

Cryopreservation of Pancreatic Islets

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Abstract

Diabetes is a progressive disease with significant health and economic impacts. Pancreatic islet transplantation is being developed as an alternative treatment for type 1 diabetic patients. This treatment is currently limited by availability of pancreas and isolated islets from deceased organ donor and need for chronic immunosuppression. There is also the need to transplant the isolated islets within 1-2 days after isolation. To address this issue, cryopreservation of islets may offer the potential to bank islets for implantation at a later time, being able to provide the transplant tissue as a non-emergency procedure and allow recipient to receive pre transplant treatment. Cryopreservation, the process of adding and removing cryoprotectants, cooling, freezing, thawing, and finally the removal of the cryoprotectants introduce significant steps that affect both islet survival and function. Previous studies have proposed various techniques that could lead to increased cell survival and function following cryopreservation. The goal of this review is to critically review the techniques of islet cryopreservation, identify new strategies to improve post cryopreservation survival and function with the goal of highlighting optimization parameters that can lead to the most viable and functional islet upon recovery and/or transplant.

INTRODUCTION

Diabetes mellitus (DM) is the 7th leading cause of death in the United States with more than 252,806 individuals deceased because of complications from diabetes [1]. It is estimated that over 9.4% of the population has diabetes as of 2015 with 5-10% of those having Type 1 diabetes mellitus (T1DM). In 2015, it is estimated that 1.5 million new cases of diabetes were diagnosed among US adults aged 18 years or older along with a prevalence for prediabetes of 33.9% in the same age group¹. As of 2017 the total estimated indirect and direct expenditures for diabetes in the United States was \$327 billion which is a 26% increase from estimated costs in 2012 [2].

Pancreatic β -cell function is crucial to glycemic control as it acts as a physiological sensor for elevation in blood glucose levels during the non-fasting absorptive state [3]. It is also crucial for the induction of glucose into peripheral targeted cells after which the processes of glycolysis, glycogenesis, and lipogenesis can be upregulated³. While type II diabetes mellitus (T2DM) is characterized by peripheral insulin hormone resistance and β -cell dysfunction, Type 1 diabetes mellitus (T1DM), also categorized under insulin-dependent diabetes mellitus (IDDM), is distinguished by near complete autoimmune destruction of insulin-secreting β -cells within the pancreatic islet of Langerhans which

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causes permanent blood glucose homeostatic dysregulation. In genetically susceptible individuals affected by environmental triggers, such as virus and toxins, immunomodulation can be compromised leading to β -cells destruction [4]. A loss greater than 90% of total β -cell mass will subjugate an IDDM individual to long-term reliance on exogenous insulin treatment [5]. Hyperglycemia is the acute symptoms from untreated or poorly regulated IDDM. The acute consequences of hyperglycemia can result in diabetic ketoacidosis and/or hyperosmolar hyperglycemic state [6], both of which can be life threatening. Chronic effects of hyperglycemia can manifest into a plethora of secondary vascular disorders such as coronary disease, retinopathy, neuropathy, and limb amputations [7]. Both the complications and cost of diabetes has motivated researchers in the field to find novel approaches to producing a cure.

The idea of solely transplanting the endocrine component of the pancreas has been around since the 19th century, however, it was not until the mid-1980s when the first human islet transplant occurred at Washington University [8]. In 1990, the University of Pittsburgh successfully performed human islet allografts with prolonged insulin-independence, due to improvements in immunosuppression [8]. In the year 2000, the Edmonton protocol was published in which 7 consecutive type 1 diabetic patients underwent islet transplantations and became euglycemic post-transplant [9]. In the years following the Edmonton protocol, the total number of islet allotransplants has been 1,011, [10] and continues to increase yearly. Furthermore, a T1DM patient with secondary renal failure received an islet allotransplantation with adjunct cryopreserved islet supplementation sustained normoglycemic for up to 3 months [11]. Islet transplantation as it currently stands requires cadaveric organ donors to supply the pancreas for subsequent islet isolation and implantation. Much of the work is done as time critical, depending on availability of first the organ donor tissue, islet isolation laboratory and implantation surgery within 1-2 days of islet isolation.

Establishing a long-term cryopreservation protocol for islets would have many downstream benefits on

the entire islet isolation and transplantation process. It would provide great benefit of transplant program to select ABO, and HLA matched tissue for specific recipients, to be able to pre-treat the potential recipient and to introduce immunosuppression before the actual transplant. Frozen tissue can be shipped between collaborative shipment centers prior to transplantations with reduction graft damage during transport.

In a study comparing pre-and post-Edmonton protocols for cryopreserved islet recovery and survival, we found a 19.3% higher survival rate at 24 hours (50.1% vs 69.4% respectively), and an overall higher rate at each time point for a total of 7 days [12]. Although this is an improvement over previous techniques, there needs to be a better protocol to make long-term islet cryopreservation banking feasible.

