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Evaluation of collagenase gold plus BP protease in isolating islets from human pancreata

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ABSTRACT

Selection of enzymes for optimal pancreas digestion is essential for successful human islet isolations. The aim of this study was to evaluate the efficacy and outcome of using Collagenase Gold plus BP protease (VitaCyte) (n = 8) by comparing it to two commercially available enzymes, Liberase MTF C/T (Roche) (n = 48) and Collagenase NB1/NP (Serva) (n = 15). The isolation outcomes were assessed by islet counting, viability, glucose-stimulated oxygen consumption rate (OCR), and successful graft-rate following transplantation in diabetic NOD scid mice. The pancreas donor characteristics were not significantly different between the tested enzyme groups regarding their BMI, pancreas weight, cold ischemia time (CIT) and HbA1c. The results show that digested tissue volume was not statistically significant between the VitaCyte enzyme (34.25 ± 5.4 mL) and the Roche enzyme (55.25 ± 3.42 mL, p = 0.073), however, this was significant with Serva enzyme (64.07 ± 7.95 mL, p = 0.020). Interestingly, the islet yields were not statistically different between all enzyme groups. Moreover, when islets were transplanted into NOD scid mice, the reversal rate of diabetes for the VitaCyte enzyme group was similar to all enzyme groups. In conclusion, the effectiveness of Collagenase Gold plus BP protease is comparable to the MTF C/T and the Collagenase NB1/NP enzymes; the low cost could facilitate the use of more pancreata for islet isolations.

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Introduction

Clinical islet transplantation has been shown to be an effective treatment for patients with brittle type 1 diabetes.[1] The islet yield and quality from pancreas digestion are critically important for achieving this goal.[2] Successful human islet isolation requires many critical decisions to be made by the isolation team depending on donor characteristics.[3-8] One of the main decisions is choosing an appropriate enzyme combination of collagenase and neutral proteases.[9-15] Roche MTF C/T and Serva collagenase NB1/NP are among a number of enzymes available for pancreas digestion during human islet isolations for research and clinical applications. The standardization of the islet isolation process is critically important for promoting biological licensed applications from the US Food and Drug Administration. For this context, it is highly recommended to use enzymes that are GMP grade, animal tissue-free, or recombinant products.[16,17] However, highly standardized GMP enzymes/materials are often costly, especially for research pancreata. Based on the UNOS report, a high number of pancreata were not used due to donor characteristics and/or economic concern for isolation cost.[18] Furthermore, large numbers of the pancreata procured were suboptimal and therefore were not used for either whole organ or islet transplantation. Thus, these pancreata were used for research application in the US including our center. Therefore, both efficient and cost-effective enzymes are advantageous to fulfill the increasing demand of islets for research applications.

Recently, VitaCyte has released a new product designated Collagenase Gold. This is a non-GMP grade enzyme that has not been fully characterized. The VitaCyte collagenase is prepared from *Clostridium*

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histolyticum that was grown in media containing porcine gelatin. This enzyme contains class I collagenase (73%), class II collagenase (27%), and low protease activity.[19] Class I collagenase is considered the essential component for isolating the islets.[20,21] This new VitaCyte Collagenase Gold contains the highest percentage of class I collagenase when compared to the Roche (60%) and Serva (50%) enzymes.[15] Since proteases are required to digest the pancreas, the combination of the Collagenase Gold and BP protease is indicated to free the islets. Moreover, with a significantly lower price, this enzyme combination may potentiate the utilization of more pancreata for islet isolations, especially for research applications. Therefore, in this study, we hypothesize that the Collagenase Gold plus BP protease is as effective as other standard enzymes in human islet isolations. A retrospective analysis was conducted to compare the VitaCyte Collagenase Gold plus BP protease to Roche MTF C/T and Serva Collagenase NB1/NP enzymes.

