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Risk factors for islet loss during culture prior to transplantation

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Summary

Culturing islets can add great flexibility to a clinical islet transplant program. However, a reduction in the islet mass has been frequently observed during culture and its degree varies. The aim of this study was to identify the risk factors associated with a significant islet loss during culture. One-hundred and four islet preparations cultured in an attempt to use for transplantation constituted this study. After culture for 20 h (median), islet yield significantly decreased from 363 309 ± 12 647 to 313 035 ± 10 862 islet equivalent yield (IE) (mean ± SE), accompanied by a reduction in packed tissue volume from 3.9 ± 0.1 to 3.0 ± 0.1 ml and islet index (IE/islet particle count) from 1.20 ± 0.04 to 1.05 ± 0.04. Culture did not markedly alter islet purity or percent of trapped islet. Morphology score and viability were significantly improved after culture. Of 104 islet preparations, 37 suffered a substantial islet loss (>20%) over culture. Factors significantly associated with risk of islet loss identified by univariate analysis were longer cold ischemia time, two-layer method (TLM) preservation, lower islet purity, and higher islet index. Multivariate analysis revealed that independent predictors of islet loss were higher islet index and the use of TLM. This study provides novel information on the link between donor- isolation factors and islet loss during culture.

Introduction

Although there is debate as to whether freshly isolated islets are superior to cultured islets in experimental transplantation [1,2], culturing human islets prior to transplantation provides many benefits to clinical islet allotransplantion. It provides a window of opportunity for additional quality control testing, initiating time dependent immunosuppressive protocols, and travel time for recipients living far away from transplant centers. For these reasons we have introduced culturing islets prior to transplantation, following the initial success of the Edmonton protocol, utilizing freshly prepared islets immediately after islet isolation [3]. One of the concerns with culturing islets has been the uncertainty of islet recovery. It is well documented that islets may deteriorate rapidly in culture. For example, Zhang *et al.* [4] reported that only 18% islet mass was recovered after 48-h culture. Bottino *et al.* [5] reported up to 80% reduction in DNA content in islet preparations after 24-h culture. These two reports were not dealing with islets for clinical transplantation; but major losses can still occur in clinical grade islet preparations [6,7]. Since the introduction of transplantation of cultured islets, we have encountered three of 102 (3%) preparations, which suffered a substantial loss of islet mass during the culture period necessitating the cancellation of planned transplantation. This fact prompted us to determine which factors, if any, are associated with islet loss during in vitro tissue culture prior to transplantation.

Materials and methods

Islet preparations from 125 deceased donor pancreases were cultured in an attempt to use them for transplantation between December 7, 2002 and March 5, 2007 at the

Clinical Islet Laboratory, University of Alberta. Twenty-one preparations were excluded from the study for the following reasons: islet assessment for postculture was not conducted because of short culture duration (4.2 ± 0.9 h) in 13 preparations; culture temperature at 37 °C was employed in eight, although all 21 preparations were used for transplantation. In the remaining 104 preparations constituting this study, five were not used for transplantation for the following reasons: a significant loss of islet mass after culture in three preparations (more than 50% loss), a positive cross match between a donor and a potential recipient in one, and lastly, fear of potential microbial contamination in the preparation that arose from an incidence of bowel perforation during organ recovery, in spite of negative gram staining and acceptable endotoxin level of the final product.

