryopreservation can be defined as the storage of cells and tissues at subzero temperatures that typically range from –80 to –196 °C. Table 1 illustrates the differences between the two processes.

A comparison is useful for determining which procedure is appropriate for a given therapeutic or bioprocessing application. For instance, if cells are to be shipped short distances or held in "stasis" before use, then hypothermic storage is preferred. That was the case with the aforementioned cellular therapy example and in cases such as the on-demand use of cell plates for drug testing and toxicity screening. Cell and tissue banks, including those preserving cord blood, require extended preservation intervals from months to years (**5**). Those cases of long-term banking and storage of

Table 1: Comparison of hypothermic storage with cryopreservation

	Cryopreservation	Hypothermic Storage
Storage temperature	–80 to –196 °C	4 to 10 °C
Storage time	Months to years	Hours to days
Cooling rate	Critical	Unknown (few reports)
Warming rate	Critical	Unknown (few reports)
Loss of yield	Can be significant	Variable; dependent on storage interval
Loss of function	Can be significant	Variable; dependent on storage interval
Cell metabolism	Little activity	Depressed by temperature
Cell membranes	Damage due to increased solute levels	Permeability can be affected
Cryoprotective agent	Required (e.g., DMSO)	None needed
Serum included	Often	Variable
Stress pathways	Many activated	Many activated
Proteome	Altered	Altered
Genome	Possibly altered	Unknown

Figure 1: Coronary artery endothelial cells following one-day storage at 4 °C in HTS-FRS (left panel) and cell culture media (right panel). Cells shown were labeled with MitoTracker (red) and AlexaFluor 488-conjugated actin (green). Note that the number of viable cells is lower when cells are stored in conventional cell culture media than in specialized hypothermic storage solution, HTS-FRS.

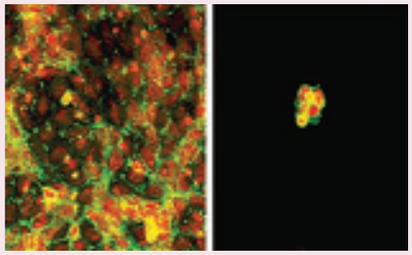
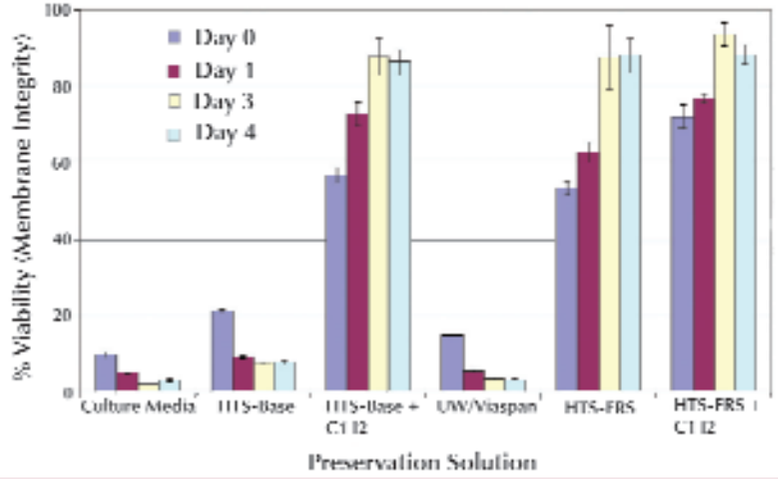


Figure 2: Coronary artery endothelial cells following two-day storage at 4 °C in cell culture media, HTS-BASE, HTS-BASE supplemented with a caspase inhibitor (C1 12), UW Solution, HTS-FRS, and HTS-FRS supplemented with a caspase inhibitor (C1 12). Cells were returned to normothermic temperatures (37 °C) for 1.5 hours (Day zero), 24 hours (Day one), 48 hours (Day two) or 72 hours (Day three). Cells were assayed for viability with calcein AM and expressed as percent control (cells not subjected to hypothermic storage). Note that cells stored in HTS FRS, HTS-BASE supplemented with caspase inhibitor, and HTS-FRS supplemented with caspase inhibitor exhibited the best levels of viability.



bioproducts demand the use of cryopreservation methodologies.

