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Biological Packaging for the Global Cell and Tissue Therapy Markets

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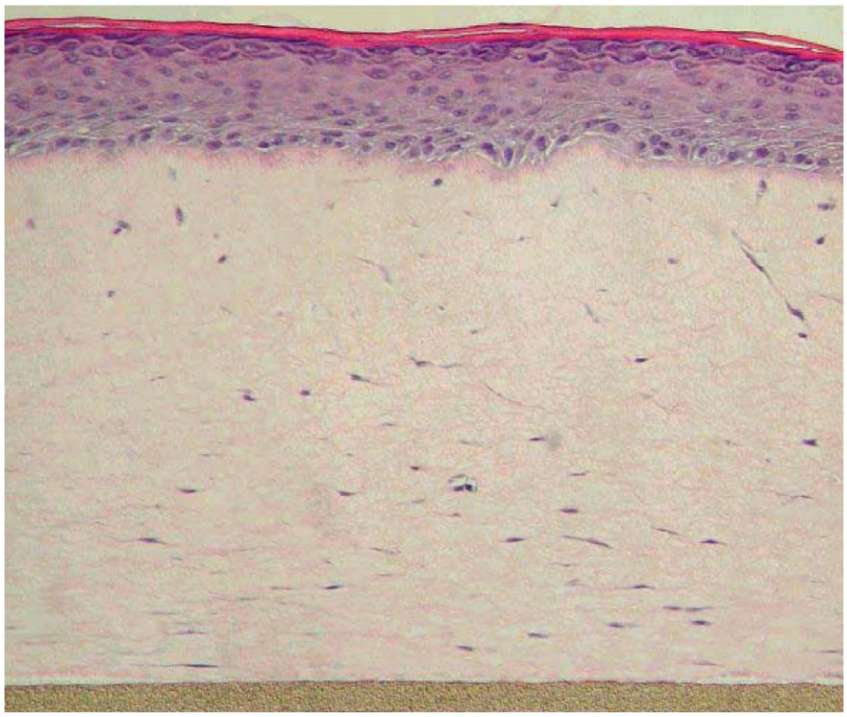

Figure 1. Engineered Tissue Constructs. Several companies provide engineered tissue constructs for clinical and non-clinical applications. The engineered skin EpidermFT (MatTek Corporation), is currently shipped worldwide for use in the product safety testing market.

The globalization and sustained growth of the biotechnology market has brought the issue of biological packaging to the fore, particularly for those companies invested in cell and tissue bioproducts, such as engineered tissues and cells used for cell therapy.[1,2] *Biological packaging* can be defined as the sum total of the physical device, temperature regulating and monitoring systems, type of preservation solution, and storage protocol(s) necessary to maintain cells or tissues in a "state of suspended animation" during transport or storage. The ideal biological package provides for the transport of cells and tissues throughout the global marketplace while maintaining both the viability and the function of the biological system at levels equivalent to those measured prior to shipment. Cells and tissues are currently shipped and stored under hypothermic (4–8° C) or cryopreserved (–80 to –196° C) conditions. These two processes have remained relatively unchanged over the past several decades, limiting their utility in the storage of modern bioproducts. However, recent evolutions in biological packaging have begun to provide scientific and financial benefits to researchers, clinicians, and corporate entities.

The goal for all cell- and tissue-focused companies is to produce a safe and effective bioproduct. This process may require transport of initial biologic material from clinical sites to research laboratories, necessitating a low temperature regime that produces minimal damage to the valuable biologic material. Following production, the final bioproduct is likely to experience further storage intervals prior to and during shipment to the end user. Therefore, the effectiveness of biological packaging is an important consideration for companies and researchers invested in the fast-growing regenerative and reparative medicine fields, *in vitro* toxicology testing arenas, and global collaborative research, as well as in clinical and transplantation applications.

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Recent Applications of Cell and Tissue Technologies

The transplantation of isolated pancreatic islets is now an option for the treatment of diabetes, and extracorporeal liver-assist devices have been successfully tested in clinical trials.[3,4] As pharmaceutical and cosmetic companies continue to employ animal replacement testing models, engineered human epidermis, such as EpidermFT (MatTek Corporation, Ashland, MA) (Fig. 1), is being utilized for such testing in the United States, Europe, Asia, and South America. Many companies, including Cambrex Bio Science (Walkersville, MD), CellzDirect (Tucson, AZ), and InVitro Technologies (Baltimore, MD) provide fresh or cryopreserved primary cell types for pharmacological testing. Other groups, such as ViaCord (Boston, MA), have begun establishing cell repositories for banking umbilical cord blood cells as an "insurance program" that may provide an autologous cell therapy option for treatment of future disease.[5] Other bioproducts in development, including engineered corneas and bioartificial kidneys, serve as clear indicators that cell and tissue therapy has a promising future.[6,7] The implementation of these cell therapy applications necessitates that improved preservation regimes be developed to address the global demands of shipping human cells and tissues worldwide.

