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Chapter

Current Advancements in Pancreatic Islet Cryopreservation Techniques

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Abstract

There have been significant advancements in the research of pancreatic islet transplantations over the past 50 years as a treatment for Type 1 Diabetes Mellitus (T1DM). This work has resulted in hundreds of clinical islet transplantation procedures internationally. One limitation of the procedure includes effective storage techniques during donor-recipient cross-matching following islet isolation from deceased donor. Cryopreservation, which is heavily used in embryology research, has been proposed as a prospective method for pancreatic islet banking to bridge the temporal intervals between donor-recipient matching. The cryopreservation methods currently involve the freezing of islets to subzero (−80/−196°C) temperatures for storage followed by a thawing and warming period, which can be increasingly harmful to islet viability and insulin secretion capabilities. Recent advances in islet cryopreservation technologies have improved outcomes for islet health and survivability during this process. The aim of this chapter is to characterize aspects of the islet cryopreservation method while reviewing current procedural improvements that have led to better outcomes to islet health.

Keywords: cryopreservation, islet, cryoprotectant, alginate, vitrification, diabetes

1. Introduction

Pancreatic islet transplantations are currently used in human clinical studies to treat Type 1 Diabetes Mellitus (T1DM); however, one of the major limitations of this therapy remains efficient and effective storage of islet prior to transplant, during donor-recipient cross-matching [1]. Islet cryopreservation has a distinctly vigorous research history as the storage, transportation, and overall preservation are critical steps in islet transportation. The first human islet allotransplantation took place at the Washington University in the 1980s, which provided proof of insulin independence following the procedure [2]. Following these early successes, clinical trials at the University of Pittsburgh observed further prolonged insulin independence using an improved steroid-free immunosuppressive regimen [3]. These initial clinical islet transplantations demonstrated the need for a preservation process to bridge a temporal gap between islet isolation from donor and transplantation of islet graft into recipients. In 1989, clinical trials at the University of Alberta were able to use both freshly isolated islets supplemented with cryopreserved islets

in two T1DM patients, which resulted in partial graft function [4]. The international trial on Edmonton protocol reported success after some of over 43 Type 1 diabetic patients achieved either partial or complete insulin independence for up to 3–5 years post-transplantation [5, 6]. After the establishment of the Edmonton protocol, the Clinical Islet Transplantation Registry (CITR) has recorded more than 1500 human islet allotransplant recipients, which is projected to increase steadily in the future [7]. The Edmonton method of islet isolation was shown to improve islet survival during islet cryopreservation as well. A study comparing cryopreserved islets before the establishment of the Edmonton protocol to human islets treated via Edmonton method observed a 24-hour survival rate increase of 19.3% (50.1 versus 69.4% respectively) with added increases recorded after 7 days of culture [8]. Although the impact of the Edmonton protocol on cryopreservation is significant, there is still vast room for improvement in the islet cryopreservation process.

A major problem of the transplantation field is the lack of human donors' sources; the islet transplantation process is particularly hard-hit from this problem since each transplant recipient must be infused with islets from multiple pancreases [9]. One recipient may also require multiple infusions of donor islets, which can further strain the donor source [10, 11]. With the epidemiologic increase in IDDM diagnosis, islet allotransplantation islet supply will be increasingly strained by the growing demand for islet replacement therapy with projected increases in the population of IDDM individuals [12]. The islet donor supply problems can be partially addressed from improvements in human islet yield, purity, and function. Although an average human cadaveric pancreas contains over 1 million islets, the human islet isolation process can be especially harsh on the isolation yield resulting in loses of up to 50% depending on the degree of success of the isolation [13, 14]. Cryopreservation techniques have been employed to address some of these islet isolation and preservation issues before islets are implanted into the recipient.

