


Assessment of Culture/Preservation Conditions of Human Islets for Transplantation

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Abstract

Islet culture before clinical transplantation has been adopted by various centers, but its effect on the survival and function of islets relative to the culture conditions and media needs further assessment. Human islets were cultured or preserved under four different conditions and three media options. Parameters such as recovery, viability, function, islet damage, and gene expressions for markers of hypoxia, and inflammation were assessed after 48-h culture or preservation. Preservation of islets was performed at 4°C in Connaught's Medical Research Lab (CMRL) and University of Wisconsin (UW) media. Islets were cultured at 22°C, 37°C, and 37°C–22°C in CMRL and PRODO culture media. Islets preserved in UW solution had visually good morphology and exhibited higher recovery with less islet damage compared with the rest of the groups, whereas islets preserved in CMRL at 4°C resulted in poor morphology, recovery, viability, and function compared with the rest of the treatment conditions. Culture at 22°C and 37°C demonstrated an increase in the expression of inflammatory and hypoxia-related genes. In conclusion, islets preserved at 4°C in UW solution showed the best overall outcomes after 48 h compared with islets cultured at 22°C, 37°C, or 37°C–22°C in PRODO. Advancement in islet culture media is warranted to reduce inflammatory gene activation and improve recovery of islets for transplantation.

Keywords

islet, culture, preservation, transplantation, inflammation, miRNA-375

Introduction

After the Edmonton protocol was established, islet transplantation was revived as a promising therapy for patients with type 1 diabetes mellitus by restoring normoglycemia and reducing hypoglycemic unawareness^{1–3}. During this period, islets were transplanted into recipients within 4 h after isolation^{3–5}. Later, it was suggested that this narrow time might limit the safety and quality assessment of the islet product before transplantation⁴.

Soon, islet culture and/or preservation before clinical islet transplantation were introduced in multiple centers because it has several advantages over transplantation of freshly isolated islets. It provides time for additional quality assessment of islets and reduces immunogenicity^{6–8}. Also, it provides additional time for recipients to initiate immunosuppressive treatment and travel to transplant centers^{9,10}. Although islets are generally cultured for 12–72 h at 37°C and/or 22°C before transplantation, most clinically transplanted islets were cultured no longer than 48 h^{9–11}. Several clinical trials adopted Clinical Islet Transplantation Consortium (CIT) culture protocols that cultured islets at 37°C for the first 12–24 h and at

22°C for the remaining time¹². Alternatively, preservation of human islets at low temperature has been reported to prevent islet loss and maintain viability and function of islets compared with culture¹³. The molecular changes and impact on the quality of the islets after culture or preservation are poorly understood. Therefore, the usefulness of islet culture and/or preservation before transplantation remains controversial.

Freshly isolated islets are still preferred by some groups because the recovery of islet mass after the culture is

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inconsistent. It is well known that islets deteriorate in culture and can lead to reduced islet mass for transplantation. Several centers have reported failure to proceed to transplantation due to the loss of islets after culture⁹. Islets isolated from donors after cardiac death have resulted in the poor recovery of islets after culture¹⁴. Islets without intrinsic vasculature are also highly sensitive to hypoxia, which may contribute to islet loss during culture/preservation^{15–17}. Due to these reasons, an appropriate culture or preservation of islets before transplantation is required to achieve successful clinical outcomes.

Transplantation of islets after culture for 24–48 h is practiced in many centers, and preservation of islets has also been recommended by some groups. In this study, we will comparatively assess the various parameters that will indicate the quality of the islets after culture or preservation for 48 h. Islets will be preserved in a refrigerated environment at 4°C, whereas islets will be cultured at 22°C, 37°C, and 37°C–22°C (37°C for 0–24 h and 22°C for 24–48 h). University of Wisconsin (UW), Connaught's Medical Research Lab (CMRL) 1066, and PRODO standard media will be used in these experiments because of the clinical applicability of these culture media for islet transplantation. UW is a standard organ preservative media that preserves the membrane integrity by preventing osmotic imbalance and reduces the formation of free radicals that can cause cell death¹⁸. CMRL is a standard islet cell culture media used in CIT trials and by many transplant centers. Islet cells are also cold-preserved for short duration in CMRL transplant media in autologous and allogeneic islet transplant cases while the recipient is prepped for infusion. PRODO media is a recently introduced alternative islet culture media for transplantation purposes^{19,20}. Outcomes of this study will provide a deeper understanding of the phenotypic nature of islets after culture and preservation.

Materials and Methods

Isolation of Islets From Human Pancreas

Human islets were isolated from the research-grade pancreas (not accepted for clinical transplantations) from deceased donors (braindead donors) through the local organ procurement organizations. Donor characteristics have been listed for all the donors used in this study (Supplemental Table 1). This study has received an exemption status from the Institutional Review Board of Virginia Commonwealth University. Islet isolation was performed using a modified Ricordi method^{21–23}. In brief, after decontamination of the pancreas, the collagenase enzyme solution was infused into the main pancreatic duct, and the pancreas was digested enzymatically and mechanically with the Ricordi chamber around 37°C. After dilution and recombination of digested tissue, islets were purified with continuous density gradient using the COBE 2991 cell processor as reported previously²⁴. Islet yield was evaluated with dithizone staining (Sigma

Chemical Co., St. Louis, MO, USA) and converted to a standard number of total islet equivalents (IEQ) based on a size of 150 μm^2 .

Islet Culture and Preservation

Freshly isolated human islets in the purity range of 80%–95% (high purity) were used in all these experiments. Islets were cultured in (1) CMRL 1066 medium (Mediatech, Manassas, VA, USA) supplemented with 2.5% Human Albumin (Baxter, Deerfield, IL, USA) and 2 mM HEPES (Mediatech); and (2) Prodo Islet Media Standard PIM(S) medium with human serum AB and glutamine/glutathione additives (Prodo Labs, CA, USA). Also, for preservation, we have used CMRL and PRODO medium, and additionally, these have been compared with (3) UW solution (Bridge to Life, IL, USA).

