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Chapter 49

Frontiers in Biopreservation Technology: Challenges for the Storage of Living Tissues and Engineered Constructs

Michael J Taylor Ph.D. and Kelvin G.M. Brockbank Ph.D.
Lead Author Michael J Taylor Ph.D., Adjunct Professor of Surgery, MCP-Hahnemann University

ABSTRACT

Modern day medicine has entered the post-genomic era when decoding of the human genome is expected to provide the basis for mapping a route that will define the genetic basis and provide the keys to the eventual cure of human diseases. This new era has also seen the emergence of new disciplines that involve manipulative engineering to provide remedies to a wide variety of conditions ranging from autoimmune diseases such as diabetes to degenerative diseases and even aging. The new discipline of tissue engineering, in its broadest sense, seeks to apply the principles of biology and engineering to produce substitutes for a variety of human tissues and organs in need of repair, restoration or replacement. The medical need for tissue replacement therapy is enormous and growing, in part because of the scarcity of donor tissues and organs, and in part because of the difficulties and complexities of overcoming immune rejection.

It is intuitively obvious that these new disciplines of tissue engineering and regenerative medicine will play important roles in the development of anti-aging medicine. Moreover, within these emerging new fields of medicine there is a crucial need for the parallel development of enabling technologies such as biopreservation. It is universally recognized that effective methods of preservation are critically important for ensuring an on-demand supply of the best quality cells, tissues and organs. Biopreservation is also important for the stabilization of natural and engineered tissues to permit transportation and extended shelf life.

Tissue engineering is an interdisciplinary field that has largely trivialized the importance of cell and tissue storage. To the best of our knowledge, issues relating to extended product shelf life of most tissue-engineered products, which are at various stages in the commercial pipeline, have still not been adequately addressed. Current practices and challenges for the storage of engineered tissues and allogeneic products will be reviewed. In addition, new technologies in development for cell and tissue storage will be presented with respect to their potential impact on the "shelf-lives" of allogeneic transplants and future tissue engineered medical products (TEMPS).

Keywords: Biopreservation; Cryopreservation; Hypothermia; Preservation solutions; Suspended Animation; Tissue Engineering

INTRODUCTION

Preservation, though it may be brief, is a necessary and essential component of any transplant procedure. It is no less important in the field of tissue engineering in which cells and tissue constructs are manipulated outside the body. The National Institute of Standards and Technology (NIST) has identified four research areas (automation and scale up, sterilization, product storage, and transportation of product) in which substantial technical innovation is required for the development of manufacturing processes [NIST Advanced Technologies Program Request for Proposals – 1997]. Concerns for the issues relating to the transition from the laboratory to the market include the major problem of preservation and storage of living biomaterials. Manufacturers and/or distributors recognize the need for maintaining stocks of their products to ensure a steady supply, while the unpredictable clinical demand for specific tissues will necessitate the creation of tissue banks at medical centers. Methods of preservation are crucial for both the source of cells and the final tissue constructs or implantation devices. Tissue preservation technology involves both hypothermic (above freezing) methods for short-term storage, and cryopreservation for long-term banking. Both approaches call for consideration of the cell in relation to its environment and as interventionalists we can control or manipulate that environment to effect an optimized protocol for a given cell or tissue. Organ Recovery Systems (www.organ-recovery.com), Inc., has research interests in both long-term preservation using sub-zero techniques, and short-term hypothermic storage in the absence of freezing

PRINCIPLES OF HYPOTHERMIC PRESERVATION

The use of hypothermia as the principal means to suppress metabolism in a reversible way and thereby approach a state of "suspended animation" is the foundation of most of the effective methods for tissue and organ storage.[1-5] Preservation by cooling is achieved by striking a balance between the beneficial and harmful effects of reducing temperature. Some of the basic principles that govern how this balance is best achieved are well understood whereas others are not. The main beneficial effect of cooling is undoubtedly the slowing of chemical reactions, and therefore the demand for oxygen and other substrates, and conservation of chemical energy. However, the details of the metabolic consequences of hypothermia are complex and not completely understood.

At the physical level, cooling has no useful effect on cell swelling or the redistribution of ions between the intracellular and the extracellular space. Maintenance of the ionic and hydraulic balance within tissues during hypothermia can be better controlled in media designed to physically restrict these temperature-induced imbalances. For example, it has been a common practice in tissue banking to use tissue culture media as the base solution for preservation media. However, there are good reasons why tissue culture media, which are designed to maintain cellular function at normal physiological temperatures, are inappropriate for optimum preservation at reduced temperatures.

