Cell Transplantation, Vol. 24, pp. 1183–1194, 2015
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0963-6897/15 $90.00 + .00
DOI: http://dx.doi.org/10.3727/096368914X681928
E-ISSN 1555-3892
www.cognizantcommunication.com

A Simple Method to Replace Islet Equivalents for Volume Quantification of Human Islets

Karthik Ramachandran,* Han-Hung Huang,† and Lisa Stehno-Bittel‡

*Likarda, LLC, Kansas City, KS, USA
†Physical Therapy Program, Angelo State University, Member, Texas Tech University System, San Angelo, TX, USA
‡Department of Physical Therapy and Rehabilitation Science, University of Kansas Medical Center, Kansas City, KS, USA

Human islets come in a variety of sizes and shapes, and the total volume of islets used for research or clinical transplants must be estimated in a manner that is simple and valid. Islet equivalent (IEQ) measurements are the standard estimate of islet volume. We published a new method (the Kansas method) for estimating rat islet volume using cell numbers that was reliable and valid. Here we modified the method for human islets. We measured the dimensions of isolated human islets showing that they are not spherical and became less so in larger islets, with an average smallest/largest diameter ratio of 0.73 in large islets and 0.85 in small islets. Human islets were individually loaded into 96-well plates, dissociated into single cells, and the total cell number per islet determined with computer-assisted cytometry. Based on the counted cell number per islet, a regression model was created to convert islet diameter to cell number with a high R^2 value (0.99). Separate regression equations for male and female donors or young and old donors were not significantly different than the pooled data and did not improve the regression values. There was an inverse correlation between the cell number per IEQ and islet size. The Kansas method was validated with ATP/cell and cell viability data. Compared to the actual cell count, conventional IEQ measurements overestimated tissue volume of large islets by nearly double. Examples of differences in results obtained from the same data sets normalized to IEQ or the Kansas method included viability and insulin secretion concentrations. The implications of the error associated with the current IEQ method of volume estimation are discussed.

Key words: Islet; Islet equivalent (IEQ); Human; Insulin; ATP; Viability

INTRODUCTION

Islets of Langerhans, containing the insulin-producing cells of the body, are often isolated for research purposes and for transplantation as a means to cure or better manage type 1 diabetes in humans. These clusters of endocrine cells come in a large range of sizes from 20 μm to more than 400 μm in diameter in humans (2). Owing to their inherent variation in size, both experiments and clinical transplants rely on an accurate method to estimate the volume of isolated islets in a preparation. In 1990, Ricordi's laboratory proposed the islet equivalent (IEQ) at the Second Congress of the International Pancreas and Islet Transplantation Association, as a means of normalizing islet volume (17). This procedure standardized islet volume measurements and greatly enhanced islet research. It is based on the calculation that one IEQ corresponds to the tissue volume of a perfectly spherical islet with a diameter of 150 μm.

Early on, the accuracy of the IEQ measurement was questioned (4,19), partially because the basic assumption that all islets are spherical was known to be incorrect. In reality, most islets are ellipsoid or irregularly shaped, both in situ and in culture (2,7,8,11,13), although little has been done to quantify that common observation.

An adjustment in the IEQ calculation was proposed in 2009, which altered the size/volume ratio, but the change was still based on the incorrect premise that islets are spherical (2). Recent digital image analysis methods improve quality and efficiency of islet volume estimations, but they also convert the images to volumes using the traditional IEQ calculations (5,9,15,18).

We developed and tested a new method for estimating islet volume based on cell numbers using rat islets (8). Our method took into account the fact that islets are not spherical. Using rat islets, we completed validity and reliability studies on the new method, called the Kansas

Received January 4, 2014; final acceptance May 13, 2014. Online prepub date: May 15, 2014.
Address correspondence to Lisa Stehno-Bittel, Ph.D., Department of Physical Therapy and Rehabilitation Science, MS 2002, University of Kansas Medical Center, 3901 Rainbow Blvd., Kansas City, KS 66160, USA. Tel: +1-913-588-6733; Fax: +1-913-588-4568; E-mail: lbittel@kumc.edu

method, and showed that it was a fast, more accurate, and reliable procedure for estimating islet volume (8). The purpose of the current study was to calculate a cell number-based volume estimate for human islets and compare it to the rat equation. We determined that separate conversion equations were necessary for the human and rat islets, and we demonstrate that the cell number conversion is more accurate than the conventional IEQ method for volume normalization of human islets. In the discussion, we address the errors associated with the current IEQ method and quantify possible error in human islet transplants.

MATERIALS AND METHODS

Islet Procurement and IEQ Calculations

Human islets were obtained from the Integrated Islet Distribution Program from May 2011 to November 2012. Table 1 summarizes the characteristic of the islet donors. There were no significant differences in age or body mass index (BMI) between male and female donors. The IEQ measurements for each preparation followed our previously published procedures (13,20). Briefly, the diameter of each islet was recorded manually using light microscopy at 40× total magnification. For irregularly shaped islets, two to four diameter measurements were taken at different locations on the islet and averaged for the final diameter measurement. The volume of each islet was calculated based on the diameter and converted to IEQ individually, where one IEQ is equal to 1.77×10^6 μm^3 (the volume of a spherical islet with 150 μm diameter) (2,17).

Islet Diameter Measurements

In order to accurately measure islet diameters in three planes (*x*, *y*, *z*), a custom glass tube chamber was placed on an inverted Olympus confocal microscope (20×) (Center Valley, PA, USA). A small air jet was used to roll the islets through the chamber while video captured their progression. Subsequent analysis of the video allowed the diameter measurements from multiple planes. The video was analyzed only if three distinct planes could be identified. Islet diameters were organized according to the largest (A), the next (B), and the smallest diameter (C). To quantify the shape, the smaller dimensions were divided by the largest (B/A and C/A) using procedures described by Avgoustiniatos (1).

Table 1. Characteristics of Donors

	N	Age	BMI
All donors	17	45.6 ± 11.4	28.7 ± 5.9
Male	11	43.7 ± 10.9	27.5 ± 5.2
Female	8	48.6 ± 12.4	30.4 ± 6.9

Age and BMI are provided as mean ± SD.