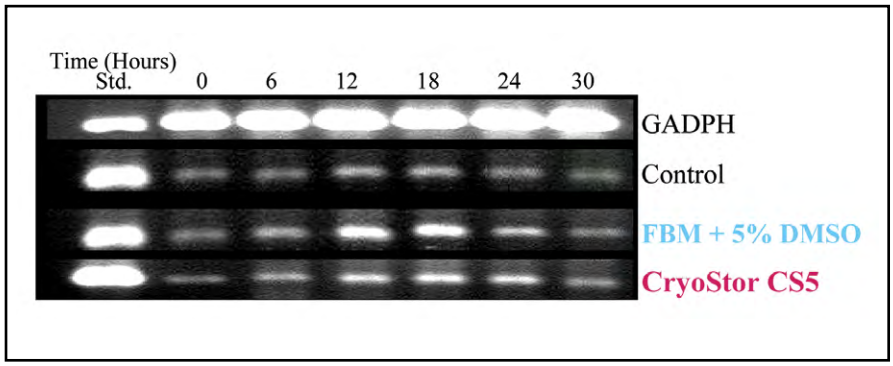

Figure 2. Molecular Alterations Following Cryopreservation. Time-course RT-PCR of cryopreserved human fibroblasts showing increased activation of caspase-3, peaking at 12 hour post-thaw.

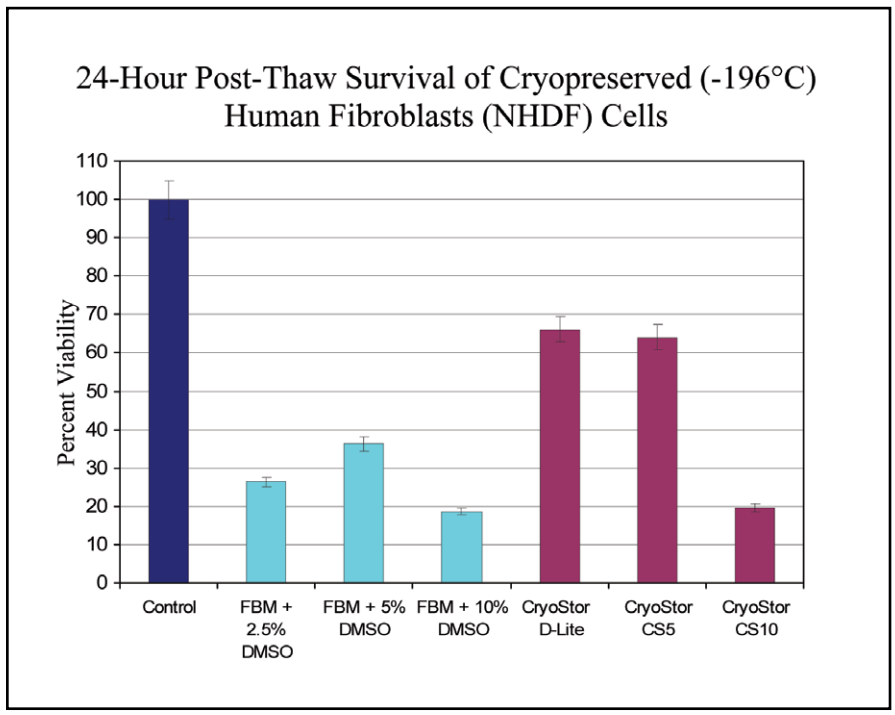

Figure 3. Cryopreservation Cap. Cryopreservation of fibroblast cells in varying concentrations of DMSO in two types of carrier solutions.