Islet isolation and culture

Islet isolations were performed using intra-ductal delivery of Liberase (Roche Applied Science, Indianapolis, IN, USA), followed by mechanical and enzymatic dissociation using the Ricordi chamber [8]. Islets were then purified with the use of continuous density gradients in a refrigerated cell processor (COBE 2991; COBE Laboratories Inc., Lakewood, CO, USA) [9]. Islet preparations were assessed by two independent observers in 93% of cases or by one investigator in 7%, for islet equivalent (IE) yield and purity with dithizone staining. Interobserver reliability for IE counting revealed a single measure intraclass correlation coefficient of 0.71 (95% CI, 0.60–0.80; Cronbach's Alpha = 0.84). Viability assessment was performed using SYTO® (Molecular Probes, Eugene, OR, USA) 13/ehidium bromide as described previously [10]. Packed tissue volume was measured after centrifugation at 300 *g* for 1 min in a 50-ml conical tube. After these initial assessments, the preparations were cultured in Connaught Medical Research Laboratory 1066 medium (CMRL; Mediatech, Herndon, VA, USA), supplemented with 0.625% human serum albumin (Canadian Blood Services, Ottawa, ON, Canada), 10 mM nicotinamide (Sigma, St Louis, MO, USA), 1% Glutamax (Gibco-Invitrogen, Carlsbad, CA, USA), 0.1% insulin-transferrin-selenium (Gibco-Invitrogen), 20 000 KIU/l Trasylol, 2% HEPES buffer, 5% sodium pyruvate, 4 mg/l Ciprofloxacin (Bayer Inc., Toronto, ON, Canada), 4.8 mg/l zinc sulfate ($ZnSO_4 \cdot 7H_2O$), and 236 mg/l calcium chloride. Culture was carried out on nontreated 150-mm dish (Fisher Scientific, Nepean, ON, Canada) at 22 °C under 95% air and 5% CO_2. At 3 h prior to scheduled transplantation time, islet preparation was recovered from dishes and subjected to assessments for IE, purity, packed tissue volume, viability, gram staining, and endotoxin level.

Donor- and isolation factors

The following donor factors were analyzed: age, cold ischemia time (CIT) defined as time from cross-clamp of aorta to initiation of islet isolation, organ preservation methods [oxygen supplementation through two-layer method (TLM) versus others], recovery team (local versus distant team), serum amylase levels (>140 vs. ≤140 U/l) and serum lipase levels (>120 vs. ≤120 U/l). Isolation factors included: digestion time, defined as time from initiation of pancreas dissociation in the Ricordi chamber to initiation of collecting tissue; collagenase activity, expressed as Wunsch units/g pancreas; neutral protease activity, expressed as Caseinase units/g pancreas; precultured viability; purity; islet morphology score; % trapped islet; the ratio of IE count to islet particle number (IPN) expressed as islet index; culture duration; and glucose stimulated insulin release, expressed as stimulation index [11]. We define major islet loss as a loss of more than 20% of IE over culture period.

Clinical assessment of islet post-transplant function

Islet transplantation was performed as previously reported [12]. Islet function was assessed 1 month after transplantation, by measuring HbA_{1c} and calculating daily insulin requirement per kilogram body weight.

Statistical analysis

Parametric data were reported as mean values ± SE while nonparametric data were reported as medians with 25% and 75% quartiles. Categorical variables were summarized with proportions. Pre- and postculture results were compared with paired *t*-test or Wilcoxon signed-ranks test. A univariate logistic regression model was used to examine the individual relationship between each variable and major islet loss. A multivariable logistic regression approach with best purposeful selection method was used to find the independent factors associated with outcome. All variables with $P < 0.2$ in univariate analyses were entered into the multiple logistic analysis. Levels of statistical significance were set at $P < 0.05$. Data analysis was performed with SPSS statistical software version 14.0 (SPSS, Inc., Chicago, IL, USA).

Results

Islet donors were characterized by average donor age of 44.9 ± 1.2 years with CIT of 7.7 ± 0.3 h. Thirty-five pancreases (33.7%) were supplied with oxygen through TLM during preservation. The remaining pancreases were simply preserved in University of Wisconsin solution

($n = 45$) or Histidine–Tryptophane–Ketoglutarate solution ($n = 24$). Thirty-three pancreases were procured by local team. Either amylase or lipase levels were elevated in 29.9% of donors. Pancreas was digested for 14.0 ± 0.4 min with enzyme blends consisting of 25.5 ± 0.6 Wunsch units/g pancreas for collagenase and 744 (662–890) Caseinase units/g pancreas for thermolysin.

After culture for 20 (16–30) h at a tissue volume density of 0.44 ± 0.02 ml/dish, islet yield significantly decreased from 363 309 ± 12 647 to 313 035 ± 10 862 IE ($P < 0.001$), accompanied by a reduction in packed tissue volume from 3.9 ± 0.1 to 3.0 ± 0.1 ml ($P < 0.001$). Postculture IPN was equivalent to preculture IPN. Consequently, islet index significantly decreased from 1.20 ± 0.04 to 1.05 ± 0.04 (Table 1). Detailed investigation of manual counting sheets revealed that larger islets (>200 μm) contributed to 55.9% of total IE before culture, while this figure decreased to 48.9% after culture. Culture did not markedly alter islet purity or the percentage of trapped islets. Islet morphology score and viability of islet preparations were significantly improved after culture.

