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Cryopreservation of Human Pancreatic Islets

Jonathan R. T. Lakey, Philip W. Burridge, and Ray V. Rajotte

Islet transplantation has the potential to permanently restore glucose homeostasis in type 1 diabetics, thereby eliminating insulin injections and possibly even reversing neurovascular complications and preventing end-stage organ failure. The severe shortage of human pancreata and the inability to consistently isolate large numbers of healthy islets have made it necessary to pool islets from several donors. Cryopreservation is cost-effective, is less labor-intensive, minimizes the chance of microbial contamination, and requires less storage space. It allows time to find the best tissue match while ensuring strict quality control. Frozen tissue stored in liquid nitrogen is easy to transport. The freeze-thaw procedures improve the purification of the islets. Cryopreservation also has a uniquely positive feature in that it modifies how the donor islets will respond when transplanted into the hostile immune environment of an unrelated recipient. But the most compelling reason for using cryopreservation is that transplant centers can store islet cells indefinitely.

ABBREVIATIONS

APC	Antigen-Presenting Cells
DC	Dendritic Cell
DMSO	Dimethyl Sulfoxide
EG	Ethylene Glycol
FCS	Fetal Calf Serum
HLA	Histocompatibility Locus Antigen
HTS	Hypothermosol
IBMX	3-Isobutyl-1-Methylxanthine
IE	Islet Equivalents
MHC	Major Histocompatibility Complex
PLM	Proinsulin-Like Molecule
STZ	Streptozotocin
UW	University of Wisconsin

Historical Perspective

Transplantation of insulin-producing tissue as the definitive treatment for diabetes was independently proposed more than 100 years ago by Williams and Ssobolew.^{1,2} Transplantation of isolated islets of Langerhans offers a physiological solution that reestablishes glucose homeostasis with precise feedback controls, leading to a permanent state of normoglycemia.

A total of 447 attempts to treat Type 1 diabetes with islet allografts were reported to the International Islet Transplant Registry between 1974 and 2000, 394 of which were performed during the last decade. Islet-kidney transplants composed the majority. The allografts were composed of freshly isolated or cultured or cryopreserved islets or a combination of freshly isolated or cryopreserved islets. In some cases, immunosuppression was induced with anti-lymphocyte serum while maintenance immunotherapy was largely based on a combination of glucocorticoids, cyclosporine, and azathioprine. As of 1999, Brendel et al. reported that 65% of grafts lost function within 1 year of transplantation. Less than 10% of patients were insulin-inde-

pendent at 1 year posttransplant, although 28% had sustained C-peptide secretion.³ Conventional immunosuppressive agents used to abrogate islet rejection and autoimmune recurrence have diabetogenic side effects, which can also cause islet graft failure.^{4,5} Using a glucocorticoid-free immunosuppression regimen of daclizumab, sirolimus, and low-dose tacrolimus, designed to prevent both autoimmune recurrence and allograft rejection while avoiding diabetogenic toxicity from highly concentrated drug delivery in the liver, the rate of insulin-independence at 1 year rose from 8% to 100%.^{6,7}

Despite significant advances during the last 30 years, inconsistency remains in the overall success of the islet isolation procedure.⁸ We, as well as others, have identified donor and islet isolation variables that affect islet recovery and in vitro function.⁹ In our study, increased donor age and body mass index and skills of the local procurement team have a positive correlation with islet recovery. In contrast, pancreas weight, hyperglycemia greater than 10 mmol/L, frequency and duration of cardiac arrest, cold storage greater than 16 h, and collagenase digestion time have a negative correlation.

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ALLOGRAFT

Transplantation of cells, tissue, or an organ from one genetically dissimilar individual to another of the same species

Data from the International Islet Transplant Registry have shown that an islet allograft in excess of 6000 islet equivalents per kilogram body weight is necessary to restore insulin independence.¹⁰ The inability to isolate large numbers of viable islets from each human pancreas has made it necessary to pool islets from several donors. Low-temperature banking of isolated islets has been one approach to supplement freshly isolated or cultured islets.¹¹