Immunofluorescence Staining

Human pancreatic sections were obtained from a private company (BetaPro, LLC, Gordonsville, VA, USA) from four donors (two males and two females), 47 ± 7 years of age. Paraffin-embedded 7- to 8-μm-thick pancreatic tail sections were deparaffinized/rehydrated in xylene (Fisher Scientific, Loughborough, UK) followed by ethanol and phosphate-buffered saline (PBS; Sigma-Aldrich, St. Louis, MO, USA), pH 7.4, using standard procedures. Antigen was retrieved using a steamer (30 min) in a Shandon plastic spill-free slide jar (Thermo Scientific, Waltham, MA, USA) containing 0.01 M citrate buffer (Sigma-Aldrich), pH 6.2, with 0.002 M EDTA (Sigma-Aldrich). After cooling for 20 min, slides were washed in PBS and permeabilized in 1.0% Triton X-100 (Sigma-Aldrich) in 0.1 M PBS for 30 min. Slides were rinsed again in PBS and regions of interest encircled with a PAP pen (ImmEdge Pen; Vector Laboratories, Burlingame, CA, USA). Sections were blocked for 30 min in 10% normal donkey serum (NDS; Jackson ImmunoResearch, West Grove, PA, USA), 1.0% bovine serum albumin (BSA; MidSci, St. Louis, MO, USA), and 0.03% Triton X-100 diluted in 0.1 M PBS. Incubation with the primary antibody mix was performed in a wet chamber at 4°C overnight, followed by incubation with the fluorophore-conjugated secondary antibodies at room temperature for 2 h in a dark chamber. The following primary antibodies were used: anti-insulin (1:200; Abcam, Cambridge, MA), anti-glucagon (1:300; Abcam), anti-somatostatin (1:300; Abcam), or anti-insulin (1:100; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Appropriate secondary antibodies were used that were conjugated with DyLight 488 (1:400; Jackson ImmunoResearch Laboratories Inc.), Alexa 555 (1:400; Molecular Probes, Eugene, OR, USA), or Alexa 647 (1:400; Molecular Probes). Both primary and secondary antibodies were diluted in 1% NDS, 1% BSA, and 0.03% Triton X-100. After washing repeatedly, slides were mounted with antifading agent Gel/Mount (Biomeda, Foster City, CA, USA). Nuclei were visualized by staining with 4′,6-diamidino-2-phenylindole (DAPI) counterstain.

Islet Dissociation and Cell Number Estimation

For single-cell assays, isolated islets were picked manually and individually distributed into 96-well plates (Thermo Fisher Scientific, Loughborough, UK) in media containing calcium–magnesium-free Hank's balanced salt solution (HyClone, ThermoFisher Scientific, Waltham, MA, USA). After recording the diameter of each islet as described above, the islets were dissociated into single cells. Briefly, after adding papain (5 U/ml) (Worthington Biochemical Corp., Lakewood, NJ, USA) in 10 mM ethylene glycol tetraacetic acid (Sigma-Aldrich) into each well, islets were incubated at 37°C for a minimum of

30 min. Subsequently, the islets were dispersed into single cells by repeated pipetting, and the dissociated cells in the wells were typically spun down in the plate with 300 rcf for 1 min at room temperature. The cell number in each well was analyzed using Celigo™ Imaging Cell Cytometer (Cyntellect Inc., San Diego, CA, USA). Every well containing single cells was photographed digitally, and the cells were counted using the automated Celigo software (V 1.3).

Electron Microscopy

The diameter of individual islet sizes was determined from electron microscopy micrographs and from confocal images. Human pancreatic samples were obtained from BetaPro (Gordonsville, VA, USA) and the National Institute of Child Health and Development Brain and Tissue Bank. The donors consisted of four females and four males with an average age of 43 ± 5 years. Electron microscopy was conducted using 2-mm sections of pancreatic tissue fixed with 2% glutaraldehyde (Electron Microscopy Sciences, Fort Washington, PA, USA), and on sections of isolated islets. All samples were rinsed twice in 0.1 M sodium cacodylate buffer (Electron Microscopy Sciences) for 10 min prior to postfixation in 1% osmium tetroxide (Electron Microscopy Sciences) for 1 h. After distilled water rinses, a graded ethanol dehydration was undertaken at 10 min each (30%, 70%, 80%, 95%, and 100%). Samples were rinsed in propylene oxide (Electron Microscopy Sciences) for 15 min prior to being infiltrated overnight in a mixture of propylene oxide (Electron Microscopy Sciences) and Embed 812 resin (Electron Microscopy Sciences). BEEM® capsules (Electron Microscopy Sciences) were used to embed the samples in fresh resin prior to curing overnight in a 70°C oven. Thin sections (80 nm) were cut using a Leica UCT ultramicrotome and placed on 300 mesh thin bar grids (Electron Microscopy Sciences). Contrast was applied by adding uranyl acetate (Electron Microscopy Sciences) followed by Sato's lead stain, composed of sodium citrate (Fisher Scientific), calcined lead citrate (Fisher Scientific), lead nitrate (Fisher Scientific), and lead acetate (Fisher Scientific) following the published formula (6). Images of human pancreatic islets were captured from random tissue sections using a JEM 140 transmission electron microscope (JEOL Inc., Peabody, MA, USA).

All images were analyzed by outlining the border of each cell and then identifying cells with clear full nuclei, thus limiting the size measurements to sections that cut through the central z section of the cell. A measurement of the largest dimension was taken and identified as x. A second measurement perpendicular to the x line was used to measure the maximum y-axis diameter. The two values were averaged for a single cell value.

Cellular ATP

Cellular ATP amounts were measured using standard luminosity measurements. Human islets were handpicked and individually placed into each well of 384-well plate in Connaught Medical Research Laboratories media (Media Tech, Englewood, CO, USA) containing 10% fetal bovine serum (FBS; HyClone, ThermoFisher Scientific), 2 mM L-glutamine (Life Technologies), and 1% antibiotic/antimycotic solution (Life Technologies). Islet diameters were measured under a microscope. The cellular metabolism was measured using the luminescence-based APTlite 1-step assay (PerkinElmer, Waltham, MA, USA) following the manufacturer's instructions. Premixed ATPlite reagent was added to the wells containing islets in a 1:1 ratio of media to reagent. The plates were sealed, spun for 2 min at 11 rcf, and luminescence was read using a SpectraMax M5 (Molecular Devices, Sunnyvale, CA, USA). Blank wells without islets were used as background measurements and were subtracted from each well value. Each plate was loaded with standards to determine a standard curve.

Cell Viability

Cellular metabolism was used as a determination of the number of live cell/islet using fluorometric measurements. Human islets were placed individually in each well of 96-well plates with Dulbecco's modified Eagle's medium + Ham's F12 supplement (HyClone, ThermoFisher Scientific) containing 10% FBS and 1% antibiotic/antimycotic solution (Life Technologies). The cellular metabolism was measured using the fluorometric alamarBlue (resazurin) assay following the manufacturer's instructions (Life Technologies). Within 30 min of loading each plate with islets, alamarBlue was added to a 10% final concentration. Islet diameters were measured on an inverted microscope (10×) (Nikon Instruments, Melville, NY, USA). Fluorescence readings (530 nm excitation, 590 nm emission) were taken at 48 h after addition of alamarBlue using a SpectraMax M5 (Molecular Devices). Blank wells without islets were used as background measurements and were subtracted from each well value.