Major islet losses occurred in 37 of 104 preparations (35.6%). Unadjusted odds ratios for the potential risk factors for islet loss are presented in Table 2. Factors significantly associated with islet loss included CIT, oxygen supplement during pancreas preservation, islet purity, and islet index. Longer CIT was significantly associated with major loss. Islets isolated from a TLM-preserved pancreas had a 2.8-fold increased risk in islet loss. Preparations with higher purity had a decreased risk of islet loss. Preparations with a higher islet index had a 4.6-fold increase in risk.

Donor- and islet-related factors associated univariately ($P < 0.2$) with the risk of islet loss were entered into a multivariate analysis using logistic regression. Islet index remained a significant predictor. Mode of pancreas preservation was also found to be independently associated with loss of islets. Islets isolated from a TLM-preserved pancreas had nearly a threefold increased risk compared with those isolated from a pancreas without oxygen supply. All other factors were not found to be independently associated with islet loss (Table 3).

There were 87 transplant procedures performed, comprising 99 islet preparations. We assessed the outcome of 87 procedures, of which 27 had suffered major islet loss over the culture period, while the remaining 60 infusions were derived from islets without major loss. For comparison of transplant outcome between these two groups, the procedures were stratified by the number of islet infusions

Table 2. Factors influencing the risk of islet loss in univariate analysis.

	Odds ratio	95% confidence interval		*P*-value
		Lower	Upper	
Donor age	1.01	0.98	1.05	0.39
Cold ischemia time	1.15	1.01	1.31	0.04
Oxygen supply during pancreas preservation	2.79	1.19	6.50	0.02
Local team procurement	0.87	0.36	2.07	0.74
Elevated serum amylase or lipase	0.94	0.36	2.46	0.90
Thermolysin dosage	1.00	1.00	1.00	0.53
Collagenase dosage	1.04	0.97	1.12	0.25
Digestion time	1.09	0.98	1.22	0.11
Islet purity	0.97	0.94	1.00	0.04
Islet index	4.62	1.47	14.51	0.01
Morphology score	0.96	0.75	1.23	0.77
Trapped islet	1.00	0.98	1.02	0.93
Viability	0.99	0.96	1.03	0.58
Culture duration	1.01	0.98	1.04	0.48
Stimulation index	1.10	0.92	1.31	0.31

Table 3. Multivariate prediction of risk of islet loss.

	Adjusted odds ratio	95% confidence interval		*P*-value
		Lower	Upper	
Islet index	4.91	1.51	15.94	0.01
Oxygen supply during pancreas preservation	2.96	1.22	7.18	0.02

Table 1. Changes in islet-related parameters during culture in 104 preparations.

	Preculture	Postculture	*P*-value
Islet yield (IE)	363 309 ± 12 647	313 035 ± 10 862	<0.001
Islet particle number	314 409 ± 10 552	316 103 ± 11 356	0.826
Islet index	1.20 ± 0.04	1.05 ± 0.04	<0.001
Viability (%)	87 (80–92)	90 (84–93)	0.020
Purity (%)	63.8 ± 1.5	65.8 ± 1.8	0.104
Morphology score	6.5 ± 0.2	7.0 ± 0.1	<0.001
Trapped islet (%)	7.5 (2.5–17.0)	5 (0.13–11.9)	0.068
Packed tissue volume (ml)	3.9 ± 0.1	3.0 ± 0.1	<0.001

Table 4. Functional performance of islet following transplantation.

