Received: 19 July 2017 | Revised: 18 September 2017 | Accepted: 3 October 2017

DOI: 10.1111/ajt.14542

BRIEF COMMUNICATION

AJT

Beneficial effect of recombinant rC1rC2 collagenases on human islet function: Efficacy of low-dose enzymes on pancreas digestion and yield

Gopalakrishnan Loganathan[1] | Venugopal Subhashree[2] | Andrew G. Breite[3] | William W. Tucker[1] | Siddharth Narayanan[1] | Maheswaran Dhanasekaran[1] | SriPrakash Mokshagundam[1] | Michael L. Green[3] | Michael G. Hughes[1] | Stuart K. Williams[1] | Francis E. Dwulet[3] | Robert C. McCarthy[3] | Appakalai N. Balamurugan[1]

[1]Center for Cellular Transplantation, Cardiovascular Innovation Institute, Department of Surgery and Endocrinology, University of Louisville, Louisville, KY, USA

[2]School of Biosciences and Technology, VIT University, Vellore, TN, India

[3]VitaCyte LLC, Indianapolis, IN, USA

Correspondence
Appakalai N. Balamurugan
Email: bala.appakalai@louisville.edu

A high number of human islets can be isolated by using modern purified tissue dissociation enzymes; however, this requires the use of >20 Wunsch units (WU)/g of pancreas for digestion. Attempts to reduce this dose have resulted in pancreas underdigestion and poor islet recovery but improved islet function. In this study, we achieved a high number of functional islets using a low dose of recombinant collagenase enzyme mixture (RCEM-1200 WU rC2 and 10 million collagen-degrading activity [CDA] U of rC1 containing about 209 mg of collagenase to digest a 100-g pancreas). The collagenase dose used in these isolations is about 42% of the natural collagenase enzyme mixture (NCEM) dose commonly used to digest a 100-g pancreas. Low-dose RCEM was efficient in digesting entire pancreases to obtain higher yield (5535 ± 830 and 2582 ± 925 islet equivalent/g, $P < .05$) and less undigested tissue (16.7 ± 5% and 37.8 ± 3%, $P < .05$) compared with low-dose NCEM (12WU/g). Additionally, low-dose RCEM islets retained better morphology (confirmed with scanning electron microscopy) and higher in vitro basal insulin release (2391 ± 1342 and 1778 ± 978 μU/mL; $P < .05$) compared with standard-dose NCEM. Nude mouse bioassay demonstrated better islet function for low-dose RCEM (area under the curve [AUC] 24 968) compared with low-dose (AUC−38 225) or standard-dose NCEM (AUC−38 685), $P < .05$. This is the first report indicating that islet function can be improved by using low-dose rC1rC2 (RCEM).

KEYWORDS
islet isolation, islet transplantation, islets of Langerhans, translational research/science

1 | INTRODUCTION

Recovery of sufficient functional islets from cadaveric [1] or severely fibrotic pancreases [2,3] is necessary for successful allo- or auto-islet

Abbreviations: AUC, area under the curve; CDA, collagen-degrading activity; GSIR, glucose-stimulated insulin release; IPGTT, intraperitoneal glucose tolerance test; NCEM, natural collagenase enzyme mixture; NP, neutral protease; RCEM, recombinant collagenase enzyme mixture; SEM, scanning electron microscopy; U, units of enzyme activity; WU, Wunsch units.

transplantation. Pancreas digestion with an optimal formulation and dose of collagenase-protease enzyme mixture is crucial for obtaining high-quality islets, as it relies heavily on the potency and consistency of these enzymes during the islet isolation procedure.[4] To ensure a safe, pure, and potent islet product, regulatory requirements require that the islet product be consistently manufactured by using a standardized and validated process.[5-7]

Although significant improvements have been made in the purification of *Clostridium histolyticum* collagenase, variations in collagenase and protease activity between different lots of the same product are common.[8,9] This variation is likely caused by degradation of class I or class II collagenase during bacterial fermentation or the subsequent purification process. Proteolysis of intact collagenase leads to variability, affecting its ability to degrade native collagen.[10,11] Purified natural collagenase is also commonly contaminated with clostripain, a sulfhydryl neutral protease (NP) with trypsin-like activity [12,13] that appears to increase endogenous protease activity via activation of pancreatic proenzymes.[14,15] Only a small portion of clostripain is active under oxidizing conditions, which further contributes to the variability in protease activity between lots of the same product.

The dose and composition of the enzymes used in the pancreas digestion process are critical factors that affect the number and quality of islets released from the human pancreas. In most islet laboratories, a collagenase dose of >20 WU/g pancreas (1 full vial of collagenase bottle) is commonly used.[4,16] Recently, it was reported that a vast amount of collagenase fails to be absorbed by the human pancreas during the digestion process, suggesting that an excess of collagenase is commonly used[17] Cross et al.[18] demonstrated that collagenase can penetrate human islets after intraductal administration. Excess collagenase may potentially harm islets during the isolation process. Conversely, islet functionality may be improved with the use of lower doses (<20 WU/g) of natural collagenase. However, lower doses result in poor islet recovery, due to higher percentages of undigested tissue.

