

Transport Limitations in Islets of Langerhans Culture

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Abstract

Introduction: Islet transplantation has become a promising treatment for type I diabetes mellitus due to recent success since the development of the Edmonton Protocol. Islet culture prior to transplantation is standard practice in several clinical islet programs. High-density islet culture is desirable because it reduces space and handling requirements during culture, but it exacerbates oxygen limitations and causing a reduction in islet viability. We investigated the effect of tissue density on total tissue recovery, viable tissue recovery, and tissue purity for conventional normoxic culture on a polystyrene dish. To improve islet quality in high density culture, we explored use of elevated pO₂ or culture on an oxygen-permeable silicone rubber membrane. We applied a theoretical O₂ transport model to investigate how O₂ transport changes for each culture condition and then compared our predictions to the experimental data to determine whether O₂ is limiting during high density culture using these new techniques.

Methods: Human islets were cultured for 34-60 hr at densities varying from 20-5300 IE/cm² on either 500- μ m silicone membranes or solid bottom dishes in a humidified incubator with 5% CO₂, 19% or 56% oxygen at 37°C. The quantity, viability, and purity of tissue were quantified using nuclei counts, oxygen consumption rate (OCR) measurements, and insulin immunostaining. Theoretically predicted profiles of oxygen partial pressure and islet viability were obtained by solution of the species conservation equation with the finite element method.

Results: Low density islet culture on a polystyrene dish resulted in tissue adherence, which decreased as density increased. The adherent tissue had a lower fraction of original insulin-positive cells collected from culture as compared to the non-adherent tissue. The combined non-adherent and adherent tissue collected from low density culture accounted for nearly 100% of the original viable tissue placed into culture and had purity similar to that for tissue prior to culture. With conventional culture, recovery of total OCR decreased sharply as viable tissue density increased. At all densities, the fraction of original viable tissue collected from culture was higher with culture on polystyrene dishes in 56% oxygen and even higher with normoxic culture on silicone rubber. Theoretical predictions were qualitatively similar to experimental results but in general over predicted the amount of viable tissue recovered.

Conclusions: In low density culture all original viable tissue can be recovered as both free and adherent tissue. In high density culture, recovery of viable tissue (1) decreases as culture density increases on a polystyrene surface, (2) increases with increasing external pO₂, and (3) increases substantially with culture on silicone rubber by removing O₂ limitations.

Introduction

Though many complications remain [1], islet transplantation is beginning to become a promising option for treating type 1 diabetes due to recent success since the development of the Edmonton Protocol [2]. After islet tissue is mechanically and enzymatically isolated from a donor pancreas [3, 4] it may be transplanted directly or cultured for a period of time prior to transplantation [4, 5]. Islet culture prior to transplantation is desirable for many reasons and is standard practice in several clinical islet programs [5]. Culture provides time for islet tissue to recover from the harsh isolation process and allows islets to be maintained over time while tissue quality is assessed for purity, viability, and sterility [5-7].

Islet culture has been the subject of much research but remains an area that needs improvement. Conditions including islet density, culture temperature, and culture duration vary between islet centers with relatively little published justification making it difficult to determine which technique is optimal for islet recovery, viability, and purity, yet still practical in a clinical setting. Though a lot of literature is available investigating various media formulations and additives (see Murdoch et al [5] and Clayton et al [7] for comprehensive reviews on media supplementation), very little is available investigating oxygen (O₂) transport and the effect of islet density on islet quality [5]. Gaber et al [8] noted that islet density and size might be related to necrotic death and then Matsumoto et al [6] demonstrated that low islet concentrations resulted in higher recoveries and higher stimulation indexes compared to culture with high islet concentrations. This decrease in tissue quality is believed to be due to insufficient nutrient transport to the core of the islet, which is supported by observations that indicate higher necrotic death in islets at 37°C compared to lower temperatures where metabolic rates are reduced [9].