The human islet isolation and culturing process involves several steps that vary in temperature, each of which has its own benefits and deficits regarding the health of the islets [13]. Islet procurement from a whole donor pancreas first exposes islet to 4°C (histidine-tryptophan ketoglutarate solution) during sterile transportation to an approved clinical islet center. During the pancreas digestion and purification, the islets are exposed to varying temperatures between 4°C and room temperature. Preservation of islet currently involves the cooling of isolated islets in a temperature-regulated solution at 4°C prior to culturing as prolonged warm-ischemia will increase islet death and subsequent decrease in islet yield [13, 15, 16]. The final step of islet preparation involves a 24- to72-hour culture in approved islet media at 37°C, which has been shown to improve islet yield and functions with reductions in dead/apoptotic islet cell mass [17, 18]. Isolated human islets are preserved while donor islets of similar cross-matching biocompatibility are compiled and matched to an islet recipient [13]. During this critical time period, cryopreservation has been suggested as a preservation method for use during the pre-transplantation period to improve islet health. Improvements in the islet cryopreservation field can translate directly to improvements in human islet cyro-banking as well. This book chapter outlines the history of islet cryopreservation, current techniques in freezing/thawing periods, and cryoprotective additives.

1.1 History of islet cryopreservation

Following the discovery of the microscope, Spallanzani observed that sperm could maintain mobility even when exposed to cold temperature conditions in

1776 [19]. Research into the effects of cryopreservation on live tissue had its roots in late 1800s when scientists used this technology to preserve both spermatozoa and red blood cells (RBCs). During this time, research demonstrated weaknesses in the process which caused inconsistent results and frequent infertility caused by early embryonic death. A breakthrough occurred in the 1950s when James Lovelock discovered that the cryopreservation process caused osmotic stress in the cell by instantly freezing the liquid and causing the formation of ice crystals in RBCs. In 1963, Mazur et al. were able to characterize that process when they demonstrated that the rate of temperature change within a cell-containing medium controlled the movement of water across a cell membrane and thus the degree of intracellular freezing [20]. This together helped to improve the overall understanding of the mechanism associated with the cryoprotective process. During the 1980s, research surrounding the cryopreservation process revealed that the speed at which the freezing and thawing process occurred was the most important factor in determining the survivability of the cells [21, 22]. It was demonstrated that small, slow increments in both the freezing and thawing processes prevented the rapid formation of ice crystals and increased membrane-bound solutes associated with early cell death [23]. Another initial advance in cryopreservation occurred in the late 1940s when researchers discovered that the use of glycerol as a medium increased the survivability of spermatozoa in subfreezing (−70°C) temperatures [24]. Using glycerol as a medium effectively served to protect the cells from rapid formation of ice crystal during the preservation process. A commonly used cyroprotective agent currently employed is dimethyl sulfoxide (DMSO), which is added to cell media prior to the freezing process [25, 26]. DMSO (10%) when added to the cell media, commonly at 2 M concentration, increases the porosity of the cellular membrane, which allows water to flow more freely through the membrane [27, 28]. In addition, early research has demonstrated that nucleation is another way to prevent the rapid formation of ice crystals during freezing [28, 29]. During the freezing process, a metal rod supercooled with liquid nitrogen is applied to the meniscus of the medium containing islets wherein the liquid molecules begin to nucleate. These nucleation reactions are due to the release of latent heat of fusion from the medium, which causes the temperature to decrease more homogenously.

During the 1970s, cryopreservation technology was applied to rat islet preservation in both storage and transportation, which demonstrated maintenance of high viability and function, which showed no significant difference when compared to non-treated islets [30, 31]. In one study, T1DM rats received allogeneic islet transplants cryopreserved using 2 M DMSO (Freezing rate = 0.25°C/min; Thawing rate = 7.5°C), which caused normoglycemia for up to 3 months (**Figure 1**). Additionally, when a modified cryopreservation protocol was applied to canine allotransplantations (freezing rate = 0.25°C/min; thawing rate = 3.4°C/min), T1DM canine recipients demonstrated prolonged glycemic control for up to 18 months [32]. These results highlighted the potential use of cryopreservation for islet transplantations in both small and large animal models in addition to differences required when cryofreezing small and large animal pancreases. Recent advances in islet quality control like oxygen consumption rate (OCR), qPCR, and MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay allow researchers to study islet cryopreservation technology more effectively [33]. Islet cryopreservation research is a very active research topic with many studies aiming to characterize and improve cryopreservation freezing/thawing processing, benefits of potential cyroprotective additives, and the effects of encapsulation on islet function during cryopreservation. The following section will discuss the islet cryopreservation process (**Figure 2**).

