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Review of vitreous islet cryopreservation
Some practical issues and their resolution

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Abbreviations: AFP, antifreeze protein; CPA, cryoprotective agent; CITR, collaborative islet transplant registry; CHT, cyclohexanetriol; 1,3 CHD, 1,3, cyclohexanediol; 2,3 BD, 2,3 butaendiol; 1,2 PD, 1,2 propanediol; DMSO, dimethyl sulfoxide; EC, eurocollins solution; UHK, unisol-UHK solution; PEG, polyethylene glycol

Transplantation of pancreatic islets for the treatment of diabetes mellitus is widely anticipated to eventually provide a cure once a means for preventing rejection is found without reliance upon global immunosuppression. Long-term storage of islets is crucial for the organization of transplantation, islet banking, tissue matching, organ sharing, immuno-manipulation and multiple donor transplantation. Existing methods of cryopreservation involving freezing are known to be suboptimal providing only about 50% survival. The development of techniques for ice-free cryopreservation of mammalian tissues using both natural and synthetic ice blocking molecules, and the process of vitrification (formation of a glass as opposed to crystalline ice) has been a focus of research during recent years. These approaches have established in other tissues that vitrification can markedly improve survival by circumventing ice-induced injury. Here we review some of the underlying issues that impact the vitrification approach to islet cryopreservation and describe some initial studies to apply these new technologies to the long-term storage of pancreatic islets. These studies were designed to optimize both the pre-vitrification hypothermic exposure conditions using newly developed media and to compare new techniques for ice-free cryopreservation with conventional freezing protocols. Some practical constraints and feasible resolutions are discussed. Eventually the optimized techniques will be applied to clinical allografts and xenografts or genetically-modified islets designed to overcome immune responses in the diabetic host.

Introduction

Clinical significance and health relatedness of islet cryopreservation. Diabetes mellitus is the seventh leading cause of death in U.S. as listed on death certificates in 2006,[1] afflicting approximately 18 million people.[2] It is estimated that a further 5.7 million patients have the disease but have not yet been diagnosed; the incidence of diabetes has increased by 13% in 2007 in comparison to 2005. Data from the World Health Organization (WHO) indicates that approximately 134 million people suffer from diabetes mellitus worldwide, and that this number will rise to over 300 million by the year 2025 [www.who.int]. Currently, there is no cure for diabetes and the disease is kept in check by regular and chronic injections of insulin. In the U.S. alone, the total annual cost of diabetes in 2007 was estimated to be $174 billion, $116 billion in direct cost being attributed to medical expenditures for diabetes care, chronic diabetes-related complications and general medical costs.[2] Nevertheless, insulin therapy is imperfect, since it does not prevent the long-term complications such as blindness, heart and kidney disease, and neuropathies in the extremities.

In the crusade to find a cure for diabetes, researchers have sought ways to return normal pancreatic functioning to the body. Conceivable methods include whole pancreas transplants, human islet transplants, animal islet transplants, fetal tissue exchange, creation of artificial pancreas or beta cells, and transplantation of genetically-engineered islets, or other cells modified with proinsulin cDNA. All of these procedures have both positive and negative attributes.[3,4]

A current update on pancreatic islet transplantation,[5] emphasized that clinical islet transplantation had recently received a strong boost from the introduction of glucocorticoid-free immunosuppressive regimens. As a result, there is now a consensus that islet transplantation may be considered a viable option for the treatment of insulin-dependent diabetes mellitus. Previously, demand was not an issue, but this breakthrough enables technologies that help overcome the shortfall in pancreata for islet isolation, as part of the critical path toward maximization of the potential of islet therapy. From 1999 to 2007, a total of 717 allograft infusion procedures were performed to 378 type-I diabetes patients in the North American islet transplant centers, as reported by the Collaborative Islet Transplant Registry (CITR) in their 2007 Annual Report.[6] The majority were islet-kidney transplants. The allografts were composed of freshly isolated or cultured or cryopreserved islets, or a combination of freshly isolated and cryopreserved islets. In some cases, immunosuppression was induced with anti-lymphocyte serum while maintenance

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immunotherapy was largely based on a combination of glucocorticoids, cyclosporin and azathioprine. As of 2007, immediately following islet implantation, 72% of the 378 type-1 diabetes recipients achieved insulin independence. Of those, only 71% were still insulin independent one year post-implant. The maintenance of insulin independence was further reduced to 52% of the patients at two years post implant. Three years post-islet infusion, about 23% were insulin independent, 29% were insulin dependent with detectable C-peptide and 26% have no detectable C-peptide.[6] Although C-peptide secretion was maintained in a majority (80%) of the 65 patients treated using the Edmonton Protocol, most (90%) had reverted to using some insulin.[7] Hence, repeated islet transplantations may become necessary.

The Edmonton protocol has unequivocally and favorably changed the clinical outlook for the long-held belief that islet transplantation would provide a way to cure type I diabetes. Beyond Edmonton, a review of successful outcomes of variants of the Edmonton Protocol indicates that insulin independence has been achieved in more than 10 centers worldwide.[8,9] These collective multi-group data clearly indicate that islet transplantation now provides similar outcomes to whole pancreas transplantation, but more donors are required to deliver sufficient islet engraftment mass. Moreover, these significant clinical developments have emphasized the need for better methods of donor pancreas preservation and resuscitation after warm ischemia in addition to optimized methods of islet cell isolation and cryopreservation.

Role of islet banking. Inconsistencies in the ability to isolate sufficient numbers of islets from the cadaveric organ donor pancreas have limited transplant success.[10,11] One approach that has led to successful insulin withdrawal in some clinical islet transplant programs is through providing an increased transplantable mass of islets by using islets from multiple donor pancreases.[11,12] This was facilitated by using pooled islets from cryopreserved samples. Effective low temperature storage also provides time for sterility and viability testing of the islet preparation and for the development of donor specific tolerance.[13,14] In selected clinical islet transplants, cryopreserved human islets have been used to supplement the islet mass. The ability to select preparations of islets from a pool of preserved islets provides increased flexibility and an ability to select specific preparations of islets based on tissue matching. However, preservation of islet tissue through cryopreservation results in a loss of islet mass and subsequent reduction in islet function.[15-17] If low temperature banking of islets through cryopreservation is to realize its full potential, methods to cryopreserve islets must be improved.