	≤20% loss	>20% loss	*P*-value
1st infusion	*n* = 25	*n* = 17	
Islet mass infused (IE/kg)	5641 ± 243	5894 ± 320	0.53
Insulin usage before infusion (U/kg/day)	0.57 ± 0.04	0.58 ± 0.04	0.78
Insulin usage after infusion (U/kg/day)	0.22 ± 0.03	0.24 ± 0.05	0.62
Percent decrease in insulin (%)	62.8 ± 4.3	61.1 ± 5.3	0.81
HbA_{1c} before infusion (%)	8.3 ± 0.3	7.8 ± 0.3	0.35
HbA_{1c} after infusion (%)	7.5 ± 0.3	7.4 ± 0.3	0.77
Subsequent infusion*	*n* = 35	*n* = 10	
Islet mass infused (IE/kg)	5670 ± 229	4896 ± 308	0.10
Insulin usage before infusion (U/kg/day)	0.34 ± 0.03	0.39 ± 0.05	0.43
Insulin usage after infusion (U/kg/day)	0.14 ± 0.03	0.19 ± 0.06	0.48
Percent decrease in insulin (%)	63.5 ± 6.4	55.6 ± 9.6	0.55
HbA_{1c} before infusion (%)	6.6 ± 0.2	6.5 ± 0.3	0.88
HbA_{1c} after infusion (%)	6.1 ± 0.1	6.3 ± 0.1	0.24

*Including five and two cases of third and fourth infusions, respectively.

the patients had received. Islet mass transplanted per procedure was similar between the two groups; therefore, outcomes of transplant procedures were also similar as assessed by insulin reduction and HbA_{1c} level (Table 4).

Discussion

There has been substantial research attempting to optimize culture conditions for human islets [13–18]. Even in culture conditions optimized for cell recovery, there is a loss of islets and its degree varies [4–7]. To date, no study has adequately documented the relative influence of donor factors and isolation parameters on the loss of islet mass during the pretransplant culture period. We report some significant factors, as well as important trends that could be useful in the management of transplantation of cultured islets.

Several mechanisms responsible for a decrease in IE over culture should be considered. There is an inherent loss accompanying seeding and collecting procedures, but probably it would be negligible. Microbial contamination during culture could be a potential reason for significant loss of cells. In the field of cornea transplantation, contamination during culture is often blamed in relation to cell loss [19], whereas the incidence is considerably low during short-term islet culture [20,21]. In fact, all preparations in this study met product release criteria in terms of sterility and microbiological contamination was therefore not the underlying factor. A weak staining of islets with dithizone as a result of a reduction in insulin reserves may result in underscoring during counting. However, earlier studies indicate that beta cells are not likely to be degranulated after short-term periods of culture [22] and lower culture temperature [23]. Fragmentation of islets during culture leads to underestimation of IE; the current protocol for counting IE does not take into account small islets with less than 50 μm in diameter. Disintegration of islet mantle region during culture, as demonstrated in rodents [24], results in a decrease in islet size. Also, cell lysis during culture would be one of the important mechanisms. Islets are subjected to numerous type of stress induced by nonphysiologic stimuli during organ preservation and islet isolation. Disruption of the interaction between islets and peri-insular extracellular matrix during islet isolation (the so-called 'anoikis') compromises islet cell survival [25–28]. Islets are further exposed to metabolic changes in the culture medium, nutrient deprivation, proinflammatory molecules released by the islets themselves, and harmful enzymes released by acinar tissue [29,30], leading to islet cell death and lysis.

The most significant risk factor for loss of islet mass from both univariate and multivariate analyses was islet index: a higher islet index at preculture resulted in a greater islet loss. A higher islet index simply means that larger islets contribute more to total IE and islet index per se does not indicate anything about islet fragmentation. However, by looking a dynamic change in islet index, some information of islet fragmentation can be obtained. In fact, we observed a significant decrease in islet index postculture compared to preculture. This clearly indicates that islet fragmentation occurred during culture, resulting in a significant loss of total IE, particularly in a preparation having a higher proportion of larger islets. Because the central core of larger islets is prone to hypoxia in a nonvascularized environment, one can speculate that this could lead to cell death and a loss of cell junctions in the central area of larger islets, thereby causing fragmentation. Notably there is a growing body of literature reporting inferiority of larger islets in terms of function, survival, and graft performance [31,32].

It was somewhat surprising that we observed a significant association of TLM with islet loss. TLM has been

advocated as a superior preservation technique for increasing the success of islet isolation [33–35]. Recent large-scale studies are, however, questioning whether or not TLM provides benefit in clinical islet isolation and transplantation [36,37]. The decision as to whether or not to use TLM was based on the availability of our staff and a collaborator in a distant center. Although CIT was significantly longer in the TLM-preserved pancreases compared with non-TLM (9.4 ± 0.5 vs. 6.9 ± 0.4 h, $P < 0.001$), the multivariable logistic regression model demonstrated that use of TLM remained significantly associated with major islet loss even when controlling for CIT and other potential confounding factors. We do not have any clear explanations of why islets derived from TLM-preserved pancreas had a significant reduction in islet mass following culture. On the basis of current findings and the previous published results [36,37], our center is not currently employing TLM preservation.