Manufacturing purified recombinant collagenases may overcome problems commonly encountered with purified natural collagenase. Recombinant class I (rC1) and class II (rC2) collagenase are expressed from single genes in the *Escherichia coli* strain containing low protease activity. This minimizes proteolysis of collagenase enzymes, leading to the generation of intact rC1 and rC2 enzymes. Clostripain contamination is eliminated, improving the control of protease activity during the islet isolation procedure.

In our recent study,[19] we successfully manufactured purified rC1 and rC2 and assessed 4 different collagenase formulations to recover islets from human pancreases (n = 12, 3 per group). Varying amounts of rC1 and rC2 collagenase activity, supplemented with a fixed amount of *P. polymyxa* NP activity (BP-Protease [VitaCyte LLC, Indianapolis, IN], a dispase-equivalent NP), were tested using a statistically designed experiment in a split-pancreas model. We observed that the low-dose collagenase-protease enzyme mixture (RCEM, 100 000 CDA U, 12 WU/g tissue, in combination with 23 400 U of BP-Protease/g tissue) digested the pancreas and recovered islets as effectively as the 3 other enzyme mixtures that contained 1.38- to 1.79-fold higher mass of recombinant collagenase enzymes per g tissue. These RCEMs contained about 42% to 75% of the mass of purified natural collagenase commonly used for digesting a 100-g pancreas.

In this study we used a low RCEM dose (12 WU/g) to successfully isolate islets, with good functionality, from whole human pancreata. These results, compared with islet isolation performed using natural collagenase enzyme mixture (60:40 C1:C2 mixture containing about 530 mg collagenase to digest a 100-g pancreas) (NCEM) with equivalent (12 WU/g) or standard (20 WU/g) amounts, demonstrated better islet recovery, tissue digestion efficiency, insulin secretion, and transplantation outcomes. This is the first report indicating that islet functionality can be improved with low-dose enzyme. Overall, the quality and functionality assessments suggest that this RCEM dose has potential to be used for human islet isolations to improve clinical outcomes.

2 | MATERIALS AND METHODS

2.1 | Donor pancreases

Human cadaveric donor pancreases (n = 20) were obtained through the Kentucky Organ Donor Affiliates (Louisville, KY) from brain-dead donors after informed consent had been obtained as part of a multiorgan procurement. Pancreases were transported in cold UW solution.

2.2 | Islet isolation enzymes (rC1 + rC2 + BP-Protease)

Purified recombinant or natural collagenase enzymes were prepared as reported earlier.[19] The only animal component used in the preparation of natural collagenase was the inclusion of porcine gelatin peptone in the fermentation culture media. No animal components were used in the generation of the raw material or purification of rC1, rC2, or BP-Protease (VitaCyte). BP-Protease was purified from *Paenbacillus polymyxa* culture supernatants. The natural C1 and C2 were recovered in separate pools through anion exchange chromatography and recombined as a 60:40 C1:C2 ratio based on mass with about 4 WU/mg protein. The rC1 and rC2 were purified separately and combined.

Table 1 summarizes characteristics of the collagenases used for the NCEM and RCEM. The C1:C2 ratios between NCEM and RCEM were 60:40 and 30:70, respectively. The natural purified collagenase was contaminated with low amounts of clostripain, reflected by the tryptic-like enzyme activity.

2.3 | Human islet isolation

Islet isolations were performed as previously reported.[20] On arrival, the pancreas was trimmed, cannulated, and distended with digestive enzymes. The rC1 and rC2 collagenases (rC1 100 000 CDA U/g, rC2 12 WU/g) were mixed with BP-Protease enzymes (23 400 NP U/g pancreas) at the time of islet isolation to generate the RCEM (n = 7). Enzymes were diluted in 350 mL of HBSS for each pancreas. After ductal perfusion of the enzymes, the pancreas was digested in a Ricordi chamber (Biorep Technologies, Miami, FL) using a modification of Ricordi's semiautomated method.[21] The digested tissue was

TABLE 1 Characteristics of natural collagenase enzyme mixture (NCEM) and recombinant collagenase enzyme mixture (RCEM) used in this study

Specification	NCEM	RCEM
Source organism	*C. histolyticum*	BL21(DE3) pLysS *E. coli*[a]
C1/C2 ratio , %	60:40	30:70
Total Wunsch activity/mg protein	4.41 (n = 7)	5.86 (n = 7)
Total CDA, U/mg protein	56 768 (n = 7)	63 896 (n = 7)
Neutral protease, U/mg protein	NA	NA
Clostripain /mg protein, BAEE units	0.121 (n = 6)	Not measured
Tryptic-like activity/mg protein	0.032 (n = 6)	Not measured
Total protein /mg protein, mg	501.4	240.4
Collagenase dose used for islet isolation, WU/g	12 and 20	12 and 20
BP-Protease dose used for islet isolation, U/g	23 400	23 400
Total endotoxin burden in the system, endotoxin unit total	2377 (n = 9)	2188 (n = 2)