Current state-of-the-art. Islet banking for transplantation is regarded as a specific application within the modern era and emerging technologies of tissue engineering. This new field has reached the point where the first engineered tissue constructs are undergoing clinical trials and research and development in this arena is now poised to yield a wide variety of commercial products. 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 large 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. Short-term preservation techniques, such as refrigeration or tissue culture, have serious limitations including restricted shelf-life, high cost, risk of contamination or genetic drift. It is widely recognized that cryopreservation, the arrest of chemical, physical and biological processes at deep sub-zero temperatures, offers a more tenable option. While freeze-preservation of a variety of cell types and some simple tissues has been successfully accomplished, it is important not to assume that current cryopreservation technology can be applied universally to more complex tissues and organs for which adequate cryopreservation protocols have still to be developed. In contrast to the ability of many single cell types to survive cryopreservation, highly organized multicellular tissues present a special set of problems which we identified in 1979,[18] thus providing the basis for experimental investigation in the intervening years. As we have reviewed recently, several reasons for the refractory response of tissues to conventional cryopreservation procedures have been suggested, but the most critical is undoubtedly the effect of extracellular ice formation which is invariably innocuous to single cell suspensions.[19,20] Proof of the severity of ice formation as the predominant basis for freezing injury in multicellular tissues and organs has led to the quest for methods of ice-free cryopreservation, an approach that is the focus of this brief review.

It is important to appreciate that while sub-optimal cryopreservation techniques may be adequate for some biological systems such as cartilage and bone that call for largely structural integrity only, or tissue in which function can be achieved with only a fraction of the initial population (e.g., sperm), or tissue which can be repopulated from a small number of surviving progenitor cells (e.g., bone marrow), tissue engineering applications, including islet transplantation, invariably demand much stricter requirements on post-thaw tissue viability. An example of such high demands would be tissues comprising genetically modified cells, including genetically-engineered islets to improve their immunotolerance, for which cryopreservation must not only yield a high degree of cell survival, but continued expression of engineered phenotypes must be assured. It is such a demand for the banking of native and genetically-engineered islets that vitreous cryopreservation has significant merits as discussed below.

Synopsis of cryopreservation techniques in relation to the goal of ice-free cryogenic storage. Conventional cryopreservation techniques, which require the substitution of up to 30% of cell water by a cryoprotective compound such as glycerol or dimethyl sulfoxide (DMSO), permit storage of many types of cells at deep subzero temperatures (typically <-100°C). Cryoprotectants (CPAs) are neutral solutes of sufficiently low mol weight to penetrate into cells, very low toxicity and high solubility in water: thus, they are tolerated in sufficiently high concentrations to significantly reduce the amount of ice that forms at any given subzero temperature.[21-23] When the rate of cooling is low enough, ice forms exclusively outside the cells and the external osmolality rises, dehydrating the cells. In fact, the ice is external to the system that it is desired to conserve—the cell—and the concentrated cell contents eventually solidify as an amorphous glass—a process known as *vitrification*.[19,21,24,25] If cooling is too rapid

to permit dehydration, and the cell contents actually freeze, the cell is destroyed. It should be noted that this result shows that **cells** can tolerate the vitreous state. It has now been established beyond any doubt that the principal problem in attempting to cryopreserve tissues and organs is that ice forms *within the system that it is desired to preserve*, albeit outside the cells, and destroys both structure and function.[22,26,27] It is clear that more than cell-survival is needed in tissue preservation; complete structural integrity is vital. We have shown that some tissues and organs are severely damaged by extracellular ice and a mechanism that is adequate to account for the effect of extracellular ice in vascularized tissues—the rupture of capillaries by accumulating ice—has been demonstrated.[26-28]

Avoidance of ice. If a sufficiently high concentration of CPA is used, the formation of ice can be avoided completely. The rate of cooling and warming are then unimportant because there is no driving force for trans-membrane water movement and no ice to recrystallize during warming. The concentration of CPA necessary to avoid freezing is very high (typically ~60%) and 'compatibility' (the absence of deleterious effects of the solute itself) is the essential problem such that the concentration of solute required is unattainable at supra-zero temperatures. By taking advantage of the temperature dependence of most toxic actions, it is possible to increase the concentration progressively as the temperature is reduced. We showed several years ago that by using this approach to increase the concentration of DMSO in a stepwise manner in order to remain above the equilibrium freezing point during cooling, it was possible to recover smooth muscle tissue with a high degree of stimulated contractile function.[19,29,30]

More recently, an alternative approach has been explored based upon dynamic features to reduce the amount of ice by selecting sufficiently high cooling rates to prevent ice nucleation. This approach produces a metastable state that is at risk of devitrifying (recrystallization) during warming and ice formation during warming is just as injurious as during cooling.[24,25] Nevertheless, vitrification procedures by this technique have been developed and shown to provide effective preservation for a number of cells, including monocytes, ova and early embryos and pancreatic islets.[25,31-34] At the time that these vitrification techniques were developed Pegg and Diaper pointed out that all of these cellular systems can equally well be preserved by conventional freeze-preservation methods.[35] In such systems a vitrification method is often preferred because of practical benefits of operational simplicity avoiding the need for expensive cooling equipment. Thus, in 1990 the challenges of vitrifying complex tissues remained formidable but approaches towards ice-free cryopreservation were still regarded as the way forward. Ten years later, this barrier was eventually removed and we have reviewed elsewhere the developments that eventually led to the successful vitrification of several tissues, two of which, blood vessels and articular cartilage, were previously refractory to cryopreservation with a high degree of functional survival.[19,36]

Vitrification refers to the physical process by which a concentrated solution of CPAs solidifies during cooling without crystallization. The solid, called a glass, retains the normal molecular and ionic distributions of the liquid state and is therefore, usually considered to be an extremely viscous supercooled liquid. The difference between conventional cryopreservation and vitrification lies, not in the occurrence of vitrification in only the latter method, but in the means by which vitrification is produced—by extracellular freezing and progressive cell dehydration during cooling in conventional preservation, and by achieving a vitrifiable system at the outset in vitrification.[19,35]