As islet transplantation technology evolves and becomes more realistic, the organ donor shortage is further exacerbated due to the necessity of using islets from multiple donors [13]. During human islet isolation, donor pancreatic tissue undergo cell damage which begins during harvest and continues until the moment of transplantation [14]. Although a adult human pancreas has nearly a million islets, current isolation processes only are able to recover 30-50% of islet mass [13]. Therefore, an integral component of pancreatic islet isolation is the ability to preserve the cells once obtained from the donor. Currently, the gold standard for islet transplantation is to procure the cells from a deceased donor and rapidly maintain them to the recipient in a 4° C preservation solution [15,16]. Inherent to this process is a donor-recipient mismatch, that is, it is difficult to match islets from several recently deceased donors to a surgically-ready recipient. With each improvement in islet cryopreservation, the utility of clinical islet transplantations becomes more feasible option for type 1 diabetic patients. Preserving highly functional islets for an indefinite period of time would not only allow islet transplantations in remote areas to be possible, but also would permit more successful transplantations. The purpose of improving current methods of islet cryopreservation is to minimize the challenge of time and bridge the gap between donor

and recipient, thus improving clinical outcome and overall utility of islet transplantation in type 1 diabetic patients.

Islet preservation options include the following: conventionally tissue culturing at 22 or 37°C, cold storage at ~ 4°C, and cryopreservation at -196°C [17]. The different temperatures of preservation each have their own strengths and weaknesses regarding islet viability and function after the preservation period. For example, in one study, islets preserved in the 37°C group showed better function and less tissue death than the ones in the -80°C group at day 1, but at day 7, there's more apoptosis and a decrease in function [17]. In this review, we will go over the history of islet cryopreservation, and the various parameters that aids successful function after the freeze and thaw periods.

HISTORY OF ISLET CRYOPRESERVATION

The first cryopreservation experiments were conducted on spermatozoa and red blood cells in the 19th century. Researchers found that they could freeze human spermatozoa and later show functional recovery, but the results were inconsistent and when used for fertilization, it didn't result in viable embryo [18]. This was due to the lack of

cryoprotectants and the technique of instantaneously freezing and thawing the cells [18]. In the 1920s when James Lovelock first explained that red blood cells experience osmotic stress during freezing which leads to cellular death [19]. In 1948, Polge, Smith, and Parkes accidentally discovered the cryoprotective effect of glycerol, used for successful cryopreservation of avian spermatozoa [20]. This leads to the use of glycerol to cryopreserve human red blood cells [21].

Researchers eventually realized that the cryoprotectant, the freezing, and the thawing rate were three parameters which affected cell function after recovery [22,23]. Both freezing and thawing should occur slowly, so that cellular equilibrium is reached with presence of neither ice crystals nor high solute levels that would damage the cells [22]. If freezing happens too quickly, water is not able to flow out of the cell and forms ice within the cell.

The current most common cryoprotectant is dimethyl sulfoxide (DMSO), which is used in 10% (2M) concentration, added to cells prior to the freezing [24]. This increased membrane porosity to accelerate water flow out of the cell. Another method used to prevent ice crystal formation is nucleation (Figure 1) [25].

