Pancreatic islet isolation variables in non-human primates (rhesus macaques)

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Abstract

Background Non-human primates (NHPs) are important preclinical models for pancreatic islet transplantation (PIT) because of their close phylogenetic and immunological relationship with humans. However, low availability of NHP tissue, long learning curves and prohibitive expenses constrain the consistency of isolated NHP islets for PIT studies. To advance preclinical studies, we attempted to identify key variables that consistently influence the quantity and quality of NHP islets.

Methods Seventy-two consecutive pancreatic islet isolations from rhesus macaques were reviewed retrospectively. A scaled down, semi-automated islet isolation method was used, and monkeys with streptozotocin-induced diabetes, weighing 3–7 kg, served as recipients for allotransplantation. We analysed the effects of 22 independent variables grouped as donor factors, surgical factors and isolation technique factors. Islet yields, success of isolation and transplantation results were used as quantitative and qualitative outcomes.

Results In the multivariate analysis, variables that significantly affected islet yield were the type of monkey, pancreas

preservation, enzyme lot and volume of enzyme delivered. The variables associated with successful isolation were the enzyme lot and volume delivered. The transplant result was correlated with pancreas preservation, enzyme lot, endotoxin levels and COBE collection method.

Conclusions Islet quantity and quality are highly variable between isolations. The data reviewed suggest that future NHP isolations should use bilayer preservation, infuse more than 80 ml of Liberase into the pancreas, collect non-fractionated tissue from the COBE, and strictly monitor for infection.

Keywords Islet isolation · Islet transplantation · Non-human primates

Abbreviations

IEQ islet equivalent
NHP non-human primate
PIT pancreatic islet transplantation
UW University of Wisconsin solution

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Introduction

Pancreatic islet transplantation (PIT) can be effective in the treatment of type 1 diabetes in humans [1, 2]. However, major obstacles to be overcome before PIT becomes a routine clinical therapeutic method include the need for multiple donors, various transplantation procedures and chronic immunosuppression. Consequently, strategies to solve these problems must be developed first in the preclinical setting [3]. Non-human primates (NHPs) are an important preclinical animal model because of their close phylogenetic and immunological relationship with humans. That is why many centres perform preclinical PIT in NHPs [3–7], but consistent

isolation of pancreatic islets from NHPs remains difficult. The difficulties relate to low availability of primate tissue, a long learning curve, high costs and limited knowledge of parameters that have a major impact on the results of NHP islet isolation. These considerations have delayed large-scale NHP studies [8].

Many factors affect the quantity and quality of isolated pancreatic islets. Among them, donor characteristics, organ procurement, pancreas condition, and digestion and purification procedures are the most important variables influencing islet yield, viability and functionality [9]. To improve the efficacy of islet transplantation, further examination and enhancement of current islet isolation methods are essential. The identification of additional important factors will help modify protocols, improve transplantation results and speed up the transition into clinical practice. The purpose of this study was to review our experience with PIT in NHPs and assess the variables that determine a successful outcome.

Methods

Animals Between 1999 and 2007 we performed 72 pancreatic islet isolations from rhesus macaques of Chinese ($n=21$) and Indian ($n=51$) origin (Covance Research Products, Alice, TX, USA). The average donor age was 5.4 ± 1.9 years and body weight 6.9 ± 2.6 kg. A total of 18 donor monkeys had a positive past medical history because they had been included in previous studies with different drug protocols or surgical procedures. Rhesus macaques weighing 3–7 kg served as recipients. Diabetes was induced with a single high dose (110–140 mg/kg) of streptozotocin administered i.v. The dose used was $\leq 1,200$ mg/m², which has been shown to induce stable diabetes safely in rhesus macaques [10]. All recipient–donor combinations underwent prospective molecular typing for MHC class I and II alleles and were selected to have multiple MHC mismatches as described previously [7]. Experiments were conducted under the standards set by the National Institutes of Health (USA) and were approved by the local Institutional Animal Care and Use Committee.