UNISOL Concept – A Platform for Optimizing Low Temperature Biopreservation

Organ Recovery Systems, Inc., has a broad interest in developing a new series of solutions designed for optimum cell, tissue, and organ preservation under both hypothermic and cryopreservation conditions. The "UNISOL" concept for which a US patent has recently issued (pending elsewhere in the world) is entitled "System for Organ and Tissue Preservation and Hypothermic Blood Substitution".[3] It seeks to develop a unified solution system for both conditions based upon established principles that have emerged over several decades of understanding cellular responses to cold. [2,6-9] Hypothermia is the foundation of all useful methods

of preservation and has proven to be most effectively applied by directly controlling the extracellular environment of cells, and controlling the intracellular environment indirectly, during cold exposure. Conceptually, our goal is to develop a base formulation, the design of which will take into account biophysical requirements and minimal biochemical components that can be standardized for all applications. This base solution (Unisol™) will then be used as a vehicle for a range of additive "cocktails" which will evolve into a system of solutions optimized for the diverse biological requirements. Two principal Unisol formulations have been designed, an "intracellular-base" composition for application at profound hypothermic temperatures (<15°C), and an "extracellular-base" composition for application at moderate hypothermia (~25°C). [2,8-9] By design Unisol contains components: (a) to minimize cell and tissue swelling; (b) to maintain appropriate ionic balance; (c) to prevent a state of acidosis; (d) to remove or prevent the formation of free radicals, and e) to provide substrates for the regeneration of high energy compounds and stimulate recovery upon rewarming and reperfusion.[2-4,10]

The optimum temperature for hypothermic storage of mammalian tissues is not well established and therefore, the temperature at which an "intracellular-type" solution is more appropriate than an "extracellular-type" to offer the best cytoprotection, is ill defined. Organ Recovery Systems is currently funded by the National Heart, Lung, Blood Institute of the National Institutes of Health to study this question in relation to the preservation of vascular grafts.[11]

The unified solution system for preparing multiple solutions, designed and optimized for different cells, tissues, and organs, as well as for the various stages of organ or tissue procurement-preservation-transplantation, and/or bloodless surgery procedures, is based on the concept of a commercial kit comprising two alternative base solutions (Unisol – intracellular base, and Unisol-extracellular base), to which a variety of cytoprotective agents can be added for optimization. We recognize that there are many classes of potential additives that could augment the preservation properties of these new base solutions. Some of the key classifications of potentially efficacious biochemical and pharmacological additives are listed in Table 1.

Table 1. Biochemical and Pharmacological Additives for Preservation Media*
• Anti-platelet aggregation/vasoactive agents
• Calmodulin inhibitors
• Calcium Channel Blockers
• Protease and phospholipase inhibitors
• Anti-oxidants/free radical scavengers
• Iron chelators
• Membrane Stabilizers
• Cytoprotective agents
• Anti-apoptotic agents
• Metabolic Substrates
• Sugars
• Nucleotide precursors (HEP enhancers)
• Oxygen-carriers
• Trophic Factors
* Illustrative examples not intended to be a comprehensive list of potentially efficacious additives for preservation solutions

Experimental Examples

Hypothermic storage of isolated cells

We have undertaken an extensive series of studies to evaluate the relative merits of the Unisol solutions in a variety of systems that span the spectrum of biological complexity from single cells, through tissues and organs, to whole body protection during hypothermic blood substitution.[3,10-13] For hypothermic preservation at temperatures below 10°C, intracellular-type solutions, such as the new Unisol (UHK) medium, provide superior short-term hypothermic protection compared with extracellular-type solutions such as culture media. This is illustrated in Figure 1 by reference to studies involving two cell types examined *in vitro* for cell viability using a metabolic indicator assay at different time points following hypothermic storage. The vascular smooth muscle cell line (A10) and a bovine pulmonary endothelial cell line (CPAE) were used in these experiments. Viability was measured at two points, either immediately after exposure, or for six consecutive days after exposure at 4°C.