BAEE, Nα-benzoyl-l-arginineethylester hydrochloride; CDA, collagen-degrading activity.
[a]rC1 or rC2 expressed by separate *E. coli* clones and purified separately.

then purified by continuous iodixanol (OptiPrep; Axis-Shield, Oslo, Norway) density gradient with a COBE-2991 cell processor (Terumo BCT, Lakewood, CO). The purified islets were cultured overnight in CMRL-1066–supplemented medium (Corning/Mediatech Inc., Manassas, VA) before being subjected to quality control assessment. Islet isolations with NCEM (VitaCyte CIzyme Collagenase HA) were performed using either 12 WU/g (n = 5) or 20 WU/g (n = 8) in combination with BP-Protease (23 400 NP U/g tissue).

2.4 | Islet morphology, histology, and viability assessment

Islets were stained with dithizone, and islet morphology was assessed and scored. Islet histology assessment was performed according to previously published methods.[22] Islet viability was assessed by using the fluorescein diacetate–propidium iodide method.[23]

2.5 | Islet surface assessment by scanning electron microscopy

Scanning electron microscopic (SEM) images of isolated islets with either standard NCEM dose or low RCEM dose were obtained, to compare surface morphology. Freshly isolated pure islets were fixed in 2% glutaraldehyde and dehydrated by using successive increases in ethyl alcohol percentages (70-100%). Hexamethlydisilazane was then applied over these dehydrated islets for chemical drying. Islets were mounted on SEM stubs, sputter coated with gold (Au), and processed for SEM imaging. Isolated islets from NCEM served as the control.[24]

2.6 | Basal insulin release before and after enzyme exposure

Islet basal insulin release into culture medium (48 hours) was evaluated after islets were isolated with a low or standard dose of RCEM or NCEM.[25] In subsequent studies, islets were cultured for an additional 48 hours under normal conditions. These cultured islets were further exposed to low and standard RCEM and NCEM doses for 30 minutes. After multiple washing with serum-containing medium, the cells were cultured again for 48 hours before assessment of basal insulin release. These results were compared with those obtained with the untreated control. Insulin was measured by using an ELISA kit (Mercodia, Winston-Salem, NC).

2.7 | Glucose-stimulated insulin release by static and dynamic methods

After overnight culture, islet insulin secretion was assessed by in vitro static and dynamic glucose challenge using low (2.8 mmol/L) and high concentrations (28 mmol/L) of glucose. Krebs Ringer bicarbonate was used as base media.[26]

Under static conditions, hand-picked human islets (150- to 200-μm islets) were placed in transwell plates and preincubated in Krebs medium containing 2.8 mmol/L glucose for 30 minutes at 37°C. After 30 minutes of preincubation, the islets were switched into Krebs medium containing 2.8 mmol/L glucose for 1 hour, followed by a high glucose stimulus consisting of 28 mmol/L for 1 hour.

In the dynamic glucose challenge test, the pre- and basal glucose concentration was maintained at 2.8 mmol/L for 30 minutes each, with a glucose stimulus consisting of a single-step increase in glucose concentration to 28 mmol/L for 30 minutes, followed by reduction to 2.8 mmol/L glucose for another 30 minutes. Insulin concentration in supernatant was assessed by using the Mercodia ELISA kit. The stimulation index was calculated as a ratio of the insulin value obtained after high glucose stimulation over the insulin value obtained after low glucose stimulation.

2.8 | Enzyme-exposed islet transplantation

The effect of enzyme exposure on islet function was monitored in diabetic nude mice by assessing the in vivo islet insulin secretion. Two thousand (IEQs) handpicked islets were transplanted in nude mice after enzyme exposure to low-dose RCEM (12 WU/g), low-dose NCEM (12 WU/g), standard-dose RCEM (20 WU/g), or standard-dose NCEM (20 WU/g). These results were compared with an untreated control. After enzyme exposure for 30 minutes, these islets were washed multiple times with serum-containing medium and transplanted under the kidney capsule of diabetic nude mice [27] in compliance with guidelines from the Institutional Animal Care Committee at

the University of Louisville (n = 15, 3 in each condition). Mice were considered diabetic (streptozotocin induced) once their blood glucose levels were 350 mg/dL or higher for 2 consecutive days. Post transplantation, the mice blood glucose levels and body weight were measured daily for the first week, then every 3 days until the completion of study. Return of normoglycemia in cured mice was identified by 2 consecutive drops of blood glucose levels to 200 mg/dL or less. At 30 days post transplantation, a left nephrectomy was performed in the normoglycemic mice to confirm a return to hyperglycemia, validating causality.

2.9 | Assessing transplanted islet graft function by intraperitoneal glucose tolerance test

Enzyme-exposed islets were challenged with high glucose by use of an intraperitoneal glucose tolerance test (IPGTT0).