Organ procurement Briefly, the splenocolic and splenorenal ligaments were divided and the tail of the pancreas was completely mobilised from the major curvature of the stomach. After liberating the duodenum and pancreatic head from the retroperitoneum, we identified, ligated and transected the bile duct, the gastroduodenal junction and the proximal jejunum. The pancreatic area was packed with ice and approximately 750 ml of cold, filtered University of Wisconsin solution (UW) was infused through the abdominal aorta while the cava vein was transected for exsanguination. This reduced warm ischaemia time to almost zero. The pancreas was then separated from the duodenum, spleen and

surrounding tissues. The pancreatic duct was cannulated with a 24-gauge catheter, and accessory ducts were identified and cannulated or ligated depending on their diameter. Intra-operative findings or events that could affect the procurement were recorded. The harvested pancreas was preserved with cold UW ($n=64$) or the two-layer technique [11] ($n=8$). Rapid transfer to the laboratory for processing ensured an average cold ischaemia time of 25–30 min. The pancreas was weighed and assessed before Liberase infusion and placement in the digestion chamber. Pancreas quality was determined by subjective evaluation of its macroscopic characteristics (oedema, presence of fat, haemorrhages and burns).

Islet isolation Donor islets were prepared by the semi-automated Ricordi technique [12] with minor modifications as previously described [7]. Reconstituted Liberase HI (0.47 mg/ml) (Roche, Indianapolis, IN, USA) with ($n=44$) or without ($n=28$) DNAase (0.6 mg/ml DNAse I; Sigma, St Louis, MO, USA), was injected into the pancreas through the cannulated duct (ductal infusion). Liberase solution was infused with a peristaltic pump set at 44 ml/min in 17 cases or manually in 53 cases. Enzyme was injected directly into poorly distended areas (interstitial infusion). The pancreas was placed into the digestion chamber filled with enzyme and connected to a circuit and pump for recirculation. Digestion was performed at 30–35°C with gentle agitation. The extent of digestion was tracked by regular sampling; digestion was stopped by adding 150 ml of cold heat-inactivated fetal bovine serum (FBS; Sigma Aldrich, St Louis, MO, USA) when three 150 μ m dithiazone-stained acinar-free islets were observed in the sample. Average digestion time was 15.5 ± 5.45 min. The digestate was diluted with cold RPMI 1640 (750 ml; Mediatech, Herndon, VA, USA), HEPES solution 1 mol/l (25 ml; Sigma Aldrich) and FBS solution (250 ml), with ($n=29$) or without ($n=26$) Pefabloc (4 mmol/l; Roche, Indianapolis, IN, USA), and centrifuged at 8°C, 1,000 rpm for 5 min. After three washes, all pellets were combined and put in Eurocollins (Mediatech, Indianapolis, IN, USA) containing 20% FBS. The digestate was purified in a COBE blood processor (COBE Laboratories, Lakewood, CO, USA) on a discontinuous Euroficoll gradient (Mediatech, Indianapolis, IN, USA): 100 ml at a density of 1.037, 125 ml at a density of 1.096, and 250 ml at a density of 1.108. Effluent was collected from the COBE in three fractions ($n=19$) or more than three fractions ($n=53$). After two washes in cold CRML-1060 (Sigma Aldrich), FBS (100 ml/l), ciprofloxacin (1.0 μ g/ml), insulin–transferrin–selenium (10 ml/l; Mediatech, Herndon, VA, USA), vitamin E (10 μ mol/l) and ZnSO₄ (16.7 μ mol/l) were added. The isolated islets were cultured in Miami Medium #1A (900 ml; Mediatech, Indianapolis, IN, USA), FBS (100 ml), ciprofloxacin (1.0 μ g/ml) and reduced glutathione (10 mg/l) at 37°C and 95% O₂.