Several methods for the preservation of human islets have been proposed, but cryopreservation is the safest and most effective method.¹¹ Long-term subzero storage has several advantages. Pooling islet preparations makes it easier to create the critical β -cell mass necessary to reverse hyperglycemia.¹²⁻¹⁴ A tissue bank of cryopreserved islets with a diverse variety of ABO and HLA (histocompatibility locus antigen) phenotypes allows time to optimize matching between donor and recipient before transplantation.¹⁵ Cryopreservation permits time to ensure strict pretransplant quality control testing of islet viability and for microbiological sterility.¹⁵ Frozen tissue stored in liquid nitrogen is easy to transport between collaborative transplant centers.¹⁶ The freezing and thawing procedures improve the purification of the endocrine tissue by selective removal of exocrine tissue.^{17,18} Cryopreservation modulates isolated pancreatic islet immunogenicity^{17,19-22} by eliminating antigen-presenting (dendritic) cells^{23,24} or by down-regulation of islet MHC (major histocompatibility complex) Class I antigen expression.^{25,26} But the most compelling reason to pursue human islet cryopreservation is that it enables clinical transplant centers to store islet allografts indefinitely while specific immune unresponsiveness (tolerance) is induced in the recipient, thereby eliminating the need for posttransplantation immunosuppression.^{17,27,28}

Cryopreservation of Animal Islets

Several cryopreservation protocols²⁹⁻⁴⁰ utilizing widely different free-thaw conditions have been developed since 1977 when Rajotte et al.⁴¹ and Ferguson et al.⁴² first demonstrated that frozen-thawed islets could normalize hyperglycemia in streptozotocin-induced diabetic rats. Protocols have been developed for rodent,^{27,41-44} dog,⁴⁵⁻⁵¹ pig,⁵²⁻⁵⁶ and human islets,^{30,33} with postcryopreservation viability demonstrated both in vivo and in vitro.

In 1989, Rajotte et al. compared a variety of reported cryopreservation protocols using dimethyl sulfoxide (DMSO) and found that slow cooling to -40°C and rapid thawing from -196°C resulted in similar in vitro function as assessed by perfusion and transplantation.⁵⁷ This was confirmed by Rich et al. who, when comparing various protocols, found that slow cooling to -40°C in combination with rapid thawing from -196°C resulted in the best in vivo survival.⁵⁸ Current methods used to cryopreserve human islets have been extrapolated from empirically derived protocols originally developed for rodent islets. Many of these protocols reported a loss of 20% to 40% of recovered viable islets, impaired insulin secretion in response to glucose stimulation, and reduced survival in nude diabetic mice.⁵⁹⁻⁶¹ Further loss of islets after cryopreservation, coupled with reductions in viability, indicates that islet cryopreservation protocols can be refined.

Using a modified protocol developed by Sakonju,⁶² Korbitt et al. demonstrated that a new permeating cryopreservative, ethylene glycol (EG), permitted a high recovery of rodent islets following cryopreservation.⁶³ EG-cryopreserved islets also contained more viable glucagon-secreting α -cells, suggesting that α -cell function in the rat is critical to β -cell function, particularly in vivo.^{63,64}

Cryopreservation of Human Islets

Lakey et al. developed an automated stepwise cryopreservation method using DMSO and a single 500 ml freezer bag.^{65,66} This method of bulk cryopreservation is cost-effective, less labor-intensive, minimizes the chance of microbial contamination, and requires less dewar storage space. Recently, Miyamoto et al. introduced a computer-controlled cryopreservation procedure using EG and a single 250 ml freezer bag.⁶⁷

A comparative study of the effects of DMSO and EG on human islets by Lakey et al. demonstrated that DMSO provided superior survival and preservation of postculture in vitro function, regardless of the addition protocol (Table 1).⁶⁵ This observation may be attributable to qualities inherent to human islets or to differences in the storage, transport, or islet isolation procedures. Furthermore, there was no difference between groups of islets treated with either 1.5 M DMSO or the standard concentration

TOLERANCE

Donor-specific immune unresponsiveness can be induced by manipulating key pathways of T-cell activity (usually using monoclonal antibodies).

Author: Please verify Table headings.