Measurement of cell viability immediately after low temperature storage does not necessarily give a true indication of cell survival. Over time, cells may undergo further changes, including the repair of sub-lethal injury or cell death through the processes of apoptosis and necrosis. Whatever the eventual fate of hypothermically exposed cells may be, the processes require time for full manifestation, and cell survival curves during the days following return to physiological temperatures are informative about the true state of viability. For example, the cells may be metabolically active for the first two days or so after hypothermic exposure as they follow the apoptotic pathway resulting in eventual cell death. In light of such issues, we routinely evaluate post-hypothermic exposure viability over prolonged periods in culture at 37°C.

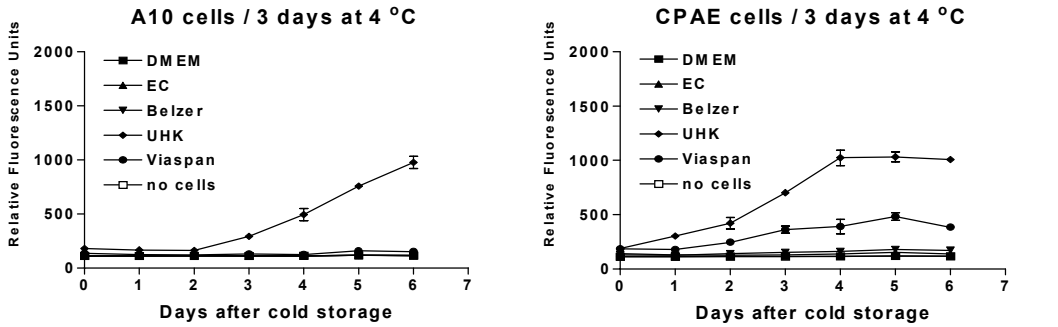

Figure 1. Comparative post-hypothermia cell proliferation curves for A10 and CPAE cells after three days of cold storage in the various preservation solutions. Viability was followed for six days after hypothermic exposure. Each data point is the mean (±SEM) response of four experiments. DMEM: Dulbecco's Minimum Essential Medium. EC: EuroCollins. Belzer: Belzer Machine Perfusion Solution. UHK: Unisol-UHK. Viaspan: UW solution.

Figure 1 illustrates that; in general, Unisol™ (UHK) provided equivalent, or better, preservation, than the other solutions. Both "intracellular-type" solutions – Viaspan™ and Unisol™ – proved superior to the other solutions tested. After three days of hypothermic storage, only CPAE cells stored in Viaspan™ or Unisol™, and only the A10 cells kept in Unisol, were able to proliferate in culture.

Hypothermic storage of whole organs flushed or perfused with a preservation solution is common practice in clinical transplantation. This procedure leaves the vascular endothelial cells in direct contact with the preservation medium during the cold ischemic period. The effect of

storage conditions on the integrity of vascular endothelium is therefore, of crucial importance for the quality of preservation of intact organs.

Hypothermic storage of vascular tissue

The importance of these effects is illustrated by experiments we conducted comparing the microscopic changes in tissue morphology that occurred when excised blood vessels were immersed and transported in either the new Unisol-UHK hypothermic preservation solution or Dulbecco's Minimum Essential Medium (DMEM) – a common culture medium used to incubate and transport tissues *ex vivo*. The total cold ischemia time in these experiments was relatively short, 34.5 ± 9.5 minutes for samples transported in DMEM, and 37 ± 4 minutes for samples in Unisol-UHK.

Tissue immersed and transported in DMEM on ice exhibited microscopic changes within the tunica intima and tunica media, as shown in Figure 2A. The intima was intact, but there was extreme vacuolization (indicated by the arrows) of the underlying basal lamina causing, in turn, extrusion of the endothelial cells into the lumen and giving a "rounding-up" appearance. Apart from this vacuolization, the endothelial cells had a near normal appearance. The smooth muscle cells (SM) had a somewhat shrunken appearance with irregular contours. The tunica adventitia was essentially normal.

In contrast, jugular veins transported in Unisol™ demonstrated little if any histological changes compared with the DMEM group as shown in Figure 2B. The tunica intima was intact with little evidence of vacuolization of the underlying basement membrane. The smooth muscle cells (SM) did not appear shrunken and were in a normal, horizontal orientation.

In summary, the *in vitro* studies that have examined both the function and structure of hypothermically stored blood vessels have clearly shown that optimum preservation of vascular grafts is achieved by selection of the type of storage medium in relation to time and temperature. Synthetic preservation solutions such as Unisol, designed specifically to inhibit detrimental cellular changes that ensue from ischemia and hypoxia are clearly better than culture medium or blood and saline, which are commonly used clinically.