Dionne et al [10] noted that transport is crucial for maintaining islet viability and function. Since blood vessels collapse during static culture [11, 12], O₂ is supplied to the islets only through diffusion resulting in the formation of O₂ gradients in and around each islet. If islets are too large or too close together, the O₂ is depleted before it reaches the center of the islet causing the core to become hypoxic and leading to the development of a necrotic core [9, 10, 13]. A uniform surface coverage of no more than 0.4% or 21 IE/cm² in 3 mm of medium at 37°C and an atmosphere of 95% air/5% CO₂ is needed to remove all O₂ limitations during culture [13]. This low-density culture requires large space and handling requirements. Assume that 400,000 islets are obtained from an isolation that have to be cultured prior to transplantation. Culture at 21 IE/cm² would require an area of about 1.9 m² for 400,000 islets. Using T-175 (surface area 175 cm²) T-flasks would require use of 109 flasks in order to remove all O₂ limitations. This would require 2-3 incubators and someone to consolidate all 109 flasks prior to transplantation which is impractical for clinical use. As a result a tissue density is chosen that is practical at the expense of the viable tissue. Current techniques include culture densities up to 400 IE/cm², which based on previous predictions could result in a 20% loss in viable tissue [13]. Thus, a high density culture technique that maintains tissue quality is desirable in order to keep tissue densities practical without sacrificing viable tissue. This study will focus on two methods for alleviating O₂ limitations in high density culture. They are culture in elevated gas pO₂ and culture on silicone rubber membranes.

In this study we investigate the effect of tissue density on the fraction of original tissue collected from culture, the fraction of original viable tissue collected from culture, and the fraction of original insulin positive cells collected from culture for conventional culture on a polystyrene dish. We explore high density culture techniques by culturing islets either in an elevated pO₂ or on an O₂-permeable silicone rubber membrane. Finally we use a theoretical O₂ transport model to investigate how O₂ transport changes for each culture condition and then compare our predictions to the experimental data to determine whether O₂ is limiting during high density culture using these new techniques.

Methods

Islet Culture. Upon receipt a portion of the human islet sample was analyzed and the remainder was placed into culture on either untreated polystyrene culture vessels or 500 μm thick silicone rubber membranes (Wilson Wolf Manufacturing, Inc. New Brighton, MN) at 37°C in a humidified environment with 95% air / 5% CO₂ (pO₂ = 142 mmHg) at densities varying from 20-5300 IE/cm² for 34-60 hr over which no medium change occurred. Islets were cultured

in supplemented RPMI (Mediatech Inc., Herndon, VA) with 10% FBS (Mediatech Inc.). For high-density experiments (>30 IE/cm² assuming 1560 nuclei/IE) islets were cultured in 0.3-2.2 cm of medium as specified. For low density experiments (<30 IE/cm²) islets were cultured in 1.3 mm of medium. For high O₂ culture, islets were cultured at 37°C in 56% O₂ (428 mmHg). After culture free and adherent tissue were collected and analyzed. For low-density culture, trypsinization was required to remove tissue that adhered to the culture flask. At higher densities trypsinization was not required because tissue did not adhere. Only non-adherent tissue was analyzed at an OCR density > 0.5 nmol/min cm², whereas adherent and non-adherent tissue was combined before analysis when the OCR density was < 0.5 nmol/min cm².

Cell Enumeration by Nuclei Counting. Equal volumes of sample and lysis solution were combined, vortexed, and incubated at room temperature. The islet mixture was rapidly forced through a needle to liberate the nuclei. Isolated nuclei were diluted with Dulbecco's phosphate buffered saline (D-PBS, Invitrogen, Carlsbad, CA), stained with 7-aminoactinomycin D (7-AAD, Molecular Probes, Eugene, OR) for at least 2 min at room temperature, and then analyzed using a flow cytometer (Guava Personal Cell Analysis (PCA) system, Guava Technologies, Hayward, CA).