Advantages of the vitrification approach. Cryopreservation by the complete vitrification of the tissue suspension offers several important advantages compared with procedures that allow or require crystallization of the suspension. Firstly, complete vitrification eliminates concerns for the known damaging effects of intra- and extracellular crystallization. Secondly, tissues cryopreserved by vitrification are exposed to less concentrated solutions of CPAs for shorter periods of time. For example, during a typical cryopreservation protocol involving slow freezing to -40°C, or -70°C, cells are exposed to solutions whose concentration increases gradually to 21.5 and 37.6 osmolal respectively. In contrast, cells dehydrated in vitrification solutions are exposed for much shorter periods of time to <18 osmolal solution, although the temperature of exposure is higher (see (Rall, 1987)[37]). Thirdly, unlike conventional procedures that employ freezing, vitrification does not require controlled cooling and warming at optimum rates—cooling and warming need only be rapid enough to prevent crystallization and this can generally be achieved without the need for specialist equipment. It is widely anticipated therefore, that for many integated multicellular tissues, vitrification may offer the only feasible means of achieving cryopreservation without ice damage, and for some tissues such as pancreatic islets that appear to withstand cryopreservation by either approach, vitrification offers a number of practical advantages that will be attractive in tissue engineering, as indeed they have for embryo banking.[37] On this basis we are committed to pursue vitrification techniques for pancreatic islets and to ultimately evaluate these in relation to the efficacy of banking genetically-engineered islets. The composition of the vitrification medium in terms of 'compatibility' and glass-forming tendency is crucial for development of an optimum technique, but this has not yet been adequately studied for pancreatic islets. In recent years, we have begun the process of systematically optimizing the various critical stages of vitreous cryopreservation of islets. For example, we have described the importance of the composition of the vehicle solution used to impregnate the islets with cryoprotectants during cryopreservation.[38] More recently we have begun to focus on alternative mixtures to promote safe vitrification of islets and here we described some practical issues and their resolution.

Technical challenges and a proposed resolution. Based upon our experience at developing successful vitrification procedures for a variety of tissues in recent years[19,36,39-45] we set out to apply the established principles and techniques of tissue vitrification to pancreatic islet samples. Attempts to adapt our baseline vitrification protocols[36,40] to isolated islets identified a variety of technical challenges that needed to be addressed:

Handling and processing islets. Islets are intermediate between isolated cell preparations and defined pieces of intact tissue that impacts the ways in which they need to be handled during

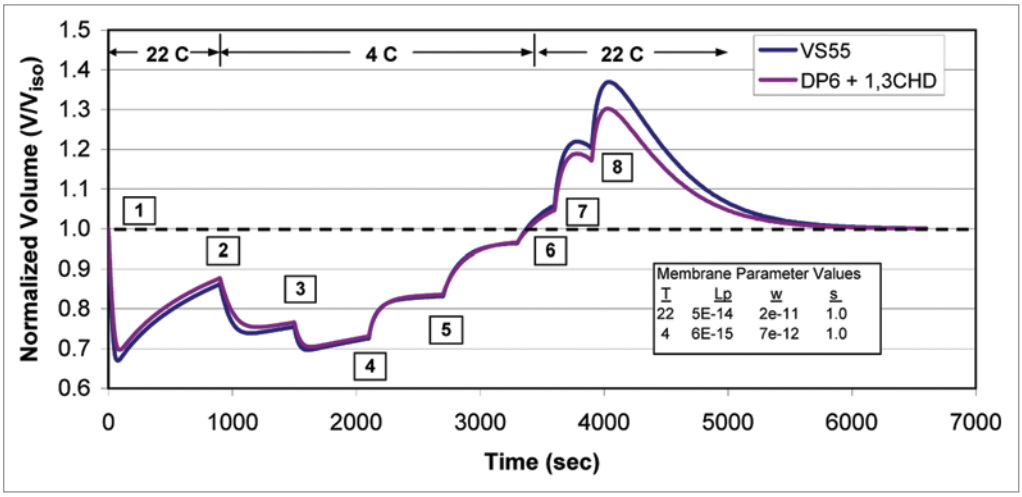

Figure 1. Plots of normalized cell volume showing the transient volume changes in islets during cryoprotectant addition (steps 1–3) and removal (steps 4–8) for the vitrification solutions VS55 and DP6-1,3 CHD. The protocols for both vitrification solutions do not exceed osmotic tolerance limits for islets of 0.6 V/V_{iso} during CPA addition and 1.53 V/V_{iso} during removal.

preservation and assaying for viability. The major distinctions between processing cell suspensions compared to intact tissues relate to sample size to effect heat and mass transfer and the practical issue of retaining cells during multiple manipulations from isolation, adding and removing CPAs, cooling and warming, and assaying for subsequent structure and function. With the exception of the possible impact of fracturing during vitreous cryopreservation (reviewed in refs. 19 and 46), tissues such as blood vessels, heart valves or pieces of cartilage retain their integrity and are conveniently handled as a unit for transfer between solutions and containers during preservation, assaying and transplantation. In marked contrast, isolated cells require repeated centrifugation and resuspension at the various stages of manipulation with the inherent risk of additional stress or loss of material during the processing. Similarly, islets although they are multicellular require sedimentation in order to exchange solutions or transfer the islets between containers.

Cryopreservation. Due to the requirement for high concentrations of CPAs to achieve vitrification these protocols call for processing the islets through a series of solutions of increasing solute concentration and hence viscosity. Moreover, the need for multiple steps of addition and dilution of CPAs in the process often necessitates the transfer between smaller to larger containers again with the potential loss of tissue due to the need to recover the sample from increasingly large volumes of viscous solutions. It became apparent in the early stages of developing protocols for the islets based upon our baseline tissue protocol that this was a significant problem that led to a loss of material irrespective of the quality of the preservation of the individual islets. In an effort to resolve this problem the protocol was revised and adapted to the point where a method was developed that permitted islets to be retained in a single container throughout all processing steps for the addition and removal of the vitrification solutions as well as for the vitrification process itself. Details of the revised scheme are described in the methods section below. Moreover, computational analysis of the osmotic responses of islets subjected to the new protocol was carried out to confirm that the islets did not exceed the tolerable limits of volume excursion during the addition and removal of the cryoprotectant cocktails.

Results

Computer simulations of the revised vitrification protocol. As outlined in the Methods section, a common cryoprotective additive protocol was established for the addition and removal of VS55 and DP6 + 1,3 CHD in order to simplify experimental procedures. The protocol employed a three step addition and five step removal process (**Tables 1 and 2**) to minimize volumetric excursions and consequently reduce the probability of osmotic induced injury. Simulations of the protocol using these two vitrification solutions were conducted with the conditions specified in **Table 3** and membrane parameter values for islets previously obtained by other researchers.[62] The resulting plots of transient volume changes for the islets during these protocols are shown in **Figure 1**. The important observation from these simulation curves is that for both cryoprotective additive treatment protocols the normalized volumes of the islets (V/V_{iso}) does not exceed the published osmotic tolerance limits of 0.6 V/V_{iso} during CPA addition and 1.53 V/V_{iso} during removal.[60]