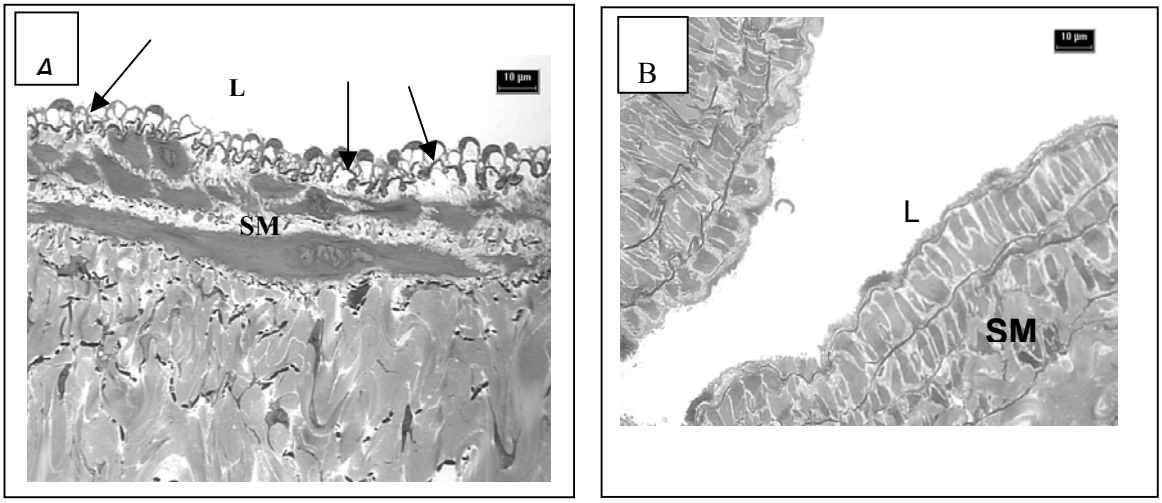

Figure 2. Light microscope histology of jugular vein segments after a period of cold ischemia in either DMEM culture medium (A) or Unisol-UHK hypothermic preservation solution (B). Details are described in the text. L = Lumen; SM= smooth muscle cells.

Corporplegia – Whole Body Preservation

We alluded earlier to the importance of solution design in the context of hypothermic blood substitution for whole body protection during extended periods of cardiac arrest for bloodless surgery.[2,7-8] This novel approach to clinical suspended animation (or "corporoplegia" meaning literally "body paralysis") has recently been explored for resuscitation after traumatic hemorrhagic shock in pre-clinical models relevant to both civilian and military applications.[12,14-16] In exsanguinating cardiac arrest (CA) conventional resuscitation attempts are futile. Safar's group at the University of Pittsburgh have recently introduced the use of cold aortic saline flush at the start of CA to rapidly induce protective hypothermia during prolonged CA (120 min) for hemostasis followed by resuscitation.[12,15]

Using a canine model they showed that a saline flush to a brain temperature of 10°C resulted in normal survival after 90 min, but not consistently after CA =120min. However, an additional study in which Unisol plus the antioxidant Tempol were evaluated as a comparative "optimized flush", showed a markedly improved outcome after 120 min CA compared with the saline flush.[12] Safar et al concluded that an optimized single large volume cold aortic flush at the start of CA can achieve normal survival with minor histologic damage after a no-flow time of 120 min.

In a completely separate study, we have collaborated with Rhee et al at the Uniformed Services University of the Health Sciences to develop a porcine model of uncontrolled lethal hemorrhage in which a combination of Unisol I-base and Unisol E-base were used to effect profound hypothermia and prolonged cardiac arrest (60min), with resuscitation after surgical repair of the vascular deficit induced to effect exsanguinations. In the most recent study, this group has demonstrated that both learning and memory is preserved after induced asanguineous, hyperkalemic, hypothermic arrest in this pre-clinical swine model of traumatic exsanguinations.[16] This provides further evidence for the protective properties of Unisol solutions used for global tissue preservation during whole body perfusion in which the microvasculature of the heart and brain are especially vulnerable to ischemic injury.[2] Moreover, the application of solution-design for clinical suspended animation under conditions of ultraprofound hypothermia, places the Unisol solutions in a unique category as a universal preservation media for all tissues in the body. In contrast, all other preservation media, including the most widely used commercial solutions, such as UW and Viaspan, are established for specific organs, or groups of organs, e.g. UW for abdominal organs and Celsior, Cardiosol, or Custodiol for thoracic organs.[10,17]

