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Human Islet Oxygen Consumption Rate and DNA Measurements Predict Diabetes Reversal in Nude Mice

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Abstract

There is a need for simple, quantitative and prospective assays for islet quality assessment that are predictive of islet transplantation outcome. The current state-of-the-art athymic nude mouse bioassay is costly, technically challenging and retrospective. In this study, we report on the ability of 2 parameters characterizing human islet quality: (1) oxygen consumption rate (OCR), a measure of viable volume; and (2) OCR/DNA, a measure of fractional viability, to predict diabetes reversal in nude mice. Results demonstrate that the probability for diabetes reversal increases as the graft's OCR/DNA and total OCR increase. For a given transplanted OCR dose, diabetes reversal is strongly dependent on OCR/DNA. The OCR and OCR/DNA (the 'OCR test') data exhibit 89% sensitivity and 77% specificity in predicting diabetes reversal in nude mice (n = 86). We conclude that the prospective OCR test can effectively replace the retrospective athymic nude mouse bioassay in assessing human islet quality prior to islet transplantation.

Keywords

Islet potency; oxygen consumption rate; nude mice; quality assessment

Introduction

Rapidly accumulating evidence suggests that allogeneic islets can become an effective therapy for a subpopulation of patients with type 1 diabetes (T1D) (1-5). Continued research on technical aspects of pancreas procurement and preservation, and on islet isolation, culture, shipping and transplantation is needed to further improve the risk–benefit ratio and the availability of this procedure to qualified recipients (3,4,6-8). Exposure to hypoxic, mechanical, oxidative, osmotic and thermal stresses, disruption of cell–cell and cell–matrix interactions (9), and removal of growth and survival factors that occur during pancreas procurement, preservation and processing into islets amplify the effects of brain death (10) and compromise the islet product in an unpredictable fashion. Validated, real-time

quantitative islet potency assays that can be used prospectively to characterize islet preparations will position the field to (a) avoid transplantation of preparations that will fail to result in diabetes reversal (DR), (b) examine the impact of donor factors and (c) optimize pancreas procurement and preservation as well as islet processing, culture and shipment protocols, ultimately contributing to higher islet transplantation (ITx) success rates (6-8).

The athymic nude mouse bioassay (NMB) is currently considered to be the most reliable and perhaps the only assay that correlates with clinical transplantation outcome (11,12). However, it is costly and technically challenging to implement; it requires several days to weeks for an outcome and thus cannot be used prospectively to exclude preparations of poor quality from being transplanted. Despite its limitations, since it is the only potency assay that correlates with clinical ITx outcome, the NMB is expected to be used as the benchmark for validating new assays under development (13).

The objective of the current study was to examine the ability of 2 parameters characterizing human islet quality: (1) oxygen consumption rate (OCR), a measure of viable islet volume; and (2) OCR per DNA content (OCR/DNA), a measure of fractional viability (FV), to predict DR in nude mice and thus to be used as a reliable prospective surrogate to the NMB.

The use of OCR in tissue quality assessment is supported by a large body of literature. OCR has been extensively applied in several areas of transplantation, such as liver, heart and skin (14-16). Prior reports have been also published on its use with β -cells and islets (17-21). OCR and OCR/DNA measurements are easy to conduct, quantitative, operator-independent and relatively inexpensive; they have low islet requirements (500–2000 islets per measurement) and can be completed in 2–3 h. The fast turnaround of results allows real-time OCR measurements and thus prospective use in quality assessment. Important unique aspects of the present study are: (1) the coordinated use of OCR and OCR/DNA as measures of viable islet volume and islet FV and (2) the prospective evaluation of their ability to predict DR in the NMB.

Methods

Measurements of OCR and OCR/DNA

Human islets purified from exocrine tissue (purity >85%) used in the experiments described here were provided by the islet cell resource (ICR) centers supported by the National Center for Research Resources (NCRR). ICRs that contributed islets were Columbia University, the Joslin Diabetes Center, the University of Miami, the University of Pennsylvania, Washington University and the University of Minnesota (UMN).

