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Experience of islet isolation without neutral protease supplementation

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Key words: collagenase, cold ischemia time, islet isolation, islet transplantation, diabetes, pancreatic tissue derived protease

Abbreviations: CIT, cold ischemia time; DMC, dimethylcasein; IE, islet equivalent; NP, neutral protease; PZ, 4-phenylazobenzyloxycarbonyl-l-prolyl-l-leucylglycyl-l-prolyl-d-arginine

We have reported improved islet isolation outcomes using a new digestion protocol where the pancreas is perfused only with collagenase, and neutral protease (NP) is administered during the digestion phase. Since the inception of this protocol, we have had some cases where administration of NP was not required. Our new protocol was utilized in 94 islet isolations. The timing of adding NP was dependent on the progression of digestion but in 10 cases the progression was rapid and most islets in the assessment samples were free from the exocrine tissue. As a result NP was not added at all for these isolations (no-NP group). In the remaining 84 isolations, NP was added during digestion phase (control group). Pancreata in the each group were digested with a similar collagenase dose. Digestion time was shorter in the no-NP (15.0 $\pm$ 1.8 vs. 19.5 $\pm$ 0.6 min, p = 0.004). At post-digestion, the no-NP had fewer trapped islets (10.9 $\pm$ 2.8 vs. 28.1 $\pm$ 2.4%, p = 0.009). Post-purification islet yield was similar (355 $\pm$ 45 x 10^3 vs. 318 $\pm$ 17 x 10^3 IE, p = 0.29). Five preparations in the no-NP were used for clinical transplantation, leading to a 64.3 $\pm$ 15.2% reduction in insulin usage. Interestingly, cold ischemia time was longer in the no-NP (10.3 $\pm$ 0.9 vs. 7.9 $\pm$ 0.4 h, p = 0.04). One particular collagenase lot having the highest NP activity contamination was used in 7 isolations in the no-NP. Our experience indicates that supplementation of collagenase with NP is not always necessary for effective isolation. Cold ischemia time and NP contamination should be evaluated for optimal NP dosage.

Introduction

The enzymatic dissociation of the pancreas represents a critical step in islet isolation for clinical transplantation. Collagen is the major structural protein constituting the islet-exocrine interface in the pancreas.[1,2] Collagen is not generally degraded by ordinary proteases but can only efficiently be degraded with high specificity by collagenase.[3] Thus, collagenase is a critical component of the enzyme product used in the islet isolation process. However, the use of purified collagenase alone is known to decrease islet yield resulting from inadequate pancreas dissociation.[4,5] The presence of non-collagenolytic enzyme appears to be required to enhance collagenase-induced dissociation of the pancreas.

Currently, most manufacturers provide purified collagenase and non-collagenolytic enzyme in separate vials,[6-10] as opposed to previous products which contained both collagenase and non-collagenolytic enzyme at a predetermined ratio in a vial.[11,12] These new products allow users to digest the pancreas in a more flexible way.[13] One example is our modified digestion protocol in which the pancreas is perfused only with collagenase, and subsequently during the digestion phase neutral protease is administered.[6] Since the inception of this protocol, we have encountered cases where administration of neutral protease was not required at all for the desired digestion. We report our experience of islet isolation without supplemental neutral protease.

Results

Outcomes of islet isolations without supplemental neutral protease (the no-NP group, n = 10) were compared with those of isolations where neutral protease was administered during digestion phase (the control group, n = 84) (**Table 1**). We digested pancreata in each group with a similar collagenase dose (27.9 $\pm$ 2.2 in the no-NP group vs. 29.4 $\pm$ 0.8 PZ-U/g of pancreas in the control, p = 0.54). However, digestion time was significantly shorter in the no-NP group compared with the control (15.0 $\pm$ 1.8 vs. 19.5 $\pm$ 0.6 min, p = 0.004). This was likely due to the fact that digestion progression was rapid in the no-NP group. For the control group, we added neutral protease at a dose of 1.02 $\pm$ 0.03 DMC-U/g pancreas (or 90.9 $\pm$ 1.2 DMC-U/pancreas) to the circulation system at 14.1 $\pm$ 0.5 min of the digestion phase. The percentage of undigested tissue was comparable between the two groups. Following recombination of the pancreatic digest, the two groups yielded a similar islet