Long-Term Preservation

Cryopreservation – using the protective effects of low temperatures – has been notably effective for banking and shipping of isolated cells, but much less so for more complex, integrated multicellular systems. We now have a broad understanding of the mechanisms that injure cells during freezing and thawing, and techniques have been developed that limit or prevent this injury, so that very low temperatures can now be used to preserve, virtually indefinitely, many cell-types with very high recovery rates.[18-20] These techniques are all aimed at preventing intracellular freezing and minimizing the damaging changes that occur in the remaining liquid phase as a consequence of the separation of water to form ice. However, in tissues and organs it is not sufficient to maintain cellular viability, it is also important to maintain the integrity of the extracellular structure on both a micro- and macro-scale. Techniques that are effective for the cryopreservation of cell suspensions do not always do this because highly organized multicellular tissues present a special set of problems; foremost amongst these is the effect of extracellular ice formation which disrupts the tissue architecture.[20-24] On the other hand, extracellular ice cannot explain all the problems encountered in moving from single cells to tissues. For example, the detachment of the corneal endothelium has a different, and currently unknown, cause;[25] prevention of this detachment is crucial for corneal viability. The effects of cryopreservation on the mechanisms of cellular adhesion have not yet been studied widely. To

date, there have been very few studies focused on the effects of cryopreservation on anchorage-dependent cells and, in general, these have shown that post-thaw recovery of adherent cells is lower than comparable cells frozen and thawed as cell suspensions.[26-27]

The science of cryobiology has defined many factors that must be optimized in order for cells to be stored for lengthy periods at low temperatures. Survival of cells from the rigors of freezing and thawing in cryopreservation procedures is only attained by using appropriate cryoprotective agents and in general, these techniques are applicable to isolated cells in suspension or small aggregates of cells in simple tissues. More complex tissues and organs having a defined architecture are not easily preserved using conventional cryopreservation techniques; this is principally due to the deleterious effects of ice formation in an organized multicellular tissue.[20,23] Moreover, the effect of cryopreservation on adherent cells has not been studied extensively, thus the application of cryobiological principles to engineered tissue constructs is likely to be fraught with similar problems to those identified during the course of trying to extrapolate the successes of freezing cell suspensions to organized tissues and organs. Avoidance of ice damage has therefore become the principal focus in research to develop effective storage techniques for multicellular tissues and organs. This article will review some of the underlying principles of the various approaches to "ice-free" cryopreservation with particular emphasis on vitrification, which has recently been shown to provide a practical solution for the cryopreservation of complex tissues that cannot be adequately preserved by freezing/thawing methods.[28-29]

NEW APPROACHES TO BIOPRESERVATION

Tissues are much more than simple aggregates of various cell types; they have a highly organized, often complex, structure that influences their response to freezing and thawing. The formation of extracellular ice, in particular, which is generally regarded as innocuous for cells in suspension, is known to be a hazard to structured tissues and organs. Cryopreservation is a complex process of coupled heat and mass transfer generally executed under non-equilibrium conditions. Advances in the field were modest until the cryoprotective properties of glycerol and dimethyl sulfoxide (DMSO) were discovered in the mid 1900's.[30-31] Many other cryoprotective agents (CPAs) have since been identified. Combinations of CPAs may result in additive or synergistic enhancement of cell survival by minimization of intracellular ice during freezing.[32]

Restriction of the amount and size of extracellular ice crystal formation during cryopreservation can be achieved by using high concentrations of CPAs that promote amorphous solidification, known as vitrification, rather than crystallization.[28-29] Vitrification is a relatively well-understood physical process, but its application to the preservation of biological systems has not been without problems, since the high concentrations of CPAs necessary to facilitate vitrification are potentially toxic. To limit toxic effects it is necessary to use the least toxic CPAs at the lowest concentrations that will still permit glass formation (at cooling rates that are practical for bulky mammalian tissues).[28-29] Comparison of the effects of vitrification and conventional freeze-cryopreservation upon venous contractility in cryopreserved blood vessels demonstrated that vitrification is superior to conventional freezing methods.[11] Vitrification has more recently been used effectively for a variety of other tissues including myocardium, skin, and articular cartilage.[29] Table 2 summarizes the viability data contrasting the outcome of vitrification with conventional cryopreservation involving freezing. Interestingly, the functional survival of vitrified tissues was approximately 80% or higher, whereas the frozen counterparts yielded less than 30% survival. This marked contrast (80% vs. 30%) appears consistent irrespective of the nature of the tissue, or the method of assay.