Table 1 | EFFECT OF CRYOPRESERVATION ON HUMAN ISLET RECOVERY^a

EXPERIMENTAL CONDITION	N	ISOLATED ISLET FRACTION ISLET RECOVERY (%)		STIMULATION (INSULIN SECRETORY ACTIVITY [% CONTENT]) INDEX		
		POST-THAW	POST-CULTURE	2.8 mM GLUCOSE	20 mM GLUCOSE	HIGH:LOW
DMSO 2.0 M stepwise	6	70 ± 4	62 ± 4	0.9 ± 0.1 [†]	3.9 ± 0.3	4.5 ± 0.5 [†]
DMSO 1.5 M stepwise	6	80 ± 2*	74 ± 3*	0.7 ± 0.1	3.9 ± 0.3	6.0 ± 0.4* [†]
DMSO 1.5 M one-step, 30 min	6	82 ± 2*	69 ± 3	0.6 ± 0.1	4.0 ± 0.3	6.5 ± 0.8*
DMSO 1.5 M one-step, 45 min	6	—	53 ± 5	0.9 ± 0.1 ^{††}	1.9 ± 0.2 ^{††}	2.2 ± 0.4* ^{††}
EG 2.0 M stepwise	6	69 ± 3	52 ± 4	1.2 ± 0.1 [†]	3.9 ± 0.6	3.4 ± 0.7 [†]
EG 1.5 M stepwise	6	71 ± 3	64 ± 5	0.7 ± 0.1	2.5 ± 0.4 [†]	4.0 ± 0.4 [†]
EG 1.5 M one-step, 30 min	6	68 ± 5	51 ± 7	1.0 ± 0.1 [†]	3.3 ± 0.4 [†]	3.5 ± 0.5 [†]
EG 1.5 M one-step, 45 min	6	—	46 ± 5*	1.1 ± 0.1 [†]	2.0 ± 0.3 ^{††}	1.8 ± 0.3* ^{††}
Nonfrozen control	6	—	—	0.6 ± 0.0	4.7 ± 0.4	8.2 ± 0.9

a. Values are mean ± SEM of 6 independent experiments. Statistical significance of differences was calculated with an unpaired student's *t*-test: **P* = 0.05 vs. DMSO 2.0 M stepwise, [†]*P* < 0.05 vs. nonfrozen controls, ^{††}*P* < 0.05 vs. 1.5 M cryoprotectant one-step added at 30 min.

of 2.0 M DMSO added in either a stepwise or one-step protocol, suggesting that the cryoprotectant could be added in a single step, perhaps by automatic means, without significantly compromising islet survival and function. Although EG permits a high recovery of functionally viable rodent islets following cryopreservation,⁶³ it did not improve the recovery of human islets.⁶⁵

Cryopreservation and Clinical Islet Transplantation

Despite considerable success transplanting cryopreserved islets in small and large animal models, insulin independence in humans has not been achieved with transplantation of cryopreserved islets alone. Transplants have been performed using combinations of freshly isolated and cryopreserved islets when insufficient islet yields from a single human donor necessitated the pooling of islets from multiple donors.^{68,69} According to data from the International Islet Transplant Registry, only 18% of recipients transplanted with a combination of freshly isolated and cryopreserved islets were insulin-independent at 1 year, whereas 11% of cases were insulin-independent when only freshly isolated islets from a single cadaveric donor were transplanted.⁶⁹ To date, the longest duration of insulin-independence in a Type 1 diabetic receiving an islet transplant simultaneously with a kidney transplant received a combination of freshly isolated and cry-

opreserved islets.^{14,69} However, the ideal scenario would be to transplant an adequate number of functional islets from one carefully matched human donor to one human recipient, thereby eliminating the need for future supplementation, and minimizing host cell-mediated destruction of the graft by avoiding the adverse effects of chronic immunosuppressive therapy.

Human Islet Cryopreservation Protocol

Standard procedures of islet cryopreservation utilize DMSO, slow cooling, and rapid thawing.^{70,71} The method used to cryopreserve human islets in our research laboratory is based on a preclinical canine model (Fig. 1).^{11,72,73}

Islet Isolation, Purification, and Quantification

After obtaining informed consent, the pancreas is recovered from a cadaveric donor following in situ vascular perfusion with cold University of Wisconsin (UW) solution as part of a multiorgan procurement.^{74,75} The pancreas is immediately transported to the islet isolation laboratory for processing. Islets are isolated using previously described techniques of controlled Liberase perfusion via the pancreatic duct and Ficoll purification.⁷⁶⁻⁸²

Islet Culture

Following tissue culture in supplemented medium for 24 h, islets are recombined and duplicate

DIABETES MELLITUS

A metabolic disease characterized by hyperglycemia that results from defects in insulin secretion, insulin action, or both.