Oxygen Consumption Rate (OCR). Islets were suspended in serum free DMEM (Mediatech Inc) and sealed in a 200- μ l stirred titanium chamber (Instech Laboratories, Plymouth Meeting, PA) equipped with a fluorescence-based oxygen sensor (Ocean Optics, Dunedin, FL). The time-dependent pO₂ within the chamber was recorded, and the data at high pO₂ were fit to a straight line. The maximal OCR was evaluated from $OCR = V_{ch}\alpha(dpO_2/dt)$, where V_{ch} is the chamber volume and α is the Bunsen solubility coefficient.

Immunostaining and Light Microscopy. Cultured tissue samples were washed twice with D-PBS prior to fixing for one hr in 10% formalin (Sigma Aldrich). After fixation the tissue was washed, pelleted in 2% agarose, incubated for 1 hr at room temperature in 10% formalin, and then stored in D-PBS until embedding in paraffin. One- μ m sections were taken from the pellet. The sections were stained by immunoperoxidase [14, 15] for β cells as previously described [16]. The primary antibody used in the staining procedure was guinea pig anti-bovine insulin (Linco, St. Charles, MO) and the secondary antibody was goat anti-guinea pig (Cappel, Irvine, CA). The peroxidase-anti-peroxidase (PAP) antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) was applied prior to staining in 50 ml of a 2 mM 3,3'-diaminobenzidine tetrahydrochloride (Sigma Aldrich) solution activated with 25 μ l of 30% hydrogen peroxide (EMD Chemicals Inc, Gibbstown, NJ). The slides were counterstained with hematoxylin (Sigma Aldrich).

Original tissue samples were analyzed using morphological analysis carried out by light microscopy. Aliquots from the islet preparation were processed using a standard procedure, during which they are fixed, dehydrated, cleared, embedded, cured, and trimmed to produce 1- μ m sections in epoxy resin. This process took about 3 days.

One- μ m sections were analyzed by stereological point counting [17] in which point intercepts of tissue with a grid covering adjacent, non-overlapping fields were counted under a light microscope. In order to compare the purity measurement using light microscopy and that with immunoperoxidase insulin staining we converted the volume fraction of islet tissue from light microscopy to the volume fraction β cells excluding vascular space using previously described mathematical relationships [3].

Statistics. Each measurement was run in triplicate unless otherwise specified. Data are expressed as mean \pm standard deviation. Statistical significance was determined by a two-way Student t-test for $p < 0.05$ assuming either paired data.

Finite Element Analysis. The finite element simulations using our theoretical model were carried out using the commercially available package COMSOL Multiphysics in conjunction with Matlab. We required the mesh to contain more than 1000 nodes and set a convergence criterion such that the error estimated by COMSOL Multiphysics program, which took into account the current and previous solution in the iterative solver, was less than 10^{-2} [18].

Theoretical Model

We modeled islet culture on O_2 -impermeable and O_2 -permeable dishes assuming the OCR of the tissue follows Michaelis-Menten kinetics. The solution to the diffusion-reaction equation

$$0 = D_{O_2} \alpha_{O_2} \nabla^2 P_{O_2} - \frac{V_{max} P_{O_2}}{K_m + P_{O_2}}$$

where α_{O_2} is the solubility of O_2 , pO_2 is the partial pressure of O_2 , D_{O_2} is the diffusivity of O_2 , V_{max} is the maximum reaction velocity, and K_m is the pO_2 at which the reaction rate is $\frac{1}{2} V_{max}$, yields the pO_2 profiles inside and outside the islet. All the tissue (islet and non-islet) was assumed to be in perfect spheres with uniform diameter (D) that were arranged in a square array in a stagnant medium of height h (Figure 1). The islet density varied between simulations and therefore the center-to-center distance (d) between islets varied. Table 1 contains the values and references for the parameters used in the simulations for both the O_2 -impermeable and O_2 -permeable dishes. As the tissue density increases, the minimum pO_2 decreases until it reaches P_{crit} at which the tissue dies and a necrotic core begins to develop. As density increases further the non-viable tissue volume increases (Figure 2).