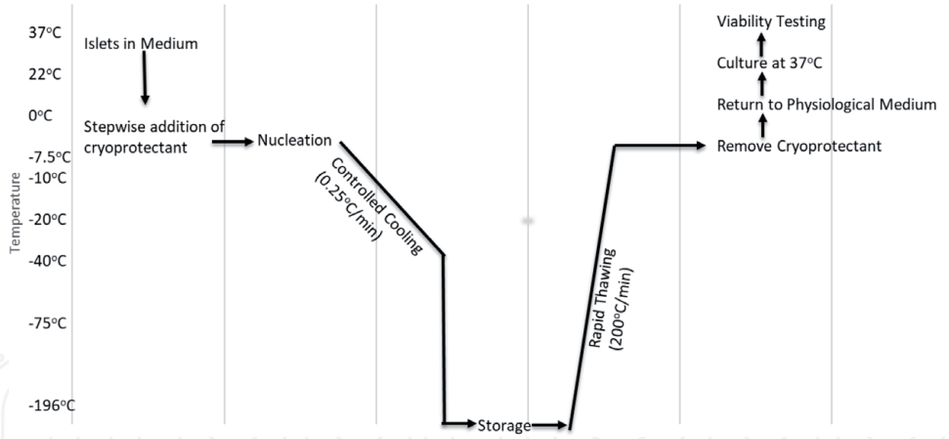

Figure 1.
Sample image of encapsulated human islets stained with dithizone for 15 min, taken at 2× magnification with objective lens 20/40 PH. Scale bar represents 2 mm. Imaging performed at UCI laboratory under supervision of Dr. Jonathan Lakey PhD.

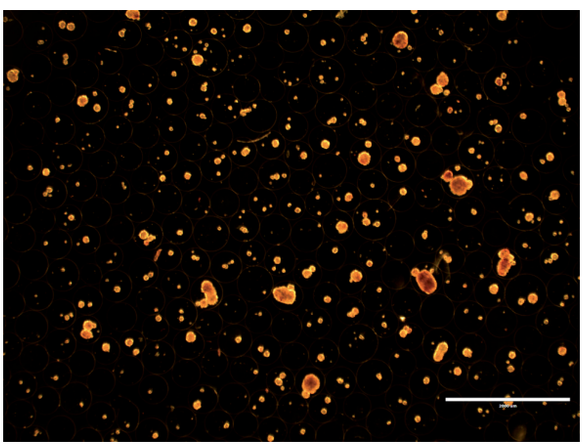

Figure 2.
Flowchart of cryopreservation. This chart describes the range of temperature, rate of temperature change, and the procedure involved during cryopreservation.

2. Characteristics of the islet cryopreservation process

2.1 Background

A crucial aspect of cryofreezing islets is the rate of freezing and thawing, which can have major effects on the islet health and morphology. The freezing process describes the process of cooling the islet-containing medium to around −196°C. If the freezing process is done too rapidly (>0.25°C/min), the liquid in the medium will freeze too quickly and crystal ice structure will form within cell membranes. Conversely, if the freezing process is performed too slowly (<0.1°C/min), then innate/adaptive immune cells, such as macrophages, dendritic cells, and lymphocytes which are present within the islet medium survive in greater numbers and can contribute to foreign body response (FBR)-mediated graft rejection upon transplantation [3, 34]. Taylor et al. demonstrated increase in macrophage viability (91%) cryofreezing is done at a rate of 0.1°C/min compared to 72–75% viable macrophage when the rate between 0.1–20°C/min [35]. Therefore, a key aspect of cryofreezing is the use of an optimal freezing rate based on islet type and volume to prevent ice crystal formation and immune cell survival. Over the years, many studies have described varying optimal freezing rates, which has made it difficult to compare between freezing/thawing methods. A consistent freezing/thawing