Islets isolated at the UMN were cultured for 2 days prior to ITx in CMRL 1066 media formulated for islet culture (Mediatech, 99-603), further supplemented with 10% fetal bovine serum (FBS) and heparin (10 U/mL), whereas islets provided by the other ICRs were shipped to the UMN and transplanted shortly after they were received. Islet OCR and OCR/DNA were assessed immediately prior to transplantation in nude mice. For these measurements, 3 or 4 samples of islets were taken from each preparation. Each sample usually contained 1000–2000 islet equivalents (IE), a number adequate for less than 1% sampling error in 95% of all samples (22). Each sample was placed in a different OCR chamber equipped with fiberoptic sensors (Instech Laboratories, Plymouth Meeting, PA) that measure the declining oxygen partial pressure (pO_2) over time. OCR measurements were conducted in culture media equilibrated at 37°C (20,21).

The liquid volume in each chamber was known, so the OCR (mol O_2 per unit time) of the islets could be estimated from the linear slope of the measured pO_2 . OCR is reduced when

local pO_2 in the islet core drops to levels comparable to the K_m value for mitochondrial respiration (which is below 1 mm Hg). For this reason, slopes were determined at bulk pO_2 values above 80 mm Hg. In these conditions, even for islets as big as 250 μm in diameter, reduction in OCR is usually negligible. The slopes thus obtained invariably had correlation coefficients over 0.999. Each slope measurement was usually completed in 5–15 min. The sensors were calibrated at atmospheric and zero pO_2 before each measurement and were found to be stable afterward.

To measure the DNA content in the chambers, the islets and supernatant were removed and the chambers were washed with media to ensure that all tissue was collected. The collected tissue suspension was then sonicated, diluted and stained with a fluorescent DNA stain. Fluorescence was read with a 96-well fluorometer and compared to a standard curve (Quant-iT PicoGreen dsDNA kit, Molecular Probes, Eugene, OR) to produce the DNA concentration of the sample. OCR was normalized by the DNA content of the islets in each chamber to produce an OCR/DNA (nmol O_2 /min—mg DNA) value for each chamber. The OCR/DNA values for the 3 or 4 samples were then averaged to produce a mean OCR/DNA value for each preparation. The coefficient of variation is usually less than 15% and averaged $13.2 \pm 5.2\%$ for the islet preparations used in this study. The total OCR doses for ITx were set by using the OCR/DNA, measured as described above, and the DNA content per unit volume of the culture medium, measured from aliquots taken from the culture flasks.

Islet potency in vivo

Islet potency *in vivo* was assessed with the NMB. All animal procedures were approved by the Institutional Animal Care and Use Committees (IACUC) and were performed by the Islet Processing Core at the Diabetes Institute for Immunology and Transplantation at the UMN. Nude mice were maintained in microisolator cages under specific pathogen-free conditions. All staff who entered the rooms wore protective disposable garments. Bedding was changed under laminar flow workstations. Mice had free access to food and water and were checked by veterinary staff on a regular basis. All surgery on mice was performed using general anesthesia. Analgesia was continued throughout the postoperative period. Streptozotocin was injected intraperitoneally or intravenously under anesthesia to induce diabetes. During the course of the experiments, any mice that were sick, that appeared to have discomfort, or that were sacrificed in the course of the experiment were euthanized using either a lethal dose of anesthesia or CO_2 . This method is consistent with the recommendations of the Panel of Euthanasia in the Veterinary Medical Association.

OCR/DNA and DNA content per media volume for each islet preparation tested were assessed immediately prior to ITx under the kidney capsule of streptozotocin-induced diabetic nude mice (>350 mg/dL blood glucose [BG] pre-ITx). Islet dosing was based on total OCR, estimated from the product of OCR/DNA and the DNA content in the media volume containing the islets to be transplanted. Up to 5 OCR doses, ranging from 0.5 to 7.5 nmol/min, were used from each preparation. Three mice were targeted for ITx per OCR dose tested for each preparation, but in some cases only 1 or 2 mice were transplanted per OCR dose because of limited islet availability. The mouse bioassay outcome could, in principle, vary—and was indeed found in several cases to vary—among mice transplanted with the same intended dose of islets from the same islet preparation. BG and mouse weight were monitored daily for about 10 days posttransplant and roughly every 3 days after that until nephrectomy, which was conducted no later than day 42 posttransplant. DR, defined as 2 consecutive posttransplant BG measurements below 200 mg/dL, was validated with return of hyperglycemia postnephrectomy (2 BG measurements over 200 mg/dL). When transplanted mice were hyperglycemic before nephrectomy, follow-up was not necessary

postnephrectomy and was not conducted. Alternative DR criteria, requiring longer periods of normoglycemia, were also assessed.