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Table 1. Islet isolation results

	no-NP group (n = 10)	Control group (n = 84)	p
Collagenase dosage (PZ-U/g pancreas)	27.9 ± 2.2	29.4 ± 0.8	0.54
Digestion time (min)	15.0 ± 1.8	19.5 ± 0.6	0.004
Neutral protease dosage (DMC-U/g pancreas)[a]	0	1.02 ± 0.03	-
Time, neutral protease added (min)	-	14.1 ± 0.5	-
Undigested tissue[b] (%)	24.7 ± 2.4	26.5 ± 1.7	0.86
Prepurification islet mass (x10^3 IE)	457 ± 49	491 ± 27	0.82
Prepurification islet mass/g pancreas (IE/g)	5,016 ± 729	5,165 ± 218	0.89
Trapped islet (%)	10.9 ± 2.8	28.1 ± 2.4	0.009
Postpurification islet mass (x10^3 IE)	355 ± 45	318 ± 17[d]	0.29
Postpurification islet mass/g pancreas (IE/g)	4,007 ± 706	3,373 ± 167[d]	0.40
Recovery rate after purification (%)	75.9 ± 5.0	67.4 ± 2.4	0.14
Viability (%)	84.9 ± 1.9	83.7 ± 1.0[d]	0.88
Islet purity assessed with dithizone stain (%)	57.0 ± 4.0	55.8 ± 2.0[d]	0.90
Percent islet volume[c] (%)	17.4 ± 2.2	15.4 ± 0.9[d]	0.13
Packed tissue volume (mL)	4.0 ± 0.4	3.9 ± 0.1[d]	0.82
Transplant rate	5/10 = 50%	31/84 = 37%	0.50

[a]Neutral protease activity contamination in a collagenase vial is not taken into account. [b]Undigested tissue (%) = undigested tissue weight/pancreas weight x 100. [c]Percent islet volume = Postpurification IE x 1.766 x 10^{-6}/packed tissue volume (mL) x 100. [d]One isolation did not proceed to the purification stage (n = 83).

Table 2. Donor related variables

	no-NP group (n = 10)	Control group (n = 84)	p
Age (yr)	55.4 ± 2.5	50.4 ± 1.1	0.17
Gender (male %)	50%	45%	1.00
Body mass index (kg/m^2)	28.2 ± 1.8	27.1 ± 0.6	0.37
Body surface area (m^2)[a]	1.96 ± 0.12	1.92 ± 0.03	0.76
Cold ischemia time (hr)	10.3 ± 0.9	7.9 ± 0.4	0.04
Cause of death (craniocerebral trauma %)	2/10 = 20%	12/84 = 14%	0.64
Elevated serum amylase (>140 U/L) or lipase (>120 U/L) level	1/10 = 10%	28/84 = 33%	0.17
HbA_{1c} (%)	5.73 ± 0.09	5.50 ± 0.05[b]	0.09
Pancreas weight (g)	102.6 ± 10.5	95.0 ± 2.7	0.46

[a]Body surface area = sqrt[body weight (kg) x height (cm)/3,600] (ref. 14). [b]Data not available in 9 cases (n = 75).

mass (457 ± 49 x 10^3 vs. 491 ± 27 x 10^3 islet equivalent (IE), p = 0.82). Expectedly, the no-NP group had fewer trapped islets at post-digestion (10.9 ± 2.8 vs. 28.1 ± 2.4%, p = 0.009). As a result, recovery rate after purification was higher in the no-NP although not statistically significant (75.9 ± 5.0 vs. 67.4 ± 2.4%, p = 0.14). Postpurification islet yields in total IE and IE/g of pancreas were similar between the two groups (355 ± 45 x 10^3 vs. 318 ± 17 x 10^3, p = 0.29 and 4,007 ± 706 vs. 3,373 ± 167 IE/g pancreas, p = 0.40). Viability, purity and packed tissue volume were also similar between the two.

Five of 10 batches in the no-NP group were infused intraportally into 5 patients with type 1 diabetes as an initial transplant for 2 individuals and a subsequent transplant for 3. The potency of the 5 batches was confirmed by remarkable decreases in insulin requirements (from 0.35 ± 0.08 to 0.15 ± 0.06 units/kg/day, 64.3 ± 15.2% reduction) at 3 month post transplant, with insulin independence being achieved in 2 individuals. These clinical outcomes were comparable with those from 31 transplanted batches in the control group (from 0.43 ± 0.05 to 0.14 ± 0.03 units/kg/day, 71.9 ± 6.1% reduction).

To investigate any contributing factors for no requirement of neutral protease during the digestion phase in the no-NP group, we retrospectively reviewed donor records for all 94 islet isolations. All pancreata were preserved in histidine-tryptophane-ketoglutarate solution except for 2 cases stored in University of Wisconsin solution in the control group. Interestingly, cold ischemia time (CIT) was significantly longer in the no-NP group than in the control group (10.3 ± 0.9 vs. 7.9 ± 0.4 h, p = 0.04). Of note, 8 of the 10 pancreata in the no-NP group exceeded 10 h in cold ischemia. Other donor related factors were similar between the two groups (**Table 2**).