Results

Initially, results from 100 consecutive islet isolations were reviewed and 71 isolation outcomes were ultimately analyzed, as the others were excluded because the donor had an HbA1c >6.5%, was DCD or had cardiac arrest >30 minutes. The choice of enzymes for pancreas digestion using Roche Liberase MTF C/T (Roche) (n = 48) or VitaCyte Collagenase Gold plus BP protease (VitaCyte) (n = 8) were alternately selected, while younger donors were digested using Serva collagenase NB1/NP (Serva) (n = 15). Overall, with the exception of age, the donor characteristics for the three different enzyme groups were not significantly different between the tested enzymes (Table 1). However, the donors in the Serva enzyme group (25.6 ± 1.8 yrs) were significantly younger than that in the VitaCyte (50.1 ± 3.3 yrs) and Roche (48.5 ± 1.6 yrs) enzyme groups ($p < 0.0001$, Table 1).

The optimum pancreas digestion time (switch time) was not significantly different between the VitaCyte enzyme (12.63 ± 0.67 min) and the Roche enzyme (12.31 ± 0.30 min, $p = 0.896$), as well as between the VitaCyte enzyme and the Serva enzyme (12.92 ± 0.27 min, $p = 0.936$) (Fig. 1A). However the VitaCyte enzyme digested significantly less tissue (34.25 ± 5.40 mL) when compared to the Serva enzyme (64.07 ± 7.95 mL, $p = 0.020$) but not the Roche enzyme (55.25 ± 3.42 mL, $p = 0.073$) (Fig. 1B). Digestion percentage showed significant difference between the VitaCyte and Roche enzymes (67.94 ± 4.50% vs 79.12 ± 1.58%, $p = 0.026$), as well as between the VitaCyte and Serva enzymes (67.94 ± 4.50% vs 84.17 ± 2.67%, $p = 0.004$) (Fig. 1C).

For the VitaCyte enzyme, the islet yields in total IEQ at pre-purification, post-purification, and post-culture were 277312 ± 51932, 204010 ± 56891, and 163016 ± 47656 IEQ, respectively. The islet yields in IEQ/g at pre-purification, post-purification, and post-culture were 4741 ± 745, 3503 ± 729, and 2752 ± 574 IEQ, respectively. There were no significant differences with regard to islet yields both in total IEQ and IEQ/g when compared the VitaCyte enzyme to either the Roche or Serva enzyme (Fig. 2). Additionally, both islet purity and packed cell volume post-purification of the three enzymes used were not significantly different (Fig. 3). The results of islet assessments showed that there was a significant difference in the viability of the islets obtained with the VitaCyte enzyme (98.13 ± 0.40%) compared to those obtained with the Roche enzyme (94.43 ± 0.41%, $p = 0.005$) and the Serva enzyme (94.93 ± 0.73%, $p = 0.020$) (Fig. 4A), though this difference did not

Table 1. Donor characteristics and enzymes used for islet isolations.

	VitaCyte	Roche	Serva	*p* value
Number of donors	8	48	15	N/A
Donor age (yrs)	50.1 ± 3.3	48.5 ± 1.6	25.6 ± 1.8	VitaCyte vs Serva: <0.0001 Roche vs Serva: <0.0001
BMI (kg/m^2)	26.8 ± 1.5	30.7 ± 0.7	31.4 ± 1.9	0.13
Donor HbA1c (%)	5.6 ± 0.2	5.4 ± 0.1	5.3 ± 0.1	0.32
CIT (hrs)	7.0 ± 0.1	7.0 ± 0.0	6.9 ± 0.1	0.99
Pancreas weight (g)	84.1 ± 4.8	100.3 ± 3.1	99.7 ± 8.3	0.2
Cost for each isolation ($)	1130	2075	4952	N/A

BMI, body mass index; HbA1c, hemoglobin A1c; CIT, cold ischemia time; N/A, not applicable.
All *p* values were calculated by ANOVA tests; the *p* values for donor ages were calculated by Tukey's multiple comparisons test following ANOVA; data expressed as mean ± SEM (standard error of mean).