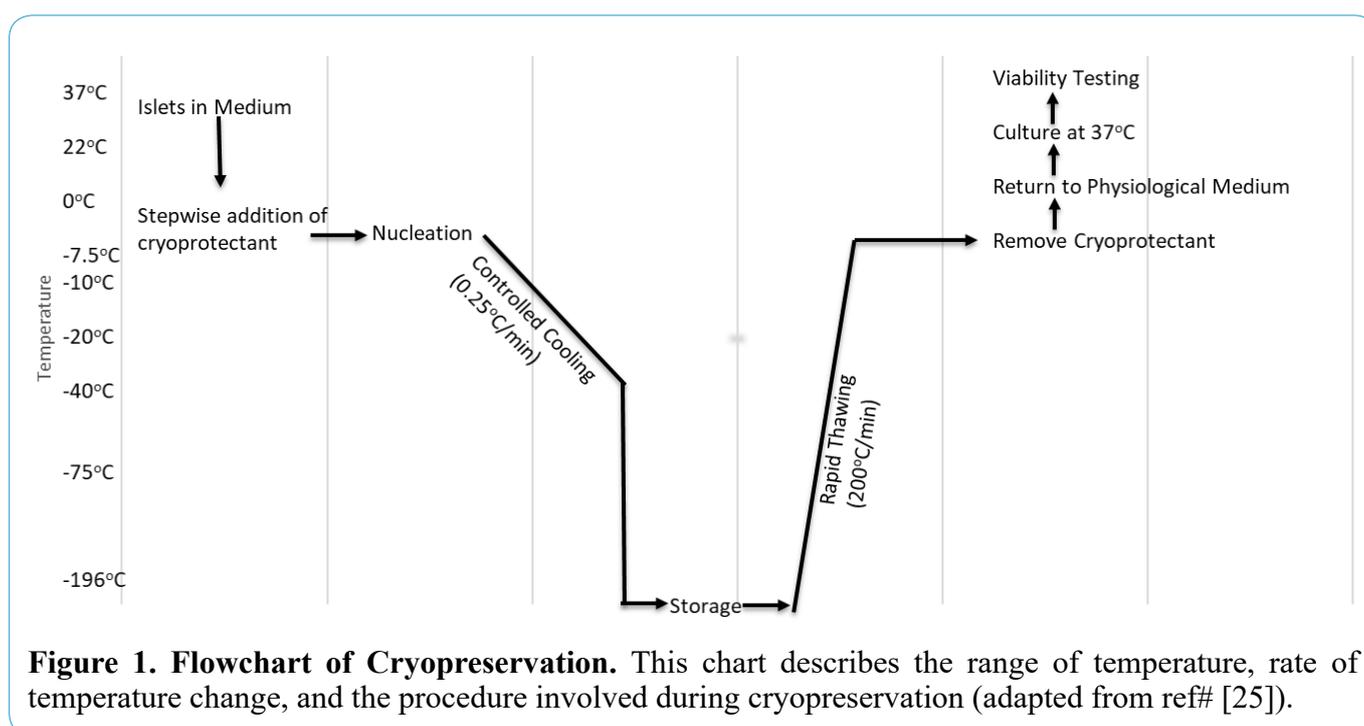


Figure 1. Flowchart of Cryopreservation. This chart describes the range of temperature, rate of temperature change, and the procedure involved during cryopreservation (adapted from ref# [25]).

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This is done by touching the meniscus of a -7.5°C test tube with a metal rod pre-cooled in liquid nitrogen, thus allowing the latent heat of fusion to be released causing a more uniform temperature within the test tube [25].

Beginning in the late 1970s, protocols for the optimal cryopreservation of islets were written with rat islets [26]. These original protocols focused on finding effective freezing/thawing rate and cryoprotectants [27-29]. In 1977, Rajotte et al transplanted cryopreserved rat islets through the portal vein into the rat liver. They froze the islets using DMSO, and after thawing and removing the DMSO, transplanted the islets into the liver. These islets started working by the 13th week after transplant [30]. This study was limited by its sample size of 1 and the relatively short 16-week follow-up period. In 1981, Nakagawara saw similar results when using 10-20% DMSO, and $1\text{-}2^{\circ}\text{C}$ cooling per minute to -80°C [31].

ADVANCEMENTS IN CELL SURVIVAL FOLLOWING CRYOPRESERVATION

Freezing and Thawing

Background

The rate of temperature change during cryopreservation is very influential on cell function and morphology. As freezing rate gets slower, this gives more time for the liquid in the cell to

reach equilibrium with the outside of the cell and preventing destructive ice crystal formation inside the cells. However, if the tissue is frozen slowly ($<1^{\circ}\text{C}/\text{min}$), more immunostimulatory cells such as macrophages, lymphocytes, and dendritic cells survive, which can negatively affect transplant results [32,33]. As such, it's important to define the optimal rate of freezing to balance islet freezing with minimal stimulation of the immune cells. Foreman et al conducted one of the first studies in 1992 comparing the effects of three rates of freezing on islets. Namely, slow cooling ($0.3^{\circ}\text{C}/\text{min}$) (Figure 2), and rapid cooling ($20^{\circ}\text{C}/\text{min}$, and $70^{\circ}\text{C}/\text{min}$) (Figure 3 and 4). The results of the study showed that if the islets were cultured in 1M DMSO for thirty minutes at room temperature, followed by 2M DMSO for ten minutes at 0°C , then the in vitro insulin secretory ability of the rapidly cooled islets was improved after thawing [34]. Other studies showed similar findings, showing that the rate of cooling becomes irrelevant if the islets have enough time to equilibrate with the cryoprotectant [35,36].

Vitrification

Permeating cryoprotectants (CPAs) such as DMSO and ethylene glycol (EG) makes islets more viable and robust to the cryopreservation process, however they also have toxic effect at high concentration. There are two methods to combat this toxicity, slow cooling, and vitrification. During the vitrification

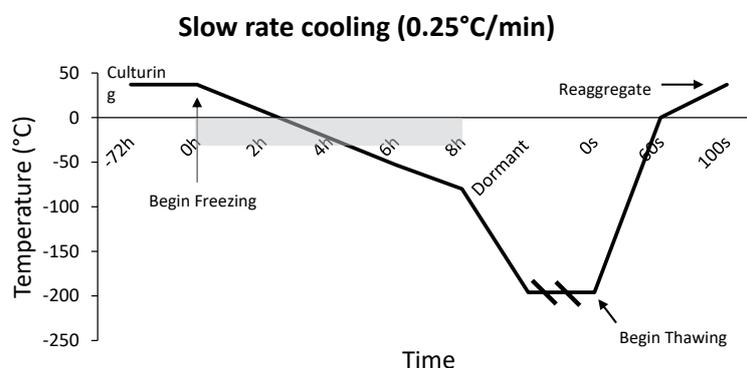


Figure 2: Slow Rate Cooling Temperature Diagram: Temperature Diagram showing the current standard in cooling rate, which is $0.25^{\circ}\text{C}/\text{min}$. This slow rate allows the tissue to reach thermal equilibrium, and allows time for water displacement, thus preventing intracellular ice crystal formation. Outlined is the burden of time which occurs due to such slow rates of cooling.