protocol uses slow freezing from −40 to −196°C followed by a rapid thawing starting from −196°C [36]. An early study aimed at characterizing differences between cooling and thawing rates exposed islets to several freezing rates between 0.3 and 100°C/min and thawing rates of 10 or 50°C/min. Highest survival rates were detected at 0.3°C/min rate with slight decreases observed between 60 and 1000°C/min rates [37]. The study also demonstrated the critical nature of using DMSO as a medium to protect the islet viability and function from cooling process. Cyroprotective agents (CPAs) like DMSO are neutral solutes of both low toxicity and molecular weight that replace up to 30% of the cell water and provide optimal conditions for subzero temperatures [38]. While a variety of DMSO concentrations have been tested, the most popular one used is 2 M DMSO, which is added in a stepwise fashion (1 M DMSO to 2 M DMSO) during pre-freezing [36]. One study found that when islets were exposed to 1 M DMSO for 30 min followed by incubation in 2 M DMSO for 10 min before cooling phase, then the islet insulin secretory patterns were improved after thawing [39]. More recently, studies have shown that the rate of cooling is much less important than the use of cryoprotective additives during the pre-freezing phase [36, 39].

2.2 Vitrification

The process of exposure and equilibration of permeating cyroprotective additives to islets is known as vitrification, which was first described by Rall et al. [38, 40]. While the use of CPAs reduces the risk of rapid ice crystal formation during the cooling phase, cryoprotective agents, such as DMSO and ethylene glycol, have been shown to be toxic to islet viability and function when concentrated in the medium [38, 41, 42]. Vitrification is used to slow the exposure of islets to CPAs by adding the CPAs in a stepwise fashion, usually in ascending concentrations of CPA, thereby allowing the CPAs to slowly permeate and form a solute equilibrium across the cell membrane. The vitrification process also causes water to flow extracellularly where, during cooling, vitreous water crystals slowly form outside the islet cells [38]. This vitreous medium exists in a solid-liquid transition state that is maintained at a supercooled temperature (≤100°C), thus having the structure of a liquid but behaving mechanically like a solid [43]. In addition to reducing ice crystal formation, vitrification involves exposing the CPAs in a stepwise fashion (1 M DMSO followed by 2 M DMSO), thus reducing the toxicity of the CPAs as the cooling process proceeds. Once the cooling process is finished, the cryopreserved islets will be stored at −196°C liquid nitrogen freezer until use.

2.3 Thawing

When the supercooled islet is in −196°C storage, the vitreous medium is still locked in a liquid-solid transition state; however, once the warming process begins, there is risk of ice recrystallization within the medium, which can damage the islets [36, 43]. A widely accepted procedure includes the use of rapid thawing from −196°C at a rate of 150–200°C/min. Mechanical agitation is applied to thawing samples in a 37°C water bath until all ice is gone (0°C). At 0°C, the samples are then placed in an ice slush bath (0°C) until thawing is completed [36]. Following the warming phase, it is important to remove the cryoprotectant from the medium. After thawing is complete, samples are spun down at 1500 RPM and supernatants containing most of the cryoprotectants are removed. The pellet is then resuspended in an isotonic buffer (0.75 M sucrose) to dilute out the remaining CPAs.

3. Factors that affect islet cryopreservation process

3.1 Background

While the use of CPAs is a critical step, there are several factors that play a significant role in the success of an islet cryopreservation process. Technological advances in these areas can help reduce the stress of the process on the islets while more effectively processing the islets tissue during cryopreservation.