Tolerance of islets to exposure to vitrification solutions without subzero cooling. Using the protocols outlined in the Methods section, the research summarized here set out to compare the survival of islets after vitrification using one of two CPA formulations of current interest. The first is our baseline VS55 vitrification solution, which has been used extensively in other systems, and the other is a more recently developed solution, DP6, containing a synthetic ice blocking molecule, 1,3 cyclohexanediol (1,3CHD).[19] The latter has a lower total concentration of CPAs than the VS55 solution (6.5 M vs. 8.4 M respectively). Moreover, this study included a comparison of two vehicle solutions for the vitrification cocktails since this is also known to be an important factor in determining the ultimate survival of cryopreserved cells.[38,66] In this study we compared EuroCollins (EC) with the more recently formulated Unisol (UHK). The latter was developed primarily as a universal solution for multiple low temperature applications.[67-71]

Metabolic activity of the islets after exposure to the various CPA cocktails shown in **Figure 2** was measured using the Alamar Blue assay. Some significant effects were observed in these pilot experiments. For example, measurments immediately

after exposure revealed that islet viability was significantly higher after exposure to DP6 compared with VS55 when EuroCollins (EC) was used as the vehicle solution, but was not significant when UHK was employed. Allowing 24 h recovery before measuring the metabolic activity showed that the viability of islets exposed to DP6 was not significantly different to VS55 when exposed in EC vehicle but DP6 was significantly less toxic than VS55 when exposed in UHK. It is also noteworthy that exposure to the more concentrated VS55 cocktail resulted in a significantly lower viability index immediately after exposure in EC compared with the UHK vehicle solution. With respect to insulin secretory function, **Figure 3** shows that islets tolerated exposure to these solutions well with retention of their ability to secrete insulin after stimulation with high glucose in a manner that was comparable to untreated controls. Moreover, the mean normalized stimulated-insulin secretion was not significantly different between the vitrification solutions irrespective of the base vehicle solution used (EuroCollins (EC) or Unisol (UHK)). These findings serve to illustrate the complexities of interactive variables in a cryopreservation protocol that has often been observed and reported in other systems as well as in islet cryopreservation.[49,66,72,73]

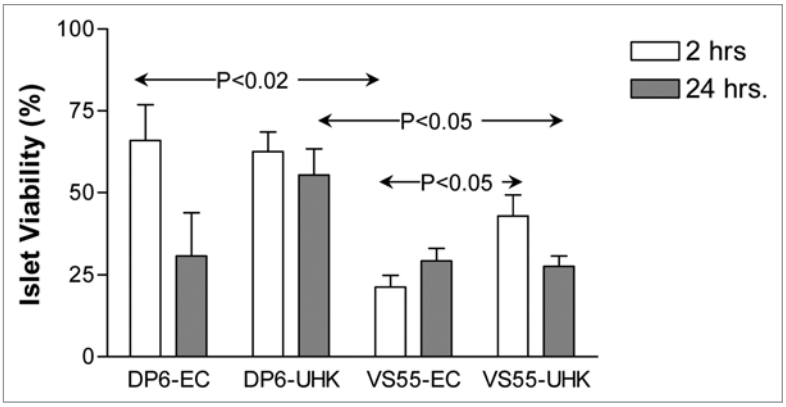

Figure 2. Metabolic activity of isolated rat islets normalized to untreated controls after either 2 hr or 24 hr recovery following exposure to combinations of the VS55 or DP6-1,3 CHD CPA cocktails prepared in either EuroCollins (EC) or Unisol-UHK vehicle solutions.

Vitrification compared with conventional cryopreservation. *Islet recovery and morphological integrity.* **Table 4** shows that the recovery of islet numbers in these experiments was of the order of 65–70% in agreement with published values for cryopreserved islets.[38] It would appear that there are no clear advantages of vitrification over freezing in terms of the percentage of islets recovered after the cryopreservation procedure.

Figure 4 shows the comparative integrity of the islets from each group after rewarming. It is clearly apparent that frozen islets had a much looser gross structure and appeared less compact than the vitrified islets. We hypothesize that this is likely to be due to the effects of ice formation within the islet structure during freezing and that as with other tissues, ice-free methods cause less distortion and disruption of the tissue architecture during cryopreservation. We plan to examine this using a cryosubstitution technique that we have used extensively in other studies to demonstrate the amount and location of ice within tissue samples at cryostorage temperatures.[40,41]

Functional recovery. The insulin secretory function of islets following cryopreservation was impacted by the method used. **Figure 5** shows that relative to untreated controls, cryofrozen islets had a lower mean stimulated insulin secretion index than either of the vitrified groups. Moreover, the relative difference was even greater 24 hours after rewarming when islets vitrified using either VS55 or DP6-1,3CHD were significantly better than the cryofrozen group. After 24 h in culture the cryofrozen islets were clearly substandard compared to untreated control responses, but the responses for vitrified samples were not significantly different to controls. It is not readily apparent why the vitrified samples have a normalized index that is higher than 1.0 when compared to untreated controls, but we suspect that it is related to the variability that is introduced into the index by calculating insulin secretion on a per islet basis. Since the size of islets varies considerably, it is likely in small sample sizes that the variations in islet size will bias the mean insulin secretion index.

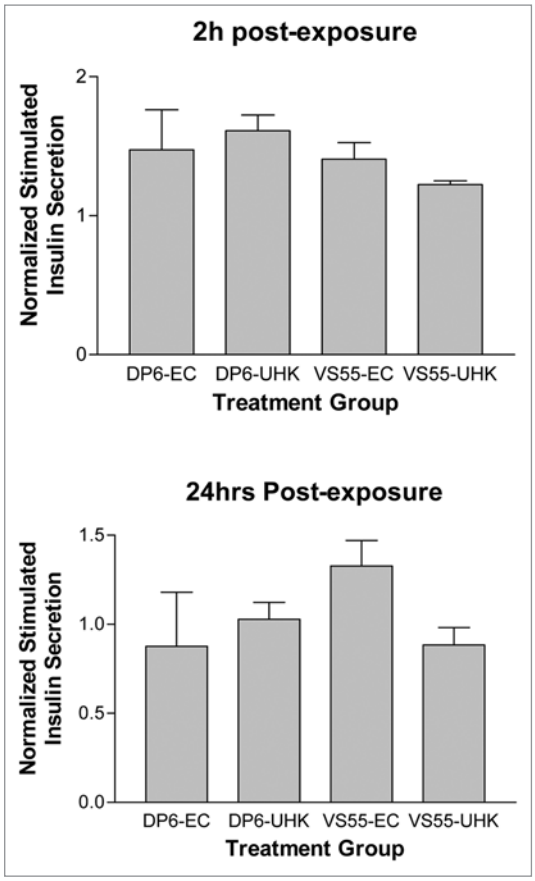

Figure 3. Islet tolerance to CPA exposure without subsequent subzero cooling and vitrification. Data is plotted as mean (±SEM) normalized stimulated insulin secretion after either 2 hr or 24 hr recovery.