Table 2. Contrasting survival of tissues after cryopreservation in either the frozen or ice-free state

Model Tissue	*Taenia Coli smooth muscle (Guinea pig)*	*Jugular vein (rabbit)*	*Carotid artery (rabbit)*	*Articular cartilage (Rabbit)*
Assay	Histamine-induced contractility	Various contractile agonists	Various contractile agonists	Esterase activity (Calcein AM)
Survival Outcome	21%	6-22%	<30%	13%
	78%	84-87%	>80%	80%
Reference(s)	Taylor and Pegg 1983 22	Taylor et al 1999 33	Song et al 2001 34	Song et al 2001 35
		Song et al 2000 28		Brockbank et al 2003 (submitted)
				Metabolic activity (alamarBlue)
Frozen				11%
Ice-free				85%

This phenomenon serves to emphasize that avoidance of large amounts of ice in organized tissues can improve the outcome of cryopreservation from a meager 20% survival to a respectable 80%. It will be interesting in future studies to see whether the magnitude of this difference is also manifest in other tissues and whether the maximum recovery can be further improved by optimization of the vitrification protocols.

In recent years, molecular approaches to minimize damage due to cryopreservation in cells and tissues have been conceived and research initiated. Molecular ice control techniques are currently in development using designer molecules that specifically interact with ice nuclei, resulting in either prevention of ice nucleus development or modification of ice crystal phenotype,[29,36] These molecules promise to have benefits in both freezing and vitrification preservation protocols by rendering ice crystals either less damaging or by permitting reduction of CPA concentrations, respectively. In addition, a better understanding of molecular signaling pathways involved in cell injury due to cryopreservation is required. It is likely that both inhibition of apoptotic cell death and stabilization of cell membranes during cryopreservation may have significant benefits. The idea that there is a preservation threshold for the cryopreservation of viable tissues is depicted schematically in Figure 3. This schematic was constructed from the data given in Table 3, which shows for a variety of tissues that survival after cryopreservation could be increased from ~30% to ~80% by employing ice-free techniques. While this represents a marked improvement that elevates the preservation technique from one that yields inferior survival using freezing to one that provides respectable recovery using vitrification, it nonetheless represents a threshold that provides room for further improvement. The ultimate goal is 100% survival and this might be approached by further optimization of ice-free technologies and/or attention to the "molecular paradigm" that has recently been purported to be a way forward to remove the so-called preservation cap.[37-39]

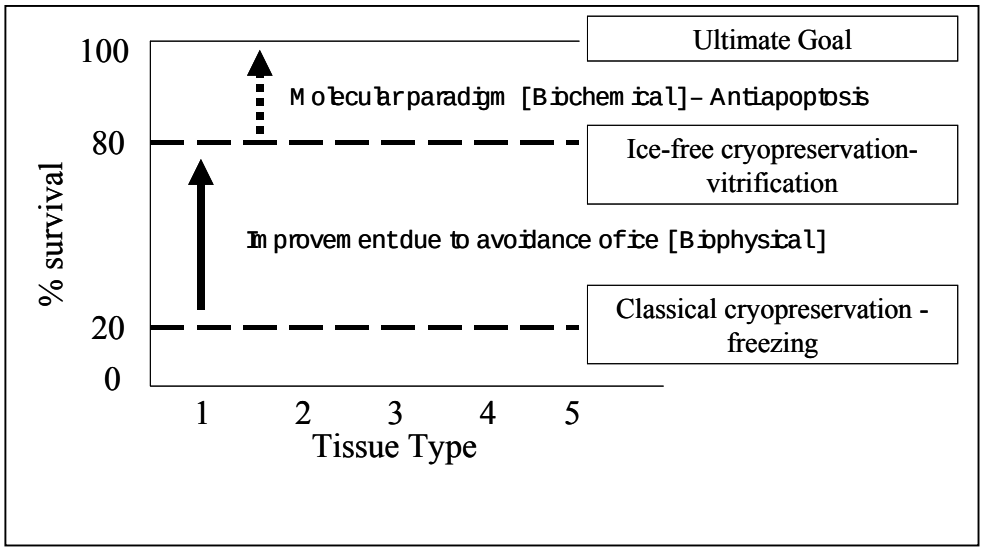

Figure 3. Improving the Outcome of the Cryopreservation of Multicellular Tissues

Biopreservation by Drying

Both conventional freezing and vitrification approaches to preservation have limitations. First, both of these technologies require low temperature storage and transportation conditions. Neither can be stored above their glass transition for long without significant risk of product damage due to ice formation and growth. Both technologies require competent technical support during the rewarming and CPA elution phase prior to product utilization. This is possible in a high technology surgical operating theater, but not in a doctor's outpatient office or in third world environments. In contrast, theoretically, a dry product would have none of these issues because it should be stable at room temperature and rehydration should be feasible in a sterile packaging system.

