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## Histidine-Tryptophan-Ketoglutarate and University of Wisconsin Solutions Demonstrate Equal Effectiveness in Preventing Cellular Edema in Preserved Human Pancreata Intended for Islet Isolation

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### Abstract

We have previously reported that both Histidine-Tryptophan-Ketoglutarate solution (HTK) and University of Wisconsin solution (UW) provide equal preservation of the pancreas for islet isolation, based upon the assessment of islet yield and function. In this study, we further assessed the ability of each solution to prevent cellular edema. This large-scale, retrospective analysis included the assessment of islet isolation outcomes influenced by cellular edema, such as pancreatic digestion efficacy, purification outcome, and islet size distribution. Multi-variable linear regression analysis, adjusted for donor age, sex, BMI, cold ischemia time, and enzyme, demonstrated similar results for the HTK group (n = 95) and the UW group (n = 157), including post-purification islet yields (the HTK: 289,702 vs. the UW: 283,036;  $p = 0.76$ ), percentage of digested pancreatic tissue (the HTK: 66.9% vs. the UW: 64.1%;  $p = 0.18$ ), and islet loss from post-digestion to post-purification (the HTK: 24,972 vs. the UW: 39,551;  $p = 0.38$ ). Changes in islet size between the post-digestion and post-purification stages were comparable within each islet size category for the HTK and the UW groups ( $p = 0.14 - 0.99$ ). Tissue volume distribution across purification fractions and islet purity in the top fractions were similar between the groups; however, the HTK group had significantly higher islet purity in the middle fractions ( $p = 0.003 - 0.008$ ). Islet viability and stimulation indices were also similar between the HTK and the UW groups. In addition, we analyzed a small sample of patients transplanted either with HTK (n = 7) or UW (n = 8) preserved islets and found similar outcomes. This study demonstrates that HTK and UW solutions offer comparable pancreas preservation and are equally efficacious in the prevention of pancreatic tissue edema in islet transplantation. Future studies assessing *in vivo* islet outcomes in larger samples are needed for complete analysis of the effects of HTK on islet transplantation.

### Keywords

HTK; UW; Organ Preservation; Cellular Edema; Human Pancreas; Islet Isolation; and Islet Transplantation

### Introduction

The quality of pancreas flush and preservation is one of the most important determining factors for the successful grafting of both whole pancreata and isolated islets. At present,

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two preservation solutions are primarily utilized for abdominal organ preservation: University of Wisconsin (UW, DuPont Pharma) and Histidine-Tryptophan-Ketoglutarate (HTK, Essential Pharma). Each preservation solution possesses a unique composition, as seen in Table 1. While UW has been the preferred preservation solution for more than 20 years (1, 2), HTK, originally developed by Brestchneider *et al.* as a cardioplegic solution (3), has been gaining favor as an alternative to UW. Several major studies and clinical trials have demonstrated the clinical equivalence of HTK when compared to UW in abdominal organ preservation and transplant outcomes (4-7), specifically for whole pancreas transplantation (8-10).

In the preservation of pancreata intended for islet isolation, a number of studies comparing the efficacy of HTK with that of other preservation solutions have been conducted since 1995, demonstrating results similar to those obtained in other abdominal organs (11-14). However, these studies have been limited either by small sample size or incomplete analysis of isolation outcomes. Islet transplantation is complicated by an intricate islet isolation process consisting of four primary stages: organ preservation, enzymatic perfusion, pancreas digestion and dissociation, and islet purification. The overall success of an islet isolation is dictated by the success of each successive step. Thus, the quality of the initial pancreas flush and preservation directly impacts the results of all of the following isolation steps.

In a previous small-scale study, we demonstrated that HTK is equivalent to UW in preserving pancreata for islet isolation, with regard to general isolation outcomes, including islet yield, viability, and *in vitro* function (15). In this single-center, large-scale study, we further examined isolation outcomes and evaluated the impact of the preservation solution, either HTK or UW, on the development and progression of cellular edema, a vital factor in isolation success, through the evaluation of pancreatic digestion efficacy, purification outcomes, and isolated islet size distribution.