Cryopreservation

As discussed previously, cell therapy and tissue engineered products shipped long distances currently are either hypothermically stored or cryopreserved. Each process has its advantages and limitations. The discipline of cryopreservation was born in 1948 when Polge, Smith, and Parks found that glycerol facilitated the frozen storage of fowl sperm to temperatures of –70° C.[8–11] A number of cryoprotective agents, including ethanediol, hydroxyethyl starch, dextran, and propylene glycol, has since been identified as prerequisites for successful cryopreservation.[12–15] The cryoprotective agent most often employed for cryopreservation is dimethyl sulfoxide (DMSO).[16] DMSO, a wood industry byproduct discovered in the 19th century, has been used as an industrial solvent for more than 100 years but wasn't incorporated into biological applications until the 1960s when Lovelock and Bishop reported on its cryoprotective properties.[17] DMSO is an amphipathic molecule with a highly polar domain and two apolar groups that allows it to be soluble in both aqueous and organic solutions. Current cryopreservation protocols originate from research established over the last several decades that specifies freeze rates of –1° C/min in solutions, coupled with cell culture media containing high concentrations of DMSO and animal-derived products (e.g., serum, albumin).

In order to successfully cryopreserve and thaw a biological specimen, several risk elements must be addressed. For example, formation of ice crystals may cause irreparable damage to cell structures. Osmolality changes during the process can cause extreme changes in cell volumes and ionic concentration balances, which in turn affect the pH of the system. Additionally, many cryoprotective agents, including DMSO, have varying toxic effects on cells.

Three major concerns related to cryopreservation have served to focus the development of typical cryopreservation protocols. First, the formation of ice crystals can physically damage membranes; the freeze concentration of solutes can add further insult. Second, multi-layered complex tissues are difficult to cryopreserve and subsequently achieve full restoration of viability and function upon return to normothermic temperatures (due to cryoprotective agent permeability and cooling/warming rate issues associated with sample size and thickness). Finally, DMSO has been shown to be cytotoxic, resulting in a myriad of serious side effects including renal failure, hypertension, cardiac arrest, pulmonary edema, nausea, and hemolysis.[16] A recent study comparing DMSO-infused and DMSO-depleted peripheral blood or bone marrow stem cell autografts demonstrated substantially fewer side effects and sustained engraftment in patients receiving DMSO-depleted cells.[18] Additionally, utilizing reduced concentrations of DMSO for peripheral blood progenitor cells cryopreservation protocols yielded greater percentages of viable CD34+ cell and provided significantly lower levels of apoptosis and necrosis.[19] At the cellular level, DMSO exposure may lead to the collapse of the mitochondrial transmembrane potential, release of cytochrome *c* from the mitochondria, and activation of caspases-9 and -3, launching apoptosis (programmed cell death).[20] Thus, one of the challenges of the biological packaging industry is to develop new cryopreservation techniques that reduce or eliminate DMSO and other cytotoxic agents, and animal-derived products, while maintaining equal or better results than current processes produce.

In addition to issues with cytotoxic cryoprotective agents, the base carrier medium should also be considered. Current cryopreservation protocols utilize standard cell culture media supplemented with cryoprotective agents such as DMSO. This media is not designed to maintain or support cellular function at low temperatures and cannot protect the biologics from drastic temperature-related changes (e.g., pH shifts, ionic imbalances, biochemical uncoupling) experienced during the cryopreservation process. Recently developed specialized solutions designed to counterbalance the hypothermic transition and molecular cascades activated during the process are facilitating the improved cryopreservation of complex bioproducts. For example, the incorporation of molecular biological approaches to solution design has led to solutions such as CryoStor™ (BioLife Solutions, Inc., Owego, NY) which modulate cellular stress responses, such as caspase activation (Fig. 2), to the cryopreservation process. These new solutions improve post-thaw cell survival and recovery while allowing for a reduction in the level of cryoprotective agent, and eliminates the need for inclusion of animal proteins and serum (Fig. 3).[21–23]