Three different mediums for culture (CMRL and Prodo) and preservation (CMRL, Prodo and, UW) were tested in four different culture conditions: (1) 4°C for 48 h; (2) 22°C (95% air and 5% CO₂) for 48 h; (3) 37°C (95% air and 5% CO₂) for 48 h; and (4) 37°C (95% air and 5% CO₂) for 24 h followed by 22°C (95% air and 5% CO₂) for 24 h. Islets were seeded at 100–150 IEQ/cm² dish area in a 100-cm² culture dish, and the height of the media was 1.5–2.0 mm.¹³

Viability Assay

The viability of islets was assessed by performing staining with 10 $\mu\text{g}/\text{ml}$ Hoechst 33342 (Sigma-Aldrich, St. Louis, MO, USA) and 20 $\mu\text{g}/\text{ml}$ propidium iodide (PI) stain (Sigma-Aldrich) for 10 min at 37°C before imaging via fluorescent microscopy¹⁷. Fluorescent micrographs were merged in Image J (NIH Bethesda, MA, USA) and the PI-positive area was divided by the Hoechst-positive area to calculate islet viability.

Glucose-Stimulated Insulin Secretion Test

Islet function was determined using a glucose-stimulated insulin secretion test (GSIS). Using a microscope equipped with an eyepiece reticle, 10 islets in the size range of 150 μm were hand-picked per replicate. Triplicate of each group was performed in each of the donor preparations separately. Hand-picked islets were incubated with low (1.67 mM) and high (16.7mM) concentrations of glucose in functionality/viability medium CMRL 1066 (Mediatech, Manassas, VA, USA) for 1 h each at 37°C. For islets preserved at 4°C, the cells were cultured at 37°C for 2 h to acclimate to physiological temperature before performing GSIS. Insulin concentrations were measured with an enzyme-linked immunosorbent assay (ELISA) kit (ALPCO Diagnosis, Salem, NH, USA). The stimulation index was calculated as the ratio of insulin levels in high and low glucose concentrations based on three independent measurements.

Table 1. Donor and Isolation Characteristics.

Variable	Values
Donor characteristics	
Age	48 ± 11
Gender ratio (male:female)	6:6
Body mass index	30.3 ± 6.7
Cold ischemia Time (h)	9 ± 4
HbA1c	5.7 ± 0.4
Islet characteristics	
Collagenase enzyme	Serva collagenase NBI + Neutral protease NB
Collagenase (Wunsch units/g of pancreas)	19.8 ± 0.45
Neutral protease (DMC units)	0.88 ± 0.11
Pancreas weight (g)	97.0 ± 28.0
Islet equivalent total (IEQ)	389,382 ± 115,805
Viability (%)	93.6 ± 3.3
Islet fragmentation (%)	20.4 ± 8.9
Islet purity (%)	87.8 ± 4.9
Stimulation index	6.0 ± 4.0

Total Small RNA Extraction, cDNA Synthesis, and LNA-Based qPCR miR-375 Assay

RNA extraction from 200 µl of samples of culture or preservation media was performed using the miRCURY™ RNA isolation Kit-Biofluids (EXIQON Inc., Woburn, MA, USA). Extracted RNA was treated with recombinant DNase to remove any DNA contamination. Complementary DNA (cDNA) strand synthesis was performed using the miRCURY LNA™ Universal RT microRNA PCR (EXIQON Inc.). Then, quantitative polymerase chain reaction (qPCR) was performed using a locked nucleic acid (LNA)-based specific primer for miRNA-375 (EXIQON Inc.). cDNA was briefly diluted 1:40 using nuclease-free water and mixed with PCR master mix. The absolute quantity was calculated based on the standard curve prepared by synthetic miRNA (miRNA 375 sequence: 5'-uuuguucguucgg-cucgcguga-3') as previously described². All measuring samples were triplicated.

Quantitative PCR for Inflammatory Cytokines, Chemokines, and Hypoxia Gene Expression Analysis

Total RNA was extracted from islets using TRIzol (Thermo Fischer Scientific, Waltham, MA, USA) and was reverse transcribed into cDNA using a High Capacity cDNA Reverse Transcription kit (Thermo Fischer Scientific). Template cDNA was mixed with SYBR® Green PCR Master Mix (Thermo Fischer Scientific) and primers of interleukin (IL)-1β, tumor necrosis factor (TNF)-α, IL-6, IL-18, chemokine C-C motif ligand 2 (CCL2), C-X-C motif ligand 8 (CXCL8), high-mobility group box protein 1 (HMGB-1), tissue factor (TF) (Invitrogen, Carlsbad, CA, USA), or hypoxia-inducible

factor 1-alpha (HIF-1α) (Qiagen Inc., Valencia, CA, USA). The reverse transcription PCR was run using SimpliAmp™ Thermal Cycler (Thermo Fischer Scientific) with the following program: 95°C for 10 min and 40 cycles of 95°C for 15 s and 60°C for 60 s. Quantitative analysis was performed with 18S ribosomal RNA (Thermo Fischer Scientific) as an internal control, and relative expression levels were calculated with the $2^{-\Delta\Delta Ct}$ method. All measuring samples were triplicated.

Statistical Analysis

Results were expressed as means ± standard deviation. All data were analyzed using GraphPad Prism 7 (GraphPad Software, Inc., La Jolla, CA, USA). One and two-way analyses of variance (ANOVAs) were used for data analysis of more than two groups, and a Tukey post hoc test was used to determine significant differences between treatment groups. *P* values <0.05 were considered statistically significant. A total of 12 different donor islet preps were used in this study; however, not all analyses included all 12 donors. The donor and the islet isolation characteristics are listed in Table 1. The number of donors used for each experiment has been described in the figure legends.

Results

Islet Morphology Assessment

Isolation of islets results in a considerable amount of damage to islets²⁶. Islet damage can be visualized morphologically by assessing the three-dimensional (3D) shape, fragmentation/disintegration, non-rounded/irregular surface of islets, or islet clumping due to DNA released from damaged cells. The culture of islets has been suggested to improve the