3.1.1 Oxygen treatment

Changes in ambient atmospheric oxygen concentration can have a negative effect on islet viability and function via ATP metabolite depletion. In hypoxic conditions, β-cells are put under oxidative stress followed by ROS production, which facilitates islet death [44]. The thawing and rewarming phases have been shown to place hypoxic-related oxidative stress on cryopreserved tissues [45]. During the thawing/rewarming period, as islets are brought back from sub-freezing temperature, cellular enzymes begin to function and increase oxygen consumption. This process can cause a reduction in adenosine 3-phosphate (ATP), a cellular metabolite, which can lead to islet death [45]. To address this issue, researchers hypothesized that hyperbaric conditions might improve islet recovery. Human islets were exposed to both normal oxygen conditions (21% O_2, 74% N_2, and 5% CO_2) and hyperbaric conditions (50% O_2, 45% N_2, and 5% CO_2) at 22°C for 45 min followed by 37°C for 45 min. Short-term (post-rewarming) and long-term (2-day culture) islet function analysis was conducted via GSIS, qPCR ischemia-gene analysis, and islet metabolism via oxygen consumption rate (OCR) assay. Long-term culture also compared normal and hyperbaric culture conditions. No significant short-term function and metabolic differences were observed between conditions and non-treated islets. However, the hyperbaric conditions were shown to suppress the increases of inflammation detected in untreated cryopreserved islets. Islet recovery after long-term culture was significantly better under hyperbaric conditions and was shown to increase after hyperbaric 2-day culture. A recent advancement in organ oxygenation known as persufflation involves the perfusion of humidified oxygen into the vasculature of the pancreas before and after cryopreservation. This technique could potentially mitigate the islet loss from hypoxia and ischemia during cryofreezing, thawing, and rewarming phase.

3.1.2 Cryopreservation storage duration

Development of an islet tissue bank will require islets to be cryopreserved for long periods of time, years if necessary. Generally, islet cryopreservation studies will only cryopreserve islets for short periods of time (1–90 days) [45–47]. For human islet cryopreservation, the upper limit of storage duration was set by Fox et al. in 2015. Human islets were cryopreserved at −196°C for an average of 17.6 ± 0.4 years. Between 2012 and 2014, human islets were then thawed and warmed, after which islet electrophysiology and function were analyzed. After measurement of β-cell excitability via path-clamp assay, similar Ca^{2+}-influx conductance patterns were observed between cryopreserved and fresh islets [48]. However, insulin stimulation index was significantly lower for cryopreserved (1.90 ± 0.24) compared to fresh islets (9.53 ± 0.92). However, after cryopreserved islets were transplanted into STZ-induced mice, partial normoglycemia was observed for 60 days with improvements to glucose tolerance [48]. More research

is needed to infer a limit to cryopreservation storage duration to ensure that islets remain functional post-thawing.

3.1.3 Islet structure limitations

Difficulties with the cryopreservation of whole organs are partially due to the non-homogenous temperature distribution within the large tissue structure of organs. Pancreatic islets exist as a spherical cluster, with an average diameter of 100 μm, of several thousands of cells connected by a dense network of connective tissue [49]. The cells that lay within the islet core are susceptible to hypoxia-related stress, particularly during the cryopreservation process [50, 51]. A recent study aimed to address this structural problem by reducing islets to single cells followed by cryopreservation [52]. Islets were reduced to single cells and cryopreserved with 10% DMSO and stored at −196°C (1°C/min) for four or more weeks. After a rapid thawing and warming phase, islets were reaggregated at 37°C [52]. Reaggregated islets were recovered at a rate of 80% and had similar diameter to intact cryopreserved islets. The viability of reaggregated islets was significantly higher than intact islets (80 versus 25% respectively) post-thawing. No significant differences in GSIS function were detected between reaggregated islets and intact islets. Upon allotransplantation of reaggregated islets into omentum of STZ-induced diabetic rats, normoglycemia was achieved in 24-hours and was sustained for 10-months. Intact cryopreserved islets failed to achieve normoglycemia. Graft volume necessary to achieve diabetic correction was lower for reaggregated islets (5–8500 IE/kg) than fresh islets (10–12,000 IE/kg) [52].