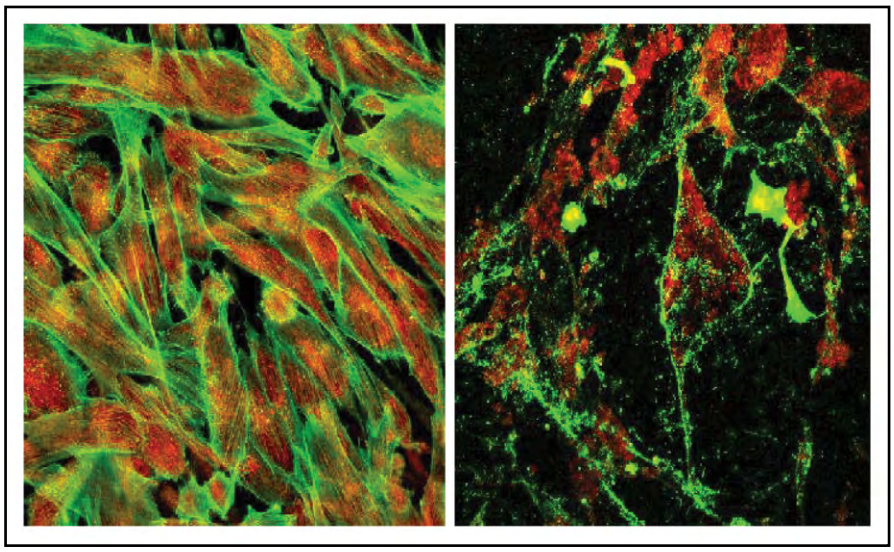

Figure 4. The Importance of Preservation Solutions. Normal Human Epidermal Keratinocytes were hypothermically stored in either a commercially available preservation solution (left panel) or in standard cell culture media (right panel) for two days at 4° C. Micrographs following the hypothermic storage period examining mitochondrial activity (MitoTracker) and cytoskeletal elements (AlexaFluor 488-actin) demonstrate the failure of cell culture media to preserve the cells and the intact monolayer and respiring cells of those stored in appropriate storage solutions.

Hypothermic Storage

Hypothermic preservation is designed to lower the metabolic activity of the cells or tissues for short time intervals, ranging from hours to days, to facilitate shipment and storage of the biological material. Although successful, a mere reduction in temperature is not sufficient to protect systems from a loss of cell viability and function. This fact was noted nearly 25 years ago and was one of the driving factors in the development of the University of Wisconsin solution (ViaSpan®; Barr Laboratories, Pamona, NY), which is considered the current "gold standard" for organ preservation. A number of cold storage solutions has been developed in the last two decades, but ViaSpan commands the largest market share in the U.S. organ preservation market. Celsior® (SangStat, Fremont, CA), and Custodiol® HTK (Odyssey Pharmaceuticals, East Hanover, NJ) are more prominent in the European market.[24–26]

More recently, a new platform of hypothermic preservation solutions — the HypoThermosol® (BioLife Solutions, Inc.) family — has been developed based on investigations into the stress pathways activated during extended hypothermic storage.[11,27–30] The base formulation of these solutions was originally designed as a whole-body hypothermic perfusate solution.[27,28] Investigations into the signaling pathways of cell death cascades activated during and following hypothermic storage have led to marked improvements in preservation solution efficacy, allow-

ing for an extension of the storage time interval while maintaining the viability and function of systems in a state similar to that of fresh systems. This is a welcome improvement on traditional hypothermic preservation approaches in which systems can succumb to stress associated with the preservation process, resulting in cell death and system failure (Fig. 4)

Cellular Cardiomyoplasty

The importance of biological packaging is best illustrated by the cellular cardiomyoplasty program at BioHeart, Inc. (Weston, FL).[31] Cellular cardiomyoplasty has recently emerged as a promising method to regenerate functional myocardium *in situ*.[31–33] The recent translation of this technology from an academic ischemic animal model to human clinical trials has demonstrated that cellular cardiomyoplasty can restore heart function. The global importance of BioHeart's dual biological packaging process is illustrated in Figure 5.