## Materials and Methods

### Pancreas procurement and isolation activities

Organ procurement organizations (OPO) provided pancreata, with consent from donors. The organs were flushed with either HTK (n = 95) or UW (n = 157), depending upon the protocols used by individual OPO, and transported to the University of Illinois at Chicago (UIC). The islet isolation procedure, including digestion, purification and culture, was performed for all pancreata according to the previously described protocol (16-18). Upon arrival, the pancreas was surface-decontaminated and trimmed of excess fat. The pancreas was then perfused, via the pancreatic duct, with the digestive enzyme, Collagenase. Tissue digestion and islet dissociation were achieved using a modified Ricordi semi-automated method (19).

After digestion was complete, the collected tissue was washed to remove traces of enzyme and incubated in UW, on ice, for 30 min. The refined UIC-UW/Biocoll (UIC-UB) continuous density gradient (20), consisting of a mixture of a high density solution (1.078 g/mL: 40% Biocoll (Cedarlane) and 51% UW) and a low density solution (1.068 g/mL: 30% Biocoll and 70% UW), was used for the purification procedure. Up to 45 mL of tissue were purified in a single operation of the COBE 2991 Cell Separator (CARIDIAN BCT). Following the centrifugation process, the tissue was collected in 12 fractions. The first two fractions were discarded due to minimal tissue volume (often less than 0.01 mL) and being primarily composed of ductal and adipose cells. In each of the remaining 10 fractions, corresponding to the aforementioned continuous gradient from 1.068 g/mL to 1.078 g/mL, a fluid and tissue volume of 30 mL was collected and then recombined based on the percentage of islet purity. Recovered tissue with an islet purity of > 70%, 40-70%, and

40% were defined as the top, middle, and bottom fractions, respectively. A small percentage of isolations required multiple sequential purifications due to a post-digestion tissue volume of greater than 45 mL.

### Assessment of islet yield, size distribution, purity, and tissue volume

Islet yield, size, and purity assessments were manually performed, using Dithizone (a zinc chelating agent) staining under light microscopy, at two time points: post-digestion and post-purification. Islet yield was measured both in actual islet number and islet equivalent (IEq), a volumetric quantification of islet mass, where larger islets contribute more to the total IEq count than smaller islets. Eight discrete categories were designated for islet size quantification: 50-100, 100-150, 150-200, 200-250, 250-300, 300-350, 350-400, and >400  $\mu\text{m}$ .

The islet purity of each post-purification fraction was determined by the estimated ratio of islet to exocrine tissue of the total tissue composition. The absolute tissue volume of each fraction was visually approximated, and the relative tissue volume was calculated as the percentage of tissue volume of each fraction versus the total collected tissue volume.

### Assessment of islet quality and function

Post-purification islet viability was determined using inclusive and exclusive fluorescent staining with Syto-Green (Invitrogen) for live-cells and Ethidium Bromide (Sigma) for dead-cells (17, 21). A static glucose-stimulated insulin secretion (GSIS) assay was used to evaluate islet function, as previously described (22). Briefly, 10 purified islets were hand-picked and pre-incubated with Krebs-Ringer buffer (KRB) solution containing 1.67 mM (low) glucose and 20 mM Hepes for 1 hr. The islets were then transferred into KRB solution containing 16.7 mM (high) glucose for another 1 hr. The secreted insulin levels of each incubation phase were measured using conventional enzyme-linked immunosorbent assay (ELISA, Mercodia); a stimulation index (SI) was calculated by dividing insulin release during the high glucose stimulation (16.7 mM) by insulin release during the basal glucose (1.67 mM) stimulation.

### Exclusion criteria for analysis

Isolations that were not completed due to technical errors or equipment malfunction, as well as isolations in which complete data for all primary variables were unavailable, were not included in the analysis (approximately 28% and 25% of the isolation data from the HTK-preserved pancreata and the UW-preserved pancreata, respectively).