After overnight fasting, the animals were injected intraperitoneally with glucose dissolved in 0.9% sodium chloride solution (2 g glucose/kg body weight). Blood samples were obtained before (0 minute) and 15, 30, 60, and 90 minutes after glucose injection and analyzed for blood glucose measurement.[28]

2.10 | Statistical analysis

All results were expressed as the mean ± SD. Islet yield comparisons and insulin secretion were analyzed by using the nonparametric Kruskal–Wallis test followed by the post-hoc Dunn method for multiple comparisons and the Friedman test followed by the Dunn correction, respectively. Statistical significance for the analysis was set at $P < .05$.

3 | RESULTS

3.1 | Islet isolation and functional outcome

Islet isolation outcomes using the low-dose RCEM are summarized in Table 2. Table 3A compares these preparations with islets isolated using a standard dose of RCEM or a low or standard dose of NCEM. The low dose of NCEM gave significantly lower islet yields (2582 ± 925 IEQ/g pancreas) and a higher percentage of undigested pancreas (37.8 ± 3%) compared with those isolations using low-dose RCEM (5535 ± 830 IEQ/g pancreas and 16.7 ± 5%, $P < .05$). There were no significant differences in islet yield or percent digested tissue between low-dose RCEM or islets isolated with the standard-dose NCEM or RCEM.

To validate effectiveness of low-dose RCEM on islet isolation, basal insulin secretion and glucose-stimulated insulin release (GSIR) response were measured and compared with values from other enzyme formulations. The functional outcomes of islets isolated using different enzyme mixture doses are summarized in Table 3A and 3B. Islets isolated with low-dose RCEM gave significantly higher basal insulin release after 48 hours of culture compared with islets isolated with the standard dose of NCEM (2391 ± 1342 vs 1778 ± 978 μU/mL; $P < .05$). There was no significant difference in basal insulin release when comparing

TABLE 2 Human islet isolation outcomes using a low-dose RCEM (100 000 CDA U, 12 WU/g tissue) supplemented with 23 400 U/g tissue of BP-Protease

No. of isolations	1	2	3	4	5	6	7	Average
Age, y	28	37	31	38	37	19	19	30 ± 8.3
Height, cm^2	180	180	165	170	170	185	189	177 ± 8.8
Body weight, kg	81	84	75	109	101	75	64.5	84 ± 15.6
Body mass index, kg/m^2	24.8	25.8	27.5	37.8	35	21.9	18.2	27 ± 7.0
Trimmed pancreas weight, g	43.7	83	55	71	63	71	50	62 ± 13.7
Digestion time, min	20	17	20	19	17	21	23	20 ± 2.1
Undigested tissue, g	5	16	8	14.5	11.2	17	5	11 ± 5.1
Undigested tissue, %	11.4	19.2	14.5	20.4	17.7	23.9	10.0	16.7 ± 5
Digest islet equivalent/pancreas	250 226	505 138	303 160	456 246	370 125	423 395	389 315	385 372 ± 87 639
Digest islet equivalent/g pancreas	5726	6086	5512	6426	5875	5963	7786	6196 ± 757
Embedded islet, %	30	30	30	10	25	50	40	31 ± 12.4
Tissue volume, mL	20	35	20	40	28	40	25	30 ± 8.7
Postpurification islet equivalent/pancreas	201 503	416 743	282 040	362 952	335 727	424 836	362 184	340 855 ±78 165
Postpurification islet equivalent/g pancreas	4926	5021	5128	5112	5329	5983	7243	5535 ± 830
Viability assessment by fluorescein diacetate–propidium iodide, %	91	86	85	90	89	90	92	89 ± 2.6

RCEM, recombinant collagenase enzyme mixture; CDA, collagen-degrading activity.

TABLE 3 Human islet isolation and functional outcome using different doses of enzyme blends

	Islet isolation with RCEM low dose (12 WU/g)	Islet isolation with NCEM low dose (12 WU/g)	Islet isolation with RCEM standard dose (20 WU/g)	Islet isolation with NCEM standard dose (20 WU/g)	*P*-value
A					
Undigested pancreas, %	16.7 ± 5*	37.8 ± 3#	15.7 ± 1	20.1 ± 9	*vs # <.05
Final IEQ/g pancreas	5535 ± 830*	2582 ± 925#	5455 ± 918	4946 ± 974	*vs # <.05
Basal insulin release in 48 h after islet isolation, μU/mL	2391 ± 1342 for 50 islets*	2097 ± 976 for 50 islets	2007 ± 1,012 for 50 islets	1778 ± 978 for 50 islets#	*vs # <.05
Stimulation index after GSIR	3.8	3.1	3.3	2.5	NS

	Control (no exposure)	RCEM low-dose enzyme exposure (WU/g)	NCEM low-dose enzyme exposure (WU/g)	RCEM standard-dose enzyme exposure (20 WU/g)	NCEM standard-dose enzyme exposure (WU/g)	*P*-value
B						
Basal insulin release in 48 h after additional enzyme exposure, μU/mL	1993 ± 973 for 50 islets	1868 ± 582 for 50 islets*	1651 ± 602 for 50 islets	1708 ± 579 for 50 islets	1479 ± 691 for 50 islets#	*vs # <.05

RCEM, recombinant collagenase enzyme mixture; NCEM, natural collagenase enzyme mixture; GSIR, glucose-stimulated insulin release.
*,# Values generated for each condition in using NCEM and compared with values generated using RCEM.

islets isolated with low- or standard-dose RCEM or low-dose NCEM. All islet preparations gave similar GSIR stimulation indices when assessed by static or dynamic insulin secretory assays (Table 3A).