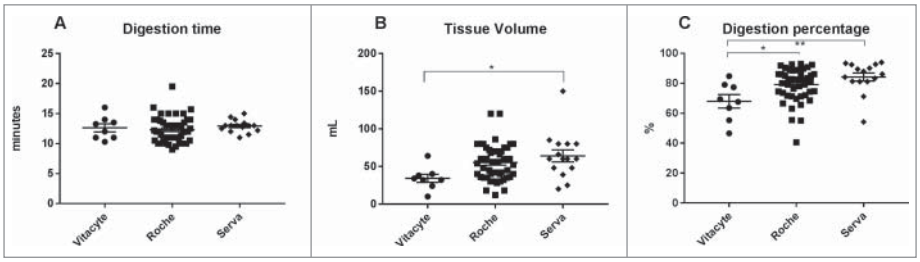

Figure 1. Pancreas digestion time (A), digested tissue (B), and digestion percentage (C) for three enzyme groups. VitaCyte (n = 8), Roche (n = 48), and Serva (n = 15). There was no significant difference in digestion time among three groups (Fig. 1A). Digested tissue volume showed no significant difference between VitaCyte and Roche, but was significant between VitaCyte and Serva ($^{*}p = 0.020$) (Fig. 1B). Digestion percentage showed significant difference between VitaCyte and Roche ($^{*}p = 0.026$), as well as between VitaCyte and Serva ($^{**}p = 0.004$) (Fig. 1C).

correlate to a significant difference in OCR consumption as the VitaCyte enzyme was 1.48 ± 0.06 and the Roche enzyme was 1.45 ± 0.03 (p = 0.943), while the Serva enzyme was 1.45 ± 0.06 (p = 0.955) (Fig. 4B). After islet transplantation into diabetic NOD scid mice, there were no significant differences found between the VitaCyte enzyme group and any of the two other enzyme groups in terms of the reversal rate of diabetes (Fig. 5); however, there was a significant difference between the Roche and Serva groups (p = 0.008). The cost per each enzyme preparation for isolation was lower when VitaCyte enzymes are used ($ 1130) compared to Roche ($ 2075) and Serva ($ 4952) enzymes (Table 1).

Discussion

In recent years, the request of islets for research has been rising. Therefore, reducing the cost of processing research pancreata would have a great impact on fulfilling the increasing demand.[7,22] The cost associated with non-GMP products is significantly lower than the cost of GMP products. Accordingly, the expense of each isolation using VitaCyte Collagenase plus BP protease was 54% and 23% of the cost compared to Roche MTF C/T and Serva collagenase NB1/NP, respectively. Research pancreata accepted for isolations have been strictly selected due to the cost burden of using expensive GMP products, like the Roche and

