Published in final edited form as:
Transplant Proc. 2014 ; 46(6): 1953–1955. doi:10.1016/j.transproceed.2014.06.011.

Islet Preparation Purity Is Overestimated, and Less Pure Fractions Have Lower Post-Culture Viability Before Clinical Allotransplantation

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Abstract

Background—Replacement of β-cells with the use of isolated islet allotransplantation (IT) is an emerging therapy for type 1 diabetics with hypoglycemia unawareness. The current standard protocol calls for a 36–72-hour culture period beforeIT. We examined 13 clinical islet preparations with ≥2 purity fractions to determine the effect of culture on viability.

Methods—After standard islet isolation and purification, pure islet fractions were placed at 37°C with 5% CO_2 for 12-24 hours and subsequently moved to 22°C,whereas less pure fractions were cultured at 22°C for the entire duration. Culture density was targeted at a range of 100-200 islet equivalents (IEQ)/cm^2 adjusted for purity. Islets were assessed for purity (dithizone staining), quantity (pellet volume and DNA), and viability (oxygen consumption rate normalized to DNA content[OCR/DNA] and membrane integrity).

Results—Results indicated that purity was overestimated, especially in less pure fractions. This was evidenced by significantly larger observed pellet sizes than expected and tissue amount as quantified with the use of a dsDNA assay when available. Less pure fractions showed significantly lower OCR/DNA and membrane integrity compared with pure. The difference in viability between the 2 purity fractions may be due to a variety of reasons, including hypoxia, nutrient deficiency, toxic metabolite accumulation, and/or proteolytic enzymes released by acinar tissue impurities that are not neutralized by human serum albumin.

Conclusions—Current clinical islet culture protocols should be examined further, especially for less pure fractions, to ensure the maintenance of viability beforetransplantation. Even though

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relatively small, the difference in viability is important because the amount of dead or dying tissue introduced into recipients may be dramatically increased, especially with less pure preparations.

Introduction

Despite the recent progress made in the field of human clinical allograft islet transplantation (IT) for the treatment of type 1 diabetes, the majority of recipients do not become fully insulin independent after a single transplantation [1). Factors associated with lower purity, such as competition for oxygen and other nutrients between islet and nonislet tissue during in vitroculture and after transplantation, may have a major impact on the success of IT. The standard method to estimate the purity fraction of islets is by staining with dithizone (DTZ) and examining by light microscopy, which is operator dependent and can lead to overestimation of purity[2,3]. Not accounting for this overestimation (ie, appropriately adjusting the culture surface density) can be detrimental to the viability and function of the islets, which in turn may reducethe chance of insulin independence in the recipient [2-7]. There is a need for standardization and optimization of purity measurements and culture methods to minimize or eliminate this chance. The present study was designed to retrospectively examine IT preparations by various methods of cell quantification and to evaluate the effect of purity on islet viability after culture with the use of oxygen consumption rate normalized to DNA content (OCR/DNA) and membrane integrity stains.

Methods

We retrospectively examined all human clinical islet allograft preparationswith at least 2 purity fractions (n = 13) after 36-72 hours of the standard culture method of IT[8].After routinehuman islet isolation and purification, pure islet fractions (≥70% purity estimated by DTZ staining) were placed in an incubator at 37°C with 5% CO_2 for 12-24 hours and subsequently moved to 22°C. Less pure (30%-69%purity) fractions were cultured at 22°C with 5% CO_2 for the entire duration. Culture density per T-175 flask was targeted at a range of 100-200 islet equivalents (IEQ)/cm^2 adjusted for purity (ie, 50-100 IE/cm^2 fora 50% pure preparation). CMRL-1066 media volume was 30mL per T-175 flask, with 20mL being replaced after 12-24 hours of culture[9]. After the culture period, islets were assessed for purity, quantity, and viability.

Purity was estimated by removing 2 samples of 100-200 μL each from the well mixed and presumed homogeneoustissue suspension and adding DTZ. Samples were examined under a light microscope, and the percent of red-stained cells was estimated as the purity [2,3,7].