Drying and vitrification have previously been combined for matrix preservation of cardiovascular and skin tissues but not for live cell preservation in tissues or engineered products. However, nature has developed a wide variety of organisms and animals that tolerate dehydration stress by a spectrum of physiological and genetic adaptation mechanisms. Among these adaptive processes the accumulation of large amounts of disaccharides, especially trehalose and sucrose, are especially noteworthy in almost all anhydrobiotic organisms including plant seeds, bacteria, insects, yeast, brine shrimp, fungi and their spores, cysts of certain crustaceans, and some soil-dwelling animals.[40-42] The protective effects of trehalose and sucrose may be classified under two general mechanisms: (1) "the water replacement hypothesis" or stabilization of biological membranes and proteins by direct interaction of sugars with polar residues through hydrogen bonding, and (2) stable glass formation (vitrification) by sugars in the dry state.

The stabilizing effect of these sugars has also been shown in a number of model systems, including liposomes, membranes, viral particles, and proteins during dry storage at ambient temperatures.[43-45] On the other hand, the use of these sugars in mammalian cells has been somewhat limited, mainly because mammalian cell membranes are impermeable to disaccharides or larger sugars. Recently, a novel genetically modified pore former has been used to reversibly permeabilize mammalian cells to sugars with significant post-cryopreservation and to lesser

extent drying cell survival.[46] Such permeation technologies, that may also include use of pressure or electroporation, may provide some of the most likely opportunities for preservation of tissues in the five to ten year vision – either by permitting cryopreservation with non-toxic cryoprotectants or drying. However, it should be noted that most organisms that reach a dried state during dormancy, and drought, do so by air drying (not freeze drying), which suggests this may be innocuous to cells under certain conditions. Studies of anhydrobiotic organisms may suggest methods for conditioning mammalian cells for storage by either cryopreservation or drying in the tissue engineered products of the future.

CONCLUSIONS

Within the confines of this brief article, it has only been possible to summarize the approaches currently being pursued for the development of biopreservation techniques as an enabling technology for tissue engineering and regenerative medicine. Some of these preservation technologies are sufficiently developed to provide a practical basis to meet the basic needs of transplantation science today. However, others are considered developments for the future to provide either better short-term preservation using hypothermic techniques, or to generate new techniques for long-term banking of complex tissues and organs that are refractory to conventional cryopreservation methods. Molecular approaches to ice-free cryopreservation and vitrification have recently achieved a breakthrough in the long-term storage of living tissues and anhydrobiosis offers some intriguing possibilities for the future. New possibilities for significant advances in the arena of biopreservation are limited only by our imagination. We were recently invited to exercise our imaginations and outline our vision for the development of biopreservation technologies during the next 50 years. Readers of this article might find our projections intriguing, challenging and thought-provoking.[47]

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About the Author

Michael J Taylor PhD. is Vice President for Research and Development, Organ Recovery Systems (ORS) and Adjunct Professor of Surgery, MCP Hahnemann University of Pennsylvania. Prior to joining ORS in 1998, he was Director of the Cryobiology and Hypothermic Medicine Program at Allegheny University of the Health Sciences, Pittsburgh from 1992, and Senior Scientist with the Medical Research Council at Cambridge University in the UK before that. Dr Taylor has more than 25 years experience in the field of low temperature biology and medicine, and is credited with more than 150 publications and patents in this field. He has also served multiple terms on the Board of Governors for the international Society for Cryobiology and is a member of the editorial boards of the three major journals in the field (Cryobiology; Cry Letters and Cell Preservation Technology).

Correspondence: Michael J Taylor PhD, VP Research and Development, Organ Recovery Systems, Inc., 701 East Bay Street, Suite 433, MSC 1119, Port City Center, Charleston, SC 29403. USA. E-mail: mtaylor@organ-recovery.com. Website: www.organ-recovery.com.