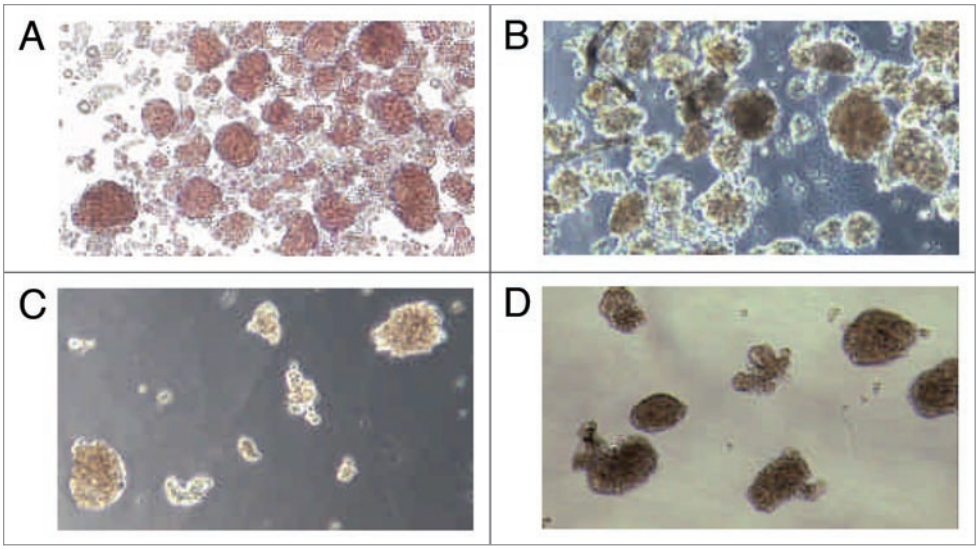

Figure 4. Gross morphology of isolated rat islets showing their comparative integrity following cryopreservation and compared with untreated control islets (A). Islets were cryopreserved using either the conventional freezing/thawing method (B) or vitrification in DP6-1,3 CHD (C) or VS55 (D).

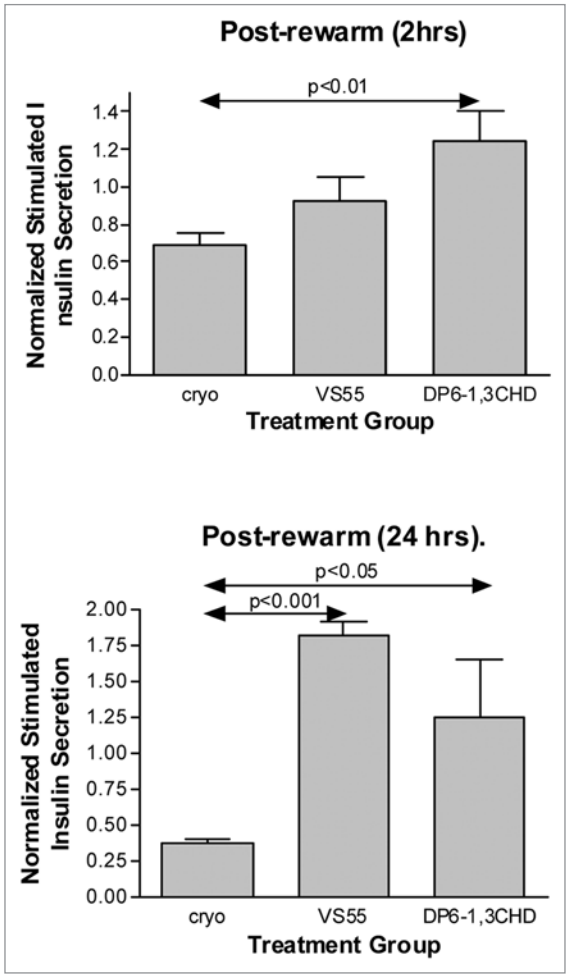

Figure 5. Functional recovery of batches of rat islets following vitrification in either VS55 or DP6-1,3 CHD media compared with conventional freezing and thawing in 2 M DMSO (CRYO). Islet function is shown as the mean (±SEM) stimulation index normalized to untreated control islets. Batches of islets were assessed for stimulated insulin secretion either within 2 hr of rewarming to 37°C or after 24 hr in culture.

This problem can be minimized by attempting to standardize the islet mass in a given sample by re-calculating islet sizes in terms of a defined "islet equivalent",[74] or by measuring the amount of islet material in each sample by some quantitative measure. For example, the measurement of total DNA in the islet sample is a more rigorous metric of the islet mass. This measure is independent of islet cell viability and will provide an ideal basis for unifying insulin secretion data. The Cyquant assay for measuring DNA in cell samples has been used extensively and we are currently adapting this for measuring the total DNA in islet samples. We have encountered some technical difficulty related to the efficient disruption of the islets to expose the total DNA from all cells, but have recently resolved this by combining repeated freeze/thaws in water with ultrasonication. This is work in progress and resolution has not yet been fully implemented or applied to the data in this pilot study. Nevertheless, the conventional index calculated on a per islet basis and used extensively in prior studies is sufficiently robust to allow the qualitative conclusion that vitrification offers significantly better recovery of cryopreserved islets compared with conventional freezing and thawing.

Discussion

Vitrification media. A variety of existing and potential vitrification solutions have been selected for evaluation in our studies. VS1, is the original vitrification solution developed by Rall and Fahy in 1985 for the vitrification of embryos,[75] and which was used by Jutte et al. for the only previously recorded attempts to vitrify islets.[31,32] Since that time, a number of other solutions have emerged including VS3,[37] and VS55;[76] the latter designed specifically to meet the stringent demands of vitrifying an entire organ such as the kidney. Attempts to understand the requirements of vitrification solutions at the molecular level have led to identification of new solutes with physical properties that would promote the vitreous state during cooling. A promising class of compounds is the polyalcohols such as 1,2-propanediol (1,2PD) and butane-2,3-diol (2,3 BD), the optical isomers of which have been shown to vitrify at significantly lower concentrations than any other known CPA.[77,78] This is important to minimize the toxic effects of using high concentrations of CPAs. Moreover, it is now established that both the concentration needed to vitrify, and the toxicity can be reduced by incorporating a wide variety of

non-permeating disaccharides or polymeric compounds.[78,79] The vehicle solution used as a carrier for the CPA cocktails has also proved to be an important consideration for optimum preservation as we have reviewed elsewhere.[19,20]