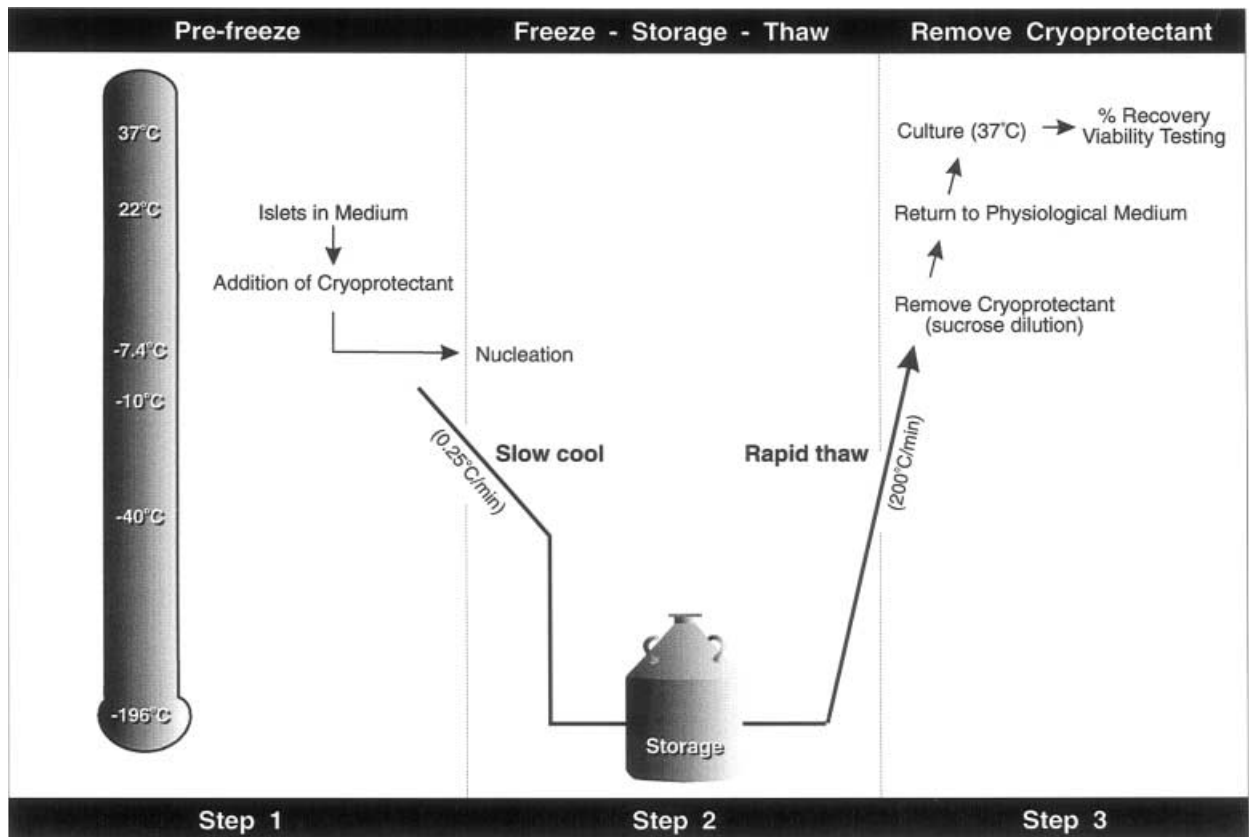


Figure 1. Flow diagram indicating the three distinct steps that are needed for cryopreservation of islets.

aliquots are stained with dithizone and assessed for quantity and purity.

Islet Cryopreservation

During cryopreservation, there are 3 distinct steps (Fig. 1): 1) a prefreeze phase during which the islets are equilibrated with the cryoprotectant; 2) freezing, storage, and thawing; and 3) removal of cryoprotectant and return of the islets to a physiological medium.¹¹

Prefreezing Phase

The islets are suspended in freeze media and the cryoprotectant (EG or DMSO) is added in stepwise fashion to bring the final concentration of cryoprotectant to 1.5 M or 2.0 M, or added in a single step to a final concentration of 1.5 M.¹¹

Stepwise Protocol

When the cryoprotectant is added in a stepwise protocol (Fig. 2), islets are equilibrated with 0.67

M cryoprotectant for 5 min followed by equilibration with 1.0 M cryoprotectant for 25 min at 22 °C. The concentration of cryoprotectant is then increased to 1.5 M or 2.0 M at 22 °C. The islets are allowed to equilibrate for 15 min before proceeding.