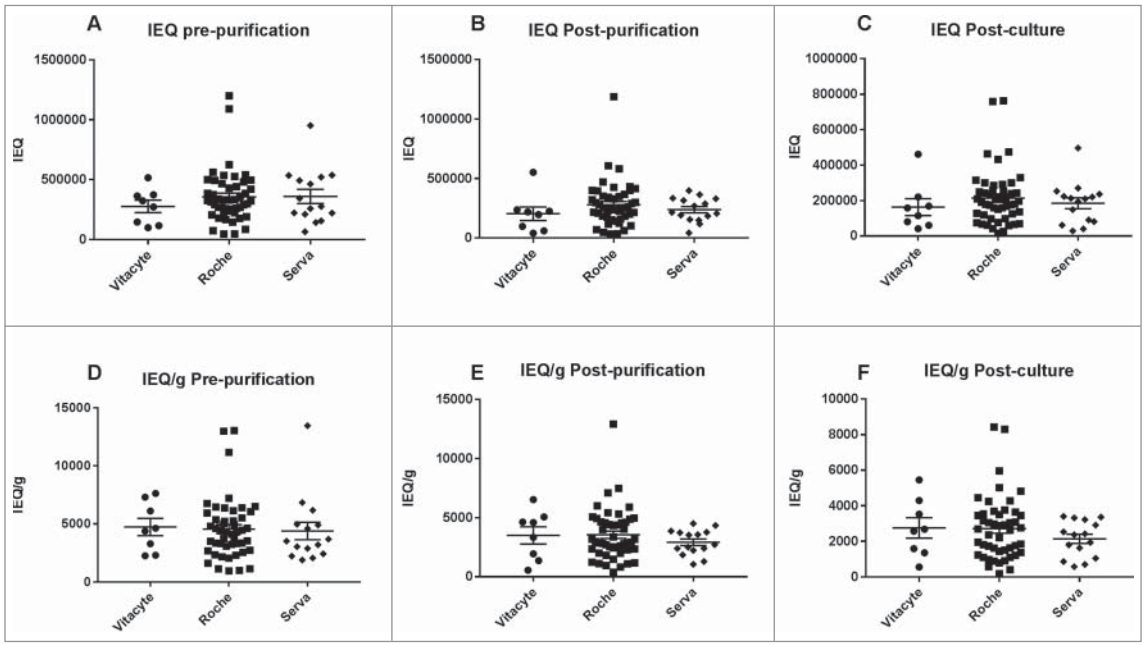

Figure 2. Islet yields for the three enzymes used. VitaCyte (n = 8), Roche (n = 48), and Serva (n = 15). Total IEQ: pre-purification (A); post-purification (B); post-culture (C). Results for IEQ/g digested pancreas tissue: pre-purification (D); post-purification (E); post-culture (F). No significant differences were found between enzymes in terms of islet yields expressed in total IEQ or IEQ/g, at pre-purification, post-purification, and post-culture.

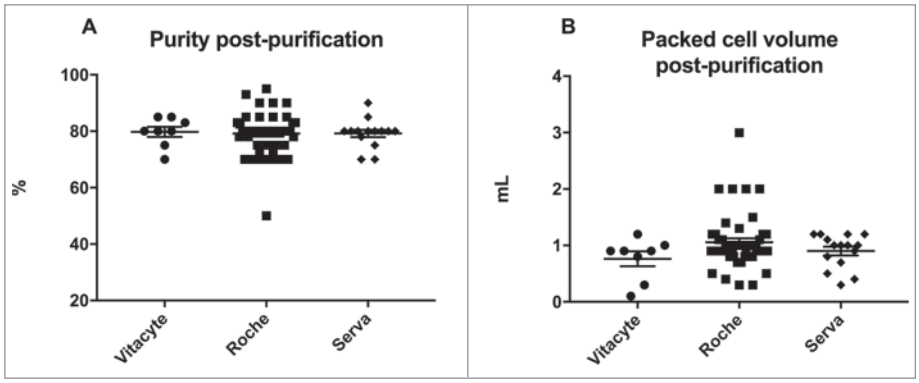

Figure 3. Purity post-purification (A) and packed cell volume post-purification (B) of the three enzymes used. VitaCyte (n = 8), Roche (n = 48), and Serva (n = 15). There are no significant differences in both purity post-purification and packed cell volume post-purification between any two groups of enzymes.

Serva enzymes.[23] In this context, using cost-effective enzymes, like VitaCyte, may facilitate utilizing more organs for islet isolations specifically for research applications. Therefore, this pilot study was initiated to explore the possibility of using less expensive, but indeed effective, enzymes for research applications.

Determining the optimal concentration of collagenase and thermolysin/NP is critical to optimize pancreas digestion and free the islets. While there are many manufacturing variabilities, and even lot-to-lot variability, there is a lack of common assays to detect enzyme activity, which limits standardizing the digestion process across different islet transplant centers.[13,24-26] In this case, one-on-one systematic comparisons of the currently available enzymes manufactured by Roche, Serva and VitaCyte are highly important in terms of selecting the ideal enzyme(s). In this study, we evaluated the performance of the VitaCyte Collagenase Gold plus BP protease in human islet isolations. Using VitaCyte Collagenase Gold plus BP protease, we were able to achieve comparable results in terms of islet yield and quality when compared to the Roche Liberase MTF C/T and the Serva collagenase NB1/NP, but with a lower cost (Table 1).

