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Clinical islet isolation outcomes with a highly purified neutral protease for pancreas dissociation

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Abbreviations: ECM, extracellular matrix; IE, islet equivalent; GMP, good manufacturing practice; PCV, packed cell volume; NP, neutral protease

Background: Pancreas dissociation is a critical initial component of the islet isolation procedure and introduces high variability based on factors including the enzyme type, specificity and potency. Product refinement and alterations to the application strategies have improved isolation outcomes over time; however, islet utilization from donor organs remains low. In this study we evaluate a low endotoxin-high activity grade neutral protease in clinical islet isolation.
Materials and Methods: The use of a non-collagenolytic enzyme, either thermolysin or high active neutral protease, was randomized in clinical islet isolations to evaluate efficacy. Additionally a retrospective comparison to neutral protease NB was conducted.
Results: The thermolysin group had lower trapped islet population and increased purity and post-culture islet mass in comparison to high active grade neutral protease. Comparison of neutral protease NB GMP grade to high active neutral protease displayed no measurable difference in islet mass or viability and transplantation outcomes at 1 mo post-transplant were favorable for both groups.
Conclusions: High activity neutral protease can generate clinical grade islets and may prove beneficial to islet function and viability based on a reduced endotoxin load but dosing of neutral protease requires ongoing optimization.

Introduction

Islet transplantation efficacy for the treatment of type 1 diabetes mellitus has improved significantly over the last decade.[1] Enhanced strategies utilizing novel immunotherapy have contributed significantly to improved and sustained graft function[2] while refinements in islet isolation, including the pancreas dissociation products, have led to increased islet mass recovery and organ allocation efficiency.[3-6] Despite these advances, rate of clinical islet isolations yielding a product suitable for transplantation remains low with the majority of sites reporting transplant rates less than 50%.

Among the challenges islet isolation laboratories face is the complexities of the pancreatic extracellular matrix (ECM). The cell–matrix and cell–cell interactions and specific roles of non-collagenolytic enzyme, such as thermolysin and neutral protease (NP), have in degrading the ECM are not completely understood in human pancreata.[7] Studies have shown that NP is integral to effective pancreas dissociation and islet recovery, but a delicate balance remains between islet mass, function and viability.[8-10] A recent study detailed islet isolation experience void of NP altogether.[11] The current alternative to NP, thermolysin, has also reported favorable outcomes.[12] Compounding potential negative effects of enzyme exposure, predominantly from non-collagenolytic enzymes, for islet viability and function is the potential introduction of endotoxin contamination from these enzymes. Derived from bacterial cultures, the enzyme products introduce large amounts of endotoxin contamination, which has been shown to have potential implications in clinical outcomes through the activation of cytokine production and apoptosis.[13] Regardless of non-collagenolytic enzyme used, the dissociation of the pancreas ECM remains a highly influential component of successful islet isolation and is associated with considerable variability. Furthermore, product refinement in islet isolation and enhancement of functional viability may have longstanding implications for islet transplant outcomes.

The manufacturing of a highly purified, low endotoxin neutral protease (haNP) (Neutral Protease NB High Active Grade; Serva Electrophoresis) product could lead to unique opportunities for islet transplantation if shown to be effective in isolating clinical grade islets. We herein study efficacy of haNP in comparison to thermolysin (Liberase MTF Thermolysin; Roche Diagnostics) in

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Table 1. Donor and isolation variables based on non-collagenolytic enzyme type used

	Thermolysin (n = 10)	High active neutral protease (n = 10)	P
Donor age (y)	46.0 ± 3.5	42.7 ± 5.2	0.603
Donor body mass index (kg/m^2)	27.9 ± 1.1	25.9 ± 1.5	0.276
Cold ischemia time (h)	10.0 ± 1.0	8.35 ± 1.6	0.400
Pancreas weight (g)	113.0 ± 21.1	82.5 ± 8.2	0.195
Collagenase dose (PZ-U/g pancreas)	24.6 ± 2.3	32.5 ± 4.6	0.143
Digestion time (min)	16.8 ± 1.3	16.6 ± 1.3	0.913
Undigested pancreas weight (g)	23.1 ± 5.9	13.3 ± 3.8	0.179
Collagenase lot allocation†			
20530005	4	4	-
20580005	2	4	-
20620205	4	2	-

a clinical setting. Additionally we reviewed retrospectively historical use of standard NP (sNP) (Neutral Protease NB GMP Grade; Serva Electrophoresis) in clinical islet isolations in comparison to the refined haNP.