Insulin Secretion

Perifusion experiments were conducted on small and large islets, preincubated for 90 min in Roswell Park Memorial Institute (RPMI)-1640 medium (Life Technologies) containing 10% FBS and 3 mM glucose (Sigma-Aldrich) at 37°C with 5% CO_2. After preincubation, the islets were placed in a custom-designed perifusion chamber with a constant flow rate (500 µl/min) at 37°C for 90 min including 30 min of low glucose (3 mM) followed by 30 min of high-glucose concentration (20 mM) and 30 min of low-glucose concentration (3 mM). During the perifusion, samples of medium with insulin released by islets

were collected from the output fraction every 10 min. Each fraction was tested in triplicate. Islet equivalency values were calculated for each well, and medium samples were withdrawn from each well to assay for insulin content using ELISA kit (ALPCO, Mercodia, NH, USA).

Static Insulin Secretion

Human islets were separated into large and small groups based on the average islet diameter. Islets were placed in triplicate wells and assigned to basal glucose (3 mM) and high glucose (20 mM). All wells were pre-incubated with RPMI-1640 containing 3 mM glucose for 30 min at 37°C and 5% CO_2. Subsequently, media were removed from each well and basal or high-glucose solutions were added. After a 30-min static incubation at 37°C and 5% CO_2, the islets were sedimented and the condition medium collected and frozen at −80°C until the insulin concentration was determined by ELISA (ALPCO) using our published protocol (13).

Statistics

The exact number of islets or replicates is shown in each figure legend. Results were expressed as means of each group or cell population ± standard error and were compared using the Student's *t*-test. The Pearson product-moment correlation was used to test the correlation between multiple volume calculating techniques. When comparing regression equations, analysis of covariance (ANCOVA) was used with Sidak-corrected post hoc comparisons. All statistical analysis was completed using SigmaPlot/SigmaStat software (San Jose, CA, USA). All figures and tables include means ± standard error, unless otherwise stated. Significant differences were defined as $p < 0.05$.

RESULTS

Human Islet Dimensions

Nonspherical islets have been described previously in situ and in vitro (3,7). Figure 1A illustrates the in situ staining of large and small islets, showing an example of the ellipsoidal shape of large human islets. After isolation from the pancreatic exocrine tissue, the small islets remained more spherical (Fig. 1B), while the large islets maintained a pancake-like shape (Fig. 1C). In order to quantify these observations, 62 isolated human islets of varying sizes were placed in a customized rolling chamber

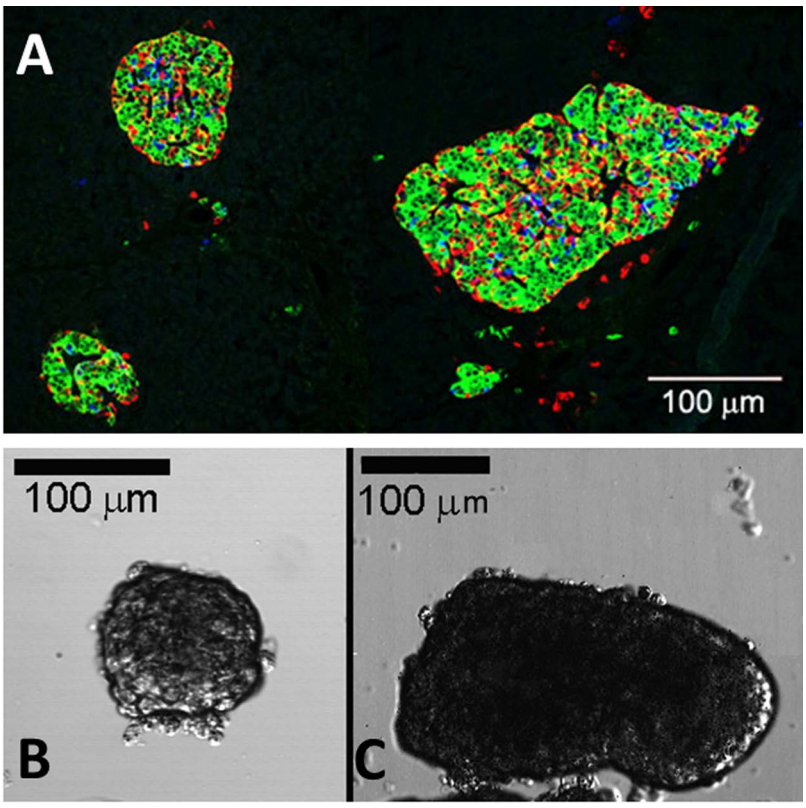


Figure 1. Human islets are ellipsoidal in shape. (A) Human pancreatic sections were immunostained with antibodies against insulin (green), glucagon (red), and somatostatin (blue). The small islets were more spherical in shape. (B) Isolated islets were placed in a rolling chamber where measurements were taken in three dimensions. Small islets (under 125 μm in diameter) were relatively spherical. (C) Example of ellipsoidal large islet.

where they were videotaped. Analysis of the video determined the *x*-, *y*-, and *z*-axis dimensions of the islets. In the largest dimension, islets ranged from 52 to 336 μm, while in the shortest dimension, the sizes ranged from 48 to 280 μm. Following a previously published protocol (1), we calculated the ratio of the three dimensions (B/A and C/A, with A>B>C) to quantify the elliptical shape of human islets. Table 2 summarizes the results. All islets illustrated an elliptical shape, but the smaller the islets (under 100 μm), the more spherical their shape. Contrastingly, the large islets had greater variation in their three dimensions as illustrated in Figure 1.

Cell Number per Islet

Three thousand six hundred sixty-three human islets from 17 donors were placed in individual wells and dissociated into single cells, which were counted using computer-assisted cytometry. The total cell number per islet from 25-μm-size bins is summarized in Table 3. Fewer islets were found at the extremely large sizes. There was an average of 929 cells in a 150-μm-diameter islet. Based on the measured cell numbers, a second-order polynomial regression trend line was the best fit with the equation:

$$y = 0.0202x^2 + 3.6543x - 95.9668$$

where *y* equals the total cell number and *x* equals the islet diameter (μm). The R^2 value was 0.990, indicating the regression trend line fit the data well (Fig. 2A).

Data were analyzed based on the donor sex, and male/female curves were produced (Fig. 2B). Two separate second-order polynomial curves fit the data best:

$$\text{For males: } y = 0.0211x^2 + 4.4710x - 137.765$$

$$\text{For females: } y = 0.0137x^2 + 3.8753x - 10.416$$

By separating the groups into males and females, there was no statistically significant difference in the two curves. Further, the R^2 values did not statistically improve (0.993 for males and 0.980 for females). Thus, an equation based on the combined data could be used to convert islet diameter to cell number, without a need to separate by sex.