Collagenase and NP are produced from *Clostridium histolyticum*,[24] while Thermolysin is purified from *Bacillus thermoproteolyticus rokko*.[25] Collagenases (class I and II isoforms) supplemented with either Thermolysin or NP are currently used for pancreata digestion.[27] VitaCyte Collagenase Gold contains low protease activity and a high percentage of class I collagenase (73%).[19] Additionally, VitaCyte Collagenase Gold retains the flexibility of being combined with different types of proteases to digest pancreata for islet isolations. In our hands, we used VitaCyte Collagenase Gold supplemented with BP protease in human islet isolations. The digested tissue volume and digestion percentage were significantly lower in pancreata

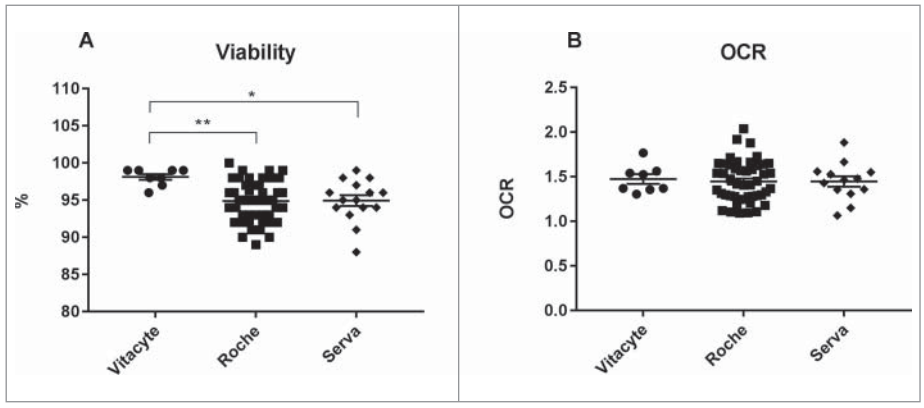

Figure 4. Viability (A) and OCR (B) results of the three enzymes used. VitaCyte (n = 8), Roche (n = 48), and Serva (n = 15). There is a significant difference in viability between VitaCyte and Roche ($^{**}p = 0.005$), as well as between VitaCyte and Serva ($^{*}p = 0.020$) (Fig. 3A). OCR data showed no significant difference between any two groups of enzymes (Fig. 3B).

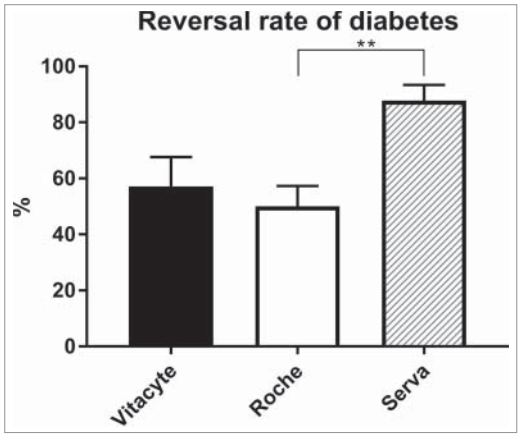

Figure 5. Reversal rate of diabetes in NOD scid mice transplanted with islets isolated using three groups of enzymes. VitaCyte (n = 7), Roche (n = 34), and Serva (n = 13). No significant differences were found between VitaCyte enzyme group and any of the two other groups of enzymes. There was a significant difference between the Roche and Serva groups (**p = 0.008).

digested with Collagenase plus BP protease compared to the digested tissue volume produced from pancreata digested with Serva. However, islet yields were not statistically different among the three tested enzymes. It is conceivable that the use of BP protease with Collagenase Gold may have contributed to digesting pancreatic tissues more efficiently which resulted in lower digested tissue volumes. The VitaCyte enzyme group had a similar reversal rate of diabetes in mice compared to the Roche and Serva enzyme groups, albeit islets isolated using the VitaCyte enzyme maintained better viability post culture. However, the Serva enzyme group had better in vivo function than the Roche group; this may be due to the fact that a higher percentage of young donor pancreata were digested using Serva enzymes supporting that islets from young donors have superior in vivo function.[28] It has been reported that islets from young donors are difficult to free from acinar tissue since most of the islet are embedded/mantled.[15] It is suggested that the Roche MTF C/T contains 60% of class I and 40% of class II collagenases (protein content by weight), while the Serva Collagenase NB1 contains 50% of class I and II collagenases (protein content by weight), respectively.[15] The difference in vivo islet functions may also stem from the fact that a neutral protease was used with the Serva enzymes while thermolysin was used with the Roche enzymes. These neutral proteases were produced by different bacterial sources, *Clostridium histolyticum* and *Bacillus thermoproteolyticus rokko*, respectively. Hence, the mode of action to digest pancreatic tissue may be different. As mentioned previously, the VitaCyte BP protease is produced by *Bacillus polymyxa*, which is suggested to be a Dispase equivalent enzyme.[19] It is conceivable that the enzymes used may have contained different Class I and II collagenases and different proteases, which might have an effect on the islet yield, viability and digested tissue volume. The percentages of class I and class II collagenases present in each vial supplied between different companies are still debatable, thus further studies are need to substantiate the optimal enzyme concentrations. Overall, the VitaCyte Collagenase Gold plus BP protease were found to be as effective as two high-quality digestion enzymes when tested in human islet isolations.