One-Step Protocol

When the cryoprotectant is added in a one-step fashion (Fig. 3), islets are held at 22 °C for 30 or 45 min, at which point 1.5 M cryoprotectant is introduced in a single addition step (Fig. 3). When the cryoprotectant is added at 30 min, 15 additional minutes are allowed for the final equilibration to occur. When the cryoprotectant is added at 45 min, the islets are transferred directly into the 4 °C ice bath without an equilibration period.

Freezing, Storage, and Thawing

After addition of the cryoprotectant (Fig. 4), the tubes are transferred to a 4 °C ice bath for 15

IMMUNE REJECTION

A process initiated by naturally occurring antibodies in the recipient's sera that bind to antigens on the surface of endothelial (and other) cells within the graft, which, in turn, activates a complex series of events that results in the rapid destruction of the transplanted organ or tissue.

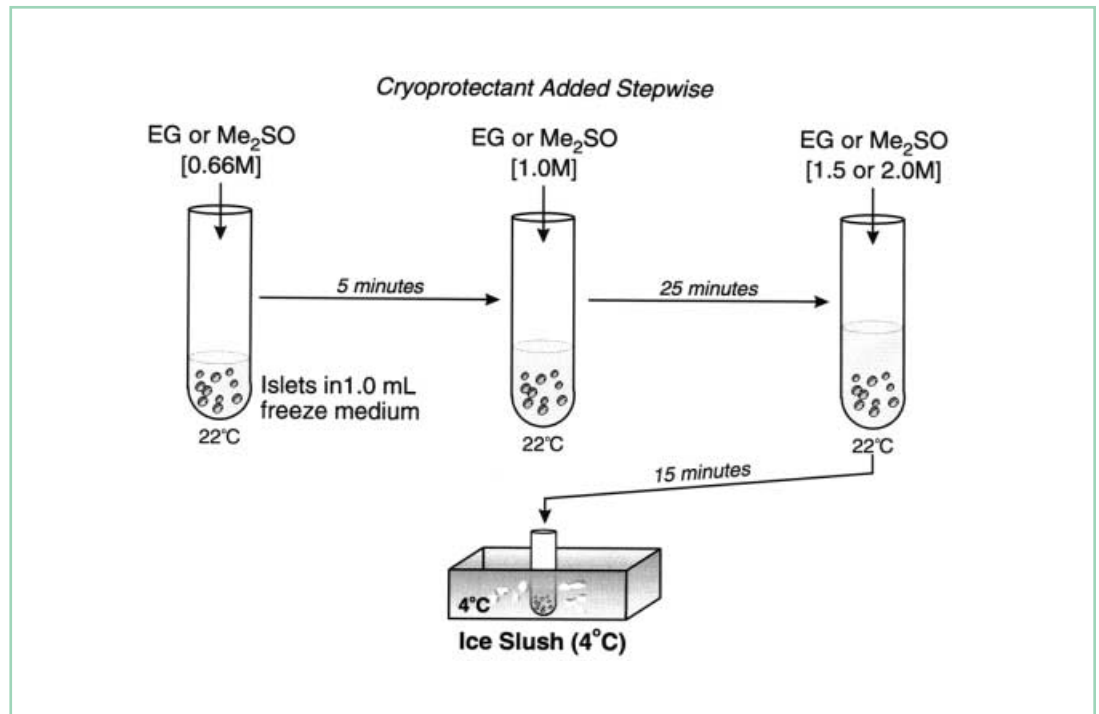


Figure 2. Protocol for stepwise addition of cryoprotectant.