Islet characterisation Islet preparations were evaluated for yield, size, morphology, viability and purity on the day of isolation before transplantation. Isolated islets were counted as described previously [13], adjusted to ~500 islet equivalents (IEQs) normalised to 150 µm diameter (IEQ150) per millilitre, and by the IEQ/g of pancreas weight before digestion. The residual undigested pancreas weight was not used for this calculation. Islet viability was examined by ethidium bromide/acridine orange staining and expressed as the ratio between green (living) cells and orange (dead) cells in each islet examined [14]. Purity was determined as the ratio between islets (stained with dithiazone) and exocrine tissue (unstained by dithiazone) [14]. Routine static incubation for insulin release by glucose stimulation was not used [14], nor was in vivo transplantation of NHP islets into chemically diabetic immunodeficient mice [4].

Isolations were classified as successful ($n=43$) or non-successful ($n=29$). A successful isolation was defined as any isolation that met the following criteria: (1) islet yield >8000 IEQ/g of pancreas; (2) adequate islet morphology evaluated independently by two observers. Three 200 µl samples of the islet suspension were taken after purification and examined with an inverted phase-contrast microscope with a calibrated grid in the eyepiece (the isolation outcome evaluated the shape, border, integrity, stain and diameter of the islets, giving an overall morphology rating of good or bad [11]; thus, no graded scale was used); (3) viability and purity both >90% [14]; and (4) sterility (negative Gram stain and conventional 24 h culture) or endotoxin level <1.0 EU/ml (QCL-1000 Chromogenic LAL Endpoint Assay; Cambrex, Charles City, IO, USA) [15, 16].

We combined islet yield and islet quality into one broad definition, and in this way we were able to measure both elements of the isolation outcome. Measuring islet quality is not easy and is very subjective, but it is used frequently in the islet isolation literature with wide acceptance of its advantages and disadvantages. In our experience these criteria differentiated the results of the isolations and helped in the transplantation decision for the islets.

Pancreatic islet transplantation A total of 48 pancreatic islet allotransplants were completed. One donor was used for one recipient in 25 cases, one donor was used for two recipients in 11 cases, and two donors were used for one recipient in one case. At laparotomy, islet infusion was performed by gravity flow through a no. 8 French catheter placed in the portal vein [7]. A mean of 22.754 ± 8.401 IEQ/kg per recipient was infused. No significant haemodynamic or portal vein pressure changes were observed. Transplantation was considered successful when primary non-function did not occur. Graft function was measured using blood glucose levels and insulin requirements. Only the first 2 weeks after transplantation were considered for this analysis because of

differences in the immunosuppressive protocols used. A transplant was considered to have full function when blood glucose levels were below 11.1 mmol/l and no exogenous insulin was needed. Islet allografts were considered to have partial function when blood glucose levels were maintained with more than 50% reduction of preoperative exogenous insulin requirements. Acute rejection was considered to have occurred when normal postoperative function was achieved but there was a slow return to preoperative blood glucose levels and exogenous insulin requirements.

Statistical analysis Outcomes that were examined in this study were: (1) islet yield (IEQ); (2) successful versus non-successful isolation; and (3) transplantation outcome. The variables analysed for association with these outcomes were: (1) donor variables (age, sex, weight, medical history, type of monkey); (2) procurement variables (surgery findings, pancreas weight, pancreas quality, pancreas preservation); and (3) isolation variables (enzyme lot, pancreas infusion method, volume delivered, ductal or interstitial infusion, time and temperature of exposure to the enzyme, use of DNAase or Pefabloc, COBE delivery). A complete definition of each variable is given in Table 1.

The mean and standard deviation were used to describe continuous variables and frequencies were used to summarise categorical variables. First, a univariate analysis using ANOVA was done to determine which variables affected islet yield and transplantation outcomes. Multiple regression was then performed to explore the effects of all the variables on islet yield and obtain a regression equation that described the prediction of the criterion variable. Next, multiple logistic regression was used to evaluate the prognostic abilities of the variables with respect to isolation success and transplantation outcome, and to estimate the OR with the 95% confidence interval (CI). The OR compared the relative odds of the outcome condition of the exposed and non-exposed groups, and the 95% CI is the range or variance where 95% of the OR results may be. An OR with a 95% CI below or above 1 was considered to be a protective or favouring factor. Comparison between successful and non-successful isolation groups was performed using the Mann–Whitney and Fisher tests for continuous and categorical variables, respectively. All comparisons were two-tailed and $p \leq 0.05$ was considered statistically significant. All calculations were performed using SPSS for Windows, Version 15.0 (SPSS, Chicago, IL, USA).