Statistical analysis

Data analyses were performed using the JMP Statistical Discovery Software (SAS Institute, Cary, NC) by the Administrative and Bioinformatics Coordinating Center for the ICR Center Consortium, City of Hope National Medical Center, Duarte, CA. Comparisons of DR rates between groups were done using a 2-sided Fisher's exact test. The DR outcomes for all mice included in the analysis were fitted using a nominal logistic regression model of the form

$$DR_{\text{probability}} = 1 - \frac{1}{1 + \exp(a + b \times \text{OCR} + c \times \text{OCR/DNA})}$$

where a , b and c are optimization parameters, OCR is expressed in nmol/min, and OCR/DNA is expressed in nmol/min—mg DNA, producing predicted outcomes as a function of OCR and OCR/DNA. An interaction term of the form $d \times \text{OCR} \times \text{OCR/DNA}$ was not found significant and was omitted from the model. Model predictions were compared to the observed outcomes to generate sensitivity and specificity values reported herein. Sensitivity is the probability that a noncured mouse is predicted by the model not to cure, and specificity is the probability that a cured mouse is predicted by the model to cure. For the logistic regression model, the mean of the 3 or 4 OCR/DNA samples taken from each islet preparation was used as a point estimate for the OCR/DNA value for that preparation. This estimate minimized the theoretical effect of intrapreparation OCR/DNA correlation when applied in our sampling scenario.

Results

A total of 92 mice were transplanted with islets from 7 isolations. Of the 92 mice, 6 were excluded from the analysis because of death before an outcome was determined, insufficient pre-ITx hyperglycemia, or, in cases of DR, postnephrectomy normoglycemia. OCR/DNA varied by preparation, ranging from 87 to 206 nmol/min—mg DNA—a range representative of most human islet preparations. This range corresponds to an FV of 19–46% based on our best estimate for OCR/DNA for fully viable islets.

Figure 1 depicts BG levels versus time for diabetic nude mice transplanted with islets from 2 of the preparations examined. One preparation had a low OCR/DNA (left panel), while the other had a substantially higher OCR/DNA (right panel). DR rates increased with OCR dose, and for the same OCR dose, DR was generally higher in mice transplanted with islets from the preparation with the higher OCR/DNA value.

Figure 2 depicts DR data for all transplanted mice across 3 OCR dose groups—low (0.5–1.5 nmol/min), medium (2.0–2.5 nmol/min) and high (5.0–7.5 nmol/min)—stratified by tissue of low (OCR/DNA <125 nmol/min—mg DNA) and high (OCR/DNA >125 nmol/min—mg DNA) FV. These groups were defined for presentation purposes only and did not play any role in the logistic regression model. For medium OCR doses, DR rates were 13% for low FV preparations and 54% for high ($p = 0.08$). For high OCR doses, DR rates were 25% for low FV preparations and 86% for high ($p = 0.0009$). These data demonstrate that at medium and especially high OCR doses, OCR/DNA has a very strong impact on DR. At low OCR doses, however, DR rates converged to zero (1 out of 10 for low FV preparations and 1 out of 20 for high FV preparations), resulting in nonsignificant differences between the 2 FV groups.

Nominal logistic regression methods were used to fit data and predict the probability of DR as a function of OCR dose and OCR/DNA. The model predictions are depicted in the form of a contour plot in Figure 3 for the optimization parameter values of $a = -6.74$, $b = 0.585$, and $c = 0.0276$. These data clearly demonstrate the aforementioned trends as well as the interplay between the amount of viable tissue transplanted (OCR dose) and the FV (OCR/DNA) in determining ITx outcome. OCR and OCR/DNA (the 'OCR test') data exhibited 89% sensitivity (95% Confidence Interval [CI] = 81–97%) and 77% specificity (95% CI = 63–92%) in predicting DR in nude mice ($n = 86$).

To assess the effect of DR criteria on our results, we explored the retrospective use of a more strict requirement of 8 normoglycemic (<200 mg/dL) out of 10 consecutive measurements for DR. With this criterion, the OCR/DNA effect was even more pronounced for both medium ($p = 0.03$) and high ($p = 0.0002$) OCR doses. As expected, the OCR test had a higher sensitivity (95%) and a lower specificity (61%) for the NMB based on this stricter DR criterion.

Discussion

In this study we examined the ability of 2 parameters characterizing human islet quality: (1) OCR, a measure of viable volume and (2) OCR/DNA, a measure of FV, to predict DR in nude mice.