In this study, VitaCyte Collagenase Gold plus BP protease were found to be effective for pancreas digestion from donors >38 years of age. It is tempting to speculate that such an enzyme may also be efficient to digest pancreata from younger donors, especially when multiple proteases such as Clostripain were used.[29] Further investigation is required to substantiate this hypothesis. Roche MTF C/T and Serva NB1/NP enzymes are GMP products and are often the most suitable for clinical use. Comparatively, VitaCyte Collagenase Gold is sterile but is a non-GMP product and therefore used as a research grade enzyme. However, in this study, the Collagenase Gold/BP protease was filtered in the GMP facility prior to use according to our SOP and Quality Assurance regulations at City of Hope. Nevertheless, improving islet isolation outcomes need to be continuously enhanced, so that ultimately every pancreas could be utilized for clinical applications; this can only be achieved with GMP enzymes once the initial results of low cost enzyme(s) show positive outcomes. In fact, the VitaCyte Company has made progress in developing a recombinant collagenase class I and II, in addition to Collagenase Gold, which would show great potential for isolating islets.[30,31]

The data generated from this study is promising because 8 isolations using Collagenase Gold/BP protease resulted in comparable outcomes as those obtained utilizing GMP grade enzymes, indicating the potency of Collagenase Gold/BP protease. However, more data from Collagenase Gold/BP Protease would further substantiate that using these enzyme combination would be beneficial to isolate islets from suboptimal

pancreata with low cost, especially from type 2 diabetic and younger donors.

In conclusion, we have shown that using Collagenase Gold plus BP protease results in equal islet quality and yield as the counterpart of Liberase MTF C/T and Collagenase NB1/NP enzymes. Furthermore, due to the lower cost, more pancreata can be utilized for isolations for advancing islet research.

Materials and methods

Study design

In this study, outcomes of 100 consecutive research-designated human islet isolations (June 2015 to April 2017) from pancreas donors were retrospectively analyzed. Isolations were categorized into three groups based on the enzymes used for digesting the pancreas: i) Collagenase Gold Plus BP protease (VitaCyte, Indianapolis, USA); ii) Liberase Mammalian Tissue Free Collagenase/Thermolysin (Liberase MTF C/T) (Roche Diagnostics, Roche Applied Science, Indianapolis, IN, USA); iii) Collagenase NB1 with Neutral Protease (SERVA Electrophoresis GmbH, Heidelberg, Germany). For the VitaCyte enzyme, one vial of Collagenase Gold (Specific FALGPA Activity 1138 Units in 1 g amount, Cat # 011–1060) plus two vials of BP protease (each vial contains 1.1 million NP activity, Cat # 003–1000) were used for each isolation according to manufacturer recommendation. For the Roche enzyme, average amount of MTF collagenase and thermolysin used in each isolation were 2862 Wunsch units and 163288 units respectively. For the Serva enzyme, average amount of collagenase NB1 and neutral protease used in each isolation were 2542 Wunsch units and 266 units respectively. Donors with an HbA1c >6.5%, donation after cardiac death (DCD) or a downtime >30 minutes were excluded from this study. Research consent was obtained from donor nearest relatives or other person authorized legally to make the consent decision. In addition, the study was approved by the Institutional Review Board of Beckman Research Institute of the City of Hope.