To further confirm the functional ability of low-dose RCEM over NCEM, isolated islets from 1 preparation were stabilized for 48 hours in culture and then subjected to various enzyme doses to measure islet function. This allowed us to evaluate enzyme effect on islet function by minimizing discrepancies arising due to donor-to-donor variability. Isolated islets after stabilization were exposed to either low- or standard-dose RCEM and NCEM and cultured for an additional 48 hours. Basal insulin release measurements from these culture islets were similar with the exception of a significant difference between low-dose RCEM and standard-dose NCEM (Table 3B, $P < .05$), suggesting that using low-dose RCEM results in better functioning islets.

3.2 | Morphology, histology and SEM assessment of isolated islets

Islets isolated with RCEM (12 WU/g) or NCEM (20 WU/g) demonstrated no difference in morphology after hematoxylin and eosin staining (not shown). However, RCEM isolated islets under SEM revealed a smooth surface with few single cells exposed (Figure 1A). In contrast, more visible surface damage was observed (Figure 1B) after islet isolation with NCEM. Overall, our results so far suggest that islets isolated using RCEM retain better functionality and morphology over islets isolated using NCEM.

3.3 | In vivo assessment of islet function

To determine the in vivo effects of islets isolated using RCEM, we performed the nude mouse bioassay. Islets (2000 IEQ) were transplanted in nude mice after enzyme exposure to low-dose RCEM (12 WU/g), low-dose NCEM (12 WU/g), standard-dose RCEM (20 WU/g), or standard-dose NCEM (20 WU/g). These results were compared with an untreated control. Our data suggest that none of the enzyme-exposed islets completely reversed diabetes except for the untreated control, but low-dose RCEM–treated islets maintained better glycemic control (area under the curve [AUC] = 24 968) and released a greater amount of insulin during IPGTT (Figure 2A). In Figure 2B, the AUC was calculated to measure the level of insulin release in response to glucose, a higher glucose clearance corresponding to lower AUC value, and vice versa. The difference was statistically significant ($P < .05$) for low-dose RCEM compared with islets exposed to low-dose NCEM (AUC = 38 225), standard-dose RCEM (AUC = 31 538), and standard-dose NCEM (AUC = 38 685) (Figure 2B).

4 | DISCUSSION

The key findings in this report are that low-dose RCEM gave significantly higher human islet yields than comparable low-dose NCEM and significantly better in vivo islet function after transplantation into nude mice. In addition, SEM images indicate less surface damage to

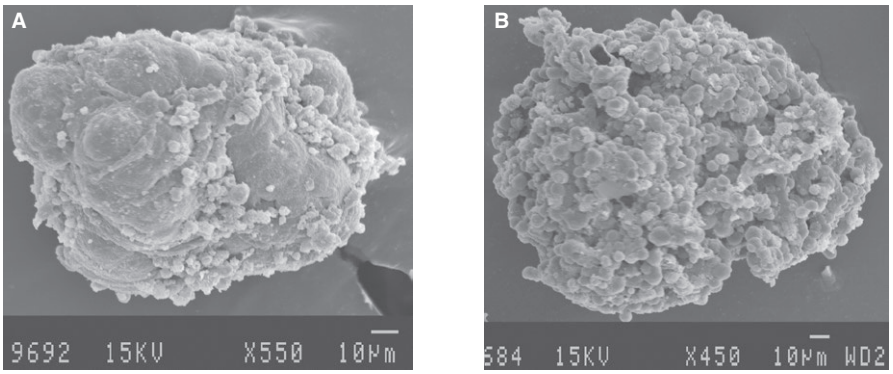

FIGURE 1 Islets isolated using low-dose (RCEM) result in less surface damage. Scanning electron microscopy (SEM) images indicate that islets isolated using (A) low-dose RCEM have an intact islet surface compared with islets isolated using (B) standard-dose (NCEM), resulting in a disrupted islet surface. The images in figure 1A and 1B are representative of several SEM images taken