Next, donors were separated into young (under 45 years old) and elderly (over 60 years old). Islets under 175 μm were not different in the mean cell number per size between the young and old groups (Fig. 2C). Above diameters of 200 μm, the islets from older individuals had a higher number of cells per islet. However, there was greater variation in cell count/islet in the largest islet diameters, and R^2 values were actually worse when the young and elderly donor curves were created. In contrast, when comparing the calibration curve generated for human islets to our previously published rat islet conversion (8), it was clear that rodents and humans required separate conversion calculations, especially in the large sizes where rat islets had significantly more cells/area than human islets (Fig. 2D).

Cell Number Compared to IEQ

Ricordi's original calculations were based on the assumption that all islets are spherical (17). We created a theoretical cell number per islet, based on the volume of an average islet cell. First, we measured the dimensions of individual cells within islets, without regard to the type of cell, based on electron micrographs (EM) of pancreatic sections, EM of isolated islets, and confocal images of isolated islets. Lines were carefully drawn around the border of cells in which a clear and large nucleus was present. By only measuring cells in which the nucleus was bisected, portions of cytoplasm were not counted as a full cell. Table 4 summarizes the findings with an average diameter of 9.83 μm from all three procedures. These diameter measurements are in line with previously published values (14).

With the cellular dimensions known, we calculated the number of cells that could fit into an islet of a specific diameter by dividing the total islet volume, based on a geometrical sphere, by the average cellular volume. Figure 3A shows that the geometry-based calculation overestimates the number of cells in an islet compared to the Kansas method based on actual cell counts. The larger the islet, the greater the difference between the two calculations. The expected result can be explained by the fact that larger islets have open spaces for blood vessels and a complex structure that increases surface

Table 2. Comparison of Islet Dimensions Based on Largest Value

	Largest Dimension (μm)	B/A	C/A	Comparison of B/A to C/A
Large islets	150–350	0.82±0.03	0.73±0.03	$p<0.001$
Small islets	50–100	0.91±0.02	0.85±0.02*	$p<0.05$
All islets	50–350	0.86±0.18	0.76±0.02	$p<0.001$

The *x*, *y*, and *z* measurements of islets were captured on video, and the islet grouped into large and small sizes based on the dimension of their largest value. A third grouping of the smallest islets under 100 μm in length was compared. The ratio of each of the dimensions were compared to the largest dimension (A), with A>B>C. There was a significant difference in the B/A and C/A ratios within groups. Between groups, the C/A ratios between the large and smallest islet groups were statistically different ($p<0.05$).

Table 3. Average Cell/Islet

Average Diameter (μm)	*N*	All Cells/Islet	Female Cells/Islet	Male Cells/Islet
50	91	188 ± 19	188 ± 21	194 ± 35
75	127	275 ± 17	271 ± 22	284 ± 29
100	127	398 ± 22	421 ± 34	388 ± 30
125	166	696 ± 32	671 ± 52	610 ± 44
150	146	929 ± 52	928 ± 58	946 ± 64
175	133	1,120 ± 52	1,061 ± 77	1,181 ± 73
200	116	1,561 ± 84	1,528 ± 161	1,586 ± 106
225	99	1,668 ± 116	1,586 ± 196	1,825 ± 149
250	125	2,094 ± 95	1,920 ± 124	2,268 ± 143
275	60	2,407 ± 146	2,434 ± 231	2,514 ± 201
300	62	2,878 ± 188	2,637 ± 309	3,094 ± 256
325	21	2,955 ± 293	2,509 ± 455	3,747 ± 360
350	20	4,071 ± 289	4,329 ± 453	3,932 ± 319

Human islets were separated, based on their average diameter, and dispersed into single cells, which were counted. The average cells/islet are listed for the group and separated into cells/islet from female and male donors.