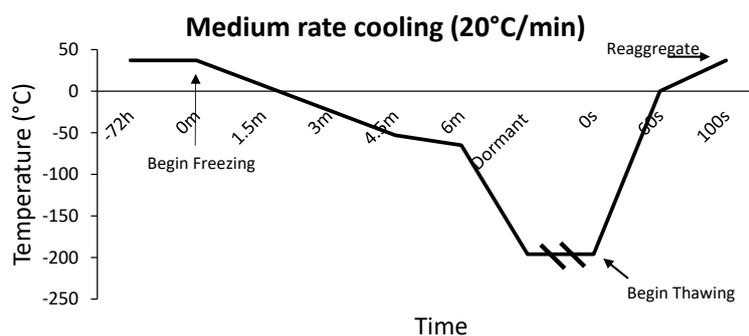


Figure 3: Medium Rate Cooling Temperature Diagram: Temperature diagram showing the significant difference in time when cooling at a rate of 20°C/min. This fast rate of cooling, although untraditional, was found to arrest immunostimulatory agents better than slow freezing.

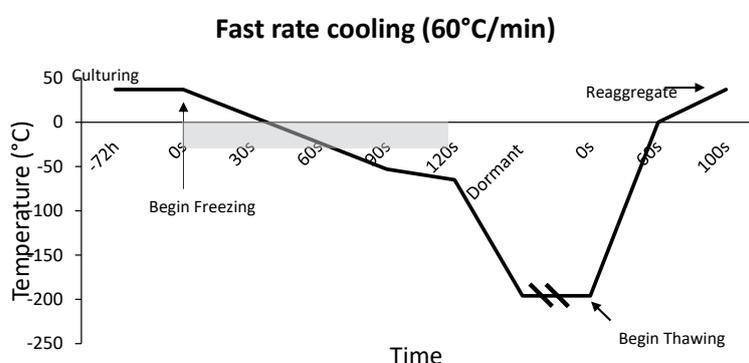


Figure 4: Fast Cooling Temperature Diagram: Temperature diagram showing the significant difference in time when cooling at a rate of 60°C/min. This fast rate of cooling, although untraditional, was found to arrest immunostimulatory agents better than slow freezing.

process, the CPAs prevent ice formation inside the islets, and instead they allow ice to form solely on the outside causing the islets to be arrested in a vitreous, glass-like phase [37]. This occurs because the CPA permeates the cellular membrane, and allows water to travel extracellularly during the freezing process.²⁵ The toxicity of a CPA is temperature dependent, and each CPA has a different osmotic load which it imposes on the cell membrane. To protect the islets from the toxic effects of the CPAs, CPAs need to be added in a stepwise fashion as the temperature decreases, thus reducing the toxic effects to the cells [38].

Thawing

The rate of thawing dictates whether the islet can recrystallize while it warms up. The current standard

of thawing is to rapidly agitate the samples in a 37°C H₂O bath which heats the samples at a rate of 150-200°C/min. Next, the sample is spun at 1500RPM, the supernatant is removed, then 0.75M sucrose buffer is added, and the sample is kept at 0°C for 30 minutes to remove the CPA [39].

Cryoprotectant Additives

There are two types of CPAs, permeating, and non-permeating. Historically, EG and DMSO have been the standard for permeating CPA, while various sugars such as trehalose and raffinose have been considered the gold-standard non-permeating CPAs for cryopreservation [40]. Permeating CPAs enter the cell while non-permeating do not [41]. Historically, permeating CPAs have been shown to be more effective, but is more toxic to the cells at high

concentration [41]. Although effective CPAs do not have cellular protective abilities against oxidative stress [42], glucotoxicity, [43] and endoplasmic reticulum stress [43] which lead to islet apoptosis. In the last ten years, many researchers have been experimenting with different CPAs which could also protect the islet from cryopreservation induced apoptosis.

Dimethyl Sulfoxide (DMSO): DMSO toxicity towards islets has been shown to be minimal at concentrations used during the freezing phase (normally 2M) and has even demonstrated protective capabilities against selective β -cell necrosis antagonist alloxan [44-46]. DMSO is considered the gold standard in cryoprotective additives and has been heavily used in research for the prevention of intracellular ice crystal formation. A 1999 study sought to compare the effect of DMSO-mediated cryopreservation on the recovery and function of canine islets [47]. Islets from seven consecutive canine isolations were dissociated into single cells and cryopreserved in 2M DMSO medium using a slow stepwise cooling method ($0.25^{\circ}\text{C}/\text{min}$) to 40°C followed by storage in -196°C . Following rapid thawing ($200^{\circ}\text{C}/\text{min}$), cryopreserved islets were recovered at 81.5% and showed no significant difference in insulin stimulated index (SI) when compared to non-treated canine islets (10.5 A.U. versus 12.4 A.U. respectively) [47]. Another study sought to standardize the critical removal process of DMSO from islet medium during the thawing phase. This protocol involves the slow stepwise addition of sucrose solution to dilute out the DMSO post-thawing [47]. Overall, DMSO will continue to play an important role in islet cryopreservation research.