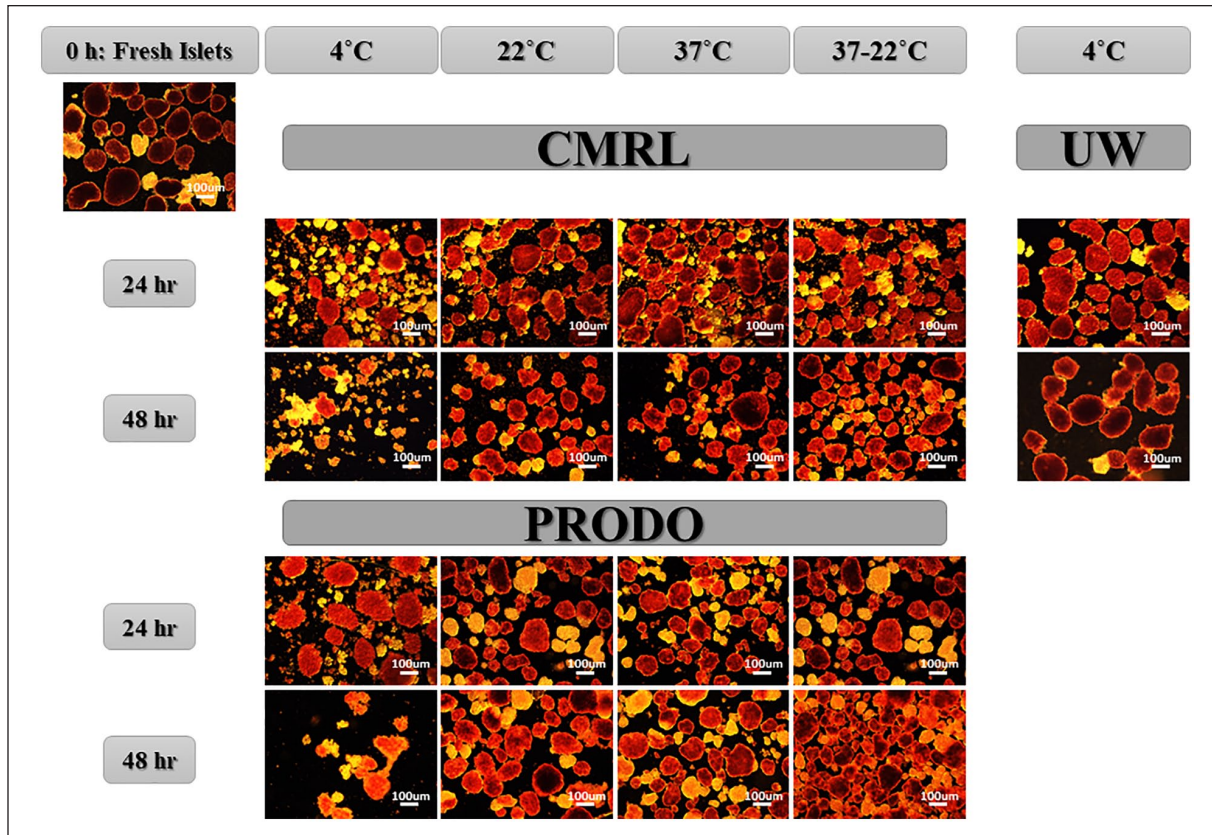


Figure 1. Temperature condition affects islet morphology during culture. Islets were evaluated for morphology using dithizone staining after 24 and 48 h of culture at different temperatures of 4°C, 22°C, 37°C, and 37°C–22°C. Images shown are from one donor islet preparation but are representative of $n = 12$ different donors. All images were captured at 40 \times magnification under the bright field microscope. CMRL: Connaught’s Medical Research Lab; UW: University of Wisconsin.

morphology of islets. Islet morphology after culture or preservation was microscopically evaluated after dithizone staining. Freshly isolated islets showed slight fragmentation (10%–15%) which is quite normal, and the surface was well-rounded with normal 3D morphology. After 24 and 48 h, islet morphology at 22°C, 37°C, and 37°C–22°C culture condition in CMRL and PRODO media was similar to fresh islets; however, we observed a decrease in dithizone staining levels compared with control, thus affecting the purity of islets at 24 and 48 h, more prominently in PRODO media (Fig. 1). On the contrary, human islets preserved at 4°C in CMRL media showed increased fragmentation and poor surface integrity, suggesting ongoing islet damage at 24 h which was significantly worsened at 48 h, whereas islets cultured in PRODO media at 4°C showed clumping and fragmentation and thus many islets could not be captured in the images. Islets completely deteriorated and very few islet clusters remained after 48 h of preservation in CMRL and PRODO medium at 4°C. Due to aggressive islet damage at 4°C, there was a significant loss of big islets at 48 h (Fig. 1). Surprisingly, the best morphology and integrity of islets was observed when islets were preserved at 4°C in UW media at both 24 and 48 h; there was no change in purity and no loss of larger

islets in this condition. Islets preserved in UW had morphology similar to fresh islets even after 48 h.

Islet Recovery After Culture

Several centers including ours have experienced a significant loss of islet counts (10%–50%) in culture after 48 h²⁷. In clinical cases, this may result in the cancellation of planned transplant procedures. Therefore, we evaluated the recovery of cultured/preserved islets at 24 and 48 h to identify an optimal environment for maximum recovery of islets. One reason for the poor recovery of islets after culture is the loss of dithizone staining in cultured islets, which may reduce the purity and counts of the preparation. Islet recovery percentage was determined by the ratio of count (IEQ) after culture/preservation to the count (IEQ) of fresh islets plated after isolation. After 24 h, preservation of islets at 4°C (CMRL and UW) had the highest islet recovery compared with the other groups ($P < 0.05$ vs 22°C and $P < 0.05$ vs 37°C–22°C) (Fig. 2A). After 48 h, the 4°C preservation in CMRL steeply declined and had the lowest percentage of recovery; this was statistically significant compared with other groups [$P < 0.05$ vs 22°C, $P < 0.05$ vs 37°C–22°C, $P < 0.001$ vs 4°C