### Statistical analysis

For the analysis of donor, pancreata, isolation characteristics, post-purification viability, and clinical outcomes the results were expressed as mean  $\pm$  standard deviation (SD) and percentages. Differences between HTK and UW groups were analyzed by using unpaired Student's t-tests and Chi-square (Fisher's Exact, as appropriate) tests. Level of statistical significance for these comparisons was set at  $p < 0.05$ .

For the statistical analysis of digestion efficacy and purification outcomes, SAS version 9.2 (Cary, NC) was used. Multi-variable linear regression was used to compare the HTK and the UW solutions, adjusting for age, sex, body-mass index (BMI), cold ischemia time (CIT) and enzyme used, for the following outcomes: digestion time and efficacy; post-digestion and post-purification IEq and the difference between post-digestion and post-purification IEq; percentage of trapped islets; post-digestion and post-purification IEq per gram of pancreas; percentage change between post-digestion and post-purification IEq in each size group; absolute and relative tissue volume; and islet purity of each density range (fraction).

Whether the effect of solution (HTK vs. UW) on absolute and relative tissue volume was modified by CIT was tested for each density layer, but no interactions were found to be significant (i.e. any differences in the effect of the HTK vs. the UW on volume did not differ across CIT strata). The effect modification by CIT was similarly explored for digestion time and efficacy, percentage of trapped islets, and IEq per gram of pancreas, and no interactions were found to be significant.

Multi-variable mixed linear regression models were used to compare distributions between the HTK and the UW for the percentage change between post-digestion and post-purification IEq across islet size groups, and absolute and relative volumes and purity across density ranges, adjusting for age, sex, BMI, CIT, and enzyme. These mixed models incorporated the correlation due to clustering by specifying the unique isolation identification number as the unit of cluster, and specified the autoregressive covariance structure with empirical standard errors. The level of statistical significance for these multi-variable models was set at  $p < 0.01$ , to minimize type 1 error with the multiple comparisons.

## Results

### Donor, pancreas, and isolation characteristics

The comparison of donor, pancreas, and isolation characteristics between the HTK and the UW groups is summarized in Table 2. No significant differences were found between the HTK and the UW groups in donor age ( $p = 0.68$ ), donor BMI ( $p = 0.36$ ), or donor sex ( $p = 0.86$ ). A significant difference was observed for CIT, for which the HTK group had a longer ischemia time than the UW group ( $p = 0.02$ ). Additionally, pancreas weight was significantly heavier in the UW group than in the HTK group ( $p = 0.02$ ). Within the 252 isolations analyzed, the frequency of enzyme used for the digestion across the four different brands of enzymes was similar between groups ( $p = 0.86$ ).

### Digestion efficacy and islet yield

The average digestion time was not found to be significantly different between the HTK and the UW groups (15.5 min vs. 14.8 min, respectively;  $p = 0.24$ ), when adjusted age, sex, BMI, CIT, and enzyme. Enzyme digestion efficacy, calculated by dividing the mass of the digested tissue by total pancreas mass, was similar between the HTK and the UW groups (66.9% vs. 64.1%, respectively;  $p = 0.18$ ).

Both the adjusted mean post-digestion and post-purification IEq were marginally higher in the HTK group than in the UW group, but were not significantly different (post-digestion IEq: 329,253 for the HTK vs. 308,008 for the UW;  $p = 0.34$ ; post-purification IEq: 289,702 for the HTK vs. 283,036 for the UW;  $p = 0.76$ ), as shown in Figure 1. The difference between mean post-digestion and post-purification IEq was similar for both groups (39,551 for the HTK vs. 24,972 for the UW;  $p = 0.38$ ). The adjusted percentage of trapped islets in the post-digestion sample was similar between the groups (the HTK: 18.1% vs. the UW: 16.9%;  $p = 0.71$ ). Additionally, the adjusted post-digestion and post-purification IEq per gram of pancreas were not significantly different between the groups (post-digestion IEq: 3,379.2 for the HTK and 3,377.2 for the UW;  $p = 0.99$ ; post-purification IEq: 2,810.8 for the HTK and 2,951.0 for the UW;  $p = 0.53$ ).