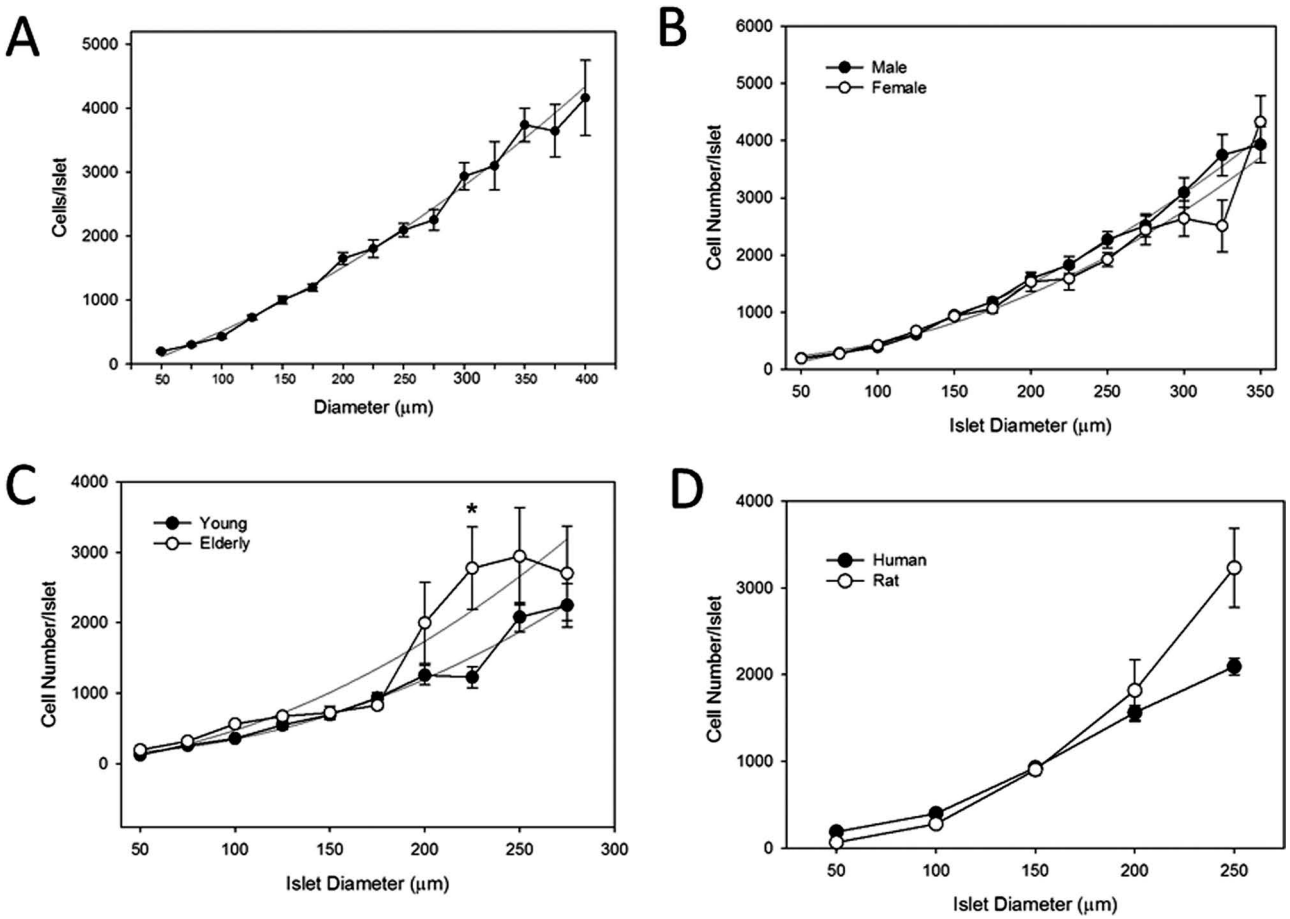


Figure 2. Correlation between mean islet diameter and cell number. (A) The counted cell number per islets was plotted against the average islet diameter, and a second-order polynomial regression trend line with a R^2 of 0.99 produced the best fit with the data. $n = 2{,}399$ individual human islets from 17 donors. (B) Cell numbers per size of the islet based on the donor sex resulted in two regression equations that were not statistically different. $n = 846$ islets from six female donors, and $n = 1{,}553$ islets from 10 male donors. (C) Separation of cell number results by the age of the donor had no effect on the cell number/islet size ratio, except for the largest islets (>200 μm). $n = 207$ islets from three elder donors. $n = 850$ islets from six young donors. $^*p < 0.05$. (D) Comparing the measured cell number/islet from human samples to our previously published rat islet data (8) illustrates the need for separate conversion equations, especially when including large islets in the population. Rat and human islets with a diameter of 250 μm were statistically different ($p < 0.05$).

Table 4. Average Islet Cell Dimensions

Condition	*x* Dimension (μm)	*y* Dimension (μm)	Average Dimension (μm)
Isolated islets, EM	11.64 ± 0.32	8.86 ± 0.26	10.25 ± 0.24
In situ pancreas, EM	9.57 ± 0.37	7.82 ± 0.39	8.66 ± 0.31
Isolated islets, confocal	11.6 ± 0.55	9.44 ± 0.13	10.53 ± 0.13

Islet cell diameters were measured from EM of isolated islets, micrographs of in situ islets within the pancreas, and confocal images of immunostained isolated islets with a DAPI counterstain to identify the nuclei. The *x*-axis was defined as the longest diameter of the cell, and the *y*-axis was measured 90° perpendicular to the *x* line.

area while creating open spaces within the islet diameter that are void of cells. In contrast, the calculated cell number based on geometry assumes no open spaces within an islet. At the lowest size (50 μm), the theoretical curve actually underestimated the number of cells/islet. However, the largest deviations were found in islets greater than 200 μm.

Next, we compared the measured cell number per islet with the conventional IEQ measurement by Ricordi et al. (17) and the refined IEQ measurement by Buchwald et al. (2).

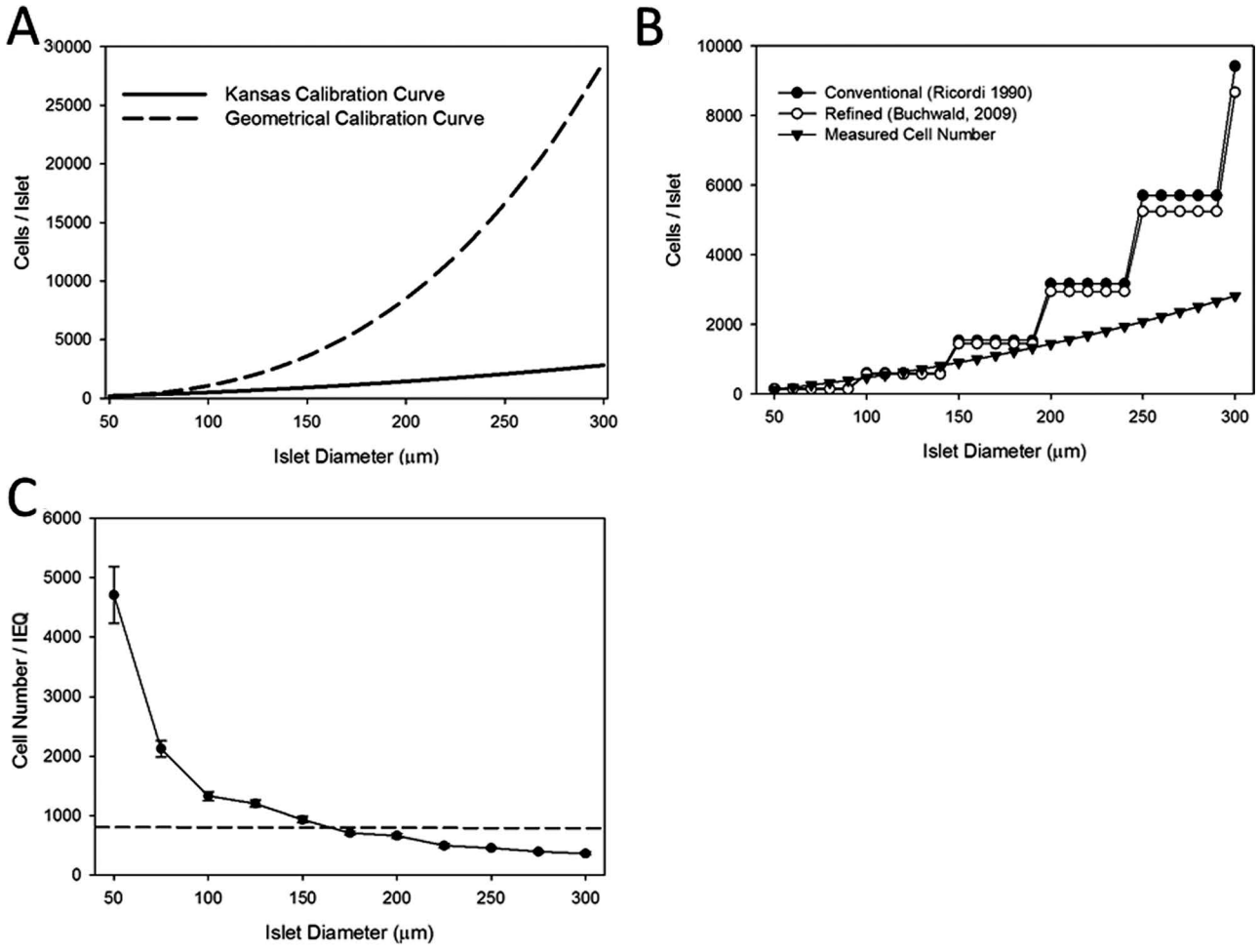


Figure 3. Comparison of cell number versus IEQ for islet volume. (A) A theoretical curve based on the geometry of an ideal islet (dashed line) was plotted. It represents the maximal number of cells that could fit into a sphere of that size. In contrast, the solid line represents that number of cells that were actually counted in each islet. (B) The measured relationship between islet size and actual cell number per islet was plotted in the black solid line. Two theoretical curves were plotted using the Ricordi's conventional IEQ measurement with different categories of sizes in a 50-μm increments (filled circles) (17) and using Buchwald's refined IEQ measurement (open circles) (2), based on our measured 929 cells per 150-μm-diameter islet (1 IEQ). (C) Correlating islet diameter to cell number/IEQ should result in a straight line (dashed line) if all islets are spherical and cell sizes and density equivalent. The measured cell/islet diameter was plotted (black circles). $n = 2{,}399$ islets from 17 donors.