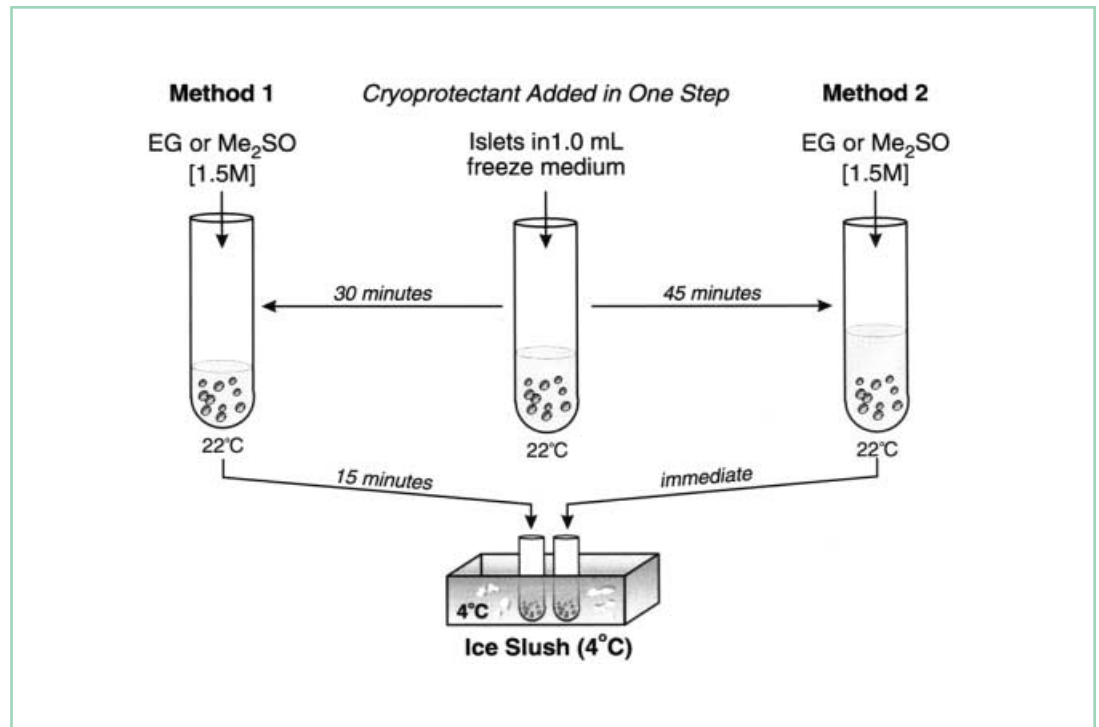


Figure 3. Protocol for one-step addition of cryoprotectant.

min^{11,65} and then supercooled to -7.4°C in an ethanol bath for 5 min and manually nucleated using a metal rod dipped in liquid nitrogen.^{11,65} After a 15-min period allowing for the release of the latent heat of fusion, the samples are slowly cooled at $0.25^{\circ}\text{C}/\text{min}$ to -40°C , and then plunged into liquid nitrogen for low temperature storage.^{11,65} After storage in liquid nitrogen for at least 1 week, the islets are thawed rapidly to 0°C in a 37°C water bath at a rate of $200^{\circ}\text{C}/\text{min}$.^{11,65}

Removal of Cryoprotectant and Return to a Physiological Medium

Following centrifugation and removal of the supernatant, the intracellular cryoprotectant is removed by the addition of 0.75 M sucrose for 30 min at 0°C , followed by stepwise addition of supplemented medium at 5-min intervals over a 20-min period.¹¹ The islets are then again centrifuged, washed in supplemented medium, and transferred to tissue culture plates containing supplemented medium. Islets are maintained for 48 h at 37°C in an atmosphere of 95% air and 5% CO_2 to allow for metabolic recovery prior to quantification and in vitro viability assessment.^{83,84}

Current Limitations of Human Islet Cryopreservation

β -Cell Dysfunction

The ability of islets to restore physiological glucose homeostasis following cryopreservation is a key prerequisite if this technology is to be utilized in clinical transplantation. In our clinical studies, we have clearly demonstrated long-term insulin release from frozen-thawed islets transplanted into Type 1 diabetics, including periods of insulin independence. Although encouraging results have been observed in other centers, clinical trials using cryopreserved islets have been limited with moderate success.