### Islet size distribution

The islet size distribution change between the post-digestion and post-purification stages, as expressed by the adjusted percentage change of islets *within* each size category, did not differ between the HTK and the UW groups for any of the eight size categories (Fig.2).

Using mixed model analysis, the results indicated that the overall distribution *across* size groups also did not differ between the groups ( $p = 0.55$ ).

### Total tissue volume and islet purity following purification

The distribution of tissue volume across purification fractions, compared between the HTK and the UW groups, is summarized in Figure 3. There were no significant differences between the two groups with regard to the tissue volume distribution *within* the purification fractions, expressed as either absolute volumes ( $p = 0.07 - 0.84$ ) or as proportions to the overall tissue volume ( $p = 0.016 - 0.94$ ). The overall volume distribution *across* purification fractions did not differ between the HTK and the UW groups (absolute volume:  $p = 0.12$ ; relative volume:  $p = 0.52$ ) in the mixed model analysis. The isolated islet purity *within* each purification fraction was also compared between the two groups (Fig. 4), with  $p$ -values ranging from 0.003 to 0.47. All fractions demonstrated similar purity values, except for fractions 6 and 7, of which the HTK group had a significantly higher purity ( $p$ -values of 0.008 and 0.003, respectively). In the mixed model analyses, the overall purity distribution *across* purification fractions did not differ between the HTK and the UW groups ( $p = 0.61$ ).

### Isolated islet quality

Islet viability after purification was similar between the two groups (the HTK: 90.6% vs. the UW: 90.2%;  $p = 0.63$ ), as were the GSIS stimulation indices (the HTK: 3.81 vs. the UW: 3.34;  $p = 0.14$ ), when adjusted for age, sex, BMI, CIT, and enzyme.

### *In vivo* islet graft function

The clinical transplant outcomes from the isolated islet preparations either preserved in the HTK ( $n = 7$ ) and the UW ( $n = 8$ ) were analyzed (Supplementary Table 1), showing that the HTK group has similar insulin independence (the HTK = 42.9% vs. the UW = 75%;  $p = 0.31$ ), HbA<sub>1c</sub> (the HTK = 5.86 vs. the UW = 6.05;  $p = 0.30$ ), and Beta-score for islet graft function (the HTK = 3.7 vs. the UW = 5.5;  $p = 0.11$ ).

## Discussion

The results from this study demonstrate that both HTK and UW preservation solutions are comparable in their ability to prevent cellular edema throughout pancreas procurement and islet isolation. This finding further supports our previous results that both solutions have an equivalent impact on islet viability and *in vitro* function.

Although considerable effort has been devoted to expanding the clinical application of islet transplantation for the treatment of patients with Type I diabetes mellitus, many variables continue to pose challenges for clinicians. Among these, the quality of pancreas flush and preservation is a key factor that determines the outcome of the islet isolation procedure. For example, poor pancreatic flush and long cold ischemia duration often result in a lower digestion efficacy, as a result of cellular edema (23). Cellular edema within endocrine and exocrine tissue develops primarily during pancreas cold-ischemic preservation, but can be further influenced by isolation factors, such as organ reperfusion and associated reperfusion injuries, enzymatic digestion, centrifugation, and purification. Due to the multiphase processes inherent in the islet isolation procedure that can exacerbate the progression of cellular edema, the selection of a suitable preservation solution is even more critical for islet transplantation than for whole pancreas transplantation. HTK and UW each have a distinct composition and differing pathways of action to prevent cellular edema. While UW primarily prevents cellular edema via the osmotically active impermeants raffinose, lactobionate, and hydroxyethyl starch, HTK works through histidine, mannitol, tryptophan, and ketoglutarate.



In the present study, we compared the impact of preservation solution on the development and progression of cellular edema during the islet isolation process through the analysis of pancreatic digestion efficacy, purification outcomes, and islet size distribution. In terms of digestion efficacy, no significant difference was found between the HTK and the UW groups, with adjustment for age, sex, BMI, CIT, and enzyme. The extent of enzymatic cleavage, based on the percentage of trapped islets (islets encased in exocrine tissue) observed after the digestion phase, also did not differ between the groups. These combined data suggest that both preservation solutions similarly impact pancreatic digestion efficacy.