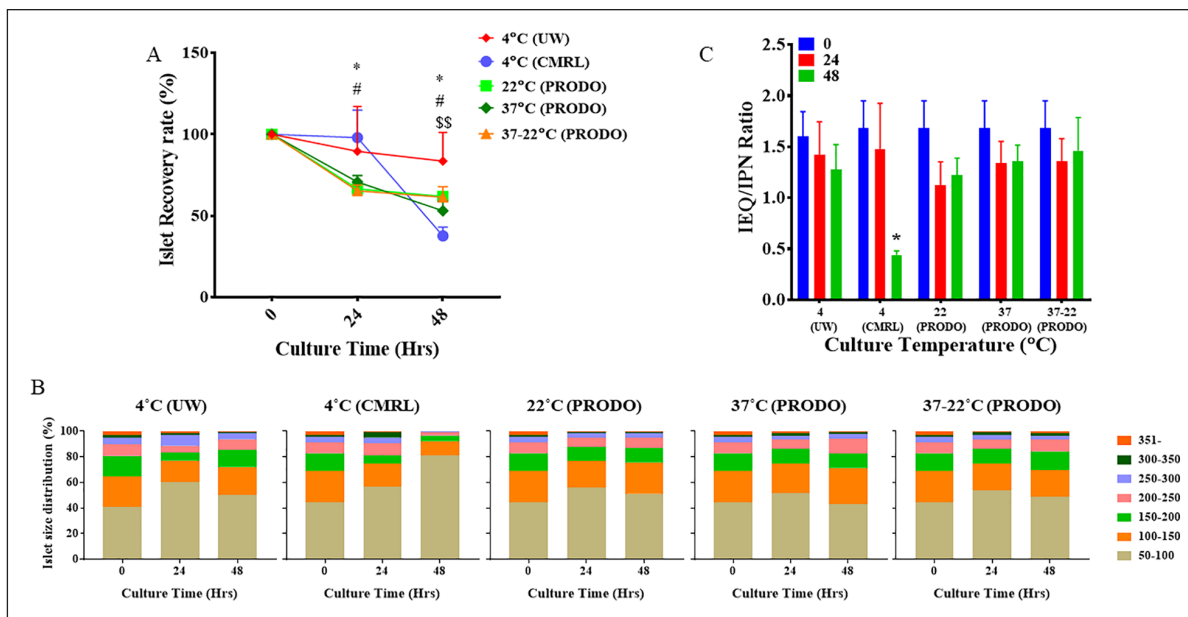


Figure 2. Postculture islet recovery rate. (A) Islet recovery rate is represented as the percentage of IEQ counted after culture to the IEQ plated for culture ($n = 9$ donors). * denotes comparison of 4°C (CMRL) versus 22°C; # denotes comparison of 4°C (CMRL) versus 37°C. (B) Islet size distribution is the percentage of islets in the particular size range (50–350+ μm) in different culture groups after 24 and 48 h ($n = 9$ donors); (C) IEQ/IPN ratio of islets was calculated after 24 and 48 h of culture ($n = 9$ donors). Groups were compared by two-way analysis of variance (repeated measures) followed by Tukey's multiple comparison test. *denotes $P < 0.05$ compared to the ratio at day 0. IEQ: islet equivalents; CMRL: Connaught's Medical Research Lab; IPN: islet particle number; UW: University of Wisconsin.

(UW)]. Preservation of islets at 4°C in UW solution had maintained the recovery percentage after 48 h, and thus had the highest recovery percentage among all groups at both 24- and 48-h time points. Our historical data of islets cultured in CMRL at 37°C for 24 h and at 22°C from 24 to 48 h showed similar recovery rate as islets cultured in PRODO media under similar conditions (Supplemental Fig. 1). However, it should be noted that the data from different donors for CMRL ($n = 5$) and PRODO ($n = 5$) have been used in this analysis, and an investigation with same donors in both groups is warranted.

The recovery rate of islets depends on the count (IEQ), and therefore, we wanted to see whether there was an increase or decrease in islets in a particular size range. The percentage of total islets counted in different size ranges (50–100, 100–150, 150–200, 200–250, 250–300, 300–350, 350+ μm) was calculated. Islets cultured in PRODO at 22°C, 37°C, and 37°C–22°C showed a gradual decline in the number of islets in the size ranges between 150 and 350+ μm and a significant increase in the percentage of islets in 50–100 μm size range at 24 h compared with 0 h (22°C: $P = 0.0036$; 37°C–22°C: $P = 0.0202$) (Fig. 2B). On the other hand, preservation at 4°C in CMRL or UW maintained the counts of larger islets (200–350+ μm) after 24 h. However, after 48 h, a significant decrease in the percentage of islets in size ranges from 100 to 350+ μm was observed, and a significant increase in the 50–100 μm size islets was noted in CMRL-preserved group but not in UW [4°C (CMRL), 0 vs 24: $P < 0.006$;

0 vs 48: $P < 0.0001$; 24 vs 48: $P < 0.0001$] (Fig. 2B). The decrease in larger islets and the increase in smaller size islets indicate persistent fragmentation/disintegration that may be reflected in the poor recovery of islets after preservation in CMRL media. Furthermore, we also evaluated the ratio of islet equivalents to islet particle number (IEQ/IPN) to verify whether it correlates with the recovery. We observed a significant decrease in the IEQ/IPN ratio at 4°C in CMRL after 48 h (Fig. 2C). Altogether, we observed a better recovery in islets preserved at 4°C in UW medium with no significant loss in large islets and excellent morphology.

Islet Viability and Function

Morphological and recovery rate assessment does not provide the overall health of the islets. Therefore, we evaluated the viability after 48 h using Hoechst/PI staining. PI-positive cells in freshly isolated islets were similar in all preps and were less than 1% (data not shown). PI+ area/islet after 48 h demonstrated a significant reduction in islet viability in the CMRL media at 4°C compared with other culture conditions [$P < 0.0001$ vs 4°C (UW), $P < 0.0001$ vs 22°C, $P < 0.0001$ vs 37°C, and $P < 0.0001$ vs 37°C–22°C] (Fig. 3A). The viability of islets at 24 and 48 h for all the culture/preservation conditions has been shown (Supplemental Fig. 2). Although the PI+ area/islet was high in the 4°C (CMRL) preservation group, the islets still have a viability of greater than 90%, namely, suitable for transplantation.