Results

Donor age, body mass index, cold ischemia time and pancreas weight were comparable between the haNP and thermolysin group as a result of the prospective randomization for group assignment (**Table 1**). In addition to randomization of the non-collagenolytic enzyme, selection of collagenase lot was separately randomized, resulting in a non-skewed distribution of collagenase lot between the groups. Processing data (**Table 2**) show that the thermolysin group had a significantly reduced percentage of trapped islets (22.3 ± 4.8% vs. 44.2 ± 6.6%, p = 0.017) prior to the purification process. Following purification an increased purity based on visual estimation (55.8 ± 3.9% vs. 44.0 ± 2.5%, p = 0.021) was seen in the thermolysin group. Similarly an increased islet concentration [calculated by dividing islet equivalent (IE) by packed cell volume (PCV) in mL] was observed in the thermolysin group (110 ± 13 × 10^3 vs. 77 ± 8 × 10^3 IE/mL, p = 0.046). Although islet mass at pre-purification and post-purification was statistically similar between groups, post culture islet quantification revealed a significantly higher islet mass in the thermolysin group (363 ± 35 × 10^3 vs. 246 ± 35 × 10^3 IE, p = 0.030) as well as the absolute recovery (post culture IE/pre-purification IE) (62.6 ± 4.6% vs. 44.8 ± 3.8%, p = 0.009). No difference was observed between groups for islet function or viability testing.

Seven of the 10 isolations using thermolysin were determined to be suitable for transplantation and 3 of 10 for haNP (p = 0.179). For the purpose of clinical efficacy we included only first transplant recipients for the haNP and thermolysin groups having 2 and 5, respectively (**Table 3**). One month following islet transplantation insulin reductions of 70.3 ± 5.4% for the haNP group and 68.7 ± 12.8% for the thermolysin group were observed with insulin independence achieved 1 mo post-transplant in two cases within the Liberase MTF group.

The haNP we used in this study possess high enzyme activity (6.95 DMC-U/mg lyophilisate) comparing to the sNP (< 2.0 DMC-U/mg lyophilisate), although both products are derived from the same organism, *Clostridium histolyticum*. Therefore, we investigated if there are any beneficial effects of haNP over sNP. To this end we retrospectively compared islet isolation outcomes using the haNP with those using sNP. Donor characteristics were similar between the two groups (**Table 4**). Retrospective review of NP dosing based on pancreas weight did differ as the historical sNP data showed 2 fold dosing increase (p = 0.038). Nevertheless results from the sNP group revealed no differences in islet isolation intra-processing parameters or outcomes when compared with the haNP isolations. There was an advantage of haNP over sNP regarding endotoxin exposure. Islet exposure to endotoxin introduced by collagenase plus NP was reduced by 39.5% when using haNP, and there was a 7.7-fold reduction in endotoxin introduced by the NP component.

Discussion

Previous studies looking at the optimization of NP suggested an increased NP dose led to a higher proportion of free islets however function and viability may have been sacrificed or that the benefit of more free islets was offset by increased damage to acinar tissue resulting in comparable purification kinetics.[9] Testing found that haNP at a similar dosing level (~1.2 DMC-U/g pancreas) to the previous studies resulted in increased percentage of trapped islets pre-purification, reduced islet purity post isolation and lower islet mass following a culture period when compared with the thermolysin group. The application of the thermolysin component, based on previous findings, performed well in comparison to the haNP group, while not statistically different a substantially higher purification recovery and islet mass was observed. Improved purity of the islet product is beneficial to patient safety[14] and may play an integral role in improving the transplanted islet product following culture.[15,16]

We further explored the function of NP dosing with historical data that utilized a higher NP dose, and no benefit was observed despite an approximately 2-fold increase in dose. Conversely, and reassuringly, no negative impact on the islet preparations was observed suggesting that dosing may be increased in subsequent studies. Ultimately it is unknown if NP dosing will have any meaningful impact on human islet isolation outcomes, but both positive and negative associations with NP dosing were observed leading us to believe further investigation is warranted.

A reduced level of endotoxin introduced into the isolation process has potential benefits based on previous reports of endotoxin mediated cytokine response in islets,[13] although in this study endotoxin levels of the two enzyme types differed by 130-fold whereas our study calculated only a 2-fold decrease in endotoxin

could be achieved. The reduction of endotoxin exposure to the islets was significant using haNP but the translational impact of this finding to reduced cytokine production or apoptosis remains unknown and further studies would be necessary to explore if this modest reduction blunts endotoxin mediated cytokine release.

While the number of islet transplants achieved in this study was too few to conduct any statistical analysis, insulin reduction rates at 1 mo post-transplant appear to be comparable between groups with no apparent detrimental or beneficial influence on graft outcomes based on enzyme used. Further to this, the SUITO index demonstrates favorable outcomes for each transplant procedure and marked reductions in HbA1c were observed. One patient continues to be free of insulin for greater than 12 mo, while another patient was able to achieve insulin independence for a period of 6.5 mo before starting on a small amount of insulin while awaiting a second islet transplant.