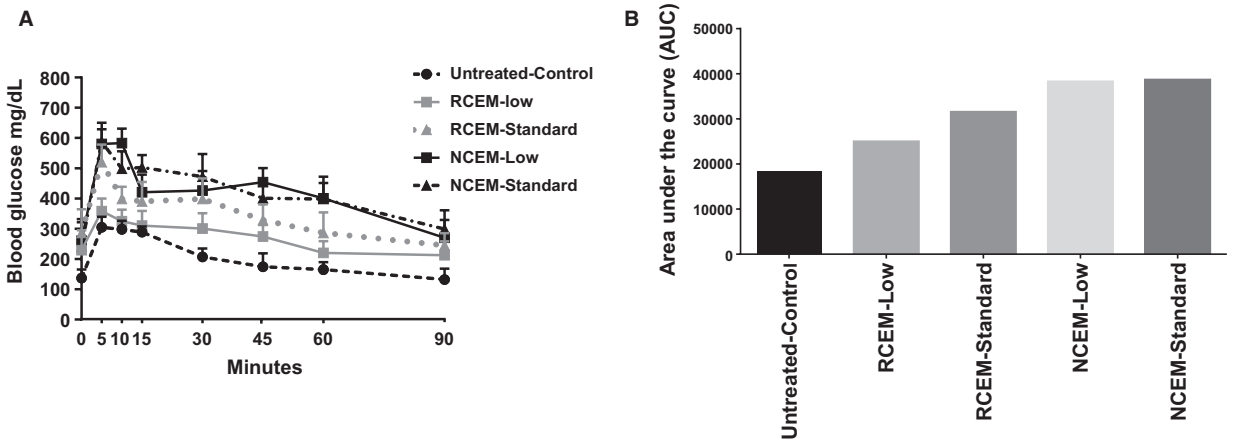

FIGURE 2 Islets exposed to low-dose (RCEM) demonstrate better glycemic function in an in vivo model. Isolated human islets (2000 IEQ) were exposed to different enzyme doses (Untreated-control, RCEM-low, RCEM-standard, NCEM-low, and NCEM-standard) and transplanted under the kidney capsule of diabetic nude mice (n = 15, 3 in each condition). After 2 weeks, an intraperitoneal glucose tolerance test (IPGTT) was performed on the transplanted islet grafts as described in the method section. (A) Comparisons between the blood glucose levels (*y*-axis) at different time points (*x*-axis) for all enzyme combinations were plotted. The blood glucose level trend for RCEM-low group (gray square) was consistently lower than that of NCEM-low (black square), NCEM-standard (black triangle), or RCEM-standard (gray triangle). The standard deviation is representative of 3 independent experimental values. (B) The area under the curve (AUC, *y*-axis arbitrary units) was calculated for each group as shown on the *x*-axis. RCEM-low maintained significantly better ($P < .05$) glycemic control (AUC = 24 968) during IPGTT testing compared with islets exposed to NCEM-low (AUC = 38 225), RCEM-standard (AUC = 31 538), and NCEM-standard (AUC = 38 685)

islets isolated with low-dose RCEM compared with standard-dose NCEM. Overall, the data suggest that purified low-dose RCEM is more effective in recovering functional human islets than purified natural collagenase.

Our previous study using the same recombinant collagenase and supplemental dose of purified BP-Protease to isolate human islets from the body-tail of research pancreata demonstrated there was no statistically significant difference in the effectiveness of recombinant collagenase to recover human islets when 4 different purified collagenase-protease enzyme mixtures were used in the isolation procedures.[19]

The success of the 30:70 rC1:rC2 ratio in achieving similar islet yields in 7 additional islet isolations using intact human pancreas in the present study supports the validity of the prior results. These results appear contradictory to previous reports that suggested that excessive class II collagenase may be detrimental to isolation outcomes and 60:40 mass ratios of C1:C2 appeared optimum for human islet isolation. However, these conclusions were drawn from isolations performed with thermolysin, not BP-Protease. Moreover, the collagen degradation activity of the natural collagenases used in these earlier studies was not characterized using an appropriate substrate, further confounding interpretation of these results. This, combined with the fact that recombinant collagenases have in vitro collagen degradation characteristics that differ from the natural collagenases, makes comparisons between studies complicated.[4,8,9]

In the present study, islet isolation results were generated from enzyme mixtures that used the same dose of BP-Protease as used

in the earlier report.[19] Here, comparable percentages of undigested tissue and islet yields per gram of tissue were obtained when either standard-dose NCEM or low- or standard-dose RCEM was used in the islet isolation procedure. All isolations performed in the present report used a standardized dose of BP-Protease, making direct comparisons between natural and recombinant collagenase more meaningful.[8] The standard dose of natural collagenase (20 WU/g tissue) gave islet yields similar to those obtained with low-dose RCEM. Reduction of natural purified collagenase (low-dose NCEM) to the activity used in the low-dose RCEM (12 WU/g tissue) resulted in significantly lower islet yields (2582 ± 925 vs 5535 ± 830 IEQ/g tissue, $P < .05$) and a significant increase in undigested tissue (37.8 ± 3% vs 16.7 ± 5%, $P < .05$). It is unlikely that differences in the C1:C2 ratios (60:40 for NCEM and 30:70 for RCEM) affected these results because our previous studies identified no significant interaction between rC1 and rC2 when the C1:C2 ratios ranged from 55:45 to 27:73.[8]