Ethylene (EG)/Polyethylene Glycol (PEG): A common constituent of car antifreeze, other permeating CPAs include both ethylene and polyethylene glycol which have been studied for islets cryopreservation [48]. These low molecular weight substances easily penetrate the cell membrane, much like DMSO, and cause solute equilibrium which osmotically drives water toward the extracellular space [44]. Once the use of DMSO as a CPA was established in islet cryofreezing, studies in rat islets began to suggest potential toxicity issues [46-49]. In one study

comparing DMSO and EG as CPAs, DMSO islets showed lower cellular DNA, insulin, glucagon, and impaired insulin secretory patterns compared to EG, which was closer to pre-freezing islets. Upon transplantation of each islet group, normal glycemic control was achieved in 100% of EG-treated and non-frozen islets but only 92% of DMSO-treated islets recipients, which also experienced delays in diabetes correction [49]. Islets cryopreserved with varying concentrations (1,2 and 3M) of DMSO, EG, and PEG were exposed to islets from canine and human sources, to quantify the permeability ($P_s = \mu\text{m}/\text{s}$). The highest P_s was achieved in canine islets when 2M EG ($2.47 \mu\text{m}/\text{s}$) was used while 2M PEG showed the highest P_s in human islets ($3.48 \mu\text{m}/\text{s}$) suggesting potential use of EG and PEG in islet cryopreservation [50].

Permeating CPA Mixtures: A mixture of ethylene glycol (EG) and DMSO, classified as EDT324, was used as a cryoprotectant during the cooling phase with rat islets. EDT324-treated cryopreserved islets showed significant increases in islet viability and insulin secretory capability compared to use of DMSO (10%) alone [51]. EDT324-treated islets were then transplanted into allogenic rat recipients and diabetic correction was achieved after 2 days. Similar results were observed after islets were treated with one of two EG/DMSO mixtures (1M DMSO + 1M EG, or 1M DMSO + 0.5M EG). Islets treated with permeating CPA mixtures achieved significantly higher yield and viability compared to islets treated with DMSO only. When transplanted into streptozotocin (STZ)-induced diabetic mice, islets treated with DMSO/EG mixtures caused normoglycemia 12 days faster on average than DMSO only treated islets [52].

Non-permeating Cryoprotective Additives:

Saccharides: Although permeating cryoprotects have been mainly used during mammalian cell cryopreservation, saccharides have demonstrated improvement to cell survival when added to the vitrification medium. Adult human islets treated with 300 mmol/L trehalose achieved 92% recovery rate compared to 58% recovery of DMSO-treated islets, in addition to 14-fold increase in insulin content within

islet grafts. More prominent differences in recovery were observed in fetal human islets treated with trehalose compared to islet only treated with DMSO [53]. More recently, an antifreeze glycoprotein (AFGP) was included to DMSO slow cooling phase medium during cryofreezing of rat islets. When compared to DMSO only protocol, AFGP-treated islets demonstrated significant increase in recovery rate ($85 \pm 6.25\%$ versus $63.3 \pm 14.2\%$) and insulin stimulation index (3.86 ± 0.43 versus 2.98 ± 0.22) [54]. These results demonstrate that saccharides and saccharide-containing substances can be used in conjunction with lower DMSO concentrations and help reduce islet toxicity.

Polymeric Compounds: High molecular weight polymeric compounds such as polyvinylpyrrolidone (PVP) and dextran have been shown to be good vitrification agent during freezing [55]. When 10% PVP was added to cryopreservation medium before cooling phase, rat islet recovery and function was significantly higher than islets treated with 2M DMSO and 3M glycerol. Islets treated with 2M PEG demonstrated comparable islet recovery and function to PVP-treated group [56]. Although use of high molecular weight cryoprotectants has been demonstrated to good results, past studies suggest that these compounds are ineffective at slow cooling temperature transitions which is a crucial step of the cryofreezing process [55].

Other Potential CPAs

There are unavoidable damaging consequences to islet health, when cells in multicellular tissues fragments are exposed to subfreezing temperatures ($> -100^\circ\text{C}$). These damages lead to apoptosis and/or necrosis after the post-thawing phase of cryopreservation [57]. Due to its fragile multicellular tissue structure, islet fragments are susceptible to various stresses included oxidative stress, osmotic stress, hypoxia, hypothermia, and inflammation induced by the cryopreservation process which can have acute and/or long-term effects on islet graft viability and function. [58,59] More recent studies aim to find CPAs which reduce stress-induced cell death associated with the cryopreservation process.