4. Advances in islet cryoprotective additive technology

4.1 Principles background

CPAs can be divided into two major types, namely permeating and non-permeating additives [53]. The main difference between the two major sub-types is whether the substance can penetrate the intracellular space during vitrification [54, 55]. Since the accidental discovery of glycerol in the 1940s [24], penetrating CPAs, such as DMSO and ethylene glycol, have shown significant benefits for islet survival in many studies [36]. These penetrating cryoprotectants, usually low-molecular weight polar aprotic solvents, penetrate the cell membrane and increase the inner volume of the cell. An equilibrium is reached across the cell membrane when the intracellular water content is lower than physiological normal range, thus reducing the probability of intracellular ice crystal formation [56, 57]. Non-penetrating cryoprotective additives like saccharides, which have a large molecular weight, remain in the extracellular space during the freezing process [56]. A build-up of these molecules in the extracellular space induces an osmotic gradient across the cell membrane, which causes water to move out of the cell. Water movement into the extracellular space helps to reduce the risk of intracellular ice crystal formation in addition to depressing the freezing point of intracellular water [58–60]. The mechanism of non-penetrating CPAs has been demonstrated to be temperature sensitive and suboptimal for certain cell types; therefore, penetrating CPAs have been traditionally favored over non-penetrating CPAs even though penetrating CPAs have increased the risk of toxicity [61, 62]. Recently, the integration of both permeating and non-permeating CPAs (e.g., DMSO with University of Wisconsin Solution (UW)) has shown improvements to post-cryopreservation islet recovery and insulin secretory behavior of islets.

4.2 Permeating cryoprotective additives

4.2.1 Dimethyl sulfoxide (DMSO)

DMSO toxicity toward islets has been shown to be minimal at concentrations used during the freezing phase and has even demonstrated protective capabilities against selective β-cell necrosis antagonist alloxan [38, 41, 63]. DMSO is considered the gold standard in islet 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 [64]. Islets from seven consecutive canine isolations were dissociated into single cells and cryopreserved in 2 M DMSO medium using a slow stepwise cooling method (0.25°C/min) to 40°C followed by storage in −196°C. Following rapid thawing (200°C/min), 81.5% of cryopreserved islets were recovered with no significant difference in insulin stimulation index (SI) when compared to non-treated canine islets (10.5 A.U. versus 12.4 A.U. respectively) [64]. 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 [64]. Overall, DMSO will continue to play an important role in islet cryopreservation research.

4.2.2 Ethylene/polyethylene glycol

A common constituent of car antifreeze, other permeating CPAs include both ethylene and polyethylene glycol, which have been studied for islets cryopreservation [53]. 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 [38]. Once the use of DMSO as a CPA was established in islet cryofreezing, studies in rat islets began to suggest potential toxicity issues when DMSO was exposed to rat islets [41, 65]. One study comparing DMSO and EG CPAs resulted in DMSO islets that exhibited lower cellular DNA, insulin, glucagon, and impaired insulin secretory patterns compared to EG, which was more like non-frozen 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 [65]. When islets cryopreserved with varying concentrations (1, 2, and 3 M) of DMSO, EG, and PG were exposed to islets from canine and human sources, the permeability (P_s = μm/s) was quantified. The highest P_s was achieved in canine islets when 2 M EG (2.47 μm/s) was used while 2 M PG showed the highest P_s in human islets (3.48 μm/s) suggesting potential use of EG and PG in islet cryopreservation [66].

4.2.3 Permeating CPA mixtures

Attempts have been made to produce mixtures of DMSO and EP (30% EP, 20% DMSO) for use during vitrification phase, which can help reduce the toxicity risks of using DMSO alone. 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 [67]. 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 (1 M ME2SO + 1 M EG, or 1 M ME2SO + 0.5 M EG). Islets treated with permeating CPA mixtures achieved significantly higher yield and viability compared to islets treated with DMSO only. When transplanted into STZ-induced mice, islets treated with DMSO/EG mixtures caused normoglycemia 12 days faster on average than DMSO only-treated islets [68].