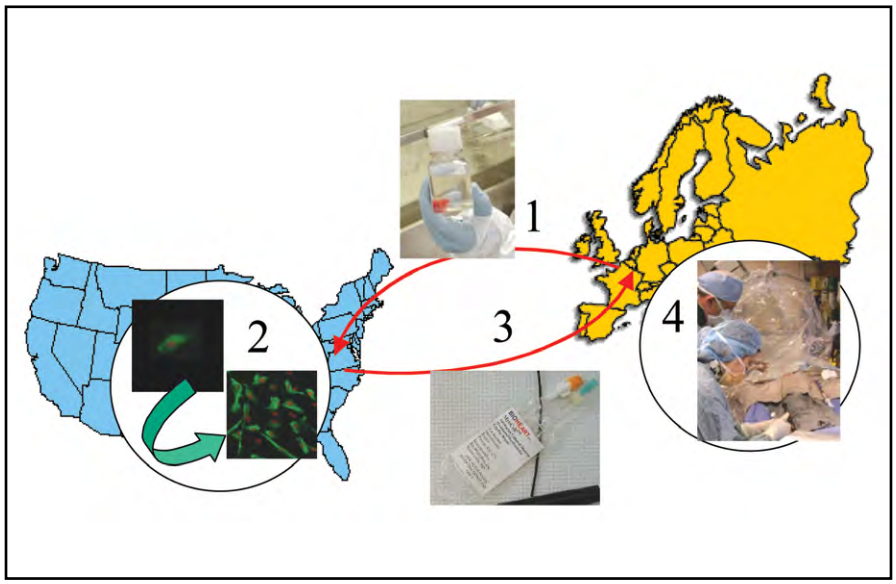

Figure 5. The Two-Way BioHeart Transport Challenge. A human skeletal muscle biopsy is retrieved from a patient in Europe (1) and transported under hypothermic conditions in an appropriate solution to Cambrex in Maryland, USA, a process which takes 36–52 hours. At the Cambrex facility, myoblasts are isolated and expanded (2). The cell slurry is then shipped back to the surgical team in Europe (3) under hypothermic conditions, and finally the cells are re-implanted via a catheter into the patient with the diseased heart tissue (4).

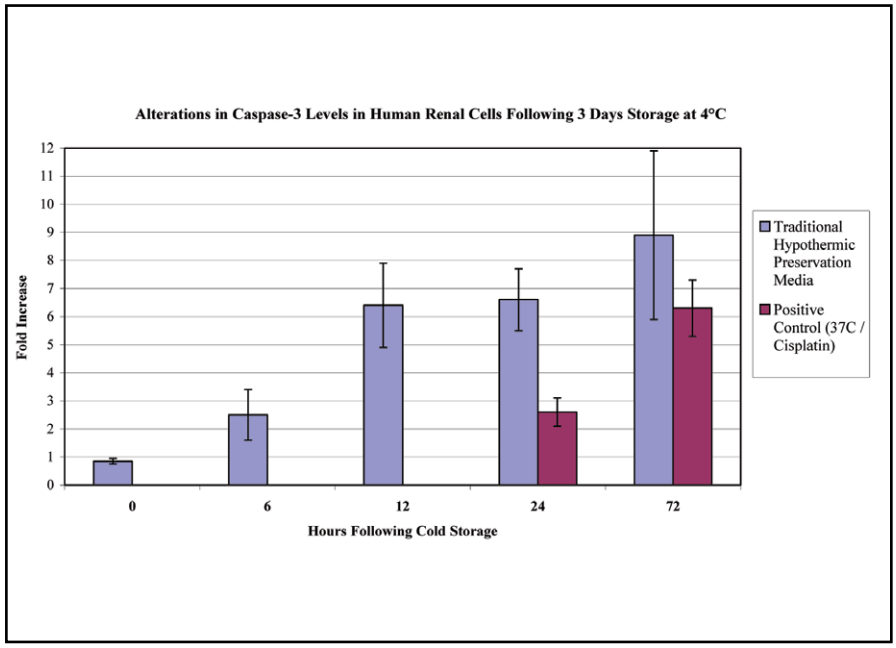

Figure 6. Apoptosis following hypothermic storage. Human renal cells were stored for three days in a conventional organ preservation solution and caspase-3 activity was monitored 0, 6, 12, 24 and 72 hours subsequent to return to normothermic temperature (37° C). Caspase 3 activity steadily increases over the observation period.

In a study that evaluated procedural efficacy in humans, patients in Europe were selected who had a previous anterior wall myocardial infarction and depressed left ventricular function. The basic procedure involved harvesting a biopsy from the quadriceps muscle of a patient, placement of the biopsy into hypothermic storage, and shipment to an isolation and expansion facility (Cambrex Bio Science) in the United States. The transcontinental transit time of the biopsy ranged from 35 to 50 hours (Fig. 5). Upon arrival, skeletal muscle myoblasts were isolated, expanded in culture, harvested and tested for appropriate immunohistochemical markers, placed into suspension in hypothermic storage, and shipped from Maryland back to clinical sites in Europe where the cells were transendocardially injected into the infarcted area of the heart.