Materials and Methods

From January 24–April 5, 2012, 20 pancreata were processed at the Clinical Islet Laboratory for the purpose of this study and for islet allograft transplantation under standard of care protocols. All 20 isolations utilized Collagenase NB 1 GMP grade (Serva Electrophoresis) from one of three different lots in combination with either thermolysin (n = 10, Lot 13489500) or Neutral Protease NB High Active Grade (n = 10, Lot 110007). Selection of collagenase lot and type of non-collagenolytic enzyme were independently randomized at the initiation of the isolation procedure. In addition to the 20 isolations, a retrospective analysis of islet isolations where sNP was utilized (n = 7) was compared with the findings in the haNP group. The sNP group only included recent isolations where the collagenase was void of degraded Class I collagenase determined by chromatography.

Islet isolation. With informed consent, pancreata were procured from deceased donors following aortic cross-clamp and in situ flush with Custodial® HTK or Static Preservation Solution and transported to our GMP facility. The enzyme was prepared by dissolving in Hanks' balanced salt solution with 3.6 mMol $CaCl_2$ with a final volume of 350 mL. Collagenase dose was 1 vial with an activity range of 2322–2453 PZ Units, haNP dosing range was 0.60–2.31 DMC/g of pancreas and thermolysin dose was < 850 TU/g of pancreas.[11] Enzyme was delivered via pancreatic duct simultaneously under controlled temperature and pressure conditions. The perfused pancreas was then transferred to a Ricordi chamber and enzymatic and mechanical dissociation was initiated by warming the closed circuit to 37.0°C. Assessment of the tissue digest was performed under microscopy as timed samples were stained with dithizone, once a sufficient number of islets were free of exocrine tissue the digestion was halted by dilution of enzyme and washing of the tissue. The collected tissue was pooled and quantified to determine islet mass and percentage of islets still encapsulated with acinar tissue. Islets separation from acinar was achieved using a UW/Ficoll continuous gradient on a COBE 2991 cell processor, density gradients of 1.062 g/mL and 1.072 g/mL were loaded into the COBE bag followed by addition of tissue digest suspended in UW solution. Following a 5 min centrifugation at 3000 RPM purity fractions were collected and again pooled to yield a pure layer and subsequent less pure layers. Islet mass, viability and purity were assessed,[17] and the determination of clinical suitability was made depending on available recipient's requirements.

Table 2. Processing variables based on non-collagenolytic enzyme type used

	Thermolysin (n = 10)	High active neutral protease (n = 10)	P
Islet mass pre-purify ($\times 10^3$ IE)	604 ± 70	543 ± 56	0.502
Islet mass pre-purify (IE/g pancreas)	6,062 ± 742	6,873 ± 618	0.412
Trapped (%)	22.3 ± 4.8*	44.2 ± 6.6	0.017
Purification recovery (%)	75.4 ± 7.0	61.7 ± 2.7	0.085
Islet mass post-purify ($\times 10^3$ IE)	429 ± 39	330 ± 32	0.064
Purity (%)	55.8 ± 3.9*	44.0 ± 2.5	0.021
Packed cell volume (mL)	4.2 ± 0.4	4.5 ± 0.5	0.587
Islet concentration ($\times 10^3$ IE/mL)	110 ± 13	77 ± 8	0.046
Viability (%)	86.4 ± 1.3	83.4 ± 2.9	0.365
Stimulation index	3.6 ± 0.4	2.8 ± 0.5	0.200
Culture recovery (%)	84.5 ± 3.8 (n = 10)	73.5 ± 5.7 (n = 9)	0.119
Islet mass post-culture ($\times 10^3$ IE)	363 ± 35	246 ± 35	0.030
Absolute IE recovery (%)	62.6 ± 4.6†	44.8 ± 3.8	0.009
β cell viability (%)	35.6 ± 4.3 (n = 7)	29.7 ± 2.2 (n = 3)	0.415
Transplant rate† (%)	7/10 = 70%	3/10 = 30%	0.179

†Fisher's Exact Test used.

Islet culture. The islets culture media formulation consisted of CMRL Supplemented Media modified with 2% v/v 25% human albumin, 0.5 mg/mL insulin-like growth factor and pH adjusted to 7.35–7.45 using 1 N NaOH. Islets were cultured at 22°C, 5% CO_2 and 95% humidity for duration of culture period, seeding density range was 0.35–0.50 mL PCV/plate with 100 mm diameter. Islet preparations that did not meet clinical suitability parameters were collected following culture duration and assessed for islet mass only.