Applications in the middle of the two extremes might include the reference to corneal preservation, in which a chondroitin-sulfate-based medium called Optisol (Chiron Vision, Irvine, CA) (6) is commonly used as the hypothermic preservation solution. Although effective for certain applications, the limited storage life-support window of such solutions does not allow corneas to be shipped transcontinentally. Thus, selection of an appropriate preservation regime depends on a number of factors, and time of storage is a key rate-limiting consideration. Other factors, including shipment method, in-house and end-user storage, and end use also can play a critical role in determining storage methodologies. Regardless of whether hypothermic storage or cryopreservation is the preferred storage regime, formulation of the storage solution is critical to consider.

Figure 1 illustrates the importance of proper storage and specialized preservation solutions. In this case, human coronary artery endothelial cells (CAEC) in vitro were stored in either a specialized hypothermic storage solution technology (left) or conventional cell culture media (right). The micrographs demonstrate that storage at 4 °C for 24 hours was sufficient to demonstrate the differences between the efficacies of those solutions in preserving cell integrity. Simply cooling or freezing cells in conventional media is insufficient to protect cell function, because these types of media were designed to support biologics at normothermic temperature and cannot provide the appropriate environments for low-temperature storage. Rather, specialized solutions are necessary to protect cell viability and function during hypothermic storage or cryopreservation.

Hypothermic storage of cells and tissues for regenerative medicine is an extension of principles and technologies used for whole-organ storage in the transplant market. In this regard, a number of storage solutions are being used worldwide including EuroCollins (7), ViaSpan (University of Wisconsin, Barr Laboratories) (8, 9), Celsior (SangStat Medical Corporation) (9), and Custodiol (Odyssey Pharmaceuticals, Inc.) (7). Most of these solutions were designed two or more decades earlier than the explosion in cell therapy and tissue engineering sciences and before our understanding of the molecular-based cell stress pathways that are activated by hypothermic storage and cryopreservation. As a consequence, the solution designs do not take into account and target the control of stress pathways, which is necessary for optimal preservation of differing cell and tissue types. This becomes increasingly important because hypothermic storage may activate different sets of stress pathways that are cell and tissue specific. Thus, although these solutions have proven to be effective for shipping/storing whole organs, they were never originally intended to be used for the hypothermic storage of cells and tissues used for cell therapy — all of which may demand unique formulations to yield optimized preservation.

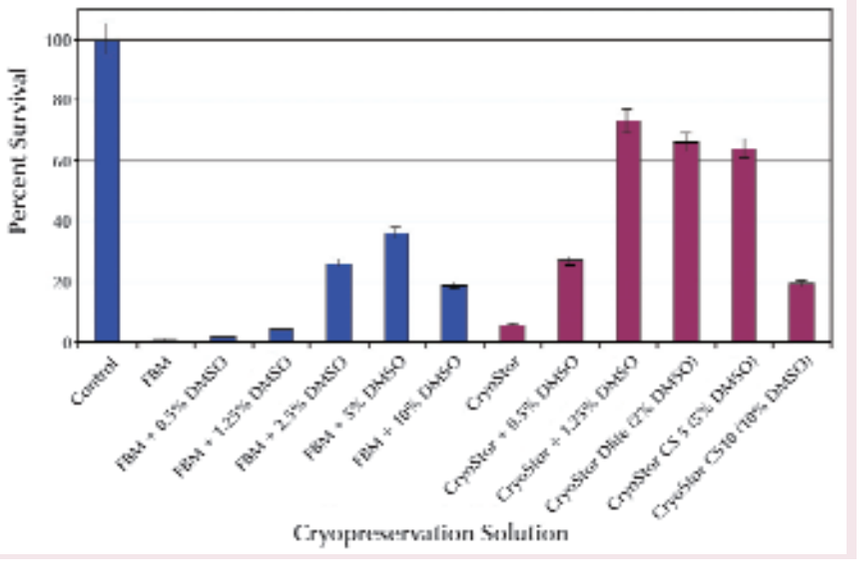
Recently the HypoThermosol (HTS) platform of solutions was introduced by BioLife Solutions, Inc. to provide enhanced preservation of biologics for use in regenerative medicine as well as in the biopharmaceutical and bioprocessing industry. Rather than a "one-size-fits-all" design such as those of the organ preservation solutions referenced earlier, the HTS platform of solutions is formulated to modulate stress pathways activated by hypothermic and cryopreservation regimes. The "multisolution hypothesis of cell preservation" concept upon which

the HTS platform strategy is formulated and its importance to the bioprocessing industry has been reported previously (**3**). It is this concept, in part, that has enabled numerous groups to market their bioproducts successfully.