The storage and transit interval of the expanded myoblasts under hypothermic storage conditions ranged from 24 to 96 hours with a validation of the bioproducts' hypothermic storage "packaged life" of at least 96 hours. Some data supported storage intervals of up to seven days — a time span that far exceeds the approved shipping time for transplanted organs.[34] Considering the product shipment parameters (time and distance) involved in this study, the integration of and reliance on biological packaging processes become quite apparent. To facilitate the shipment of specialized bioproducts, BioHeart first recognized the critical role that biological packaging played in the success of its trials and in the commercialization of its product, then turned to experts to develop specialized preservation solu-

tions that allow for extended storage and enhanced cell viability. This example demonstrates the critical nature of customized collaborative efforts, including specialized laboratories, biological packaging, and protocols in the successful development and commercialization of biotherapeutic products.

Cell- and Tissue-Matched Preservation Solutions

Most, perhaps all, common hypothermic storage solutions, including ViaSpan, were developed as whole organ transport solutions designed for hypothermic conditions at the organ level, rather than addressing the needs of the individual constituent cell types. Furthermore, the development of this generation of preservation solutions did not focus on elucidating and modulating the molecular stress and cell death pathways activated as a consequence of extended hypothermic storage or cryopreservation. In fact, an effort to understand these pathways has only been recently undertaken. It is now appreciated, however, that a variety of both apoptotic and necrotic stress pathways are activated when cells are biopreserved (whether hypothermically or cryopreserved), and that modulating these pathways can improve the preservation efficacy of hypothermic storage and cryopreservation solutions.[23,29]

Recent studies by Mathew *et al.* detail the activation of caspase-3, an apoptotic executioner protease, following hypothermic storage in a conventional organ preservation solution (Fig. 6).[29,30] These reports further describe the ability of specialized preservation solutions, designed to modulate free radical generation and caspase activation, to reduce the level of caspase enzyme activation, thereby improving preservation efficacy. In addition to these reports, numerous investigators have begun examining the roles that apoptosis and necrosis play in preservation outcome. An example of the complexity of these cell death pathways is illustrated in Figure 7, which focuses on apoptotic activation and progression revolving around the mitochondria. It is important to bear in mind that each cell type may respond differently to preservation-induced stress, thereby activating a different set of stress pathways, thus presenting a challenge to the future design of preservation solutions.