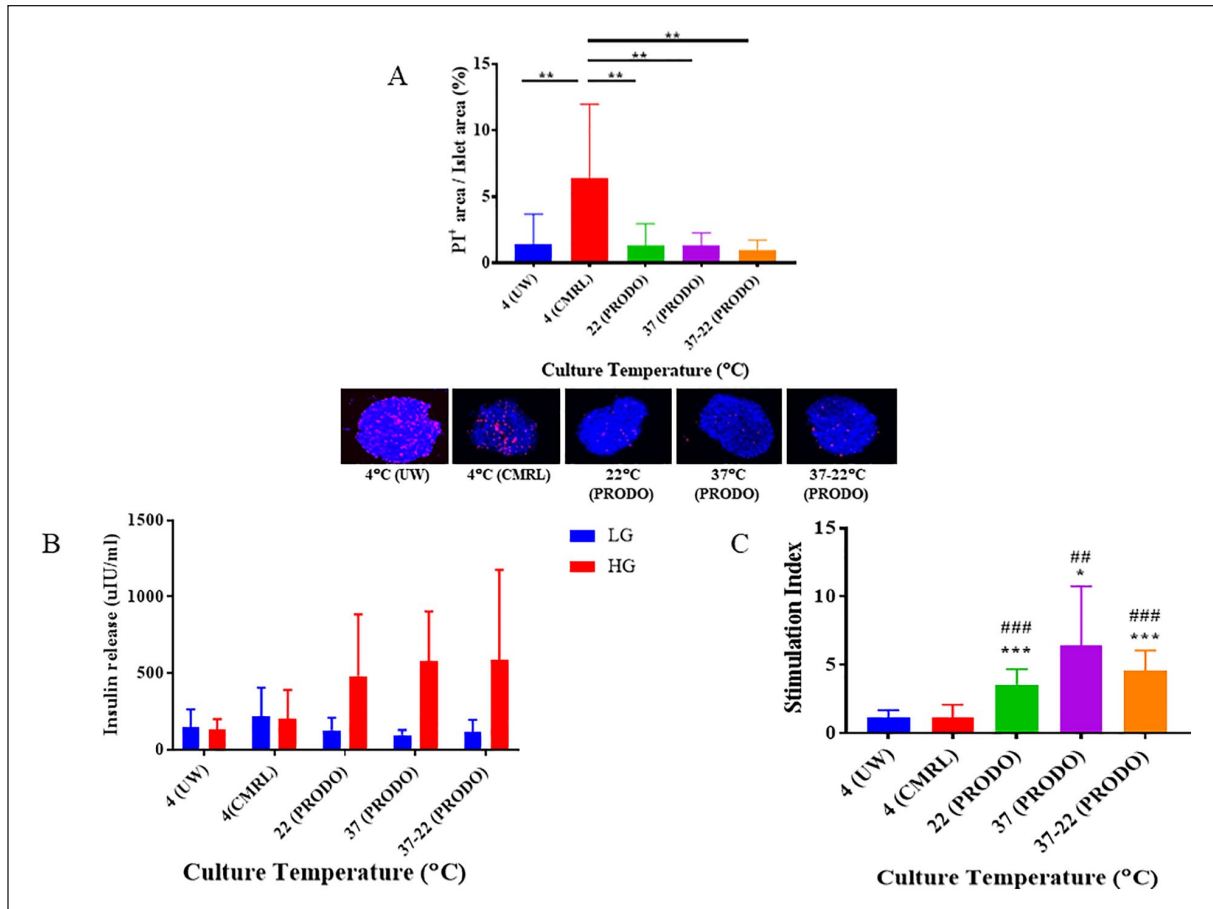


Figure 3. Culture temperature affects viability and islet function. (A) Percentage of PI+ area in islets after 48 h of culture ($n = 15$ individual islets analyzed per nine donors). Representative images of Hoechst/PI staining for each group have been shown. Purple-stained cells are dead cells. Groups were compared by one-way analysis of variance followed by Tukey's multiple comparison test. $**P < 0.01$; (B) glucose-stimulated insulin secretion: blue and red bars indicate insulin-released levels in response to low (1.67 mM) and high (16.7 mM) glucose, respectively; (C) stimulation index calculated by dividing the amount of insulin released during incubation in high glucose by that released in low glucose. Groups were compared by one-way analysis of variance followed by Tukey's multiple comparison tests [$n = 9$ different donors; $*P < 0.01$, $**P < 0.001$, and $***P < 0.0001$]. * represents the comparison with 4°C (UW); # represents the comparison with 4°C (CMRL). PI: propidium iodide; UW: University of Wisconsin; CMRL: Connaught's Medical Research Lab.

We then assessed the functional capacity of cultured/preserved islets after 48 h. Insulin secretion by static stimulation in response to low and high glucose media was measured. Human islets cultured in PRODO medium at 22°C, 37°C, and 37°C–22°C responded appropriately to low and high glucose stimulation; however, islets after 4°C preservation did not respond to high glucose stimulation (Fig. 3B), resulting in poor stimulation index. The stimulation index of islets preserved at 4°C (CMRL and UW) was significantly lower than cultured islets at 22°C ($P < 0.01$), 37°C ($P < 0.0001$), and 37°C–22°C ($P < 0.001$) (Fig. 3C).

Sensitive and Accurate Assessment of Islet Damage Using miR-375 During Culture

Damaged islets tend to release miRNA-375 (miR-375) into the extracellular environment, and this has been previously

used to measure the amount of damage caused during isolation and transplantation^{2,26}. We measured the absolute quantity of miR-375 in the medium after 24 and 48 h of culture/preservation of islets using the standard curve method (Fig. 4A). MiR-375 levels immediately after isolation were considered baseline, and an increase or decrease will determine damage or repair of islets, respectively. MiR-375 level in islets cultured in PRODO at 22°C, 37°C, and 37°C–22°C was unchanged compared with freshly isolated islets. However, at 4°C in CMRL media, a continual increase in miR-375 levels after 24 and 48 h was noted (Fig. 4B). The miR-375 level was significantly elevated at 4°C (CMRL) compared with other culture conditions [$P < 0.0001$ vs 4°C (UW), $P < 0.001$ vs 22°C, $P < 0.001$ vs 37°C, and $P < 0.001$ vs 37°C–22°C] (Fig. 4B). Thus, it suggests that CMRL-based media is not suitable for the preservation of islets at 4°C as it undergoes continuous damage after isolation which