To make the calculation, we used our measured 929 cells per 150-μm-diameter islet for the Ricordi and Buchwald calculations. The results were plotted in Figure 3B. Compared to the actual cell counts, there was a deviation of the values from the IEQ cell calculations (Ricordi and Buchwald) that was more prominent as the islets increased in diameter, especially in islets over 150 μm in size. Even though Buchwald's refined algorithm introduced a downward correction (2), the adjustment was marginal, and a significant overestimation still existed (Fig. 3B).

To further demonstrate the errors within the current IEQ calculations, the cell number within islets divided by the islet's IEQ based on the Ricordi method (17) was plotted. Figure 3C illustrates the errors within the current IEQ calculations. The dotted line indicates the cell number/IEQ that should be obtained, independent of the size of the islet, if IEQ were an accurate measure of islet volume. However, our measured values (solid line) demonstrate that the IEQ calculation underestimates the number of cells in small islets and overestimates the number of cells in large islets. With such variation in the denominator of the cell number/IEQ calculation, using IEQ as a normalization method may lead to errors in data analysis. To test this hypothesis, data normalized by conventional IEQ were compared with normalization using this new method.

Cellular ATP

ATP is present in all metabolically active cells and is used as a common marker for viable cell number. Cellular ATP levels were normalized to IEQ and to the current cell number method in human islets of varying sizes. Individual islets from 50 to 350 μm in diameter were loaded into separate wells of a 384-well plate. ATP levels were determined using an ATPlite assay. When the signal was normalized to IEQ, a steep negative curvilinear relationship was identified between the ATP levels and the diameter of the islet (Fig. 4A). However, this was not the anticipated result because each cell should have approximately the same amount of ATP. Thus, normalizing by volume (IEQ) was expected to result in a flat linear relationship between ATP amounts and islet volume, such as the result obtained when the same data were normalized using the Kansas method of cell number (Fig. 4B). In order to directly compare the ATP levels using the two methods of normalization, the results from the ATP/IEQ were divided by our calculated 906 cells/IEQ. The relationship of the Kansas method of normalization (open circles) shows a linear relationship ($R^2 = 0.98$) in comparison with the negative curvilinear relationship of the IEQ normalization method (Fig. 4B).

Viability

Viability is often measured with fluorescent or colorimetric indicators of redox reactions. alamarBlue is a reproducible measure of metabolically active cells. Individual islets from 50 to 350 μm in diameter were loaded into wells of a 96-well plate. The alamarBlue signal reading was determined 48 h later and binned in 25-μm categories according to the islet diameter. When the signal was normalized to IEQ and plotted against the islet diameter, a steep negative curvilinear relationship was identified (Fig. 4C) similar to the relationship noted with ATP when normalized to IEQ (Fig. 4A). When the values were compared to the IEQ values divided by 906 cells/IEQ (from the Kansas method conversion calculation), the flat linear relationship of the Kansas method of normalization (open circles) shows a linear relationship ($R^2 = 0.76$) with a slope of 0.003 in comparison with the negative curvilinear relationship of the IEQ normalization method (Fig. 4D).

Data Normalization Comparing IEQ to Cell Number

Insulin Secretion. In order to test the Kansas method of volume normalization, we conducted experiments with the resulting data normalized to IEQ compared to normalization by cell number. Previously, we published papers concluding that small islets secreted more insulin than large islets per volume using IEQ as the volume normalization (7,13). Figure 5A shows a typical perifusion experiment when small and large islets were exposed to high glucose from minutes 30 to 90. Small islets secreted significantly more insulin than large islets when normalized to IEQ. Using the same insulin secretion data, the response was normalized to cell number, and for this human donor's islets found no significant change in the conclusions; small islets still secreted more insulin per volume (Fig. 5B). However, two important differences were noted. The basal level of insulin secretion (in low glucose) was the same when normalized by the Kansas method but was greater in small islets using IEQ. Second, the difference in the peak insulin secretion between the large and small islets in response to high glucose was less with the Kansas method of normalization.

Static Insulin Secretion. Basal insulin secretion was measured for 1 h from groups of small and large islets. When the data were normalized to IEQ, there was a significant difference in the insulin secreted between the two groups, with great variation (SE bar) in the small islet group (Fig. 5C). However, when the same data were normalized to cell number using the Kansas method, the variation between preparations was greatly reduced, and the total insulin released by the two groups was not statistically different, although small islets still secreted more insulin/cell (Fig. 5D).

DISCUSSION

Determining the volume of islet tissue in a preparation is critically important in research and in clinical