The observation that lower islet purity resulted in greater islet loss in univariate analysis may support the view that harmful enzymes released from dying acinar cells may be deleterious to islet cell survival. Cell loss during culture is not a phenomenon specifically for islets. It is also well known that acinar tissue does not survive well in culture [38,39]. We previously reported a remarkable cell death during culture of pancreatic digest, consisting of predominantly acinar tissue, under a culture condition similar to that used in this study: only 6.8% of amylase positive cells were recovered after 4 days of culture [40]. We did not measure nonendocrine cell loss in this study, but a significant decrease in packed tissue volume without any changes in islet purity after culture suggests that nonendocrine cells, in addition to islets, were lost during culture. The observation may also support the rationale that islet preparations with higher purity should be cultured separately from lower purity layers, which we believe important to minimize loss of islets during culture.

CIT has been known to be an important donor factor that significantly determines islet yield after isolation [41–43]. Our observation of a significant association of CIT with islet loss during culture will further emphasize the importance of CIT in islet isolation and transplantation.

Duration of culture period, another time frame, may be an important factor influencing islet loss. However, culture duration did not have unfavorable effect on islet recovery in this study. This observation may be reflected by the fact that most of our preparations were cultured for a short period with a narrow range. Interestingly, a recent report from the Miami group showed that a significant islet loss was observed after 32.5-h culture, but an additional shipment of those islets did not result in a remarkable loss [7]. Taken together, a majority of islet loss during culture may occur during the early stages following islet isolation. Currently, many centers employ 2–3 days culture prior to transplantation [44–49]. Of note, in a recent report from the Brussels group, islets were preserved for up to 20 days in tissue culture prior to transplantation [6]. No data on islet loss during culture are given in the report, but the authors imply that there was a significant islet loss during culture.

We emphasize that our study did not identify the viability score using membrane integrity stains as a correlative parameter. We previously reported that an extremely low viability assessed with the same method results in a significant loss of IE mass during culture [10]. A relatively higher viability score of islet preparations in this study may explain why this measurement is not helpful to predict islet loss. The fact that our membrane integrity test cannot distinguish islets from nonendocrine tissue constitutes another explanation. Membrane integrity tests can be rapidly performed, and make them attractive for use just prior to transplantation. However, time-related constraints are not a big issue for islet potency assay prior to culture. Other assays such as beta cell specific viability may provide meaningful measurements [50].

Several limitations of this study need to be mentioned. First, our assessment of islet loss was based exclusively on manual counting of IE, which will be in error to the extent that the actual shape of the islets is not always spherical. It is also complicated by observer subjectivity. Second, this was a retrospective review and so our findings might have been confounded by unmeasured variables. Finally, the study was conducted at a single center with a relatively small number of islet transplantation procedures. All islet isolations presented in the study were performed with the use of Liberase, which is no longer used for clinical islet isolation at our center, as it has recently become apparent that this enzyme is potentially exposed to bovine brain products during manufacture. These facts may limit the generalizability of our findings to other islet isolation centers as well as to other islet isolation methods.

The price to pay for culturing islets prior to transplant is a modest 14% loss of islet mass. Occasionally, this could potentially cause difficulties if a subject received depletional T-cell antibodies during the culture period. Overall, the benefit of convenience for recipient transportation, transplant preconditioning and transplant timing during regular daylight hours outweigh the modest loss in islet yield in most cases. The reduction in packed cell volume associated with islet culture is an important benefit in terms of reduced risk of elevated portal pressure and portal vein thrombosis. This study identifies several important factors influencing islet loss during culture. Such information will help select a potential recipient. Further optimization of culture conditions to minimize islet loss should be the subject of future research.

Authorship

TK: drafted the manuscript, performed study, collected data. PS, DO'G and BR: collected data, performed study. AS: statistician, analyzed data. AMJS: performed study, gave critical input for the analysis.

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