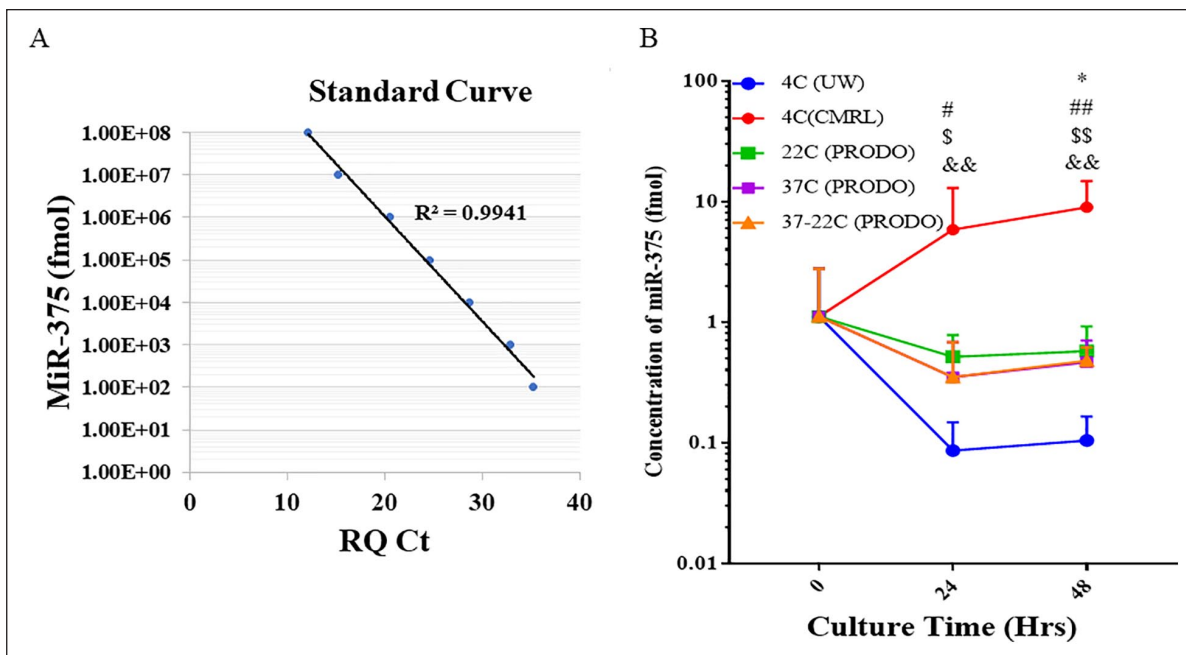


Figure 4. MiR-375 absolute quantity in the culture at various temperatures. (A) The standard curve was used to calculate the absolute quantity of miRNA in the media. (B) Absolute concentration of miR-375 in the culture media after 24 and 48 h of culture/preservation of islets. Groups were compared by two-way analysis of variance (repeated measures) followed by Tukey's multiple comparison tests ($n = 5$ donors, *4°C (CMRL) compared with 22°C, #4°C (CMRL) compared with 37°C, \$ $P < 0.05$ 4°C (CMRL) compared with 37°C–22°C, and 4°C (CMRL) compared with 4°C (UW)]. * represents $P < 0.01$, ** represents $P < 0.001$. CMRL: Connaught's Medical Research Lab; UW: University of Wisconsin.

is reflected by the increase in miR-375 level in the media. Contrary to CMRL media, UW media at 4°C showed markedly lower miR-375 levels at 24 and 48 h and it was significantly lower than all the other culture and preservation conditions (Fig. 4B).

Differential Expression of Inflammatory and Hypoxia-Specific Genes

One of the major drawbacks of culture is the upregulation of inflammatory cytokines and chemokines by islets, which can initiate acute inflammatory damage after transplantation. Hence, we pursued to determine whether inflammatory mediators were differentially expressed in islets that may cause damage during culture/preservation. Previous reports have shown an upregulation of proinflammatory cytokines in cultured islets compared with freshly isolated islets¹⁵. But the expression level of proinflammatory cytokines in different culture/preservation conditions has not been investigated. Therefore, we examined messenger RNA (mRNA) expressions of proinflammatory cytokine (IL-1 β , TNF- α , IL-18, and IL-6), chemokine (CCL2 and CXCL8), hypoxia damage markers (HMGB-1 and HIF-1 α), and TF in freshly isolated islets and after 24 and 48 h of culture or preservation. Expression levels of mRNAs for proinflammatory cytokines (IL-1 β , TNF α , IL-18, IL-6) (Fig. 5A–D), chemokines (CCL2, CXCL8) (Fig. 5E, F), and hypoxia markers

(HMGB-1, HIF-1 α) (Fig. 5G, H) were increased in islets cultured in PRODO media at 22°C, 37°C, and 37°C–22°C after 24 and 48 h when compared with fresh islets ($P < 0.05$) (Fig. 5). However, there was no significant difference in the expression of proinflammatory genes when islets were preserved at 4°C in CMRL or UW media compared with freshly isolated islets, and this may be due to the lack of metabolic activity in islets at the low-temperature condition (Fig. 5).

Discussion

Transplantation of a sufficient number of good-quality islets is essential for successful long-term clinical outcomes. Despite recent advances in isolation protocols, islets are subjected to damage and stress throughout the process of isolation and culture. Assessment of current culture/preservation protocol is necessary to identify the limitations and improve the methodology of islet culture for better transplant outcomes. In this study, we evaluated the culture and preservation of islets in four different temperature conditions and three different media preparations. We tested the preservation of islets at 4°C in CMRL, PRODO, and UW media for 48 h to evaluate whether these conditions will maintain islet viability and counts. Islet centers have practiced short-term cold preservation of islets in autologous and allogeneic transplantation while the recipients are prepped for infusion, and this can last from couple of hours to an entire day. Therefore,