Results

Islet yield The number of isolated islets varied greatly between different isolations ($78,663 \pm 42,570$ IEQ; $8,920 \pm$

Table 1 Description of the variables studied

Variable	Description
Donor	
Age	Age of donor at the time of isolation (years)
Sex	Donor sex (male or female)
Donor weight	Weight of donor at the time of isolation (kg)
Past medical history	Donor comorbidities that may influence isolation. Examples: previous surgeries or medications (positive or negative)
Type of monkey	Monkey origin (Indian or Chinese)
Procurement	
Surgery findings	Important deleterious events that occurred during pancreas harvest. Examples: multiple adhesions, intraoperative bleeding, pancreatic lesion (positive or negative)
Pancreas weight	Weight (g) of pancreas immediately after harvest and cleaning of the organ
Pancreas quality	Subjective evaluation of pancreas macroscopic characteristics before isolation. A blind evaluator considered oedema, presence of fat, haemorrhage, burns and overall pancreas aspect (adequate and inadequate). No special scoring system was used
Pancreas preservation	Technique used to preserve the pancreas immediately after pancreas harvest (two-layer technique or UW)
Isolation	
Enzyme lot	Lot number of the enzyme used during digestion. Lots were ordered chronologically and efficiency was evaluated by determining the numbers (%) of lots that allowed successful and non-successful isolation
Infusion method	Method used for infusing the enzyme into the pancreas (by hand or using a pump)
Volume delivered	Total volume (ml) of enzyme infused into the pancreas
Ductal infusion	Average percentage of enzyme volume infused into the pancreas through the pancreatic duct for digestion
Interstitial infusion	Average percentage of enzyme volume infused into the pancreas interstitially for digestion
Temperature	Mid-range temperature (°C) recorded during the process of digestion in the Ricordi chamber
Digestion time	Time (min) during which the pancreas was exposed to the enzyme in the digestion chamber
DNAase	Additive to the solutions used during the isolation process (used and not used)
Pefabloc	Additive to the solutions used during the isolation process (used and not used)
COBE delivery	Method of collecting the samples after COBE purification (<3 or >3 fractions)
Endotoxin UW	Level of endotoxin in the UW transport solution of the pancreas after harvest (EU/ml)
Endotoxin digest	Level of endotoxin in the digest (EU/ml)
Culture time	Days of islet culture before transplant

5,062 IEQ/g pancreas). In the univariate analysis, the type of monkey, pancreas quality, pancreas preservation, enzyme lot, pump infusion method, volume delivered, use of DNAase and collection of three or fewer fractions after COBE delivery were significantly associated with superior islet yield (Table 2). Univariate analysis revealed no significant relationship between endotoxin levels in UW or Liberase and islet yield. Although there was wide variation in pancreas weight, this variable was not significantly associated with islet yield in the univariate or multivariate analysis, and similar results were observed when the analysis was performed using IEQ/g of pancreas instead of IEQ alone. In the multivariate analysis, the type of monkey ($p=0.0178$), pancreas preservation ($p=0.0484$), enzyme lot ($p=0.0056$) and volume delivered ($p=0.0490$) showed a statistically significant contribution to the variability of islet yield. Pancreases from Chinese monkeys yielded about 28,870 IEQs more than those from Indian monkeys. Two-layer pancreas preservation after procurement produced 33,845 IEQs more than UW preservation. Each millilitre of extra

volume delivered into the pancreas increased islet yield by 116 IEQs. Islet yield decreased by 4,880 IEQs for each newer enzyme vial. Thus, there was a significant negative correlation between enzyme lot and islet yield (Spearman $r=-0.2551$, $p=0.033$). Analysis of the enzyme information provided by the manufacturer also showed a significant negative correlation between the enzyme lot and collagenase concentration (Spearman $r=-0.3822$, $p=0.003$) and a positive correlation between enzyme lot and endotoxin levels (Spearman $r=0.7646$, $p<0.001$). Therefore, islet yield diminished as the collagenase activity in the enzyme preparation decreased and endotoxin content increased.