Tolerance of isolated pancreatic islets to hypothermia and high concentrations of CPAs. A pilot study was originally undertaken to examine the tolerance of isolated islets to hypothermic exposure with high concentrations of permeating cryoprotectants.[80] Specifically, rat islets were exposed at 0°C to 3 mol/l of either 2,3 BD or DMSO added in incremental steps of 1 mol/l at 20 min intervals. Removal of the CPAs was by step-wise serial dilution at 0°C before the islets were resuspended in isotonic culture medium (RPMI 1640 + 10% FCS). Islet viability was assessed either 2 or 24 h after treatment using both a fluorescent membrane integrity test and in vitro insulin secretion assays. Islets in all groups retained gross structural integrity with a very high proportion of constituent cells maintaining membrane integrity. **Table 5** shows that stimulated insulin secretion was reduced in islets after brief hypothermic exposure in RPMI 1640 culture medium per se in the absence of any added CPAs, but this returned to normal control levels after 24 h of normothermic culture. This illustrated the vulnerability of islets to thermal shock and emphasizes the importance of optimizing the conditions of cold exposure. **Table 5** also shows that batches of islets treated with up to 3 mols/l of CPA (DMSO or 2,3 BD) showed marginally reduced functional indices compared with those of hypothermic controls not exposed to CPA. These earlier studies suggested that islets are able to withstand exposure to the high concentrations (~30% w/v) of DMSO and polyalcoholic CPAs such as 2,3 BD necessary to vitrify at practical cooling rates.[80] Nevertheless, it has subsequently been discovered that the purest form of 2,3 BD available commercially, contains a low concentration of the *meso* isomeric form that is both cytotoxic and hinders the vitrification process as discussed below. These findings were in large part responsible for our subsequent focus on propanediol as a more suitable polyalcohol as a component of new vitrification solutions.

Another approach we have begun to evaluate more fully is the efficacy of DMSO and polyalcohols, with or without the addition of glass-promoting solutes such as polyethylene glycol (PEG 400) and synthetic ice blocking molecules, as components of vitrification media as discussed below. Discovery of new ice inhibiting cryoprotectants for use in either classical cryopreservation or in molecular ice control techniques and vitrification, has become an important focus in recent years.[19,81] The concept of designing specific artificial chemical agents whose purpose would be to control the physics of ice was first mentioned by Fahy[82] who stated "insight into the mechanism of antifreeze protein (AFP) action opens the possibility of designing molecules which may be able to inhibit ice crystal growth in complementary ways, e.g., along different crystallographic planes." Chou[83] mentioned an intention to specifically design ice crystal growth inhibitors. However, his interest was confined to minor modifications of existing naturally occurring AFPs and did not include preparation of de novo synthetic non-protein antifreeze molecules. Historically, serendipity has been responsible for most discoveries of cryoprotectants. A major focus of our research has been rational, intentional design of synthetic ice blockers (SIBs), which will combine with conventional cryoprotectants, and possibly naturally occurring antifreeze compounds, to minimize ice nucleation and growth during deep subzero cooling. Molecular modeling techniques can be used to identify molecular conformations that might complement the atomic spacing of hydrogen-bonding sites on the prism face of an ice crystal. Hypothetically, these structures might be expected to hinder the growth of ice by lattice-matching with available sites on the basal plane surface of an ice crystal. Such considerations revealed that 1,3,5 cyclohexanetriol (CHT), or its—diol derivatives, possess the required bond angles and distances to conform with this hypothesis and were selected as lead compounds in preliminary physical studies to determine their efficacy in controlling ice growth. Two proprietary synthetic ice blockers have already demonstrated exceptional ice blocking capabilities in our early studies as we have reviewed recently.[19]

Importantly, this combination of solutes proved more effective at controlling ice crystal growth than the baseline VS55 vitrification solution. Since a major objective in developing optimized methods of cryopreservation is to minimize CPA toxicity by using the lowest concentration consistent with cryoprotection, we chose to evaluate the efficacy of DP6 + SIB as a novel new medium for ice-free cryopreservation of pancreatic islets. This was compared with our baseline vitrification solution VS55, which contains 30% greater concentration of CPA solutes than the new DP6 + SIB solution, but has been used successfully for vitreous cryopreservation of other tissues.

Selection of conventional CPAs for combination with SIB molecules to promote the vitreous state. Although 2,3 BD has been shown to possess superior glass-forming capabilities compared with other CPAs, such as DMSO and other polyalcohols,[77] it has become apparent that the highest purity samples available commercially are contaminated with a *meso* isomer that is deleterious to the process. This stems from the formation of a hydrate, which crystallizes easily during cooling and is markedly cytotoxic. Racemic mixtures of the active enantomers without *meso* contamination are not commercially available diminishing the practical interest in this compound as a useful component of vitrification media.[78,84] For this reason we recommend 1,2 PD as a practically more favorable polyalcohol for combination with DMSO and SIBs as illustrated in the preliminary work with our new DP6 solution. Moreover, we have found 1,2 PD to be well tolerated at high concentrations (up to 3 M) by other cells and tissues (data not included here).[36,40,44,45,85,86]

Methods

Islet isolation. Islets were obtained from Sprague Dawley rats (150–250 g) using the Cambridge protocol for pancreas harvest and islet isolation used extensively in our prior studies.[47-49] In brief, the pancreas was distended by ductal injection of collagenase(3 mg/ml; Sigma Type V) in situ in anesthetized rats. The pancreas was then excised and digested at 37°C and the dissociated tissue was washed in Dulbecco's phosphate buffered saline

Table 1. Protocol for handling islets during the CPA addition and elution phases of a baseline vitrification procedure

Revised scheme to vitrify islets without the need for centrifugation until the final step after rewarming and dilution
Use 2 ml glass vials with screw caps.
Start with islets (up to 500) in 0.1 ml medium (0% CPA) at Room Temp (RT)
Add 25 µl of 110% VS (VS55 or DP6 etc.,) → 125 µl of 22% VS @ RT for 15 min
Transfer to ice
Add 75 µl of 110% VS → 200 µl of 55% VS @ 0°C for 10 min.
Add 800 µl of 110% VS → 1 ml of 99% VS @ 0°C for 10 min.
VITRIFY using N_2 purge, NOT isopentane to avoid ice crystallization from water condensation.
Rewarm according to the defined protocol to vitreous melting.
Start with 1 ml 99% VS after melting and immediately add 1 ml dilution medium + 300 mM mannitol (M) → 2 ml 50% VS @ 0°C for 10 min.
Transfer to pre-cooled 15 ml centrifuge tube.
Add 2 ml medium + M → 4 ml 25% VS @ 0°C for 10 min.
Add 4 ml medium + M → 8 ml 12.5% VS @ 0°C for 5 min
Add 4 ml medium + M → 12 ml 8% VS and allow to warm to RT for 5 min.
Spin @ RT and resuspend pellet of islets in culture medium.
Count and assign to assays.