The analysis of purification outcomes served as an indirect measurement of pancreatic cellular edema. For single cells, the extent of edema is often measured by means of a cell-resistance assay. However, the same technique cannot be applied to whole islets, which possess a three-dimensional cytostructure composed of 1,000-2,000 individual cells. Thus, the analysis of islet and exocrine tissue distribution and islet purity in a continuous gradient of 1.068 -1.078 g/mL served as an indirect approach to assess the impact of preservation solutions on pancreatic cellular edema. The islet isolation procedure relies on a density-based purification, following the digestion phase, to separate islets and exocrine tissue; the intrinsic density difference between islet tissue (~1.059 g/mL) and exocrine tissue (1.059 – 1.074 g/mL)(24) is so minimal that even a slight change in the density of either tissue would have a detrimental effect on recovered tissue mass and purity. For instance, trapped islets, a product of incomplete digestion and dissociation, have a relatively high density that is similar to that of exocrine tissue. An elevated percentage of trapped islets within a digested tissue population will result in rightward shift in islet purity, and consequently, a lower overall islet purity. On the other hand, exocrine cellular edema will result in a leftward shift, and consequently, incomplete separation of these cell types during purification and lower overall islet purity. Our results revealed no significant difference between the HTK and the UW groups in tissue volume distribution, both in terms of absolute and relative volume, within purification fractions (Fig. 3). A slightly higher tissue volume was observed in the lowest density fractions (fractions 1 and 2) within the UW group, whereas a slightly higher tissue volume was observed in the highest density fractions (fractions 8-10) within the HTK group. Due to the low islet purity within these fractions, exocrine tissue comprised the majority of the volume. In order to eliminate other influencing factors, the relative tissue volume, as a percentage, was analyzed, adjusting for age, sex, BMI, CIT, and enzyme. These results paralleled those observed for the absolute tissue volume.

The distribution of islet purity across fractions also served as an indicator of purification outcome, and an indirect measurement of cellular edema. Within the top fraction (> 69%), the islet purity distribution was similar between the HTK and the UW groups. However, within the middle fraction (40 - 69%) a significantly higher purity was observed in fractions 6 and 7 of the HTK group (Fig.4). It is generally accepted that superior islet purification is only possible when the intrinsic density difference between islets and exocrine tissue is preserved during the isolation process. Based on these purification outcomes, our analysis suggests that HTK and UW have equal efficacy in preserving islet and exocrine densities, demonstrating similar top fraction islet purity, in which the majority of islets were recovered, and tissue volume distribution across purification fractions. Although, it appears that HTK may be slightly more effective in preserving exocrine tissue density, as the UW group shows lower islet purity in the middle fraction that is may caused by exocrine tissue edema.

In addition to the impact on tissue distribution and islet purity, cellular edema, by definition, will also influence islet size. Increased intracellular water retention will result in islet enlargement. Consequently, this will result in a shift in the islet size distribution of a given islet preparation, with a higher number of islets designated to the large size categories and a

lower number of islets designated to the small size categories. With this in mind, we compared the islet size distribution between the HTK and the UW groups at two time points: post-digestion (earliest possible dissociation time-point) and post-purification (latest possible time-point). No discernible difference between islet size was observed at either time-point (Fig.2), supporting the aforementioned findings regarding digested tissue distribution and islet purity. In addition to an increased percentage of large islets, it is also expected that more islets will be lost during purification in the presence of cellular edema. As a volumetric quantification of mass, IEq can also be used as an indicator of the occurrence of edema. In the presence of cellular edema, the total IEq from the post-digestion to the post-purification stage will change. In this study, no significant difference was found in total IEq between these time points. The similarity in size-related variables within this study indicates either the absence of significant in-trislet cellular edema, regardless of preservation solution used, or that edema did occur but was not significantly different between the two groups.