In 1999, a study by Piemonti et al. described the effects of cryopreservation on in vitro and in vivo long-term function of human islets.⁸⁵ First, islet recovery was about 70%, which is similar to that reported in other studies. Second, they also confirmed abnormal insulin secretion. Compared to unfrozen islets, high basal insulin secretion was ob-

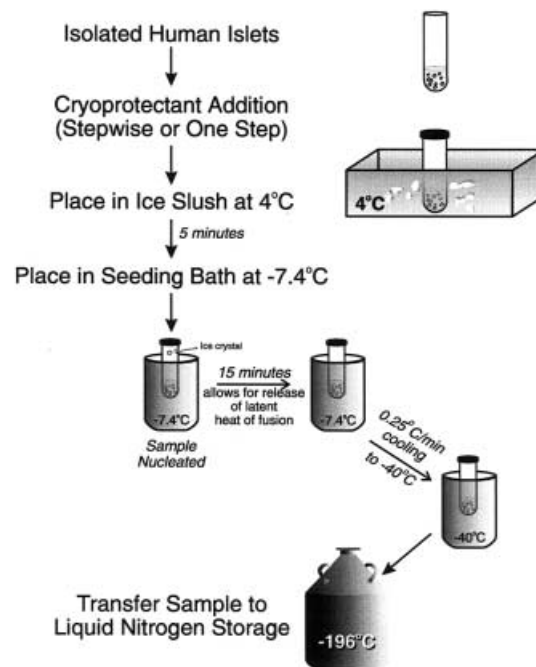


Figure 4. Freezing protocol, including nucleation, controlled slow cooling, and storage.

vested islets. This evidence strongly suggests that islet graft failure may be due to either a reduction in islet mass or a subtle decline in function over time. This latter mechanism may be due to either β -cell overstimulation and subsequent exhaustion or, more likely, an impairment of the key paracrine relationships between endocrine cell types (α -cells, β -cells, γ -cells) within the islet. The latter observation has been observed in two of our patients supplemented with cryopreserved islets who were initially insulin-independent for 2 years but had to resume exogenous insulin therapy when graft function declined.⁸⁷ However, the islets are still functioning in these patients 10 years after transplantation. Amperometric studies have confirmed that the β -cell insulin receptor can mediate positive feedback for insulin secretion. Disruption of this mechanism could explain the existence of both impaired insulin secretion and insulin resistance.⁸⁸

Refinements in Cryopreservation Protocols

Successful cryopreservation of islets requires that the addition and removal of cryoprotectants as well

as cooling and warming are carried out within certain biophysical and cell physiological tolerance limits. Cryopreservation is a complex procedure with a series of steps that subject cells to major osmotic stresses, which can result in potentially damaging changes in cell volume.⁸⁹ Cells shrink transiently upon the addition of cryoprotective agents and then swell as the cryoprotectant permeates. Cells undergo a second shrinkage when cooled at rates low enough to preclude intracellular freezing as growing extracellular ice concentrates the solutes in the diminishing volume of nonfrozen water, causing exosmosis. The cells return once again to their normal volume during warming and thawing. Finally, cells undergo a potentially damaging osmotic volume excursion during the removal of the cryoprotectant.^{89,91}

Current methods of islet cryopreservation utilize permeating cryoprotectants combined with isotonic solutions without specifically addressing issues of ionic balances, buffering capacity, or oxygen-free radicals that occur during hypothermic stresses. The standard method uses a cell culture solution, Medium 199 (Gibco, Carlsbad, CA), supplemented with 10% fetal calf serum, and a cryoprotectant, DMSO, in combination with slow cooling and rapid thawing.¹¹

Cryopreservation of islets with solutions specifically derived for cells, tissues, and organs at hypothermic temperatures have many theoretical advantages over standard tissue culture solutions. UW solution (DuPont Merck Pharmaceutical Company, Wilmington, DE) was developed in the early 1980s as an intracellular-based preservation solution. It is the universal standard vascular flush and preservation solution for kidney, liver, and pancreas.⁹²⁻⁹⁵ BioLife Solutions, Inc. (Binghamton, NY) has developed two crystalloid-colloid blood substitutes for whole organ preservation, which have been shown to greatly improve cell viability and inhibit cryopreservation-induced apoptosis (cellular suicide) and freeze-induced traumatic necrosis.⁹⁶⁻¹⁰² Hypothermosol-M (HTS-M) is a hyperkalemic intracellular-like solution specifically designed to prevent intracellular acidosis and maintain cellular integrity under hypothermic conditions. Its counterpart, Hypothermosol-P (HTS-P), is an "extracellular" flush solution designed to purge the mainte-

nance solution, accumulated toxins, and metabolic by-products from the donor organ.