Table 2. Vitrification solution addition and removal protocol

Step No.	Step description	Period (min)	Temp. [K]	CPA conc. [mOsm]	Impermeable ion conc. [mOsm]	Mannitol conc. [mOsm]	TOTAL impermeable conc. [mOsm]
1	Add 25 uL of CPA	15:00	295.15	1584	290	0	290
2	Add 75 uL of CPA	10:00	277.15	3960	290	0	290
3	Add 800 uL of CPA	10:00	277.15	7128	290	0	290
4	Add 1 mL 300 mM mannitol dilution media	10:00	277.15	3564	290	150	440
5	Add 2 mL 300 mM mannitol dilution media	10:00	277.15	1782	290	150	440
6	Add 4 mL 300 mM mannitol dilution media	5:00	277.15	891	290	150	440
7	Add 4 mL 300 mM mannitol dilution media	5:00	295.15	594	290	150	440
8	Resuspend in culture media	-	295.15	0	290	150	440

containing 10% fetal calf serum (FCS) before the free islets were purifed using a Ficoll/hypaque density gradient.

Cryopreservation protocols. *Conventional cryopreservation.* The widely used and documented cryopreservation method introduced originally by Rajotte et al.[50,51] was used as a control method against which to compare the efficacy of the vitrification approach. Briefly, this method involves slow cooling (0.25°C/min) of batches of islets in the presence of 2 M DMSO to -40°C in a programmed controlled-rate freezer (Planer Kryo 10) followed by storage in liquid nitrogen at -196°C. For rewarming, frozen samples were thawed rapidly by agitation in a 37°C water bath and the cryoprotectant was eluted in the presence of 0.75 M sucrose as an osmotic buffer.

Vitrification protocols. The vitrification method we developed and showed to be superior to conventional cryopreservation for blood vessels was adopted as a baseline method for isolated islets in this study.[36,40] This entailed a gradual infiltration of batches of islets with an 8.4 M vitrification solution (VS55) consisting of 3.10 M DMSO, 3.10 M formamide and 2.21 M 1,2-propanediol in EuroCollins solution at 4°C.[40] Precooled vitrification solution (4°C) was added in three sequential steps. After addition of the final vitrification solution, islet samples were cooled rapidly (43°C/min) to -100°C, followed by slow cooling (3°C/min) to -135°C, and finally stored in a freezer at -135°C for a minimum of 24 hours. A thermocouple was inserted into a separate dummy sample of the same vitrification solution, and its output monitored via a digital thermometer. Vitrified samples were rewarmed in two stages: First, slow warming to -100°C (30°C/min) and then rapid warming to melting (225°C/min). A slow warming rate was achieved by moving the sample to the top of the -135°C freezer. The fast warming rate was generated by placing the glass vial in a mixture of 30% DMSO/water at room temperature. This technique prevents ice from forming on the outside surface of the glass vial, thereby allowing visualization of the melting process. After rewarming, the vitrification solution was removed in a stepwise manner using a mannitol solution for osmotic buffering. The revised scheme for handling islets during the steps described above is summarized in **Tables 1 and 2**.

Vitrification using new solutions containing synthetic ice blocking molecules was attempted using similar methods of

Table 3. Islet permeability model parameters for computational analysis

Membrane parameters				
Parameter	**Parameter description**	**Value**		**Units**
T	Temperature	296.15	277.15	K
Lp	Hydraulic conductivity	5.00E-14	6.00E-15	$m^3/N*s$
ω	CPA Permeability	2.00E-11	7.00E-12	mol/N*s
σ	Reflection coefficient	1.0	1.0	-
Extracellular parameters				
Parameter	**Parameter description**	**Value**		**Units**
Cc	CPA concentration	500		mOsm
Cimp	Impermeant concentration	290		mOsm
Intracellular parameters				
Parameter	**Parameter description**	**Value**		**Units**
V	Islet volume	1.77E-12		m^3
A	Islet surface area	7.07E-08		m^2
Vb	Osmotically inactive volume	0.474		-
nw	No. moles of water	7.36E-08		mol
ncpa	No. moles of CPA	8.10E-32		mol
nimp	No. moles of impermeants	1.89E-08		mol

cooling and warming as specified for the baseline method. Steps for loading and unloading the cryoprotectant mixtures for the new DP6-protocol were derived based on the kinetics of CPA permeation and other related biophysical parameters available in the literature specifically for islets. A computer based model used to predict osmotic responses in other cell types[52] was modified slightly to accommodate pancreatic islets as described in the next section.

Viability assays. Islets were numerically counted before and after a given experimental treatment in order to determine the recovered yield. In accordance with previous studies established methods of measuring the ability of islets to secrete insulin in vitro in response to conventional secretogogues such as glucose were used as the primary assay. Insulin content of frozen supernatants from the secretion assays were determined using standard ELISA commercial kits (Alpco Diagnostics). While these tests provide a direct assay of islet function, they are nevertheless, time consuming and expensive for the routine screening of a large number of experimental variables such as those comprising a cryopreservation protocol. For this reason, the alamar Blue metabolic indicator assay, which has been used effectively with a range of other cells and tissues,[44,53,54] was also employed as a high throughput assay. The alamarBlue™ assay, is a quantitative method of non-invasively measuring islet viability in vitro and, because the reagent is non-toxic, the test can be performed on islets that are then subjected to further tests.

Computational studies. An adapted model was used as a design tool to simulate the biophysical responses of islets subjected to the addition and removal of cryoprotective solutions over a range of experimental temperatures. The diffusion of water and permeable solute across the plasma membrane of cells within the islet was modeled with the coupled membrane transport equations for water and solute as presented originally by Kedem and Katchalsky (1958).[58] Biophysical parameter values used in this model were obtained from reference data available in the literature that also include the permeability data for several cryoprotectants.[55,56]

Table 4. Islet recovery indices

Experimental group	Recovery (%) n = 3
Cryopreserved by freezing	66 ± 6
Vitrified—DP6 + 1,3 CHD	70 ± 14
Vitrified—VS55	64 ± 5