Taurine: As an antioxidant, taurine has the ability to scavenge for free radicals and also protect the membrane from oxidative damage [60]. Taurine increased islet viability when adding 0.3mM and 3.0mM taurine prior to cryopreservation. The viabilities were ($91.9 \pm 2.3\%$ for the 0.3mM, and $94.6 \pm 1.58\%$ for the 3.0mM) [60]. Likewise, taurine caused a reduction in lipid peroxidation, and a normal glucose clearance after transplantation into BALB/c mice. Hyperglycemia was reached once the graft was removed, showing that the transplanted islets were functional [60].

Metformin: Metformin is an anti-diabetic drug that increases insulin sensitivity [61]. It has been shown to have beneficial effects on islet survival [62], and thus, was further researched by Chandravanshi et al [63]. They added 100 $\mu\text{g/ml}$ of metformin to the cryopreservation solution of mice islets, for storage of the islets at low (4°C) and ultra-low temperature (-196°C) storage for 15 and 30 days respectively. Islets treated with metformin and stored at the low temperature for 15 days secreted insulin (8 ng/ml) and had a stimulation index greater than 5 indicating a high functionality and glucose sensitivity [63]. They found a similar result with metformin after a 30-day storage period at the very low temperature. In addition, they found reduced levels of reactive oxygen species (ROS) in both circumstances, when compared to islets not treated with metformin [63].

Gamma aminobutyric acid (GABA): GABA is a neurotransmitter that has also been shown to act as a neuroprotective agent [64]. This effect has also been shown on human β cells [65]. Chandrayanshi et al hypothesized that the effects of GABA would also aid in the process of cryopreservation. After 15 days in low temperature, islets treated with 100 μM GABA secreted 5.5 ng/ml, and after 30 days in very low temperature, secreted 20 ng/ml [63]. Similarly, they found reduced levels of reactive oxygen species (ROS) in both circumstances, when compared to islets not treated with GABA [63].

Eicosapeeanoic acid (EPA) and docosahexanoic acid (DHA): EPA and DHA are polyunsaturated fatty acids, with the common source being dietary intake of omega 3 fatty acids [66]. They have

been shown to have anti-inflammatory effects in asthma, inflammatory bowel syndrome, and arthritis cases [67]. Chandrayanashi et al were the first researchers to investigate the cryoprotective effects of EPA and DHA on islet cells. They added 1 μM of EPA+DHA to the islet solution listed above. The results of the glucose stimulated insulin secretion (GSIS) was 11ng/ml and 20ng/ml after 15 days at low temperature and after 30 days of very low temperature storage respectively. There was also a significant reduction in the levels of ROS [63]. The greatest insulin release following glucose challenge was achieved with a solution that had a combination of EPA, DHA, and metformin.

Curcumin: Curcumin, the main component of turmeric spice, has demonstrated antioxidant and anti-inflammatory effects in multiple cell types [68]. Curcumin has not been shown to increase insulin stimulation; however, it has demonstrated upregulation of oxidative stress reducing genes Hsp70 and HO-1 [69]. To evaluate cyroprotective abilities of curcumin, Kanitkar et al. compared the effect of 10% DMSO with and without 10 μM curcumin on islets treated with slow cooled cryopreservation (-196°C) for 7 days. Curcumin-treated islets showed increases in SI compared to non-treated cryopreserved islets but no difference from fresh islets. In curcumin-treated medium, over-expression of HO-1 and Hsp70 was observed to incrementally increase as the cryopreservation process unfolded [69].

Sericin: Sericin is a silk protein produced by silkworm (*Bombyx mori*). It was tested in 2012 by Ohnishi et al for its ability to replace fetal bovine serum (FBS) as an additive for DMSO [70]. FBS has been scrutinized due to recent epidemic burst, such as bovine spongiform encephalopathy and viral infections. Using sericin as a replacement for FBS, Ahnishi et al showed no significant differences between GSIS results between the FBS+DMSO and the Sericin+DMSO groups.

Butylated hydroxyanisole (BHA): Oxidative stress in cells produces endogenous reactive oxygen species (ROS), such as superoxide (O_2^-) and free hydroxyl (OH^-), which leads to an increase in free radical concentration intracellularly [71].

Elevated internal levels of free radicals can cause cellular damage and lead to cellular process disruptions. To combat cryopreservation related oxidative stress, one early study added butylated hydroxyanisole (BHA) to islet cryomedium while monitoring oxidative stress via glutathione redox state (GSH/GSSG). Islets treated with BHA demonstrated enhanced insulin secretory behavior (2.2-fold increase) when compared to untreated islets. In addition, exposure to alloxan, a highly damaging free radical generating agent, did not induce significant oxidative stress [72].