Islet quantity was measured by 3 methods and estimated by 1 method(1) A standard IEQcountwas based on the DTZ samples, in which islets ≥50 μm in diameter were stratified into groups of 50-μm increments and normalized to a150-μm diameter size. (2) A dsDNA fluorescent dye kit (Quant-it PicogreendsDNA kit; Molecular Probes, Eugene, Oregon) was used to measure the quantity of DNA in each purity fraction; the total quantity of DNA measured was converted to IEQ by assuming 1 IEQ = 10.4 ng DNA [2] and corrected for purity as measured by DTZ to be compared with the IEQ counts. (3) For measuring the observed pellet volume, ithe tissue was aspirated into a 10-mL glass pipette and allowed to

settle for 3-5 minutes, and the cell volume (mL) was then visually observed. (4) The IEQ measured by method (1) was then used to estimate the expected pellet volume to be compared with the observed pellet volume; the expected pellet volume (mL) based on the IEQ counts was determined by using the volume of an IEQ which, being by definition a sphere with a diameter of 150 μm, is 1.77×10^6 μm^3; for example, accounting for a ~10%void fraction, or the extracellular space between islets, a pellet size of 2 mL would be expected to contain ~1×10^6IEQ of 100% pure islets, and 4 mL would be expected to contain ~1×10^6IEQ of 50% pure islets.

Islet viability was assessed by 2 methods. The first method was by membrane integrity staining with the use of anIT release assay (viability must be ≥70%). Briefly, an aliquot of islets (80-100 IEQ) were spun down and resuspended in 460 μL Dulbecco phosphate-buffered saline solution (DPBS), 10 μL 0.46 μmol/L fluorescinediacetate(Sigma no F7378;inclusion dye in which viable cells appear as bright green fluorescent), and 10 μL 14.34μmol/L propidium iodide (Sigma no P4170;exclusion dye which enters ruptured cell membranes with a red/orange fluorescence). The cells were then washed with DPBS and viewed under a fluorescent microscope. Percentage viability was estimated as the area of green cells (live) to total tissue [2,3]. The second method of assessing islet viability was by measuring the OCR/DNA as detailed elsewhere [2,7,10] with the use of a real-time in vitroassay that has been validated as a quality assay in various animal models and the human autograft model[11-13].

Statistical Techniques

All statistical analyses were performed with the use of the SAS statistical software package, version 9.3 (SAS Institute, Cary, North Carolina) or GraphpadPrism, version 5.03 (GraphpadSoftware, La Jolla, California). Values are reported as mean ± SD. Statistical significance was tested by a paired Student *t* test and corresponded to $P<.05$.

Results

The islet puritywas typically grossly overestimated in all fractions based on the comparison between observed and expected pellet volumes, especially in the less pure fractions. This was demonstrated by the significantly larger observed pellet volumesrelative to the volumes expected from the counts ($P = .01$ for pure; $P = .0001$ for less pure; Fig 1a and b). The overestimation of purity was also evidenced by the significant difference between the actual IEQ counts and the IEQ estimated from the dsDNA assay when available ($P = .003$ for pure; $P = .0001$ for less pure; Fig 1c and d). Less pure fractions showed a significantly lower viability than pure fractions after culture as measured by both OCR/DNA and membrane integrity stains (Fig 1e and f). Mean ± SD OCR/DNA was 125 ± 28nmol O_2/min•mgDNA for pure fractionsand 99 ± 19 for less pure fractions ($P = .009$). Based on membrane integrity, percentage viability was 92 ± 5% for pure fractionsand 86 ± 4%for less pure fractions ($P = .003$).

Discussion

Among the major factors favoring successful IT, the islet yield, the purity of preparations, and the viability of the islets post-culture are critical for transplant outcome. Purity has been traditionally measured by visual estimation of preparations stained with DTZ, and this test gave 20%-30% erroneously higher values on average compared with more rigorous assessments of islet preparation fractions, such as electron microscopy or laser scanning cytometry[4,5,7]. Street et al reported that when comparing islet purity assessed by DTZ staining with results with the use of immunostaining to quantify total endocrine cellular composition, the DTZ-based purity assessment gave significantly higher results than those indicated by the endocrine immunostaining [6]. Furthermore, it is known that individual estimates from DTZ staining are subject to considerable observer variability [6,14,15].