As reviewed authoritatively recently by Mazur,[23] optimal cryopreservation protocols demand consideration of the osmotic responses of cells during the cryoprotective additive (CPA) treatments designed to minimize potential cell injury caused by excessive volumetric excursions. The addition of permeable CPAs cause transient volume changes in islets as water is replaced in intra- and extracellular spaces with CPA. Opposite effects occur during cryoprotectant removal as water moves in and CPA exits the islet. The permeation rates of water and solutes are strongly governed by temperature effects in which membrane permeability to water and CPA diminish exponentially with decreasing temperature. In conventional cryopreservation involving freezing, most dilute concentrations of cryoprotective additives (≤2 M) can safely be added or removed in single steps.[15,16,26,49,51,57] However, cryopreservation protocols in which high concentrations of CPA are required, stepwise addition and removal steps

Table 5. Comparative viability of islets after exposure to hypothermia and high concentrations of cryoprotectants

	Stimulated insulin (pg/islet/min)		Stimulation factor (stimulated/basal)	
Treatment	**2 h**	**24 h**	**2 h**	**24 h**
Normal controls (24 h; 37°C)	59.9 ± 6.8	-	8.5 ± 1.3	-
Hypothermic controls (2 h; 0°C)	42.4 ± 5.2	57.3 ± 7.6	8.5 ± 1.1	7.2 ± 0.8
DMSO (3 M; 0°C)	39.7 ± 5.0	29.7 ± 4.8	9.0 ± 1.3	6.1 ± 1.1
2,3 BD (3 M; 0°C)	33.3 ± 3.5	39.8 ± 4.9	6.2 ± 0.8	6.6 ± 0.9

are employeed to limit volume changes within a safe region of excursion. Permeation kinetics of cryoprotectants are also dependent upon cryoprotectant type. Comparison of chemicals with cryoprotectant properties reveals no common structural features although smaller molecular CPA's tend to permeate more rapidly than larger molecules.[20]

A computer based model was generated to serve as an analytical tool for predicting osmotic responses of pancreatic islets during the addition and removal of vitrification solutions used in this study. The model permits the rational design of CPA addition/removal protocols that minimize cellular injury. The model was based on transport equations from irreversible thermodynamics that describe the coupled diffusion of water and cryoprotectants across biological membranes.[58] Using a linearized form of these equations,[59] the model simulates the movement of water and cryoprotectants into and out of a single pancreatic islet across a range of temperatures and cryoprotectant concentrations. The membrane is described by three parameters that characterize the hydraulic conductivity of water (Lp), cryoprotectant permeability (ω) and the reflection coefficient (σ) which, describes the interaction of water and solute molecules as they cross the lipid bilayer.

In this study, the islet was idealized as a single, equivalent osmotic unit with an average diameter of 150 μm. The cytosolic component of the islet was modeled as an isotonic solution composed of impermeable ions with a total osmolality of 290 mOsm. Biophysical parameter values for islets were obtained from the literature.[55,56,60-63] The percentage of osmotically active water in the islet was modeled to be 53%.[64] The membrane parameters of Lp, ω and σ were obtained from rat islet permeation studies[65] conducted with DMSO at 22°C and 4°C and are shown in **Table 3** along with the other islet parameters. The volumetric excursion limits for islets in a canine model were 0.6 V/V_{iso} and 1.53 V/V_{iso}.[60]

The vitrification solutions used in this initial study, VS55 and DP6 + 1,3CHD were composed of two or more permeating cryoprotectants, each with different permeation rates. For simplification, the multiple cryoprotectants of each solution were lumped as a single cryoprotectant with the same size and membrane permeability as DMSO. Although computational algorithms that model multi-solute transport exist, they are in the early stage of development and offer marginal refinement of the results presented here for a significant increase in complexity. The selection of DMSO as the modeled cryoprotectant is an appropriate choice since membrane permeability data obtained in different islet transport studies is typically less than or equal to the permeability of 1,2 propanediol.[56,63] A slower penetrating cryoprotectant causes greater transient changes in cell volume. Excessive volumetric excursions cause osmotic injury as cellular membranes and cytoskeletal networks are compromised. The use of slower membrane permeability parameters that yield worst-case volume excursions permit the design of addition/removal protocols that prevent cellular injury when excursions are maintained within osmotic limits.

Summary and Future Directions

Transplantation of pancreatic islets for the treatment of diabetes mellitus is widely anticipated to eventually provide a cure, once a means for preventing rejection is found without reliance upon global immunosuppression. Long-term storage of islets is crucial for the organization of transplantation, islet banking, tissue matching, organ sharing, immuno-manipulation and multiple donor transplantation. Methods of cryopreservation have generally focused on conventional freezing methods involving significant ice formation in the system. Even recent work to optimize cryopreservation based on theoretical considerations, has focused on classical freezing protocols. It is now well established that long-term storage of complex multicellular tissues (the integrity of which is compromised by ice formation), will best be achieved using techniques that minimize or avoid the formation of ice. The development of techniques for ice-free cryopreservation of mammalian tissues has been a focus of our research in recent years and is incorporated here to apply these technologies to the long-term storage of pancreatic islets. More specifically, it is the general aim of these studies to test the feasibility of applying new vitrification-preservation approaches to isolated islets and to, eventually, apply optimized techniques to genetically-modified islets which are designed to overcome immune responses in the diabetic host.[87,88]

The long-term objective of this program of research is to develop an optimized method of cryopreservation for genetically-modified pancreatic islets based upon vitrification technology to restrict water crystallization and ice-induced injury. This goal is based on the premise that:

(1) an ice-free approach to cryopreservation will enhance the survival of isolated islets compared with conventional cryopreservation involving freezing, and

(2) ice-free cryopreservation technology can be applied to islets that have been genetically-engineered to enhance local immunosuppression.[89]

The preliminary studies reviewed here demonstrate the efficacy of vitreous cryopreservation even in small tissue structures such as pancreatic islets, which are amenable to cryopreservation

by either vitrification or classical freezing. The potential advantages of the vitrification approach are also reviewed highlighting some of the practical issues that must be addressed in the development of optimized techniques. A revised scheme for vitrification of isolated islets in tubes that avoids the need for multiple transfers and centrifugation steps is proposed. Moreover, computational analyses of the osmotic responses of islets during the various steps in the procedure verify that the objective of restraining cell volume excursions within tolerable limits during the addition and removal of CPAs was accomplished.

The ultimate goal of this approach is to develop a marketable package comprising cryopreserved genetically modified islets that can be removed from vitrified storage, rewarmed simply without specialist training or equipment, and transplanted to produce a well-tolerated graft with biological effectiveness that exceeds insulin injection therapy. Further developments of this approach will need to address additional practical issues relating to scale-up of the product to clinically relevant sample sizes. Inherent in this will be the need to avoid thermal stresses and fracturing that are now known to be a significant hurdle in some cryopreserved systems.[46,90-92]

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