Ascorbic acid-2 glucoside(AA2G): Ascorbic acid-2 glucoside (AA2G), a derivative of Vitamin-C, is a potent antioxidant and can deliver stable antioxidant activity into culture media [73]. AA2G (100 $\mu\text{g}/\text{mL}$) in combination with the UW islet preservation solution were used as the cryopreservation medium [74,75]. Following a 3-month storage (-80°C) the islets treated with UW/AA2G demonstrated viability maintenance ($68.3\pm 5.6\%$) and significantly increased insulin stimulation via glucose stimulated insulin secretion (GSIS) test when compared to UW alone (1.93 ± 0.5 and 1.17 ± 0.6 respectively). Transplantation of thawed AAG2/UW-treated into liver of nude mice produced engraftment with insulin-positive cells observed.

Three-Dimensional Structure

Islets in their native form are multicellular tissues containing 1,000-10,000 cells [76] with average diameter of $150\mu\text{m}$ [77]. Similar to full organs, islets is more difficult to freeze because of non-uniform temperature changes causing difference in ice formation [78]. As the islet is cooled, cells on the outside experience a greater rate of temperature change than the cells on the inside. Because of this gradient, ice crystals can form on the inside of the cells, leading to cellular death [79]. Researchers have proposed the prospect of freezing the islets as individual cells, and then reconstituting them into their natural spherical form after thawing [80].

In vitro human islet experience: Rawal et al tested the differences in function, insulin release,

and viability of human islets which were either cryopreserved as single cells or as native tissue [80]. Native islet spheroids were broken down into single cells [81], then they were dispersed in 10% DMSO, cooled at $-1^{\circ}\text{C}/\text{min}$, and frozen at -196°C [80]. The native islet tissue was frozen using stepwise DMSO addition during freezing until -196°C [24]. After the single cells were thawed, they were reformed into spheroids at 37°C and compared with the native islets in terms of structure and function [80]. After 4 weeks of cryopreservation, there was no significant difference between the average spheroid diameter or the volume. There was a significant difference in viability between the two groups, with an 80% cell death of native islets compared to only a 25% cell death for the reaggregated islet cells [80]. Using GSIS, they were unable to detect any insulin secretion from the intact native tissue. The single islet cells, however, secreted insulin and showed moderate levels of insulin sensitivity.

***In vivo* rat islet:** The same group of researchers tested the *in vivo* effects of islet cryopreservation in the native vs cellular forms. They isolated rat islets and cryopreserved them for 1-4 months in either their native islet form, or in their cellular form. When transplanted in diabetic rats, 5000-8500 islet equivalents/kg (IEQ) of freshly isolated were not able to return normoglycemia up to 30 days post-transplant. Transplanting the same IEQ/kg ratio of cryopreserved islet cells yielded far better results: the rats were normoglycemic within 24 hours of the transplant and maintained an average blood glucose of 150mg/dL one month after. They continued to be normoglycemic for 10 months after transplant. The group of rats receiving the cryopreserved native islets were unable to reverse hyperglycemia [80].

Oxygen Environment

One of the mechanisms for islet death is ATP depletion, which become increasingly significant during the rewarming process as the cytoplasmic enzymes regain their function [82]. Since ATP could be made aerobically through oxidative phosphorylation, Komatsu et al hypothesized increased oxygen in the chamber around the islets during cryopreservation can ameliorate the ATP depletion and increase islet health [83].

Human Islets: This hypothesis was tested using human islets in two groups, a low oxygen group (21%), and high oxygen group (50%) [83]. 500 IEQ of human islets were cryopreserved using the islet cryopreservation solution (ICS) for a period of at least three months. For oxygenated thawing, the islets were equilibrated under 50% O_2 , 45% N_2 , and 5% CO_2 . Once thawed, they were placed in a 24 well plate, at 250 IEQ/well on ice, and transferred to a 50% oxygen incubator [83]. The islets were incubated for a total of 90 minutes, the first 45 of which at 22°C , and the second 45 minutes at 37°C . They then were analyzed for islet volume and compared to their volume prior to the cryopreservation. They defined this as short-term recovery rate. For long-term recovery rate, they evaluated the islet's volumes after 1 or 2 days in the incubator, and compared the results to the short-term, and pre-cryopreservation periods using both the hyperoxia group, and the regular oxygen group. Other tests they conducted were RNA analysis, and GSIS from the pre-cryopreserved and post-thaw/rewarmed groups. The results showed that there was no significant difference of oxygenated thawing/rewarming on short-term islet recovery rate based on the islet volume ratio. However, there was a significant reduction in the amount of inflammatory genes expression. The long-term recovery group showed a reduction in volume loss in the oxygenated thawing/rewarming group. The results of the GSIS for each of the groups showed no significant differences [83].

Cryopreservation Duration

The purpose of cryopreservation is to metabolically arrest the islets for as long as possible. The question becomes, "How long is too long?" specifically to prevent cellular death and to preserve islet function. There have been studies assessing a three month and a two-year period of cryopreservation [84,85], with the lengthiest duration conducted by Fox et al. [86] This study tested both the *in vivo* and *in vitro* effects of using human islets which were cryopreserved for a mean of 18 years. The islets came from 43 human donors, frozen using 2M DMSO [87]. The mean age of the islet donors for cryopreservation

was 40.9 ± 2.0 years, with 47% male vs 53% female, while the fresh tissue donors were 60.5 ± 3.0 years, with 56% male vs 43% female [86].