Low-dose RCEM supplemented with 23 400 NP U BP-Protease/g tissue appears to be a suitable enzyme mixture for recovery of human islets when these results are compared with isolations using other enzyme mixtures. Different doses of enzyme-exposed islets were transplanted into diabetic nude mice. In these, low-dose RCEM enzyme mixture maintained better glycemic control and released higher amounts of insulin during IPGTT compared with islets exposed with low-dose NCEM, standard-dose NCEM, or standard-dose RCEM. This difference was statistically significant and indicates the importance of reducing enzyme activities used for islet isolation to improve islet function in vivo. This conclusion is further supported by in vitro culture studies that showed that purified islets isolated with low-dose RCEM gave significantly higher basal insulin release after 48 hours of culture compared with those isolated with the use of standard-dose NCEM (2391 ± 1342 vs 1778 ± 978 μU/mL for 50 islets). Exposure of purified islets with either the low-dose RCEM or standard-dose NCEM for 30 minutes before 48 hours of culture and measurement of subsequent basal insulin release showed a significantly higher insulin release after brief incubation with RCEM compared with those incubations that used the standard dose of NCEM. An earlier report compared the effectiveness of natural and recombinant purified enzymes to isolate human islets.[29] There were no significant differences between any of the islet isolation parameters when results from islet isolations using these 2 enzyme mixtures were compared.

Currently, natural collagenase has been the enzyme of choice for use in human islet isolations. Most of the laboratories use the entire bottle of purified natural collagenase (≥2000 WU) and supplemental protease for each islet isolation procedure. Variation in the pancreas weight leads to the use of a wide range of collagenase and NP activities, varying from 10 to 50 WU/g tissue and from 0.5 to 4.0 dimethylcasein U/g tissue. This variation can often lead to the use of unintentionally high doses of collagenase and NP, potentially affecting islet yield, quality, and function. It has been reported that the commonly used dose of collagenase usually ranges from 21 to 32 WU/g of pancreatic tissue for human islet isolation.[4,30] However, a recent study has reported that only 30% of the total FALGPA activity (measure of C2 activity) is absorbed by the pancreas during the perfusion process,[17] suggesting that a vast amount of enzyme is not used during standard islet isolation protocols.

In this context, low-dose RCEM appears to be notable advance to improve the yield and function of human islets recovered after digestion with collagenase-protease enzyme mixtures. The results summarized here strongly suggest the recombinant collagenases possess an intrinsic superiority to the naturally derived collagenases, but the biochemistry responsible for this has yet to be identified. This improvement is aligned with the evolving regulatory challenges that will likely demand the use of animal origin–free materials in the islet isolation process and economic considerations regarding the cost effectiveness of islet transplantation that justifies reimbursement by third party payers.

ACKNOWLEDGMENTS

The authors thank the Jewish Heritage Fund for Excellence for providing generous support to our program. The authors sincerely thank Kentucky Organ Donor Affiliates for the supply of human pancreases. Special thanks to Benjamin Tweed, Praneeth Goli, Aravinth Prasanth Jawahar, Ankit Patel, Jithu Pradeep, Leigh Kleinert, and Brian Gettler for their assistance.

DISCLOSURE

The authors of this manuscript have conflicts of interest to disclose as described by the *American Journal of Transplantation*. AGB, MLG, FED, and RCM are members of VitaCyte LLC. AGB is director of quality assurance, MLG is director of quality control, FED is chief scientific officer, and RCM is president and CEO. The other authors have no conflicts of interest to disclose.

REFERENCES

1. Shapiro AM, Pokrywczynska M, Ricordi C. Clinical pancreatic islet transplantation. *Nat Rev Endocrinol*. 2017;13(5):268-277.
2. Chinnakotla S, Beilman GJ, Dunn TB, et al. Factors predicting outcomes after a total pancreatectomy and islet autotransplantation lessons learned from over 500 cases. *Ann Surg*. 2015;262(4):610-622.
3. Naziruddin B, Matsumoto S, Noguchi H, et al. Improved pancreatic islet isolation outcome in autologous transplantation for chronic pancreatitis. *Cell Transplant*. 2012;21(2–3):553-558.
4. Kin T, Zhai X, Murdoch TB, Salam A, Shapiro AM, Lakey JR. Enhancing the success of human islet isolation through optimization and characterization of pancreas dissociation enzyme. *Am J Transplant*. 2007;7(5):1233-1241.
5. Hering BJ, Clarke WR, Bridges ND, et al. Phase 3 trial of transplantation of human islets in type 1 diabetes complicated by severe hypoglycemia. *Diabetes Care*. 2016;39(7):1230-1240.
6. Ricordi C, Goldstein JS, Balamurugan AN, et al. National Institutes of Health-sponsored clinical islet transplantation consortium phase 3 trial: manufacture of a complex cellular product at eight processing facilities. *Diabetes*. 2016;65(11):3418-3428.
7. Wonnacott K. Update on regulatory issues in pancreatic islet transplantation. *Am J Ther*. 2005;12(6):600-604.