Successful isolation As defined in the Methods, the successful islet isolation group showed higher islet yield, viability, purity and sterility than the non-successful islet isolation group (Table 3). Also, the successful isolation group resulted in a higher rate of transplantation. A total of 36 (84%) successful isolations were used for transplantation and only seven were not transplanted because of their use in

Table 2 Univariate analysis between isolation variables and islet yield and transplantation outcome

Variable	Number of monkeys	Characteristic	Islet yield	Transplant outcome
Donor variables				
Age (years)	72	5.4±1.9 (2–14)	0.454	0.735
Sex	72	Female, 6 (8%); male, 66 (92%)	0.255	0.729
Donor weight (kg)	65	6.9±2.6	0.508	0.057
Medical history	72	Positive, 18 (25%); negative, 54 (75%)	0.755	0.530
Type of monkey	72	Chinese, 21 (29%); Indian, 51 (71%)	0.047*	0.491
Procurement variables				
Surgery findings	72	Positive, 11 (15%); negative, 61 (85%)	0.257	0.328
Pancreas weight (g)	72	9.5±3.5 (1.8–19.6)	0.059	0.923
Pancreas quality	72	Adequate, 36 (50%); inadequate, 35 (50%)	0.001*	0.283
Pancreas preservation	72	UW, 64 (89%); two-layer, 8 (11%)	0.023*	0.042*
Isolation variables				
Enzyme lot	70	20 chronologically ordered lots were used	0.011*	0.042*
Infusion method	72	Hand, 53 (76%); pump, 17 (24%)	0.025*	0.855
Volume delivered (ml)	62	177.1±123.6 (50–380)	0.005*	0.486
Ductal infusion (%)	65	80.6%	0.650	0.135
Interstitial infusion (%)	65	19.4%	0.775	0.147
Mean temperature (°C)	66	33.6±1.2 (31.5–35.2)	0.645	0.507
Digestion time (min)	68	15.5±5.4 (5–26)	0.324	0.949
DNAase	72	No, 28 (39%); yes, 44 (61%)	0.044*	0.296
Pefabloc	55	No, 26 (47%); yes, 29 (53%)	0.499	0.831
COBE delivery	72	<3 fractions, 53 (74%); >3 fractions, 19 (26%)	0.000*	0.053
Endotoxin UW (EU/ml)	46	0.3±0.3 (0.01–1.5)	0.698	0.051
Endotoxin digest (EU/ml)	46	0.6±0.003 (0.01–1.5)	0.373	0.000*
Culture time (days)	43	0 day, 5; 1 day, 30; 2 days, 8	NA	0.356
Viability (%)	62	93.8±0.8	0.221	0.370
Purity (%)	62	94.7±0.6	0.207	0.513

NA, not applicable

* $p<0.05$

parallel studies. Only 2 of 29 (7%) non-successful isolations were combined and transplanted into one recipient. There were no differences in donor and procurement variables between the groups. Among isolation variables analysed, only volume delivered, infusion method and enzyme lot showed statistical differences. More than 80 ml of volume delivered into the pancreas was predictive of a successful isolation (OR 5.216, 95% CI 1.746–15.586, $p=0.004$). There was no association between enzyme volume delivered and pancreas quality (Spearman $r=-0.04326$, $p=0.736$). The use of pump infusion showed borderline significance as a factor favouring successful isolation (OR 4.167, 95% CI 1.071–16.216, $p=0.046$). On the contrary, the use of newer enzyme lots was a predisposing factor for poor isolation (OR 0.3846, 95% CI 0.1426–0.9123, $p=0.043$).