This present study is limited mainly by the imprecise assessment techniques that are commonly employed during the islet isolation procedure. Subjectivity is inherent in the established methods for islet yield, size, purity, and volume quantification, and the results may differ depending on the assessor. However, we believe that variation due to user error and subjectivity did not have a great effect on this analysis because all isolations were performed by experienced isolation technicians, according to a standardized islet isolation protocol. Additionally, the large number of isolations analyzed in this study (n = 252) minimizes the impact of user variation on the overall results.

An additional limitation is the potential impact of the 30-min UW incubation prior to purification on cellular edema. However, pancreatic tissue from both groups was treated identically in this manner, and the duration of this incubation was so brief that we believe any impact would be negligible. Despite the limitations, the results of this study are quite clear: HTK and UW solution function similarly to prevent cellular edema during islet isolation.

While there is a consensus that both solutions are acceptable for abdominal organ transplants, there has been new debate as to which solution offers the most comprehensive cold ischemia protection in response to recent studies (25-28). These studies indicate that HTK is associated with reduced survival of abdominal organ transplants, especially with increased cold ischemia time. Others studies suggest that while HTK-flushed pancreata appear more edematous, there is no evidence of impaired early graft function (8, 29). However, there have not been any studies conducted examining the long-term effects of human pancreas preservation with HTK on *in vivo* islet grafting. Therefore, we believe that an in-depth comparison of the effects of HTK and UW on *in vivo* islet graft outcomes is warranted to garner a complete understanding of the impact of HTK on islet transplantation although our initial patient data indicate comparable graft function and clinical outcomes.