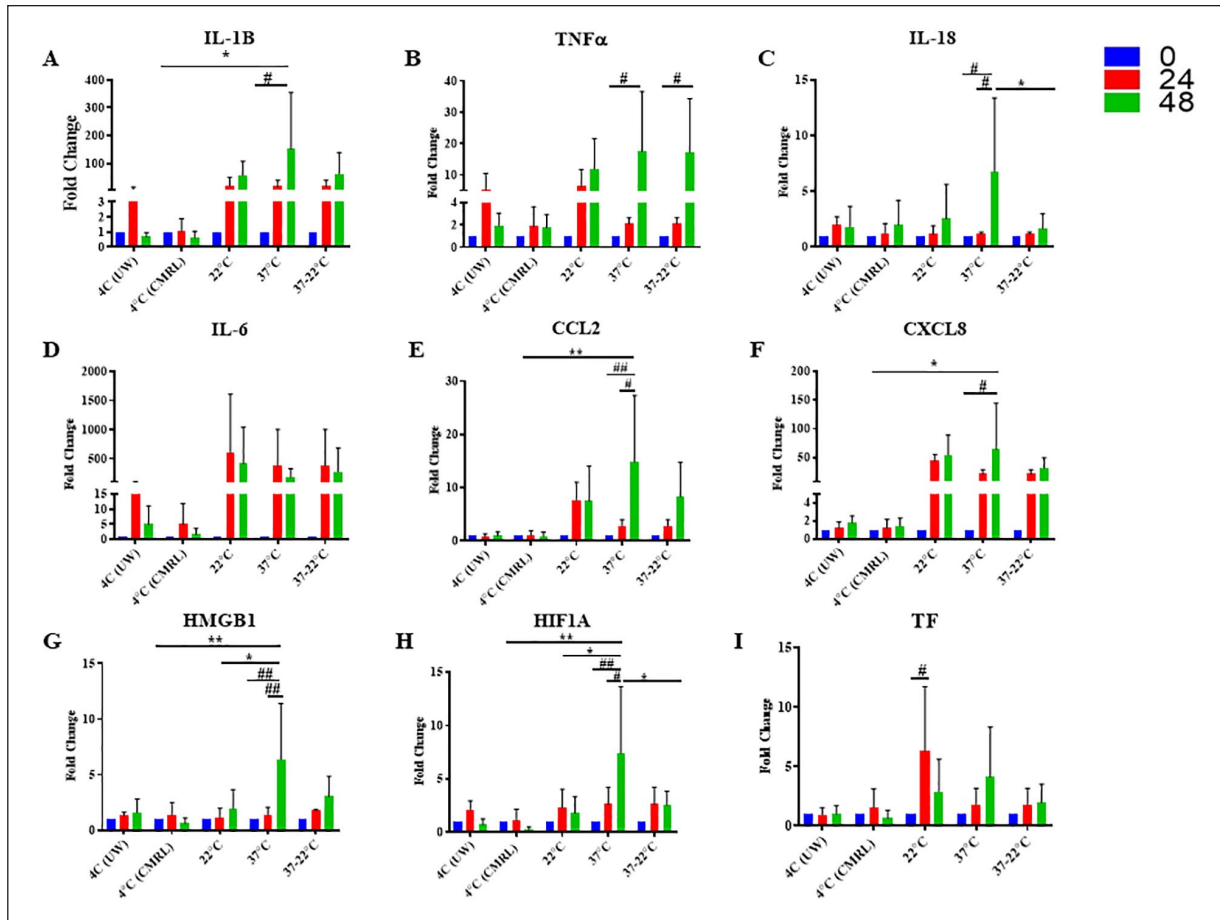


Figure 5. Differential gene expression during islet culture. Gene expression of proinflammatory cytokines, chemokines, and hypoxia in islets after culture for 24 and 48 h. (A) IL-1 β ; (B) TNF α ; (C) IL-18; (D) IL-6; (E) CCL2; (F) CXCL8; (G) HMGB-1; (H) HIF-1 α ; (I) TF. Groups were compared by two-way analysis of variance (repeated measures) followed by Tukey's multiple comparison tests ($n = 5$ donors, $*P < 0.05$, $**P < 0.01$, $\#P < 0.05$, and $\#\#\#P < 0.01$). Groups include 4°C in UW, 4°C in CMRL, and 22°C, 37°C, and 37°–22°C in standard PRODO medium. All the treatment groups were compared with 4°C (CMRL). *represents a comparison of the same time points between groups. # represents a comparison of different time points within the same group. IL-1 β : interleukin-1 β ; TNF α : tumor necrosis factor- α ; IL-18: interleukin-18; IL-6: interleukin-6; CCL2: chemokine C-C motif ligand 2; CXCL8: C-X-C motif ligand 8; HMGB-1: high mobility group box protein 1; HIF-1 α : hypoxia-inducible factor 1-alpha; TF: tissue factor; UW: University of Wisconsin; CMRL: Connaught's Medical Research Lab.

it is necessary to study the impact of cold preservation in CMRL-based transplant media. Moreover, these preservation conditions except UW have not been tested previously by others. Second, we tested the culture of islets in 5% CO₂ at 37°C for 48 h, as this has been practiced by few centers, and it is suggested that the culture of islets at this temperature will reduce immunogenicity and improve the purity of islets by eliminating acinar cells. Another group of islets was cultured in 5% CO₂ at 22°C for 48 h; once again, some groups have shown the beneficial effect of culturing islets at this temperature by causing a reduction in hypoxia and necrosis. Finally, we cultured the islets according to the CIT protocol, that is, culture in a 5% CO₂ incubator at 37°C for 24 h and then at 22°C for the final 24 h. PRODO standard culture media and CMRL 1066 media with additives have been used to test the culture at 22°C, 37°C, and 37°C–22°C. Islet cells

were evaluated based on morphology, recovery, and viability. Furthermore, islet damage during culture was monitored by miR-375 analysis. We also evaluated the expression levels of proinflammatory and hypoxia-related genes in the various conditions tested.

The isolation procedure exposes the islets to harsh conditions during enzymatic and mechanical digestions, purification, and recombination. We have previously reported that significant islet damage during isolation can affect the outcomes after transplantation²⁶. The culture of islets ensures postisolation repair and provides time for better assessment of the product's quality and patient preparation. Alternatively, freshly isolated islets will guarantee transplantation of maximum islet yield, without loss of cells in culture. It, therefore, remains controversial whether islets should be transplanted immediately or cultured before transplantation. Studies have

also reported improved quality and recovery of islets when they were preserved at 4°C in the UW solution¹³. To further clarify, we compared the effect of islet preservation on viability and function of islets when CMRL or UW media was used at 4°C. The overall goal of this study was to identify optimal culture/preservation conditions that result in improved recovery of islets for transplantation.

Islets cultured at 37°C, 22°C, and 37°C–22°C in CMRL and PRODO medium exhibited good morphology at 24 and 48 h with fewer fragmented islets and a smooth surface, similar to freshly isolated islets. The number of islets in larger size ranges was reduced compared with day 0, causing a decline in the recovery rate from ~60%–65% after 24 h and ~55%–60% at 48 h in PRODO medium. Islets preserved at 4°C in CMRL or PRODO medium had poor morphology with a ragged surface after 24 h, yet the recovery rate of islets at 4°C was highest among all the groups as the larger islets were maintained in this condition. Larger islets tend to undergo necrosis due to hypoxia at higher temperatures, but not at 4°C^{17,28}, and a small decrease in the larger islets can cause a significant decline in the overall IEQ, thus affecting the recovery of the islets which is observed at 37°C and 22°C culture condition. After 48 h of preservation at 4°C in CMRL and PRODO media, islets were extremely fragmented and dispersed and the total IEQ and recovery percentage plummeted drastically to less than 40% in CMRL media. We also saw a significant drop in IEQ/IPN ratio at 48 h compared with 24 h and 0 h, and a substantial increase in the percentage of smaller islets (50–100 µm) and a decrease in larger islets (100–350+ µm), indicating an immense deterioration of islets during low-temperature preservation with CMRL media. A sharp contrasting result was observed with islets preserved in UW solution at 4°C. The morphology and integrity of islets were far superior to any of the other culture and preservation groups. Furthermore, the recovery percentage of islets was significantly higher in UW-preserved islets compared with CMRL preservation. We also observed that islets tend to reduce dithizone uptake after being cultured in CMRL or PRODO medium, but the preservation of islets in UW solution also retained the ability to stain with dithizone. Poor staining of islets with dithizone affects the islet purity and counts, thus impacting the overall recovery of islets after culture. The lack of dithizone staining in some islets is due to a reduction in the zinc content in those islets, but the underlying cause for this change is not known. The culture of islets under conditions that can prevent hypoxia may be necessary to improve the recovery rate of islets when cultured at 37°C, 22°C, or the combination.