Initial transplantation outcome Four recipient monkeys were excluded from the islet in vivo functional analysis because they presented severe perioperative complications (bleeding, infection and embolism) and satisfactory follow-up was not possible. The transplants failed to restore euglycaemia

and to decrease the insulin requirement to >50% of pre-operative insulin in three cases (7%) because of primary non-function. Overall pancreatic islet allotransplantation success for the first two postoperative weeks was 93%. During this time, 12 monkeys (27%) were considered to have fully functional allografts, 21 (48%) had allografts with partial function and eight (18%) experienced acute rejection. There was a significant correlation between islet yield and graft function (Spearman $r=0.4002$, $p=0.0002$) and between successful isolation and graft function (Spearman $r=0.6769$, $p<0.0001$). In the univariate analysis, pancreas preservation, enzyme lot and endotoxin level in the digest were the only variables statistically associated with the transplantation outcome (Table 2). In the multivariate analysis, pancreas preservation ($p=0.0447$), enzyme lot ($p=0.0075$), endotoxin in the digest ($p<0.0001$) and COBE collection ($p=0.0166$) were independent variables contributing to the variability of the transplantation outcome. The chances of having a functional transplant increased by 25% with the two-layer preservation technique, decreased by 36% with higher endotoxin levels in the digest, and increased by up to 44% with a less fractioned COBE collection.

Table 3 Analysis of successful isolations

Variable	Successful isolation (<i>n</i> =43)	Non-successful isolation (<i>n</i> =29)	<i>p</i> value
Criterion			
Islet yield (IEQ)	95829±38904	47839±30268	<0.001*
Viability (%)	95.7±2.5	88.9±5.9	<0.001*
Purity (%)	95.6±3.4	89.7±4.9	0.001*
Endotoxin UW (EU/ml)	0.28±0.25	0.64±0.57	0.022*
Endotoxin digest (EU/ml)	0.56±0.57	0.86±0.64	0.026*
Transplantation	Yes, 36; no, 7*	Yes, 2*; no, 27	<0.001*
Donor variables			
Age (years)	5.2±1.7	5.9±2.5	0.457
Sex	Male, 39; female, 4	Male, 27; female, 2	0.638
Donor weight (kg)	6.8±2.0	7.5±2.8	0.368
Medical history	No, 33; yes, 10	No, 20; yes, 9	0.420
Type of monkey	Indian, 29; Chinese, 14	Indian, 22; Chinese, 7	0.613
Procurement			
Surgery findings	Positive, 5; negative, 38	Positive, 23; negative, 6	0.885
Pancreas weight	5.3±1.7	5.9±2.5	0.457
Pancreas quality	Adequate, 21; inadequate, 22	Adequate, 15; inadequate, 14	1.000
Pancreas preservation	UW, 36; two-layer, 7	UW, 28; two-layer, 1	0.126
Isolation			
Volume delivered	196.7±129.5	127.3±98.0	0.039*
Ductal infusion	81.5% (0–100)	81.1% (1–100)	0.894
Interstitial infusion	19.4% (1–100)	19.4% (1–100)	0.984
Infusion method	Pump, 14; hand, 28	Pump, 3; hand, 25	0.046*
Enzyme lot	66.9%	33.1%	<0.001*
Digestion time (min)	13.9±5.1	15.7±6.1	0.221
Mean temperature (°C)	33.4±1.3	33.5±1.2	0.975
DNAase	Yes, 26; no, 18	Yes, 17; no, 11	1.000
Pefabloc	Yes, 19; no, 13	Yes, 10; no, 13	0.285
COBE delivery	Complete, 34; fractioned, 9	Complete, 19; fractioned, 10	0.420

**p*<0.05

Discussion

Insulin independence after PIT is not yet a consistent achievement in many centres. Several issues in donor standardisation, organ procurement, islet isolation, engraftment and the prevention of allograft rejection still need to be addressed. In order to refine islet transplantation procedures, several methods have been tried in various animal models [17, 18]. Primates are a promising preclinical model for islet transplantation research. Unfortunately there have only been a few reports analysing the results of large-scale NHP islet transplantation [4, 18, 19]. Poor availability of NHP tissue, long learning curves and prohibitive expenses constrain the consistency of isolated NHP islets for PIT studies, which in turn impedes large-scale cooperative studies to improve islet allotransplant results. To advance preclinical studies, we attempted to identify key variables that consistently influence the quantity and quality of NHP islets.