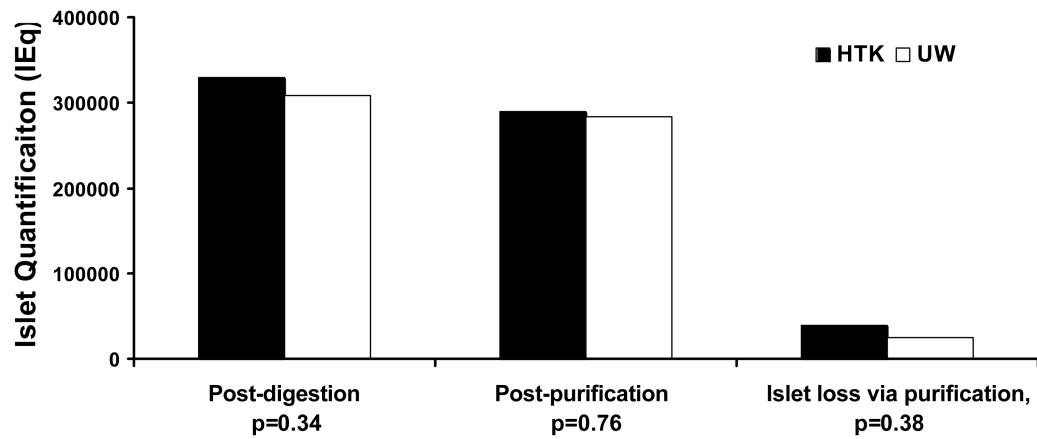
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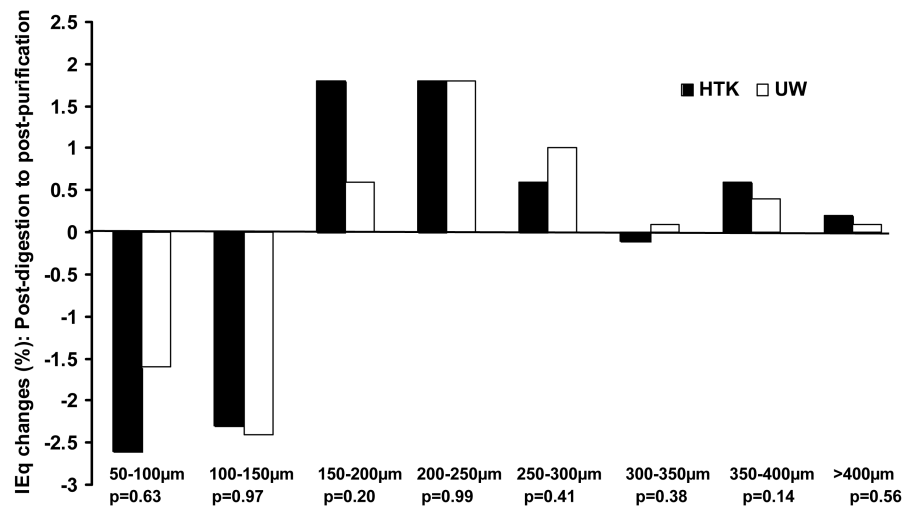
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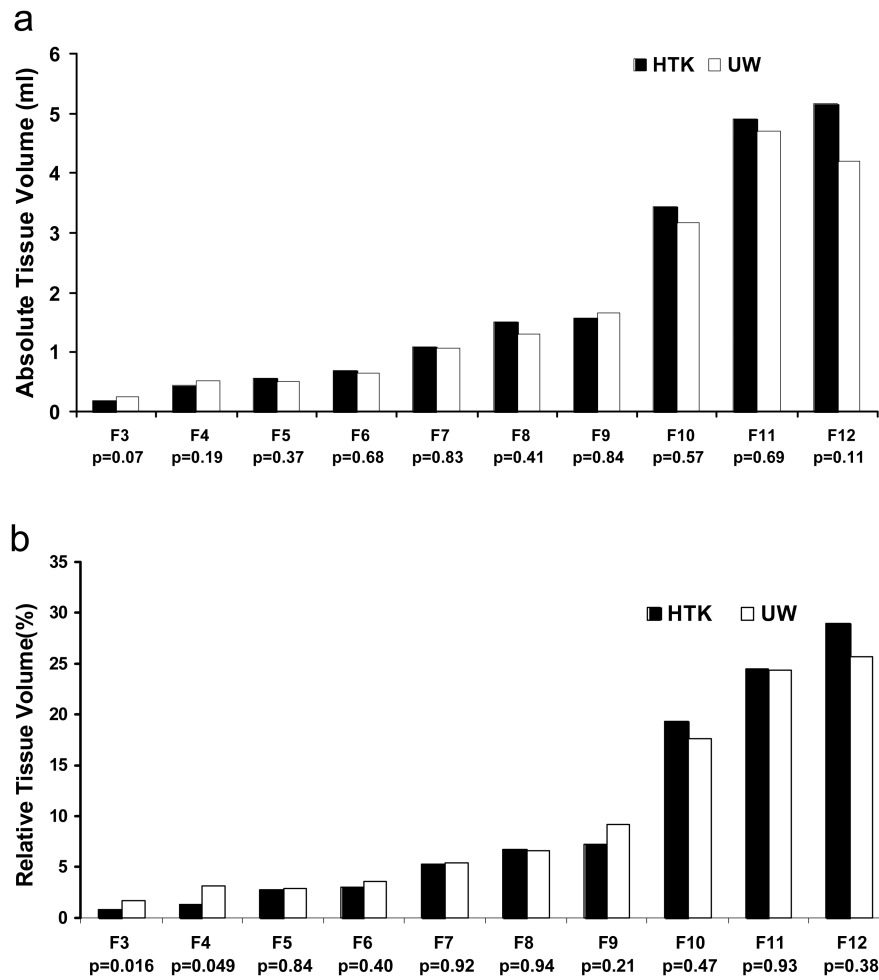


**Figure 1. Islet quantification (IEq): post-digestion, post-purification, and the difference between post-digestion and post-purification (islet loss via purification)**  
The number of isolations analyzed was 95 and 157 for the HTK and the UW, respectively. Data were adjusted for age, sex, BMI, CIT, and enzyme. Statistical significance was set at  $p$ -value  $<0.01$ .



**Figure 2. IEq change (%) between the post-digestion and post-purification stages, across the eight islet size categories**

The number of isolations analyzed was 95 and 157 for the HTK and the UW, respectively. Data were adjusted for age, sex, BMI, CIT, and enzyme. Statistical significance was set at  $p$ -value  $<0.01$ .