Islet viability after 48 h was within the acceptable range for transplantation (>70%) in all groups tested; however, an increased percentage of dead cell area was visualized in islets preserved at 4°C in CMRL but not in UW medium. These data correlated with the poor morphology and recovery of islets in CMRL-preserved islets. Functional analysis revealed that islets cultured at 37°C, 22°C, and 37°C–22°C

for 48 h demonstrated good stimulation in response to low and high glucose media, whereas islets preserved in UW and CMRL at 4°C did not show any stimulation in response to high glucose, thus resulting in poor stimulation index compared with the cultured groups. The stimulation of islets with glucose and insulin secretion response has a direct correlation with temperature. It has been reported that hypothermia inhibits insulin secretion as a result of inhibition of the availability of energetic substrates and the lack of metabolic signals²⁹. The reduced metabolic activity of islets due to preservation at 4°C may have caused the three- to fourfold decrease in insulin stimulation compared with islets cultured at 22°C, 37°C, and 37°C–22°C. It should be noted that the stimulation was carried out at 37°C after cells were normalized at this temperature for 2 h. A longer period of normalization may be required for islets preserved at 4°C, and this limitation will be explored in future studies.

Earlier studies by our group showed that elevation of miR-375 in the media is an excellent marker of islet damage and resulted in poor islet transplant outcomes^{26,30}. In correlation to poor viability, there was also a significant elevation in miR-375 level in the supernatant of islets preserved at 4°C in CMRL media after 24 h, which steadily increased up to 48 h. This suggests that low-temperature preservation in CMRL transplant media causes persistent islet damage after isolation. On the other hand, preservation of islets in UW media at 4°C had reduced miRNA 375 levels compared with fresh islets at 24 and 48 h, suggesting reduced islet cell damage in this condition. Similarly, miR-375 levels at 22°C, 37°C, and 37°C–22°C were not significantly altered after 24 h. This may also infer that islets tend to stabilize in the culture after isolation. Thus, it can be inferred that islets preserved at 4°C in UW undergo the least islet damage.

It has been reported that the enzymatic and mechanical stress during islet isolation contributes to islet reduction by the production of proinflammatory cytokines^{31,32}. Due to the lack of intrinsic vasculature, islets are subjected to hypoxic conditions during islet isolation and culture^{15–17}. Hypoxia induces activation and release of the proinflammatory cytokines and chemokines and subsequent cell death via NF-κB signaling³³. It is well known that islets produce cytokines and chemokines upon exposure to inflammatory and hypoxic conditions *in vitro* and *in vivo* as well^{34,35}. Similarly, the mRNA expressions of proinflammatory cytokines and chemokines IL-1β, TNFα, IL-18, CCL2, and CXCL8 showed a time-dependent upregulatory trend when islets were cultured at 37°C, 22°C, and 37°C–22°C. In contrast, no noteworthy change in mRNA expressions of proinflammatory cytokines and chemokines was seen at 4°C preservation in CMRL or UW condition, and this may be due to metabolic inactivity of islets at low temperature. This also suggests that the islet damage that takes place during low-temperature preservation seen in CMRL media is not caused by inflammatory mediators.

It has been reported that low temperature decreases hypoxic damage by reducing the expressions of HMGB-1 and HIF-1 α in mouse and rat models^{17,36}. Consistent with previous reports, the 4°C groups showed the lowest mRNA expression of HMGB-1 and HIF-1 α compared with other groups. Islets cultured at 37°C for 48 h had significant upregulation of HIF-1 α and HMGB-1, indicating the hypoxia pathway is activated. However, when cells were cultured at 22°C or cultured in 37°C for 24 h and then moved to 22°C, there was a considerably reduced activation of the hypoxia-related genes HIF-1 α and HMGB-1. These results indicate that culturing islets at 22°C may have some advantage by reducing inflammatory and hypoxia signaling compared with 37°C culture.

Previously our group has reported that low-temperature preservation of human isolated islets with UW preservation solution improved islet survival and function compared with islet culture¹³. We chose to study the cold preservation of islets in CMRL 1066 and PRODO media in comparison with the UW solution to evaluate the suitable media for preservation. CMRL 1066 media was tested because it is clinically used for transplantation, and short-term cold preservation is practiced as it would prevent the shock to cells from the change from other preservation solution to transplant media¹². Moreover, CMRL 1066 has been shown to prevent the growth of immune cells, such as dendritic and endothelial cells³⁷. However, to investigate the difference between CMRL 1066 and UW preservation at 4°C, we performed a comparative analysis. In our observation, we found that UW had improved morphology, viability, less islet damage, and better recovery compared with CMRL 1066 media. This may be because the UW media contains additives that prevent cold-induced necrotic injury due to impermeable and osmotically active endogenous factors¹⁸. It is also necessary to understand that cold preservation of 4°C alone may not be sufficient to improve the viability and survival of islets, but the use of an ideal preservation media such as UW solution is equally critical for low-temperature preservation of islets.

Inflammatory and hypoxic signaling in islets after culture would further augment the effect of instant blood-mediated inflammatory reaction and may result in an increased loss of islet mass after transplantation. The culture of islets in conditions that can reduce inflammatory and hypoxic signaling is highly preferable to prevent cell loss during culture and after transplantation. Novel culture methodologies such as higher oxygen supply during culture³⁸ or incorporation of anti-inflammatory molecules³⁹ or a combination of both may result in improved recovery of islets for transplantation.

Based on our assessment, culturing islets at higher temperatures maintains the quality and function of islets, but affects recovery and induces inflammation. Preservation of islets in UW solution at 4°C provides the optimum conditions that maintain islet viability, purity, integrity, and counts and prevent islet damage for up to 48 h and may be most suitable media for low-temperature preservation of islets before transplantation. However, further studies are needed

relating to the poor functional stimulation and inflammatory gene expression in islets preserved in UW solution. A table of all the experimental results has been summarized (Supplemental Table 2). Studies that test novel culture media or innovative methods to improve islet culture problems are necessary for better transplant outcomes.

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Authorship

YS, MFL, and MAK participated in the research design. YS, JK, and PBS were involved in the performance of the research and data analysis. YS, MAK, JK, and PBS participated in the writing and editing of the manuscript. MFL and MAK were involved in the research oversight.

Ethical Approval

Ethical Approval is not applicable for this article. This study has received an exemption status from the Virginia Commonwealth University Institute Review Board.

Statement of Human and Animal Rights

This article does not contain any studies with human or animal subjects.

Statement of Informed Consent

There are no human subjects in this article and informed consent is not applicable.

Declaration of Conflicting Interests

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Supplemental Material

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