4.3 Non-permeating cryoprotective additives

4.3.1 Saccharides

Although permeating cryoprotectants have been mainly used during mammalian cell cryopreservation, saccharides have demonstrated survival advantages when added to the vitrification medium. When adult human islets were treated with 300 mmol/L trehalose, a 92% recovery rate was achieved 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 [69]. 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 increases in recovery rate (85 ± 6.25 versus 63.3 ± 14.2%) and insulin stimulation index (3.86 ± 0.43 versus 2.98 ± 0.22) were observed [70]. These results demonstrate that saccharides and saccharide-containing substances can be used in conjunction with lower DMSO concentrations and help reduce islet toxicity.

4.3.2 Polymeric compounds

High-molecular weight polymeric compounds such as polyvinylpyrrolidone (PVP) and dextran have been shown to be effective at formation of amorphous glass matrix during cryofreezing phase [71]. When 10% PVP was added to cryopreservation medium before cooling phase, rat islet recovery and function were significantly higher than when islets were treated with 2 M DMSO and 3 M glycerol. Islets treated with 2 M PG demonstrated comparable islet recovery and function to PVP-treated group [72]. Although, the use of high-molecular weight cryoprotectants has been observed, past studies suggest that these compounds are ineffective at slow cooling temperature transitions, which is a crucial step of the cryofreezing process [71].

4.4 Other potential CPAs

One of the most important causes of cell damage/death during cryopreservation is due to ice crystal formation. However, there are unavoidable damaging consequences to islet health when islet cells, especially when in multicellular tissues fragments, are exposed to subfreezing temperatures (≥100°C), which can lead to apoptosis and/or necrosis after the post-thawing phase of cryopreservation [73]. Due to its fragile multicellular tissue structure, islet fragments are susceptible to various stresses including 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 [74, 75]. As research into islet cryoprotection has become more nuanced in recent years, studies have started to target CPAs, which reduce stress-induced cell death associated with the cryopreservation process (**Table 1**).

Parameter	Method	Reference
Cryoprotectant	EPA + DHA + Metformin	[40]
Cooling rate	Rapid (50–70°C/min)	[27–29]
Thawing rate	Rapid (150–200°C/min)	[32]
Oxygen environment	50% during thawing	[54]
3D structure	Freeze as individual cells, re-aggregate into spheroids after thaw	[51]
Encapsulation	1.75% Alginate encapsulation prior to cryopreservation	[64]

Assessment methods include islet viability, glucose sensitivity, and GSIS values after thawing.

Table 1.
Cryoprotection: A quick summary of the parameters that had the best outcome for the islets during cryopreservation.

4.4.1 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 [76]. 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 [77].

4.4.2 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 [78]. AA2G (100 μg/mL) in combination with the UW islet preservation solution was used as the cryopreservation medium [79, 80]. Following 3 months of storage (−80°C), the islets treated with UW/AA2G demonstrated viability maintenance (68.3 ± 5.6%) and significantly increased insulin stimulation via glucose-stimulated insulin secretion (GSIS) test, when compared to treatment with 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.

4.4.3 Curcumin

Curcumin, the main component of turmeric spice, has demonstrated antioxidant and anti-inflammatory effects in multiple cell types [81]. Curcumin has not been shown to increase insulin stimulation; however, it has demonstrated upregulation of oxidative stress-reducing genes Hsp70 and HO-1 [47]. 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 [47].

4.4.4 Taurine

An amino sulfonic acid, taurine has been suggested as a CPA because of chemical properties that allow antioxidant and osmoregulatory properties [82]. An addition of taurine to the cryopreservation medium demonstrated cyroprotective effects during islet cryopreservation (Hardikar 2001). Pretreatment of taurine prior to cryopreservation freezing phase at 0.3 mM and 3.0 mM resulted in high maintained viability of 91.9 ± 2.3 and 94.6 ± 1.58% respectively. Lipid peroxidation, which is a known indicator of oxidative stress, was reduced significantly compared to controls. Finally, normoglycemia was achieved when taurine-treated cryopreserved islets were transplanted into immunocompetent mice.