We also reviewed the certificate of analysis provided by the manufacturer (**Table 3**). For the 94 isolations, we used 7 different lots of Serva GMP collagenase (Serva, Heidelberg, Germany). Among the 7 lots, 2 were used for both groups and the remaining 5 lots were used only for the control group. Values for DMC-U per mg lyophilisate provided by the manufacturer seem to be within a narrow range among the 7 lots. However, our calculations based on the values provided by the manufacturer confirmed that one particular lot exhibited considerably higher amount of neutral protease (23.5 DMC-U in one vial of collagenase). We found that this particular lot was used in 7 of the 10 isolations (70%) in the no-NP group.

Table 3. Enzyme characteristics in each lot

Lot # (first 4 digits)	PZ-U in a vial[a]	PZ-U/mg lyophilisate[a]	Lyophilisate (mg) in a vial[b]	DMC-U/mg lyophilisate[a]	DMC-U in a vial[b]	no-NP group # Isolations	Control group # Isolations
2008	2348	5.206	451.0	0.024	10.82	0	2
2010	2695	5.242	514.1	0.028	14.40	0	3
2012	2332	5.370	434.3	0.023	9.99	0	16
2015	2709	4.795	565.0	0.025	14.12	0	12
2017	2597	5.858	443.3	0.023	10.20	0	15
2021	2523	6.069	415.7	0.030	12.47	3	27
2042	2568	4.255	603.5	0.039	23.54	7	9

[a]Actual values presented in the certificate of analysis. [b]Calculated values based on the certificate of analysis.

Pancreatic tissue derived protease activity was assessed in another series of 39 human islet isolations with the standard digestion method using Liberase HI (Roche Applied Science, Indianapolis, IN). **Figure 1** shows the changes in protease activity before and after intraductal perfusion of the pancreas using Liberase HI. Postperfusion protease activity was always greater than preperfusion activity in all 39 pancreata and varied widely. Tissue derived protease was found to be higher in the pancreas with long CIT (>6 h) than in the short ischemic pancreas, although not statistically significant (86.7 ± 18.4 vs. 48.6 ± 7.5 units/g pancreas).

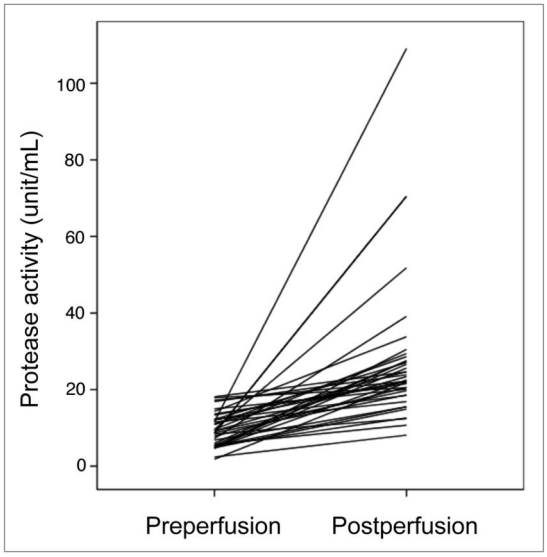

Figure 1. Protease activity in preperfusion and postperfusion samples was measured using *Bacillus thermoproteolyticus rokko* as a standard in 39 pancreata subjected for the standard digestion method using Liberase HI.

Discussion

The previous enzyme products were a mixture of collagenase and non-collagenolytic enzyme in a vial at an unalterable predetermined ratio.[11,12] Now most manufacturers provide purified collagenase in a vial along with a separately packaged non-collagenolytic component.[6-10] Consequently, users are given more strategic opportunities for efficient pancreas digestion than ever before. Our modified digestion protocol utilizes the advantage of the new product.[6] We observed very rapid digestion which precluded any requirement for addition of neutral protease in a number of isolations and compared these cases with isolations using our procedure with the sequential addition of collagenase and later neutral protease.

The interesting finding is that the CIT of the pancreata was significantly longer in the no-NP group than in the control group. The decision as to when the neutral protease should be added or indeed not to add the neutral protease, was solely dependent on the digestion assessment; CIT was not taken into consideration for the decision making in our practice. CIT has been considered one of the most important donor factors influencing islet isolation success. Previous studies have shown that extended CIT negatively influences isolation outcome.[15-17] Proposed explanations are that endogenous proteolytic enzymes are released from exocrine pancreas with extended cold storage as suggested in our tissue derived protease measurement and that these enzymes destroy or damage islets during islet isolation.[18] It should be noted, however, that other studies failed to show clear associations between CIT and islet isolation success.[19-21] Possible approaches to improve islet yield from ischemically damaged pancreata may shed some light on this disagreement. For pancreas with prolonged CIT which may release excessive amount of protease during isolation, reducing the amount of non-collagenolytic enzyme may be beneficial for optimal islet isolation.