The present work highlights the large number of variables that can influence the outcome of islet isolation and transplant. Although some of the associations found in

the univariate analysis may be secondary to confounding variables or statistical errors, the multivariate analysis did distinguish those associations that independently and significantly influenced outcome. The retrospective nature of this study, the long period of time over which it was performed, some missing data and unbalanced frequencies must be considered as possible flaws. Some variables are difficult to control, especially in a long-term retrospective study such as that presented here. Personnel, technology and methods also changed with time. We caution readers to take these factors into consideration when evaluating our observations and to challenge our findings in prospective studies, which will more accurately allow the many variables to be deconvoluted. The best efforts were made to analyse the information using three well-defined outcomes to evaluate islet quantity (islet yield) and quality (successful isolation and transplantation results), allowing us to obtain valuable information to improve PIT. A good islet count does not necessarily mean that the isolation was successful and the islets are ready to be transplanted because a successful isolation combines quantitative and qualitative

characteristics of the islets. The major parameters favouring successful NHP islet isolation and transplantation were the type of monkey, the two-layer preservation technique, volume of enzyme delivered, enzyme lot, endotoxin level and the type of COBE collection.

The type of monkey was the only donor variable that significantly influenced the islet yield but did not affect the success of isolation and the transplantation outcome. There are many differences in pancreatic isolation results between different mammals, NHPs [4] and humans [20], but we found significant intraspecies differences in favour of the Chinese rhesus macaques as the only significant donor variable. Many variables, such as donor age, BMI, cause of death and the use of vasopressors, considerably influence human PIT [9, 21]. This individual variability seen in humans is not observed in the experimental setting, where more standardised donors are used, highlighting the importance of donor standardisation in clinical practice [22, 23]. Therefore, not all the results obtained in NHP PIT may be translated to humans and one should be cautious in generalizing these outcomes. For example, the human pancreas is often fibrotic, and it may therefore be more difficult to infuse large amounts of collagenase solution and ischaemia times (warm and cold) are longer than in the experimental model. Although these differences are important, knowing their performance in different situations and animal models is useful and may have some applications in humans. Our deductions related to successful islet isolation in NHP were derived under relatively ideal conditions, but are probably more relevant to humans than to rodent studies. Translation of these NHP findings to the human cadaveric donor situation is unlikely to be straightforward for the reasons stated above. However, the basic tenets should endure because the process of obtaining islets is similar between species and certain fundamental principles are universal to any islet isolation process.

The two-layer preservation method was another factor favouring higher islet yields and better transplantation results. It has been demonstrated that the two-layer technique oxygenates and activates the metabolism of the pancreas, leading to resuscitation of pancreases with extended preservation time and/or ischaemically damaged tissue [11, 24]. This might explain the higher islet counts and the improved success rate of transplantation compared with the UW preservation method, although the two-layer technique was performed in only eight cases and cold ischaemia times were uniformly short. The small sample size and short ischaemia time could reduce the biological/clinical significance of our observation, but the statistics support the conclusion. Possibly there is a difference in sensitivity of islets taken from healthy normal NHP vs islets from brain-dead humans with respect to the two-layer preservation method variable. It is not clear in the literature whether the

two-layer technique should be used only when the condition of the pancreas is bad or in all procedures [25, 26]. Having analysed these data and the literature, we believe it is reasonable to consider this technique as part of the isolation process, but further studies are required to endorse these observations.