The concept of cell- and tissue-matched preservation solutions arose from the observation that extended preservation can launch stress pathways such as those involved in apoptosis and necrosis (**10**, **11**). For example, human coronary artery smooth muscle cells (CASMC) were stored in cell culture media, UW solution (ViaSpan), HTS-FRS, HTS-BASE, or HTS solutions supplemented with an apoptotic inhibitor (HTS-C1I2, HTS-FRS C1I2) (Figure 2). These data illustrate that inhibition of components of the caspase cascade, specifically the executioner enzymes of apoptosis, can improve solution performance. Interestingly, our research team determined that some caspase inhibitors work with some cell types but are relatively ineffective with others. For instance, in one case a select caspase inhibitor worked well to increase the hypothermic storage time of a human transformed cell line but failed to show similar levels of protection when the normal cell strain counterpart was used in place of the cell line (unpublished data). Thus, the stress pathways activated by extended storage times may be specific to both cell type and phenotype (cell line and cell strain).

It is also important to appreciate that the activation of the caspase cascade can take several days to be fully manifested once cells and tissues are removed from storage and returned to normothermic temperatures — a fact that has critical implications to regenerative medicine and bioprocessing. Full function is restored typically within the first 24 hours of return to normothermic temperatures. Cell viability and function of select cells and tissues, such as engineered human epidermis stored for extended periods, can exhibit viability similar to control levels immediately on warming, but a decline in viability to less than 10% of control levels can occur over a week following (**12**). Similarly, in the case of CAEC it is noteworthy that the viability of cells stored in three of the solutions declined following storage; whereas the viability of those stored in the three specialized solutions increased because the surviving cells retained replicative ability and began to repopulate (**10**, **11**) (Figure 2). These observations highlight the importance of examining posthypothermic storage cell viability and function for a week in vitro of cells designated for therapeutic applications as a method to predict posttransplant outcome. Thus, it is critical that these time-dependent, cell-specific stress pathways be thoroughly analyzed and understood along the path of developing improved hypothermic storage procedures for the regenerative/reparative medicine and bioprocessing markets.

Figure 3: Normal human dermal fibroblasts following cryopreservation in fibroblast cell culture media (FBM), FBM supplemented with different levels of DMSO, CryoStor, and CryoStor supplemented with different levels of DMSO. Cells were cryopreserved using a standard stepwise process to –196 °C, thawed rapidly, and then assayed 24 hours subsequent to return to normothermic temperatures for cell survival using the metabolic probe, alamarBlue. Note that (1) improved preservation was noted with CryoStor, (2) twice the number of cells survived in CyoStor compared with cells stored in cell culture media that contained two to four times the concentration of DMSO, and (3) increasing concentrations of DMSO did not reduce the "cryopreservation cap" beyond a certain threshold that was solution-specific.



Cryopreservation: The discipline of cryopreservation emerged from an academic endeavor to a commercial reality in 1948 when Polge, Smith, and Parkes found, serendipitously, that glycerol greatly facilitated the freezing of fowl sperm at temperatures of –70 °C (for a review, see **13**). Since then, a number of cryoprotective agents, such as ethanediol, propanediol, hydroxyethyl starch, and propylene glycol have appeared to be prerequisites for successful cryopreservation.

The protective agent most often used in cryopreservation is dimethyl sulfoxide (DMSO) (**14**). It was used in the wood industry in the 1800s and incorporated into biological applications in the 1960s. It is an amphipathic molecule with a highly polar domain and two apolar groups that allow it to be soluble in both aqueous and organic solutions. DMSO has been used as a therapeutic to treat amyloidosis, gastrointestinal disorders, brain edema, and a number of dermatological disorders. Its use as a cryoprotective agent is well studied.