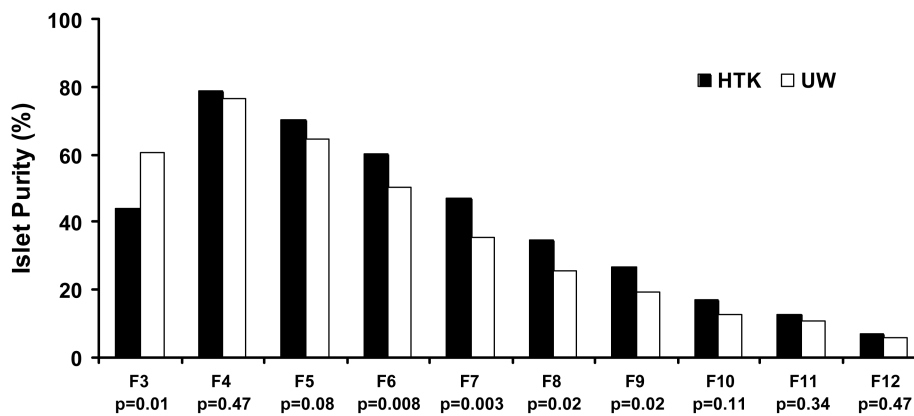


**Figure 3. Tissue volume distributions across purification fractions**

(a) Absolute tissue volume distribution (mL). (b) Relative tissue volume distribution (%).

The number of isolations analyzed was 95 and 157 for the HTK and the UW, respectively.

Data were adjusted for age, sex, BMI, CIT, and enzyme. Statistical significance was set at  $p$ -value  $<0.01$ .



**Figure 4. Islet purity distribution across purification fractions**

The number of isolations analyzed was 95 and 157 for the HTK and the UW, respectively. Data were expressed in percentage and adjusted for age, sex, BMI, CIT, and enzyme. Sample size ranged from 167-249 across fractions. Statistical significance was set at  $p$ -value  $<0.01$ .



**Table 1**  
**Comparison of organ preservation solution compositions**

HTK (g/L)	UW (g/L)
Sodium chloride: 0.87	Poly (0-2-hydroxyethyl) starch: 50
Potassium chloride: 0.67	Lactobionic acid: 35.83
Potassium hydrogen 2-ketoglutarate: 0.18	Potassium phosphate monobasic: 3.4
Magnesium chloride-6 H <sub>2</sub> O: 0.81	Magnesium sulfate heptahydrate: 1.23
Histidine · HCl · H <sub>2</sub> O: 3.77	Raffinose pentahydrate: 17.83
Histidine: 27.92	Adenosine: 1.34
Tryptophan: 0.41	Allopurinol: 0.136
Mannitol: 5.47	Total Glutathione: 0.92
Calcium chloride · 2 H <sub>2</sub> O: 0.0022	Potassium hydroxide: 5.61

**Table 2**  
**Comparison of donor, pancreas, and isolation characteristics**

Data are expressed as mean  $\pm$  SD or percentage. Statistical evaluations were performed by either two-tailed unpaired Student's *t*-test or Chi-square test, with significance at *p*-value  $<0.05$ .

Characteristics	HTK	UW	<i>p</i> -value
Isolation number (n)	95	157	
Age (y)	48.7 $\pm$ 11.1	48.1 $\pm$ 11.5	0.68
Sex (% male)	54.3	55.1	0.86
BMI (kg/m <sup>2</sup> )	29.5 $\pm$ 5.9	28.8 $\pm$ 6.0	0.36
Cold ischemia time (hrs)	9.7 $\pm$ 2.5	8.9 $\pm$ 2.8	0.02
Pancreas weight (g)	99.8 $\pm$ 28.2	108.4 $\pm$ 30.0	0.02
Enzyme used for perfusion (%)			
Serva Premium	21.7	25.3	0.86
Serva GMP	1.9	4.2	
Sigma V	19.7	12.6	
Roche Liberase	56.7	57.9	