The results from this study indicate that the purity by DTZ staining is typically grossly overestimated in all fractions and especially in less pure ones. This was evidenced by observing significantly larger settled tissue volumes and estimated IEQ from DNA quantities than what was expected based on the counts. This overestimation could be the cause of lower viability in the less pure preparations after culture. The cells may have been exposed to hypoxia and/or nutrient deficiency, because the culture density would be much higher than desired [16]. Another possible cause of the difference in viability may be toxic metabolite accumulation and/or proteolytic enzymes released by the acinar tissue impurities that are not neutralized by human serum albumin [17].In addition, there may be a preferential loss of viability in nonislet tissue, which would explain the lower viability in less pure fractions.

In conclusion, current clinical islet purity measurements and culture protocols should be examined and further refined to ensure the maintenance of viability after culture and beforetransplantation. Even though relatively small, the difference in viability observed with purity is important because the amount of total and thus dead tissue introduced in recipients may be dramatically increased, especially with less pure preparations.

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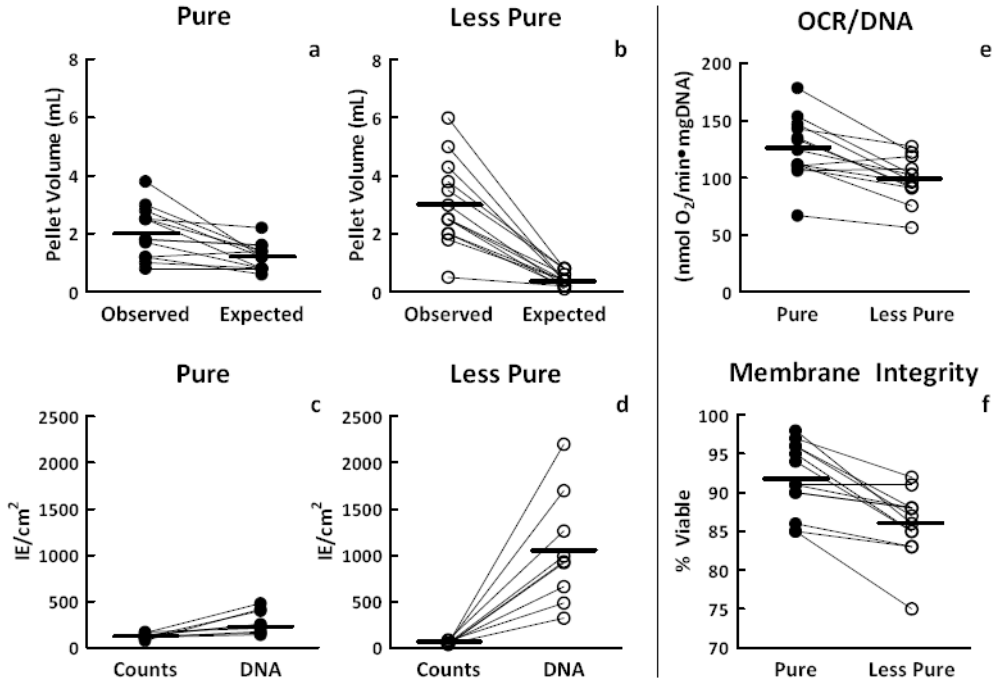

Fig 1.

Observed values plotted against theoretical expected valuesfor (a, b) pellet volume, (c,d) islet density (islet equivalents [IE]/cm^2) in culture flasks as measured by counts and DNA, and (e,f) islet preparation viability for separate purity fractions as measured by oxygen consumption rate normalized to DNA content (OCR/DNA) and membrane integrity staining for 13 clinical islet preparations.