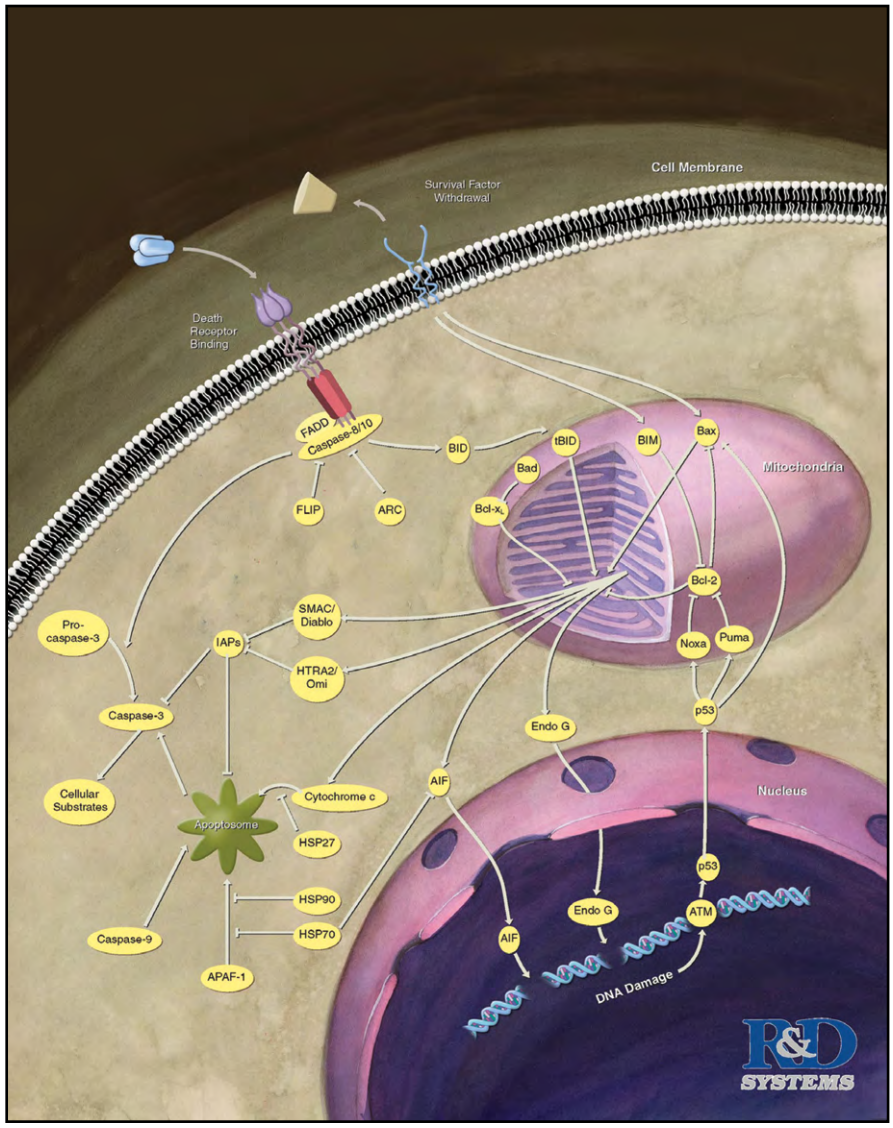

Figure 7. The Mitochondrial Paradox. Research is discovering that the mitochondria appear to play a diverse number of roles in both apoptosis and necrosis. Many of these activities involve the release of cytochome c, synthesis/translocation of the bcl-2 family of proteins, and Caspase activation (Art work by permission from R&D Systems).

Given these recent discoveries, it is now appreciated that it is critical to understand the specific stress pathways activated in cells and other bioproducts during and following preservation in order to facilitate the development of unique, cell-matched preservation solutions. This multi-solution concept differs from the "one size fits all" approach of conventional hypothermic preservation solutions and some cryopreservation protocols. This paradigm shift in preservation solution design and development stems from a natural evolution of our understanding of apoptosis and necrosis pathways and from the need for a new generation of hypothermic storage and cryopreservation solutions.

The development of the HypoThermosol family of solutions resulted from investigations of the preservation-associated cell stress response pathways, including the examination of caspase activation, anti- and pro-apoptotic protein expression, as well as extended monitoring of post-preservation cell