Human islet isolation

The human islet isolation procedures were performed in state-of-the-art cGMP facility at the City of Hope using the standard islet processing procedure previously described.[7] Briefly, the pancreas was trimmed, decontaminated, and subsequently cannulated pancreatic lobes were perfused with digestion enzymes using an automatic perfusion apparatus (BioRep Technologies, Miami, FL, USA) with one of the aforementioned enzymes. Afterwards, the pancreas was cut into 7–10 pieces and placed into a semi-automatic Ricordi's digestion chamber for digestion. Purification of the pooled, digested, tissue containing islets was performed using a COBE 2991 with continuous density gradients. Samples were taken and stained with DTZ for counting and determination of the purity of the islets microscopically; this was performed before and after purification. Islet Equivalents (IEQ) were used to express the total islets count.[32]

Purified islets were cultured at 37°C/5% CO_2 for 24–72 hours as previously described.[15] Post-culture islet samples were taken for quality assessments, including islet count, viability, glucose-stimulated oxygen consumption rate (ΔOCR), and islet transplantation into diabetic NOD scid mice.

Islet viability and ΔOCR

Viability of post-cultured islets was assessed through the use of fluorescent microscopy with FDA and PI as previously reported.[33] Islet oxygen consumption rate (OCR) was measured using a Seahorse XFe analyzer (Seahorse Bioscience, North Billerica, MA). Briefly, islets were washed with modified Seahorse XFe assay media containing 3 mM glucose and 1% FBS, and equilibrated in same assay media for 3 hrs at 37°C. Then, 70–100 IEQ islets were handpicked and plated into Seahorse XFe islet capture plates (Seahorse Bioscience). Islet OCR was measured at basal level (3 mM glucose), upon glucose stimulation (20 mM glucose), and on mitochondrial respiration inhibition (Oligomycin 5 nM). OCR fold increase was calculated by OCR upon glucose stimulation/OCR at basal level. Minimum of 4 islet samples were measured simultaneously in each experiment.

Transplantation of human islets in diabetic NOD scid mice

Islet transplantation into NOD scid mice was carried out as previously developed method.[8] Briefly, male NOD scid mice (Jackson Laboratory, Bar Harbor, ME, USA) that were 10–12 weeks of age were used as islet recipients. Mice were housed at the Animal Resources Center, Beckman Research Institute of the City of

Hope. Diabetes was induced in mice by injecting mice intraperitoneally with 50 mg/kg of Streptozotocin (STZ; Sigma-Aldrich, St Louis, MO, USA) for three consecutive days. Mice with glucose levels >350 mg/dL for at least 48 hours were used for islet transplantations. For each isolation, 2–5 mice were transplanted with 1200 IEQ/each under the left kidney capsule. We used this islet number because our empirical study showed that approximately 60% of transplanted mice reversed diabetes (unpublished data). Blood glucose levels were followed for 30 days using a glucometer (LifeScan, Inc., Milpitas, CA, USA). All glucose blood levels readings <200 mg/dL for two weeks were considered a successful transplant. For each enzyme, the success graft-rate was calculated by dividing the amount of successful transplants by the total number of recipients.

Statistical analysis

Data was analyzed with GraphPad Prism (GraphPad Software 7.1, La Jolla, CA, USA). ANOVA one-way analysis of variance was used to compare the three groups of enzymes followed by Tukey multiple comparisons test to compare the mean values between any two groups. All the values were expressed as mean ± standard error of mean (SEM).

Abbreviations

Cgmp	Current Good Manufacturing Practice
CIT	Cold Ischemia Time
DCD	Donation after Cardiac Death
GSIS	Glucose-Stimulated Insulin Secretion
HbA1c	Hemoglobin A1c
IEQ	Islet Equivalent
MTF C/T	Mammalian Tissue Free Collagenase/Thermolysin
NOD	Non-Obese Diabetic
NP	Neutral Protease
ΔOCR	Oxygen Consumption Rate
SCID	Severe Combined Immunodeficient
STZ	Streptozotocin

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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