***In vitro* human islets:** After rapid thawing at a rate of $150^\circ\text{C}/\text{min}$ to 4°C , the DMSO was removed with a sucrose buffer. Using dithizone staining, they found no significant decrease in purity between frozen and freshly isolated islets. Immunofluorescence staining showed expression of insulin positive beta cells and glucagon positive alpha cells, indicative of a healthy islet. Apoptosis was similar in both groups. Using a patch clamp apparatus, they determined that the cryopreserved group retained ion channel function and had exocytotic responsiveness like the fresh tissue group. GSIS results were similar between the two groups, with no correlation between cryopreserved time and insulin responsiveness.

***In vivo* human islets in mice:** The same islets were transplanted at 2,000-4,000 IEQ into the kidney capsule of STZ-induced diabetic mice. Of a total of 11 mice receiving the cryopreserved islets, only one could achieve similar glucose levels as the mice with fresh islet transplants. This reduction in the insulin secreting ability of the cryopreserved islets *in vivo* could be proportional to the duration of the extended cryopreservation period [88].

Other Cryopreservation Methods

Hollow fiber vitrification (HFV): HFV is commonly used for the cryopreservation of embryos [89], and has only been recently studied for the pancreatic islet model. HFV is desirable method of preserving islets because it reduces the amount of CPA use, which in turn could lead to higher islet viability and purity [90]. In 2016, Nagaya et al compared their HFV protocol to the open pulled straw method [91], and to Sasamoto et al's protocol [51]. It was shown that the new HFV method yielded the highest viability of the three protocols *in vitro* [90]. When they transplanted HFV islets into STZ mice, they were all euglycemic within 4-8 days, and returned to hyperglycemia once the kidney graft was removed after 30 days [90].

Adenosine: The addition of adenosine to UW solution during regular non-cryopreservation has

been shown to increase yield, viability, purity, and insulin release when compared to regular UW solution alone [92]. However, this has not been tested for cryopreservation use.

Alginate Encapsulated Cryopreservation: Cryopreservation with Alginate-based Microencapsulation Technology:

Alginate-based microencapsulation technologies have developed in concert with islet transplantation research where the alginate polymer forms a semipermeable immunisolating barrier around islet fragments [93-95]. Alginate-microencapsulation has recently been applied to the field of islet cryopreservation in order to characterize its effect on islet survivability [96]. Chen et al. reported the development of an oxygen sensing alginate coating to encapsulate islets prior to cryopreservation [97,98]. Islets were encapsulated with alginate coating containing ruthenium-based oxygen-sensitive fluorophore (ROF) after which the encapsulated islets were subjected to a 10% DMSO with or without 50×10^{-3} M trehalose and stored for 1-7 days. Encapsulated islets undergoing cryopreservation showed significantly higher insulin stimulation behavior than bare islets at Day 1 and 7 [97]. In addition to cyroprotective abilities, the microcapsules treated with ROS demonstrated viable oxygen sensitivity during OCR measurements. This study demonstrates the use of multiple cyroprotective parameters to mitigate potential damage during cryopreservation [97]. Recently, Kojayan et al. demonstrated that cryopreserved rat islets, which were pre-encapsulate (1.75% alginate) prior to cryopreservation, had improved post-thawing function compared to non-encapsulated cryopreserved rat islets. Upon transplantation into diabetic mice, on average encapsulated cryopreserved rat islets achieved significantly faster normoglycemia (5 days) than non-encapsulated cryopreserved islets (18 days) [99]. Other studies have demonstrated the benefits of alginate microencapsulation use during cryopreservation as the 3-D barrier is porous and can resist stress/strain associated with ice formation [97,100,101].

Table 1: Cryopreservation Parameters and Suggested Methods: A quick summary of the parameters which had the best outcome for the islets during cryopreservation. Assessment methods include islet viability, glucose sensitivity, and GSIS values after thawing.

Parameter	Method	Reference Number
Cryoprotectant	EPA+DHA+Metformin	62
Cooling Rate	Rapid (50-70°C/min)	34
Thawing Rate	Rapid (150-200°C/min)	39
Oxygen Environment	50% during thawing	83
3D Structure	Freeze as individual cells, re-aggregate into spheroids after thaw	80
Encapsulation	1.75% Alginate encapsulation prior to cryopreservation	99

CONCLUSION

The technology of islet cryopreservation has been changing and expanding over the past 50 years. Cryopreserving islets is actively researched because of there is high demand for a long-term storage bank of islets. At this time, entire organ cryopreservation is not entirely feasible, and is only shown possible after a few hours of storage at hypothermic temperatures, nothing in the cryopreservation field [102]. Based on all the reported results, we have outlined the new options with the goal of optimizing islet function after cryopreservation (Table 1). We hope eventually a standardized cryopreservation protocols would make islet bank possible.

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