Islet transplantation. Islet preparations that were determined to be clinically suitable were cultured for the duration of time required to prepare recipient for transplantation procedure. Immediately prior to transplant procedure the islets were

Table 3. Islet transplantation outcomes based on non-collagenolytic enzyme used

Patient	Islet mass (IE/kg)	Pre-Tx insulin (u/kg/day)	1 mo post Tx insulin (u/kg/day)	Basal c-peptide (nmol/L)	Stimulated c-peptide (nmol/L)	HbA1c 1 mo pre-Tx	HbA1c 1 mo post-Tx	SUITO index
haNP 1	6,410	0.65	0.15	0.72	Not done	9.6	7.2	33.2
haNP 2	6,445	0.69	0.25	0.80	Not done	7.8	6.8	49.1
MTF 1	7,188	0.46	0.00	0.81*	2.13*	10.0	7.9	93.3
MTF 2	6,699	0.71	0.38	0.48	1.02	8.6	7.5	30.2
MTF 3	7,249	0.42	0.00	0.41	0.58	8.2	7.1	35.7
MTF 4	5,937	0.51	0.25	0.47	1.19	8.9	7.7	29.6
MTF 5	6,050	0.63	0.34	0.54	1.69	8.3	6.9	34.4

*Values are from 3 mo post-transplant assessment.

Table 4. Assessment of NP dosing on human islet isolation parameters

	High active neutral protease (n = 10)	Neutral protease GMP grade (n = 7)	P
Donor age (y)	42.7 ± 5.2	37.1 ± 6.5	0.517
Donor body mass index (kg/m^2)	25.9 ± 1.5	29.2 ± 1.9	0.192
Cold ischemia time (h)	8.35 ± 1.6	7.7 ± 1.6	0.770
Pancreas weight (g)	82.5 ± 8.2	95.3 ± 3.4	0.175
Collagenase dose (PZ-U/g pancreas)	32.5 ± 4.6	25.3 ± 2.2	0.182
NP dose (DMC U/g pancreas)	1.16 ± 0.20	2.16 ± 0.4*	0.038
Endotoxin load - NP only (EU)	78.5 ± 7.1	602.4 ± 93.3**	0.001
Total endotoxin load (EU)	500.6 ± 63.8	826.9 ± 94.5*	0.015
Digestion time (min)	16.6 ± 1.3	14.7 ± 1.4	0.344
Undigested pancreas weight (g)	13.3 ± 3.8	9.8 ± 1.4	0.398
Islet mass pre-purify (×10^3 IE)	543 ± 56	607 ± 92	0.558
Islet mass pre-purify (IE/g pancreas)	6,873 ± 618	6,382 ± 900	0.661
Trapped (%)	44.2 ± 6.6	31.7 ± 6.0	0.179
Purification recovery (%)	61.7 ± 2.7	66.7 ± 5.5	0.437
Islet mass post-purify (×10^3 IE)	330 ± 32	405 ± 70	0.356
Purity (%)	44.0 ± 2.5	50.7 ± 3.4	0.134
Packed cell volume (mL)	4.5 ± 0.5	5.0 ± 0.7	0.540
Islet concentration (×10^3 IE/mL)	77 ± 8	82 ± 11	0.690
Viability (%)	83.4 ± 2.9	80.6 ± 3.4	0.549

collected from tissue culture plates and pooled. Islet mass, purity, viability, PCV, endotoxin and Gram stain were confirmed prior to release of islets to transplant site.

Concurrent with islet culture, recipients underwent Alemtuzumab (n = 8) or Basiliximab (n = 2) induction dependent on absolute lymphocyte count upon admission. The transplant procedure was performed under local anesthetic in radiology where the percutaneous transhepatic approach was used to access main portal branch. Infusion was completed with a Ricordi infusion bag containing islets suspended in a CMRL based transplant media supplemented with 20% v/v human albumin and 25 mMol HEPES. Following infusion the tract was sealed with Avitene™ paste.

Islet viability and function assessment. Islet assessment consisted membrane integrity staining, glucose stimulated insulin release, endotoxin content as described previously.[5] Beta-cell viability[18] was assessed for islet preparations that proceeded to transplantation.

Clinical graft assessment. Graft function assessment was completed at 1 or 3 mo post-transplant measuring for basal (n = 7) and stimulated c-peptide levels using mixed meal tolerance test (n = 5). SUITO index at 1 mo post-transplant was calculated as previously described.[19] Insulin requirements were managed as appropriate and HbA1c testing was done routinely.

Statistical analysis. Statistical analysis was performed using SPSS Statistical software (SPSS Inc. v14.0). Results are reported as mean ± standard error of the mean or as a percentage. Student's t-test or Fisher's exact test was employed for comparison where applicable. Statistical significance was set at $p < 0.05$.

Disclosure of Potential Conflicts of Interest

The collagenase and high active neutral protease products were donated to the Clinical Islet Laboratory by SERVA Electrophoresis GmbH for the purpose of this evaluation.

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