It has been emphasized that Serva collagenase is highly pure and contains a trivial amount of neutral protease contamination.[22] Prior to writing this report, we too deemed that neutral protease contaminant in a vial of Serva collagenase was insignificant and did not vary among different lots. As a result of retrospective review, however, we recognized that there are more than 2-fold differences in DMC-U in a vial among lots when we calculated the values as shown in **Table 3**. Furthermore, we retrospectively found that 70% isolations in the no-NP group were performed using a lot having the highest DMC-U contamination, and that use of this particular lot resulted in no requirement of neutral protease for the desired digestion in 7 of 16 cases. Careful re-evaluation of DMC-U contamination is recommended when using Serva collagenase.

The ratio between neutral protease and collagenase activities seems to be critical at least in rodent islet isolation.[23] On the basis

of our experience, it seems difficult to define the optimal ratio in human islet isolation. In agreement with this, previous studies in human islets failed to show impact of this ratio on isolation outcome.[8,9,20,24] The optimal ratio may vary depending on the quality and individual characteristics of the human donor pancreas.

In summary, our experience reported here does not preclude an important role for non-collagenolytic enzyme for effective islet isolation, but brings into question the usefulness of routine supplementation of collagenase with non-collagenolytic enzyme. CIT and pre-existing neutral protease contamination have to be taken into consideration for dose adjustment in enzyme blends (**Suppl. Fig. A**). Islet processing centers should be aware of the heterogeneity between Serva collagenase lots with considerable neutral protease contamination.

Materials and Methods

Pancreas dissociation. Between July 24, 2007 and December 27, 2009, our modified digestion protocol with Serva GMP enzymes was utilized in 94 clinical islet isolations.[6] Human pancreata were procured from deceased donors with informed consent and were processed for islet isolation with the intent to transplant. The pancreas was perfused with 350 mL of cold solution (Perfusion solution; Mediatech, Herndon, VA) containing only collagenase. The distended pancreas was transferred to a Ricordi chamber and digested by recirculating the enzyme solution through the chamber at ~37°C.[25] During recirculation, one mL samples were taken periodically to monitor the digestion process. The amount of digested tissue was evaluated by centering the tissue in a 60 mm dish. The timing of neutral protease addition to the circulating system was dependent on the progression of pancreas dissociation; typically it was added when the tissue covered approximately 5% of the area of the dish. In 10 cases, however, the progression was considerably rapid and more than 80% of islets in the first assessment sample was free from the exocrine tissue. As a result neutral protease, although prepared for use, was not administered at all.

Islet purification and assessment. Islets were purified on a continuous density gradient using a mixture of Biocoll (Biochrome AG, Berlin, Germany) and University of Wisconsin solution.[26] Islet preparations were assessed for IE and purity with dithizone staining. Viability assessment was performed as previously described.[21] Packed tissue volume was measured after centrifugation at 300 g for one minute in a 50 mL conical tube.

Enzyme products. Serva employs the Wunsch assay for collagenase activity in its product specification. The Wunsch assay uses 4-phenylazobenzyloxycarbonyl-l-prolyl-l-leucylglycyl-l-prolyl-d-arginine (PZ) as substrate. For neutral protease activity, dimethlycasein (DMC) is used as substrate. Collagenase activity and neutral protease activity were expressed as PZ-U and DMC-U, respectively.

Tissue derived protease activity. Protease activity was measured using EnzChek fluorescent kinetic micro plate assays E-6638 (Molecular Probes, Eugene, OR). This assay kit uses a fluorescence saturated casein substrate and is open-ended in terms of the type of activity, standards and controls used. We used protease from *Bacillus thermoproteolyticus rokko* (Sigma-Aldrich, St. Louis, MO) of known activity, diluted 1:50, 1:100 and 1:200, in triplicate to construct a standard curve. After reconstitution of Librease HI with 350 mL of Perfusion solution, approximately 1.0 mL sample was taken prior to initiation of intraductal perfusion of the pancreas (pre-perfusion sample). The second sample was taken from the recovered Liberase solution after 10 min of the intraductal perfusion (post-perfusion sample). Tissue derived protease activity was calculated by subtracting the first sample value from the second sample value and normalized for pancreas weight.

Clinical assessment of islet post-transplant function. Islet function was assessed by measuring daily insulin requirements per kg body weight prior to transplant and at three months post transplant. The reduction in insulin use at three months was calculated.

Statistical analysis. Data are presented as mean ± SE for continuous variables and proportions for categorical variables. Mann-Whitney U-test or Fisher's exact test was used where appropriate. A p value of <0.05 was considered statistically significant and all p values reported were two-sided. All analyses were performed using SPSS statistical software version 11.5 (SPSS, Inc., Chicago IL).

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Note

Supplementary materials can be found at:
www.landesbioscience.com/supplement/KinISLETS2-5-Sup.pdf

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