8. Brandhorst D, Brandhorst H, Johnson PRV. Enzyme development for human islet isolation: five decades of progress or stagnation? *Rev Diabet Stud*. 2017;14(1):22-38.
9. Kin T, Johnson PR, Shapiro AM, Lakey JR. Factors influencing the collagenase digestion phase of human islet isolation. *Transplantation*. 2007;83(1):7-12.
10. Balamurugan AN, Breite AG, Anazawa T, et al. Successful human islet isolation and transplantation indicating the importance of class 1 collagenase and collagen degradation activity assay. *Transplantation*. 2010;89(8):954-961.
11. Al-Abdullah IH, Bagyan K, Bilbao S, Qi M, Kalkum M. Fluorogenic peptide substrate for quantification of bacterial enzyme activities. *Sci Rep*. 2017;7:44321.
12. Mitchell WM. The contamination of purified collagenase preparations by clostridiopeptidase B (clostripain): the potential effect of studies utilizing collagenase as a highly specific structural tool. *Johns Hopkins Med J*. 1970;127(4):192-198.
13. Brandhorst H, Friberg A, Andersson HH, et al. The importance of tryptic-like activity in purified enzyme blends for efficient islet isolation. *Transplantation*. 2009;87(3):370-375.
14. Dendo M, Maeda H, Yamagata Y, et al. Synergistic effect of neutral protease and clostripain on rat pancreatic islet isolation. *Transplantation*. 2015;99(7):1349-1355.
15. McCarthy RC, Breite AG, Green ML, Dwulet FE. Tissue dissociation enzymes for isolating human islets for transplantation: factors to consider in setting enzyme acceptance criteria. *Transplantation*. 2011;91(2):137-145.
16. Kin T, O'Gorman D, Senior P, Shapiro AM. Experience of islet isolation without neutral protease supplementation. *Islets*. 2010;2(5):278-282.
17. Friberg AS, Korsgren O, Hellgren M. A vast amount of enzyme activity fails to be absorbed within the human pancreas: implications for cost-effective islet isolation procedures. *Transplantation*. 2013;95(6):e36-e38.
18. Cross SE, Hughes SJ, Partridge CJ, Clark A, Gray DW, Johnson PR. Collagenase penetrates human pancreatic islets following standard intraductal administration. *Transplantation*. 2008;86(7):907-911.
19. Balamurugan AN, Green ML, Breite AG, et al. Identifying effective enzyme activity targets for recombinant class I and class II collagenase for successful human islet isolation. *Transplant Direct*. 2016;2(1):e54.
20. Balamurugan AN, Loganathan G, Bellin MD, et al. A new enzyme mixture to increase the yield and transplant rate of autologous and allogeneic human islet products. *Transplantation*. 2012;93(7):693-702.
21. Ricordi C, Lacy PE, Finke EH, Olack BJ, Scharp DW. Automated method for isolation of human pancreatic islets. *Diabetes*. 1988;37(4):413-420.
22. Balamurugan AN, Chang Y, Fung JJ, Trucco M, Bottino R. Flexible management of enzymatic digestion improves human islet isolation outcome from sub-optimal donor pancreata. *Am J Transplant*. 2003;3(9):1135-1142.
23. Boyd V, Cholewa OM, Papas KK. Limitations in the use of fluorescein diacetate/propidium iodide (FDA/PI) and cell permeable nucleic acid stains for viability measurements of isolated islets of Langerhans. *Curr Trends Biotechnol Pharm*. 2008;2(2):66-84.
24. Williams SK, Touroo JS, Church KH, Hoying JB. Encapsulation of adipose stromal vascular fraction cells in alginate hydrogel spheroids using a direct-write three-dimensional printing system. *Biores Open Access*. 2013;2(6):448-454.
25. Loganathan G, Dawra RK, Pugazhenthi S, et al. Insulin degradation by acinar cell proteases creates a dysfunctional environment for human islets before/after transplantation: benefits of alpha-1 antitrypsin treatment. *Transplantation*. 2011;92(11):1222-1230.
26. Balamurugan AN, He J, Guo F, et al. Harmful delayed effects of exogenous isolation enzymes on isolated human islets: relevance to clinical transplantation. *Am J Transplant*. 2005;5(11):2671-2681.
27. Loganathan G, Graham ML, Radosevich DM, et al. Factors affecting transplant outcomes in diabetic nude mice receiving human, porcine, and nonhuman primate islets: analysis of 335 transplantations. *Transplantation*. 2013;95(12):1439-1447.
28. Bertera S, Geng X, Tawadrous Z, et al. Body window-enabled in vivo multicolor imaging of transplanted mouse islets expressing an insulin-timer fusion protein. *Biotechniques*. 2003;35(4):718-722.
29. Brandhorst H, Brandhorst D, Hesse F, et al. Successful human islet isolation utilizing recombinant collagenase. *Diabetes*. 2003;52(5):1143-1146.
30. Qi M, Valiente L, McFadden B, et al. The choice of enzyme for human pancreas digestion is a critical factor for increasing the success of islet isolation. *Transplant Direct*. 2015;1(4):1-9.

How to cite this article: Loganathan G, Subhashree V, Breite AG, et al. Beneficial effect of recombinant rC1rC2 collagenases on human islet function: Efficacy of low-dose enzymes on pancreas digestion and yield. *Am J Transplant*. 2018;18:478–485. https://doi.org/10.1111/ajt.14542