Yet for its versatility as a near-universal solvent and its position as the most effective cryoprotective agent, DMSO has a number of toxic effects of concern to those working in regenerative medicine and

bioprocessing. For instance, DMSO is widely used as a cryoprotectant for hematopoietic stem cells, but it is toxic to both the cells and patients (**15**). At higher concentrations, it can cause intracranial hemorrhage (**16**). Although the mechanism(s) underlying DMSO's toxicity is not yet fully understood, DMSO is known to activate heat-shock protein (HSP) 70 levels at certain concentrations (**17**).

Given these toxic characteristics of DMSO, many have searched for alternative cryoprotectants that provide the same level of protection but with less toxicity. A few alternatives have shown limited promise at the research level, but none have translated into the industrial marketplace.

Although eliminating DMSO has been a formidable challenge, there has been recent success at reducing the levels of DMSO in cryopreservation cocktails while improving cryopreservation efficacy. New cryopreservation technologies such as CryoStor can improve cryopreservation outcomes while reducing the level of DMSO necessary for successful preservation (Figure 3). In one study (**18**), normal human dermal fibroblasts were cryopreserved using a standard cocktail consisting of the cell culture medium (FBM) supplemented with different levels of DMSO or CryoStor supplemented with the same levels of DMSO. Samples stored in CryoStor showed twice the viability using one-half to one-fourth the levels of DMSO (Compare FBM + 2.5% DMSO to CryoStor + 1.25% DMSO). The data further illustrate that despite the carrier solution, there appears to be a "cryopreservation cap": a level of viability beyond which increasing levels of DMSO cannot be overcome.

Data presented elsewhere highlight the key concept that cell function and viability are intimately linked (**19, 20**). In other words, half the viability in a set of cryopreserved cells implies that the cell function (per viable cell) may also be compromised to a similar extent. Animal serum has often been added to further protect cell function and viability during the cryopreservation process, but that is unacceptable for bioprocessing and regenerative medicine applications for which serum-free formulations are essential, to preventing the spread of zoonotic disease.

Also of note is the role of apoptosis and necrosis stress pathway activation as a consequence of cryopreservation, which can lead to a several-day decline in cell viability and function upon return to normothermic temperatures (**10**). How the new approaches and technologies are serving as stepping stones toward the successful cryopreservation of complex biologics such as engineered tissues is discussed in another forum (**21**). These factors represent key issues that must be overcome to improve cryopreservation sciences and adequately fulfill the emerging demands of the cell and tissue bioprocessing industry.

New Challenges Confronting the Preservation Sciences

Recent biotechnology initiatives in cell and tissue therapy, stem-cell banking, and the use of human primary cells such as hepatocytes for drug toxicity testing demand development of improved methods of biological packaging. Military and civilian use of cells and tissues as sensor sentinels and to detect viral or bacterial infections in hospitals and other settings are on the horizon. The acute shortage of human organs for transplantation also calls for improved preservation protocols and solutions. In this quest it is clear that many current problems confronting hypothermic storage and cryopreservation must be addressed, including removal of animal serum from preservation protocols, replacing DMSO with a less toxic cryoprotective agent, inhibiting the several-day decline in cell viability often noted under extended preservation regimes once cells are returned to normothermic temperatures, and resolving the critical limitation of the cryopreservation cap.

As these and other challenges are addressed, advances in preservation technologies will be made, leading to improvements in cell and tissue therapy and cell banking practices, as well as increasing the ability to meet human organ and tissue transplantation needs.

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Robert G. Van Buskirk *is senior vice president of Cell Preservation Services (CPSI), Owego, NY, and a professor in the Department of Biology at the State University of New York at Binghamton (SUNY).* ***John M. Baust*** *is president and CEO of CPSI and a research assistant professor in the Department of Bioengineering at SUNY.* ***Kristi K. Snyder*** *is a graduate research fellow in the Institute of Biomedical Technology at SUNY and a principal scientist at CPSI.* ***John G. Baust*** *is president/CEO of BioLife Solutions Inc., Owego, NY, and professor of biology and director of the Institute of Biomedical Technology at SUNY.* ***Corresponding author Aby J. Mathew*** *is director of manufacturing at BioLife Solutions, Inc. and can be reached at amathew@biolifesolutions.com, 1-607-687-4487.*