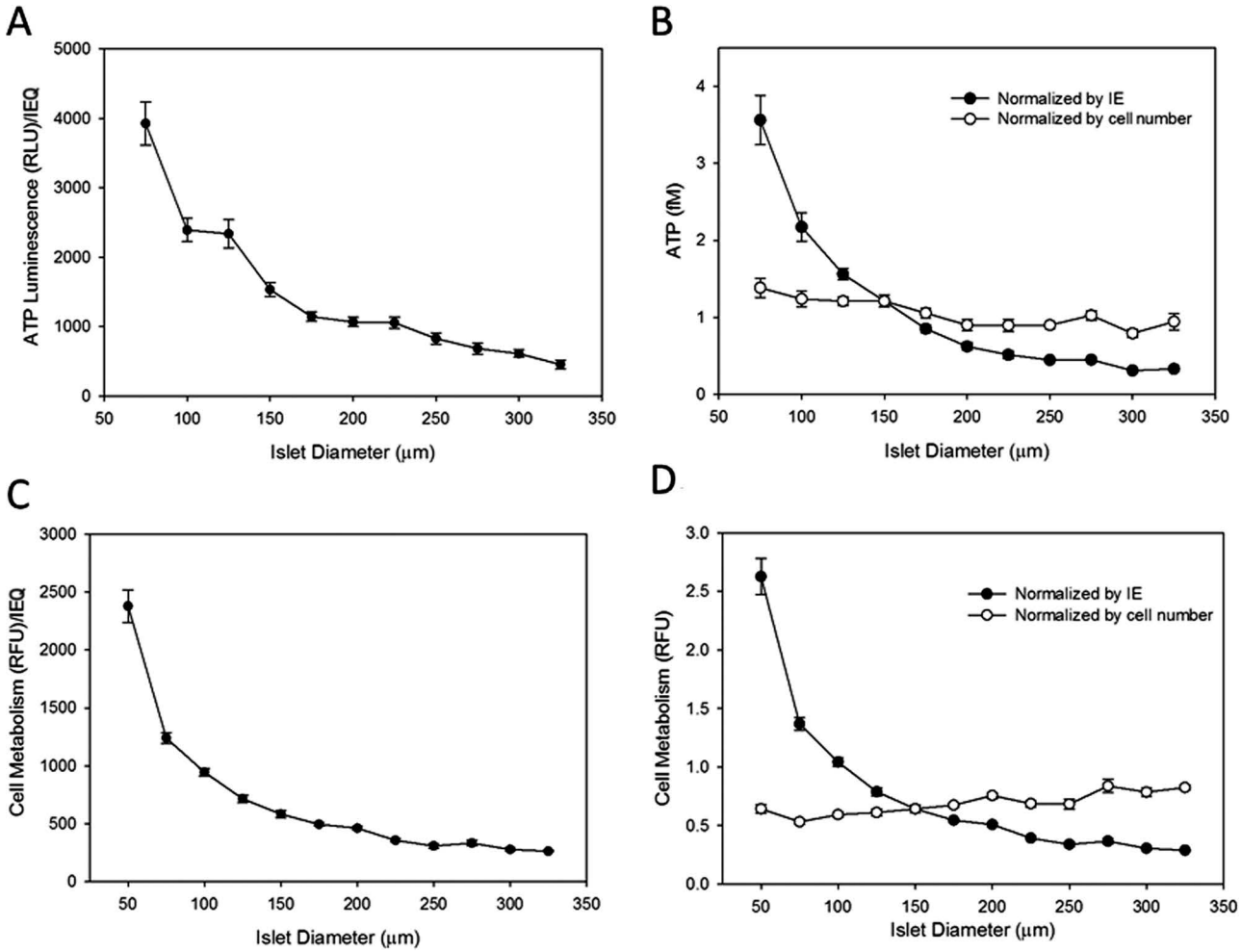


Figure 4. Validation of normalization method with ATP levels. (A) Cellular ATP was measured using the luminescent assay ATPlite (PerkinElmer), a luciferase-based reaction measured in relative light units. Individual islets were placed in single wells of a 384-well plate and the diameter of each islet measured. The cellular ATP amounts per islet volume when normalized by IEQ and a curvilinear relationship resulted with a fourth-order best fit curve ($R^2 = 0.98$). (B) Taking the results shown in (A) and dividing by the average number of cells in one IEQ (906) resulted in an ATP/IEQ/cell number curve that could be directly compared to the normalization method described in this publication. n = an average of 21 ± 2 islets/size category for a total of 236 islet measurements. (C) Cellular viability was measured using the fluorescent assay alamarBlue in relative fluorescence units. The cell metabolism per islet volume when normalized by IEQ and a curvilinear relationship. (D) Taking the results shown in (C) and dividing by the average number of cells in one IEQ (906) resulted in a curve that could be directly compared to the cell number/IEQ calculations based on the normalization method described in this publication. n = an average of 30 ± 5 islets/size category for a total of 317 individual islet measurements.

transplantation. The standard method of volume estimation is the international IEQ measurement, a method that greatly enhanced islet research by providing a common method of islet volume estimation that was simple, quick, and could be broadly applied. The IEQ calculations were based on the mathematical assumption that all islets are spherical, when in fact they are elliptical. Many research groups, in addition to us, have recognized that the shapes of islets are irregular (7,10,15). The 3D measurements of isolated islets reported here confirm the earlier measurement by Avgoustiniatos showing that the variation in the smallest to largest diameter of islets produced a ratio of 0.6 (1), where we measured a ratio of 0.7.

Since islets are clusters of cells, we proposed to estimate islet tissue volume by total cell number. The same sampling procedure and islet count methods currently utilized in clinics and research laboratories (2) can be used with the Kansas method to more accurately calculate islet volumes. Thus, this new method requires minimal changes to the standard operating procedures for islet transplant clinics or research laboratories. Only the last step of conversion to IEQ needs to be changed to normalize using our