The enzyme lot used in the digestion phase was a major factor influencing islet yield and successful isolation. Enzyme lot number was an independent variable with a significant negative correlation with islet yield (Spearman $r=-0.3707$, $p=0.0112$). Islet yield decreased significantly with newer lots but it was impossible to perform a more detailed investigation because of incomplete data. Despite this observation, the enzyme lot had a strong association with islet yield and isolation success but was more weakly related to transplantation outcome. Assessment of the manufacturer's information on each lot revealed that collagenase content decreased but endotoxin increased in the Liberase during the study period. These data suggest that enzyme quality declined over time, although some other independent factors that were not analysed, such as changes in personnel, the surgeon's skill and protocol modifications, should also be considered. These findings are consistent with recent reports that the efficiency of Liberase HI declines over time in human pancreas processing [27]. We can extrapolate from this that enzyme lots with higher collagenase activity and lower endotoxin content will be advantageous for NHP islet isolation. The duration of pancreas exposure to enzyme demonstrated no association, but the enzyme volume delivered and the method of infusion affected islet yield and isolation success. Complete separation of the exocrine and endocrine pancreas should be a rapid, accurate and uniform procedure. To accomplish this, enzymatic digestion should occur simultaneously from outside and inside the pancreas, to reach every corner of the organ in a homogeneous way [28]. This might be the reason why the volume of enzyme injected into the pancreas before digestion was such a significant factor in the multivariate analysis. The results obtained with the infusion method may be more controversial. Pump infusion demonstrated only a borderline significant association with isolation outcome; this could be due to the comparison of time series and lack of randomisation. Well conducted studies should be performed before arriving at definitive conclusions regarding the superiority of the manual or pump infusion method of introducing enzyme into the pancreas.

Sterility of isolated islets is critical for successful isolation and transplantation. Microbiological assessment and endotoxin levels were used to monitor sterility. Although non-specific, the endotoxin level was an independent factor associated with islet transplantation success. Notably, endotoxin contamination in UW and Liberase did not influence islet yield per se. This discrepancy between the effects of

endotoxin levels on islet yield and transplantation success is probably related to the brief exposure of beta cells to endotoxin at low temperature during the isolation process. In contrast, endotoxin contamination in vivo can stimulate production of proinflammatory cytokines that impair beta cell viability and function [15, 16, 29–32]. Endotoxin has been introduced into experimental models by direct contamination or indirectly via commercial products and monitoring and elimination should therefore be performed consistently [33]. Lipopolysaccharide can shift active immunity towards the priming of Th1 cells [34] by promoting activation and maturation of dendritic cells [35], which might be a confounding factor in in vitro and in vivo experimental models. In this way, lipopolysaccharide contamination might antagonise the effects of some immune-modulating agents used in induction protocols (e.g. 5-deoxyspergualin) with potential deleterious effects on tolerance induction. Therefore, lipopolysaccharide monitoring was considered an important part of immune testing in order to avoid misleading results and interpretations. Even though the endotoxin level was not associated with islet yield and isolation success, it was a significant independent factor for transplantation outcome, corroborating the above statements and the observations made by other investigators [15, 16, 29–32].

The method of collection after COBE purification was also an independent factor associated with transplantation success but it did not influence the islet yield and success of isolation. Complete (less fractioned) was superior to multi-fractioned collection in the multivariate analysis. Most experimental [4, 8, 19, 36] and clinical [9, 22, 37] studies on pancreatic isolation variables have focused on the type of gradient for islet purification and have found no differences between continuous and discontinuous methods. We used the discontinuous gradient technique, but the sample collecting method was modified during the study period. We hypothesised that collecting more fractions would make it easier to identify where the islets were but the results led us to reject our hypothesis. Since this variable has not been assessed before, it is difficult to find a good explanation for the outcomes. We believe that less manipulation, homogeneous dilutions and fast processing might be the reasons for this difference.

This study provides a guide for donor, isolation, purification and islet characterisation that may improve the efficiency and efficacy of islet transplantation in NHPs. With the caveats of a retrospective study and unbalanced frequencies of some variables, we have identified several key factors that affect the recovery of costly islets isolated from NHPs. However, we recognise that even under the most optimal conditions, islet quantity and quality will be highly variable between isolations. In this retrospective study, the data suggest that future NHP isolations should use bilayer preservation, infuse more than 80 ml of Liberase

into the pancreas, collect non-fractioned tissue from the COBE and strictly monitor for infection.

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Duality of interest J. M. Thomas is a Consultant for Genzyme Corporation. The remaining authors declare that there is no duality of interest associated with this manuscript.

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