4.4.5 Γ-aminobutyric acid (GABA)

GABA neurotransmitters facilitate the inhibitory neuronal pathways within the central nervous system and have demonstrated regulatory and protective effects on β-cells [83]. GABA has been shown to produce membrane polarization (Ca^{2+}-influx) which activates survival and growth pathways (PI_3-K/Akt) and can restore β-cells' mass in severely diabetic mice [83, 84]. Islets were treated with 50 or 100 μM GABA and were cryopreserved to 196°C for up to 30 days. At both 15 and 30 days post-thawing, islets treated with GABA exhibited similar insulin secretory behavior compared to fresh islets. When oxidative stress was measured via MTT assay, reduced ROS content was observed in GABA-treated islets in comparison to non-treated cryopreserved islets [46].

4.4.6 Metformin

Metformin is a standard-of-care drug used for the treatment of Type 2 Diabetes and has been identified as an "essential medicine" by the WHO [85]. While the complete mechanism of action is unknown for metformin, it has demonstrated insulin-sensitizing properties and reduces unfettered liver gluconeogenesis [86, 87]. When used as a cryoprotective agent at ultralow temperatures, metformin produced membrane stabilizing effects. Cryopreserved islets treated with metformin-containing cyromixtures exhibited comparable viability (90 versus 100%) to non-treated fresh islets. Improved insulin secretion was observed at 15 days post-thawing (8 ng/mL) with a stimulation index value of >5, suggesting islets were highly functional [46]. Recently, similar cyroprotective results were observed in chicken islets [88].

4.4.7 Sericin

Produced by *Bombyx mori* silkworm, this gel-like protein has previously demonstrated oxidative stress reduction properties induced by freezing temperatures in both rat islets and various other mammalian cell lines [89, 90]. When added to media, Ahnishi et al. showed no significant differences between GSIS results between the FBS + DMSO and the sericin + DMSO groups. This study demonstrated that reduction in DMSO content in cyromixtures is possible with addition of 1% sericin, which could reduce the toxic risk posed by high concentrations of DMSO.

4.5 Recent advances in islet cryopreservation technology

4.5.1 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 immune-isolating barrier around islet fragments [91–93]. Alginate-microencapsulation has recently been applied to the field of islet cryopreservation in order to characterize its effect on islet survivability [33]. Chen et al. reported the development of an oxygen-sensing alginate coating to encapsulate islets prior to cryopreservation [94, 95]. 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 $50x10^{-3}$ 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 [94]. 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 [94]. Other studies have demonstrated the benefits of alginate microencapsulation use during cryopreservation as the 3D barrier is porous and can resist stress/strain associated with ice formation [94, 96, 97].

4.5.2 Hollow fiber vitrification

Previously described for use in embryological studies, vitrification scaffolds have been suggested as a medium for cryopreservation of islets to improve the islet survival [98, 99]. This technique involves the loading of islets into a hollow fiber chamber (HFV) composed of cellulose-triacetate, which is permeable to CPAs [100]. Researchers used a combination of permeating CPAs like DMSO and EG during cooling phase of both non-vitrified islets and islets vitrified using HFV method. *In Vitro* assays demonstrated similar islet structure and insulin gene promoter expression (NeuroD, Pdx1, MafA) to non-vitrified islets; however, the insulin stimulation index was significantly decreased for islets undergoing HFV compared to non-vitrified islets (27.8 ± 8.2 and 3.5 ± 0.6 respectively). Nonetheless, after HVF and non-vitrified islets were transplanted into kidney subcapsular space, all mice were euglycemic within 4–8 days and remained so for 1 month until nephrectomy, which induced hyperglycemia.

5. Conclusion

Islet cryopreservation has come a long way in the last 40 years. Many parameters of cryopreserving islets are being actively researched because of the high demand for long-term storage. Currently, entire organ cryopreservation is not entirely feasible, and is only shown possible after a few hours of storage [101]. Based on experiments performed in our lab along with results from other research groups, we predict that future improvements in islet cryopreservation will rely on the use of a mixture of cyroprotective additives along with the use of secondary technologies like alginate encapsulation [96]. We hope that with a standardized cryopreservation protocol, islet banking would be more feasible, and ultimately, transplantation would no longer be throttled by the donor-recipient mismatch.

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