Our use of OCR/DNA instead of fluorescein diacetate/propidium-iodide (FDA/PI) to assess FV was based on these factors: (a) the FDA/PI method provides only a lower limit for the fraction of nonviable tissue, rather than an estimate of the viable tissue, as cautioned by the developers of the assay we used (23,24); and (b) we found that FDA/PI was consistently very high, even for preparations that failed to reverse diabetes in nude mice at high islet doses. Figure 4 shows that FV, based on OCR/DNA, varied between 10% and 90% for islet preparations with FV clustered around 90% based on FDA/PI. Table 1 shows OCR/DNA, NMB and FDA/PI data from another study in our lab in which mice were transplanted with 2000 IE by manual counts. The average OCR/DNA significantly differed between the 2 groups examined, and DR showed a similar trend toward statistical significance ($p = 0.077$), yet the average FV estimated by FDA/PI was virtually identical. In light of these data, we consider the FDA/PI data as generally noninformative.

The effect of the transplanted viable tissue dose (represented by OCR) on DR follows an apparently similar rationale to the standard practice of transplanting more tissue to achieve better DR rates. However, the strong effect of FV (represented by OCR/DNA) decouples the effects of total viable versus total tissue transplanted. The higher DR rates with similar doses of viable tissue when using tissue of higher FV suggest that nonviable tissue transplanted negatively affects the potency of the cotransplanted viable islets. Furthermore, for similar doses of viable tissue, the lower the FV, the more total tissue was transplanted. Thus, DR rates can drop when the total transplanted islet mass increases, if this increase is associated with nonviable tissue. Therefore, the total amount of transplanted tissue is not as good a predictor of DR as the OCR test.

The OCR test measurements that we did involved highly purified ($>85\%$) islet preparations; hence, the applicability of our results is limited to such preparations or preparation fractions. Current practice in human ITx uses islets from preparations with low purity (as low as 40%). The presence of exocrine tissue in such impure preparations may limit the use of the OCR test (or any other metabolic or apoptosis assay, as well as the NMB, which is currently limited to highly purified fractions) in characterizing islet quality (11,12). Human islet isolations produce a high and a low purity fraction, which are cultured separately prior to

ITx. OCR and DNA measurements can be conducted on the highly purified fraction, which contains the vast majority of islets in the preparation (more than 70% of human islets in clinical preparations at the UMN). The islet quality in the high purity fraction is expected to be representative of the entire preparation. This rationale is supported by data obtained with the NMB at the UMN using only the highly purified islet fraction, which correlate with ITx outcome in humans (100% sensitivity and 69% specificity, unpublished results). In our experiments only the highly purified islet fraction was used, because we observed that the presence of excessive amounts of exocrine tissue dramatically decreased DR rates in the NMB. In that regard, the use of only highly purified islets for the OCR test does not constitute a disadvantage relative to the NMB.

The OCR test can be taken a step further when combined with β -cell composition data by replacing the islet OCR dose with a β -cell OCR dose. The β -cell OCR dose can be estimated by assuming that OCR of all endocrine islet cells is equally affected by the isolation and culture processes. Development of methods for reliably assessing FV of β -cells alone may allow the relaxation of that assumption. The use of β -cell viable mass, β -cell FV and β -cell composition (encompassing purity) may become a more comprehensive and powerful model for predicting DR.

Certain conditions that affect the respiratory chain and the extent of coupling can affect OCR parameters. We anticipated that such variability may exist and be part of our experimental uncertainty. We expected that artifacts due to uncoupling would manifest themselves in the ability of the OCR test to predict DR in mice. Our results indicate that not accounting for any uncoupling effects did not prevent the OCR test from predicting NMB outcome. During OCR measurements, the islets were exposed to their respective culture media and to high intraislet pO_2 levels that were significantly higher than those affecting OCR, oxidative phosphorylation and hence ATP production rate and ATP levels. Future work is warranted to determine the level of uncoupling and to correlate OCR measurements with ATP production rate and levels.

NMB outcome, especially when transplanting large number of islets (as is the case with human islets), is subject to variability. Reasons include, but are not limited to, the following: (a) difficulties with homogeneous sampling and preparation of the islet aliquot to be transplanted, (b) injury of the islets during the transplantation process, (c) unsuccessful placement of the islets under the kidney capsule, (d) extent of the islet spreading under the kidney capsule, (e) the presence of exocrine tissue and the overall purity of the preparation and (f) the duration and severity of diabetes prior to ITx. As a result of this variability, NMB outcome is sometimes different for the exact same intended parameters. This variability cannot be captured by any predictive model based on the controlled parameters. Therefore, deviations of sensitivity and specificity from 100% may not necessarily reflect the limitations of the assay (such as the OCR test in this study) in predicting the potency of the graft, but rather reflect the variability of the NMB itself.