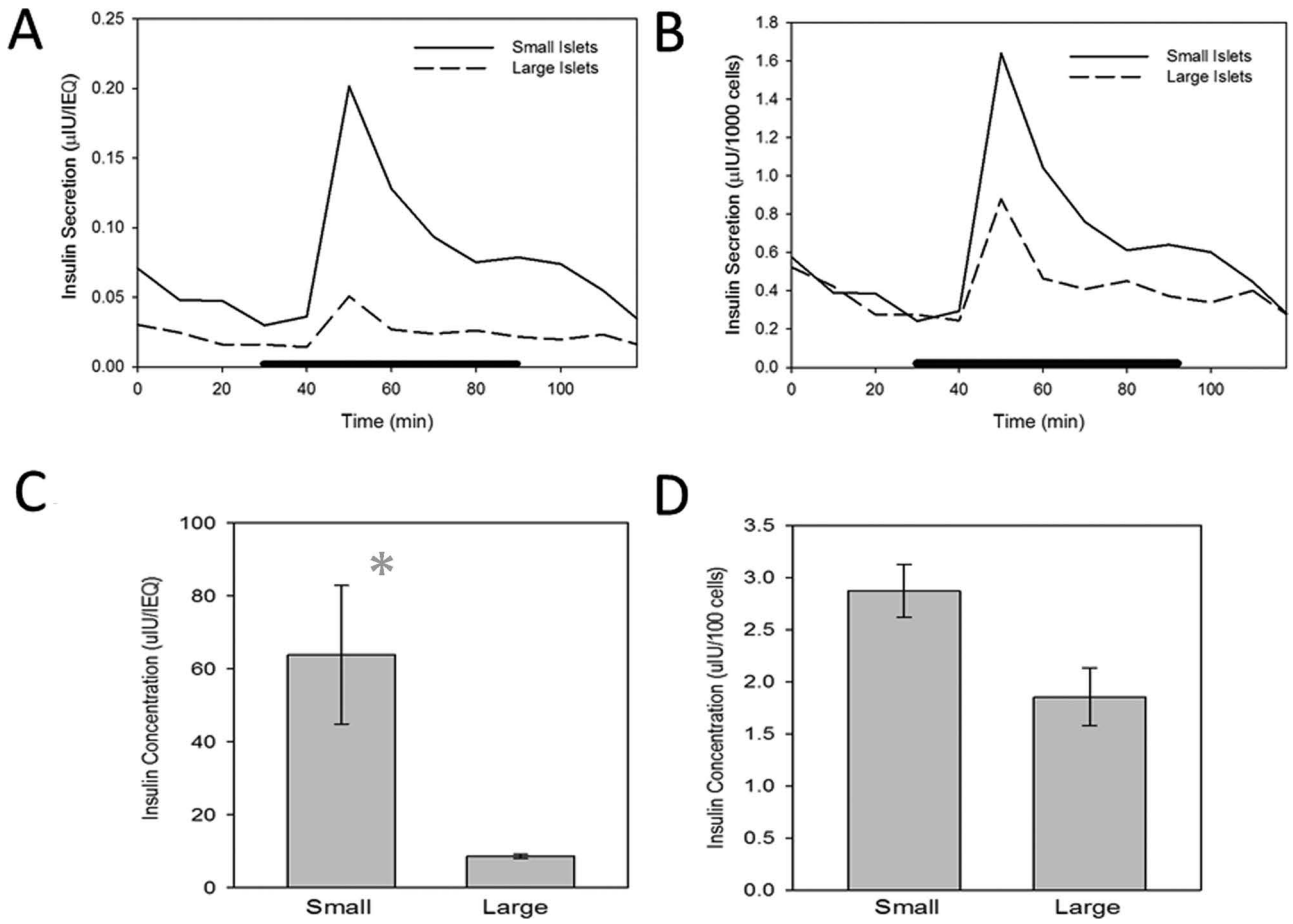


Figure 5. Insulin secretion measurements altered by normalization. (A) Perifusion experiments were performed on human islets when exposed to low glucose (2.8 mM) from 0 to 30 min. Islets were then exposed to high glucose (22.4 mM glucose) from minutes 30 to 90 (black bar), at which time they were returned to the low-glucose media. Each graph shows the results from a single experiment with a range of 150–300 IEQs per preparation. Small islets secreted statistically more insulin than large islets when normalized to IEQ at each time point measured ($p < 0.05$). (B) When the same insulin secretion data was normalized to cell number using the Kansas method, there was no difference in the conclusion of the results; small islets still secreted more insulin per volume, but the difference was not as great, and the initial insulin secretion in basal glucose was not different. (C) In a separate preparation from a different donor, insulin secretion was measured by static incubation and was greater in the small islets ($p < 0.05$). (D) However, when normalized to cell number, the variation within the small group was decreased (error bars), and the difference between the two groups was diminished so that there was no statistically significant difference.

formula. Recently, Pisania et al. estimated the islet volume by using a cell nuclei count (16). Like this report, they concluded an overestimation when using IEQ measurement and suggested the overestimation might be due to the possible space such as intraislet vessel and intracellular space not accounted for by IEQ measurements (3,12). Compared to Pisania's work, we also base our volume estimations on cell numbers, but our approach does not require the extra step of nuclei staining to estimate volume.

Importantly, the validation studies conducted, using two different methods for viable cell counts, resulted in the same findings. When ATP measurements were normalized to IEQ, the underestimation of volume in the small islets gave the appearance of dramatically more ATP/volume in small islets and with a gradual decrease with larger islets (Fig. 4A). However, when the same data were normalized to cell number using the Kansas method, the ATP/volume was nearly the same in islets of all measured sizes (Fig. 4B). The same was true when cell viability was measured with alamarBlue; an assay based on resazurin's conversion to the fluorescent resorufin in living cells (Fig. 4D). Thus, the Kansas method was validated with luminescent and fluorescent assays based on completely independent chemistry.

Current volume-normalizing methods in islet research need to be reconsidered because completely different results may be obtained, depending on the normalization method. Here, we provide examples of differences in

Table 5. Analysis of Human Transplant Data

Islet Category	Islet Size (IEQ/Islet)	Calculated Cell Number/ Islet Based on IEQ	Calculated Cell Number/Islet Based on Kansas Method
Small	0.80 ± 0.01	604	633
Medium	1.71 ± 0.04	1,540	1,109
Large	3.03 ± 0.27	3,171	1,685

Previously published human islet transplant data (11) was analyzed to determine the IEQ/islet, and recipients were grouped according to the average size islet in the transplant. The average cell number transplanted was calculated for each group based on the IEQ method, assuming all islets are spherical, and the Kansas method.

results that were obtained from the same data sets normalized to IEQ or the Kansas method of cell number. These include data from ATP levels, viability, and static and perifusion insulin secretion. Additionally, we now have produced two separate normalization calculations for rat and human. Compared to our previously published rat conversion calculation (8), the small human islets had a higher number of cells/islet diameter as shown in Figure 2D. However, 150-μm-diameter islets had approximately the same number of measured cells/islet for either species (943 for rats and 929 for humans). For larger islets, the number of cells/islet size was greater in the rat islets. The results suggest that two different conversion calculations must be used for rat and human islets, especially when including large islets in the populations.

Our findings may have a tremendous impact on assumptions made about the volume of islets used in clinical transplants. Historically, large islets have been preferentially used for transplantation due to their assumed high tissue volume. Using human islet transplant data published by Lehmann et al., the average size of the islets transplanted into diabetic recipients during the study period ranged from 0.80 to 3.30 IEQ/islet (11). Lehmann reported that patients receiving more small islets (lower IEQ/islet) had better stimulated C-peptide values after the transplant, while the total IEQ volume of transplanted islets failed to correspond with the C-peptide values. When we calculate the average size of each islet per transplant (total IEQ/number of islets) from the Lehmann et al. data, the average islet sizes fell into three categories of small, medium, and large (Table 5). If one uses the cell number/islet diameter values predicted by Ricordi's method (Fig. 3B, closed circles) the resulting cells/IEQ value ranges from an average of 604 to 3,171 cells/islet. However, if the Kansas method was used to convert the average islet size to cell number, the range was 633 to 1,685 cells/islet (Table 5). Consistent with our finding in Figure 3, the larger the islets, the greater the overestimation of the total tissue volume used in the transplantation. In the group of patients described in Lehmann's publication (11) receiving larger average islets for their transplant, the Kansas method of volume estimation suggests that they received only 53% of the volume that was reported based on IEQ.

The Kansas method has been integrated into a spreadsheet that automatically calculates cell number from any measured islet diameter between 20 and 350 μm and has been placed on our website for free downloads at http://www.ptrs.kumc.edu/kansasmethod/ or http://ptrs.kumc.edu/kansasmethod/. Different spreadsheets are available for human and rat conversions. This procedure for volume normalization no longer requires the binning of islets into conventional 50-μm size classifications. Rather, the exact diameter of an islet could be measured visually or via image software and the conversion made directly from the islet size. The Kansas method for estimating islet volume is simple, requiring only minimal changes in the current volume estimation protocol. It requires no special equipment or software, and the calculation program is offered free to the public. More importantly, the Kansas method provides a more accurate estimation of the volume of tissue in an islet, based on actual cell counts.

ACKNOWLEDGMENTS: The authors wish to thank the members of the Flow Cytometry Core Laboratory at the University of Kansas Medical Center, S. Janette Williams for insulin secretion data, Dr. Tiffany Schwasinger-Schmidt for EM images, and Dr. Lesya Novikova for immunofluorescence images. Funding for the project was provided by the University of Kansas Medical Center Biomedical Research Training Program and the Institute for the Advancement of Medical Innovation. The authors declare no conflicts of interest.

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