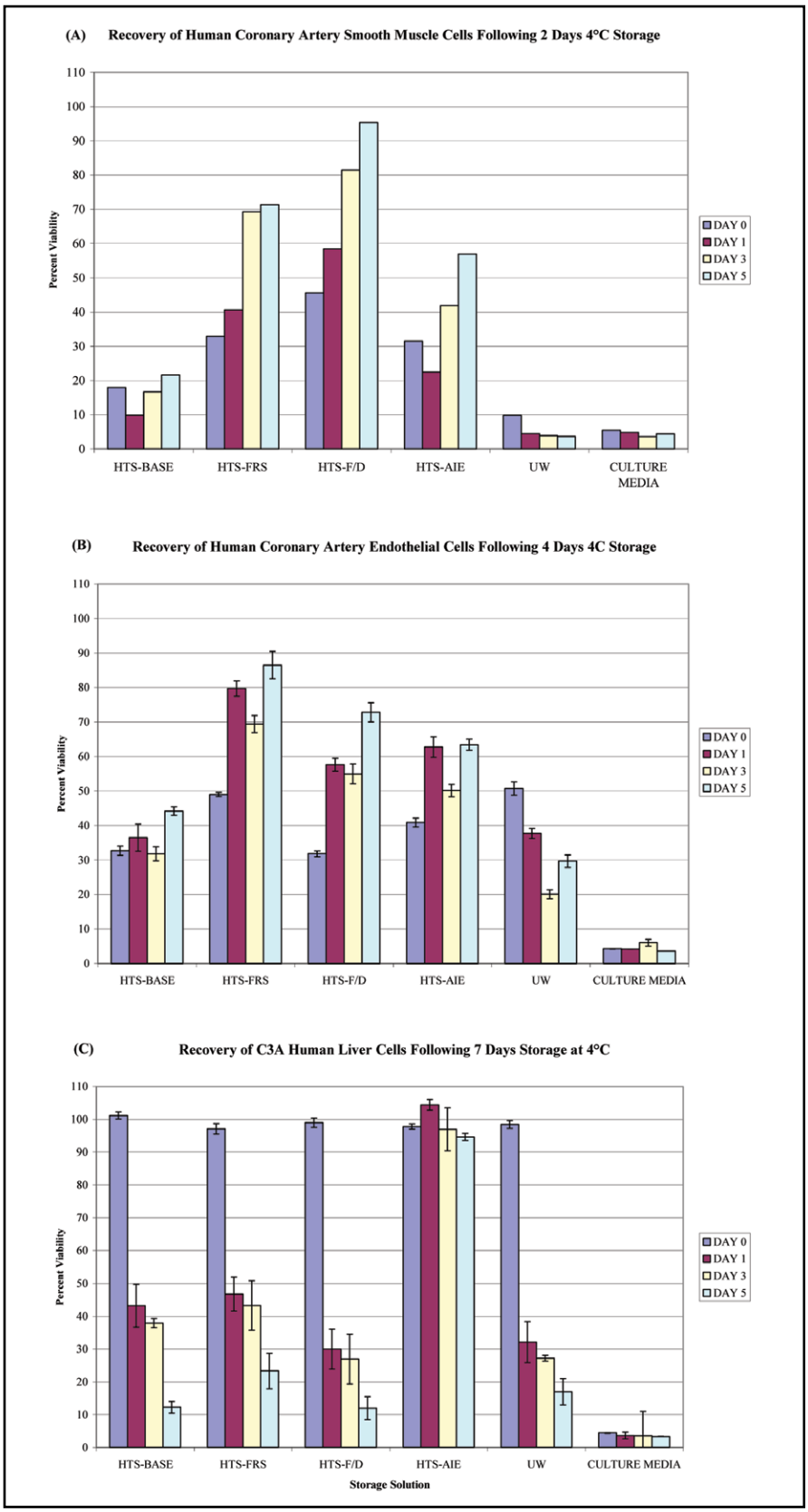

Figure 8. Multi-Solution Hypothesis for Hypo-thermic Storage. Human coronary artery smooth muscle, endothelial and hepatocarcinoma cells were stored for 2, 4 or 7 days at 4° C in a panel of solutions including HypoThermosol® variants, UW Solution (ViaSpan®) and cell culture media. Each tested cell type was best protected during hypothermic storage by a distinct preservation solution.

viability, repopulation, and function. To date, many cell types have been investigated, resulting in the development of numerous solution variants. Examples of the cell systems investigated include the hepatocyte cell line C3A, and human coronary artery smooth muscle and endothelial cells.

A recent study by Mathew *et al.* showed that each of these distinct cell types displays a differential level of survival and specific molecular responses to the preservation process.[30] In this study, a panel of preservation solutions (including HypoThermosol variants, culture media, and ViaSpan) was evaluated to assess solution efficacy (Fig. 8). A comparison of smooth muscle (A) and endothelial cells (B) derived from human coronary artery tissue demonstrates that two solutions, HTS-F/D and HTS-FRS, respectively, provided the highest level of protection to these cell systems during extended hypothermic storage. In contrast, C3A cells (used in extracorporeal liver-assist devices) demonstrated a differential stress response to preservation that did not parallel that of the smooth muscle or endothelial cells, and were therefore best preserved in HTS-AIE (Fig. 8C). Thus, this report demonstrated that, within the groups tested, each cell type was optimally preserved using different solutions that were designed, in part, based on the respective stress pathways activated as a consequence of extended preservation.

These data, as well as other recent reports, support the premise that optimal preservation requires the development of solution variants that match cell-specific stress pathways. By doing so, these cell- and tissue-matched preservation solutions will be able to support the continued development and global distribution of bioproducts utilized in cell and tissue therapy, as well as in pharmaceutical and *in vitro* toxicology applications. Examining the molecular responses of cells unable to withstand hypothermic or cryopreservation storage in standard solutions enabled the development of novel solutions that reduce the cell-specific activation of stress pathways during low-temperature storage protocols. These technological advancements have allowed researchers

to improve results where partial failure was previously accepted as "a cost of doing science."

Summary

Regenerative and reparative medicine therapies have now been proven as successful approaches to treat several disease indications. As the use and development of these biological-based technologies continues to expand and evolve, the issue of biological packaging will become increasingly important. Growing demands for the elimination of human- and animal-derived proteins and sera from preservation protocols, the development of alternative cryopreservation processes that reduce or eliminate the need for DMSO, and the extension of hypothermic storage intervals, that protect viability and function, are now exceeding the capabilities of commonplace preservation technologies, methodologies and approaches. Novel advances in the biotransport sciences will facilitate the continued progression of bioproduct development and distribution. Continued research and development in the biological packaging arena will allow the cell and tissue marketplace to continue to prosper in the global economy and will provide the keys to more efficient utilization of biologics in research, testing, and clinical trials.

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