While the OCR test may be sufficient for ensuring initial primary function of islets posttransplant, it does not provide any information on islet defense-repair capacity, which relates to future apoptosis after exposure to posttransplant stress, islet β -cell function and replication, or islet proimmune activity. It may be important to be able to assess and precisely define each islet preparation based on reliable and meaningful markers at the cellular and molecular level. Such assessments will be complementary to the OCR test and may provide information that would help define immunologic acceptance and long-term function.

In conclusion, when used together, simple, quantitative, real-time measurements of OCR and OCR/DNA are good predictors of DR in nude mice and can be effectively used as a surrogate to the NMB. Current work is directed toward correlating these parameters with clinical ITx outcome and toward further defining the islet product by combining the OCR test with information on β -cell composition and on markers of stress, apoptosis and immunogenicity assessed at the molecular level.

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Abbreviations

BG	blood glucose
CI	confidence interval
DR	diabetes reversal
FBS	fetal bovine serum
FDA/PI	fluorescein diacetate/propidium iodide
FV	fractional viability
HSA	human serum albumin
IACUC	Institutional Animal Care and Use Committees
ICR	Islet Cell Resource
IE	islet equivalents
ITx	islet transplantation
JDRF	Juvenile Diabetes Research Foundation
NCRR	National Center for Research Resources
NMB	athymic nude mouse bioassay
OCR	oxygen consumption rate

OCR/DNA	oxygen consumption rate per DNA content
pO ₂	oxygen partial pressure
T1D	type 1 diabetes
UMN	University of Minnesota

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Figure 1. Blood glucose (BG) levels and rates of diabetes reversal (DR) in athymic nude mice transplanted with 5 OCR doses

Data in the left column were obtained with islets from a preparation with low OCR/DNA, while data in the right column were obtained with islets from a preparation with a higher OCR/DNA. Asterisk (*) denotes DR rates that do not take into account mice that were excluded from the analysis either (a) because of insufficient pre-transplant hyperglycemia (left panel, OCR dose of 2.5 nmol/min) or (b) because of postnephrectomy normoglycemia in cases of DR post-transplant (right panel, OCR dose of 1 nmol/min). Time of nephrectomy is shown with a dashed line when follow-up postnephrectomy was conducted. When transplanted mice were hyperglycemic before nephrectomy, follow-up post-nephrectomy was not necessary and was not conducted.

Figure 2. Rates of diabetes reversal (DR) in athymic nude mice for 3 OCR dose groups (low, 0.5–1.5 nmol/min; medium, 2–2.5 nmol/min; and high, 5–7.5 nmol/min) when transplanted islets were of low (OCR/DNA <125 nmol/min—mg DNA) or higher viability (OCR/DNA >125 nmol/min—mg DNA)
N.S. = nonsignificant.

Figure 3. Probability of diabetes reversal (DR) in the athymic nude mouse bioassay (NMB) as a function of transplanted OCR (a measure of the transplanted viable tissue volume) and OCR/DNA (a measure of islet fractional viability)

The model was based on outcomes from 86 mouse transplants. The model equation and optimization parameters are provided in the text.

Figure 4. Islet fractional viability (FV) based on OCR/DNA plotted against islet FV based on FDA/PI for 43 human islet preparations
OCR/DNA shows a high level of variability in FV among preparations that is not reflected in FDA/PI measurements clustered around 90%.

Table 1

OCR/DNA, athymic nude mouse diabetes reversal (DR) rates, and fractional viability (FV) based on FDA/PI for clinical islets cultured with 0.5% human serum albumin (HSA) and human research islets cultured with 10% fetal bovine serum (FBS). OCR/DNA and DR rates were higher for the research islets, but FV values based on FDA/PI were almost identical for the 2 groups

	OCR/DNA nmol/ min—mg DNA	Nude mice DR%	FDA/PI%
HSA (Clinical islets)	138 ± 54 (n = 13)	47.6 (n = 21)	93.9 ± 3.5 (n = 29)
FBS (Research islets)	202 ± 87 (n = 45)	70.4 (n = 54)	93.6 ± 4.7 (n = 71)
p	0.016 ¹	0.077 ²	0.78 ¹

¹ 2-sided Student's *t*-test.

² 2-sided Fisher's exact test.