The properties that make these solutions effective for low-temperature organ storage may also augment the recovery of cryopreserved cells. Lakey et al. demonstrated that the nonpermeating cryoprotective components in UW solution and an early version of HTS did not protect canine islets from the damaging effects of cryopreservation. However, post-freeze-thaw survival improved significantly when DMSO was added to either solution.¹⁰³ Because cryopreservation of islets has been primarily based on an empirical approach,¹⁰⁴ little is known about the biophysical parameters of human islets. Recent canine islet studies of basic cryobiological parameters, including the determination of the membrane permeability to water and cryoprotectants, have enabled the development of mathematical models to predict several key cellular responses that occur during the cryopreservation process.¹⁰⁵ These simulated responses of islet cells to different osmotic environments make important predictions about key factors involved in cryopreservation, including cryoprotectant permeation, cellular volume changes, and incidence of intracellular ice formation.

These data should challenge researchers to examine new strategies for successful cryopreservation of human islets. For example, new cryoprotective additives may achieve better results with preservation of the individual endocrine cell types.⁶⁷ Combinations of permeating and nonpermeating cryoprotectants may be needed. Optimal cooling and thawing rates must be determined for human endocrine cells based on the permeability coefficient of the cryoprotectant. Protective strategies to improve insulin biosynthesis or, at least, protect against chronic oversecretion may be required. And lastly, it is important to define the factors that lead to long-term decline in β -cell function, which is more pronounced with cryopreserved islets.

Transplantation of cryopreserved islets into an allogeneic environment exposes them to the rigors of immunosuppressive drugs and cytokines. Current studies in a large animal model in our research laboratory have shown that cryopreserved islets need to be augmented to overcome the adverse effects of cryopreservation before insulin independence can be assured. Also, cryopreserved human islets are

B-CELLS

Insulin-producing cells within the islets of Langerhans of the pancreas

more susceptible to cytokines that induce extremely high levels of reactive superoxide radicals.¹⁰⁶ Human β -cells, in turn, are very vulnerable to these toxins, which may explain the poor performance of cryopreserved islets during long-term follow-up. The addition of antioxidants (glutathione) and free radical scavengers (glutathione and mannitol) to prevent β -cell destruction in an allogeneic hyperglycemic environment may be required.

Future Prospects

Restricting HLA matching to cases with zero or only one antigen mismatch is rarely encountered (except with a very prolonged waiting period), often necessitating a compromise and the acceptance of a marginal solid organ. To date, well-matched single-donor islet allografts have rarely been performed. HLA matching might not be as much a critical factor as originally thought. None of the Type 1 patients who became insulin-independent with a single-donor allograft received a 5- or 6-antigen-matched islet allograft.¹⁰⁷

Cryopreservation of human islets would allow sufficient time to modulate the recipient immune system before transplantation. Diabetic recipients of a renal transplant could receive later infusions of donor-specific islets following the induction of tolerance by an earlier renal allograft. This rationale has been validated by rodent studies.¹⁰⁸ The induction of unresponsiveness to functional extrathymic islets has been achieved with intrathymic islet transplants. This was then followed by an extrathymic graft of cryopreserved islets to normalize blood glucose.¹⁰⁹ Another method involves inducing tolerance with donor bone marrow-derived dendritic cells before transplantation.¹¹⁰⁻¹¹³ These potent "passenger" antigen-presenting cells are crucial in initiating immune responses, but evidence also exists for their tolerogenicity, particularly in the liver. This approach has great potential for juvenile Type 1 diabetics, particularly if hyperglycemia can be reversed before end-stage organ failure and neurovascular complications occur, thereby negating the need for long-term immunosuppression.

Summary

The ability to successfully bank human islets remains a promising technology. Caution must be ex-

ercised before any definite conclusions can be drawn from new research studies. First, careful comparison of cryopreserved islets with unfrozen control islets from the same donor is needed. It is well known that human islets suffer damage during prolonged pancreas cold storage as well as during the isolation process, and that these islets withstand cryopreservation extremely poorly. Second, interpretation of the outcome of cryopreservation must incorporate in vivo as well as in vitro data.

In conclusion, cryopreservation of pancreatic islets will greatly assist the application of immunomodulatory strategies in clinical islet transplantation, but further in-depth studies are required to evaluate the effective quality of cryopreserved islets and the capacity of these islets to maintain function for a long period.

Acknowledgments

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CRYOPRESERVATION

Preservation of cell, tissue, or organs by subjection to extremely low temperatures

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