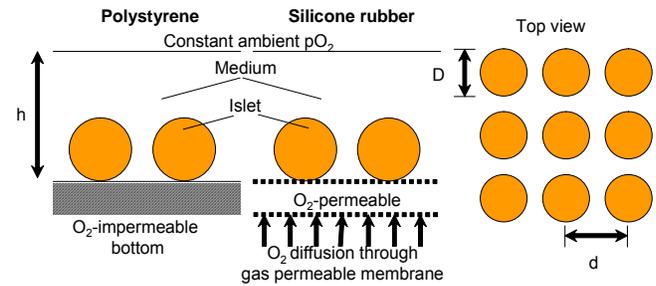


Figure 1. Islet geometry used to model culture on polystyrene and silicone rubber.

Table 1. Values and references for the parameters used in simulations.

Parameter	Value	Units	Reference
K_m	0.44	mmHg	Wilson et al. 1988
O_2 diffusivity - medium	2.78×10^{-5}	cm^2/s	Avgoustiniatos 2001
O_2 diffusivity - tissue	1.22×10^{-5}	cm^2/s	Avgoustiniatos 2001
O_2 diffusivity - silicone rubber	2.17×10^{-5}	cm^2/s	Colton et al. 1971
O_2 solubility - medium	1.27×10^{-9}	$mol/cm^3 \text{ mmHg}$	Avgoustiniatos 2001
O_2 solubility - tissue	1.02×10^{-9}	$mol/cm^3 \text{ mmHg}$	Avgoustiniatos 2001
O_2 solubility - silicone rubber	1.21×10^{-8}	$mol/cm^3 \text{ mmHg}$	Colton et al. 1971
Critical pO_2	0.1	mmHg	Avgoustiniatos 2001
Islet radius	75	μm	Based on definition of IEQ
Silicone rubber thickness	500	μm	Determined by experiment
V_{max}	540-2310	$nmol/min \text{ cm}^3$	Determined by experiment
Medium height	1.3-22	mm	Determined by experiment
External pO_2	142 or 428	mmHg	Determined by experiment

Experimental Results

Following culture only non-adherent tissue is recovered and transplanted in most, if not all, clinical islet programs. When we cultured human islets for 34-60 hours in a fully humidified environment at $37^\circ C$ (142 mmHg O_2) on untreated polystyrene we observed that the fraction of original nuclei collected from culture as non-adherent tissue (FN_{NA}) varied

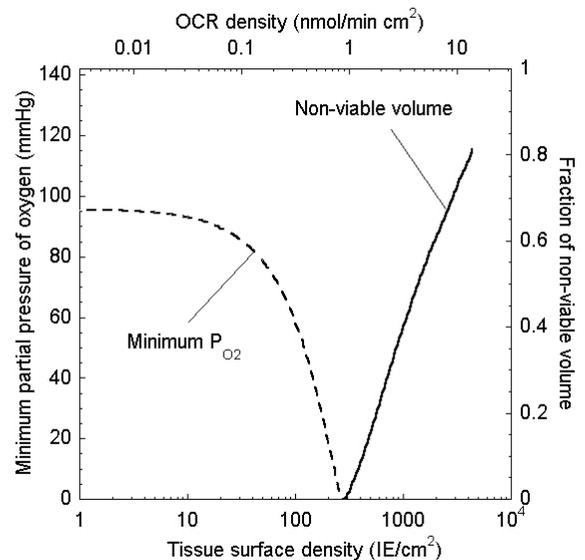


Figure 2. Theoretical prediction for minimum islet pO_2 (dashed line) and non-viable tissue volume (solid line) as a function of islet surface density and OCR density for steady state islet culture in 3 mm medium at an external O_2 concentration of 142 mmHg assuming $V_{max} = 2210 \text{ nmol/min cm}^3$.

with initial OCR density. Visual inspection revealed that tissue was sticking to the untreated culture vessels at low culture densities. The adherent tissue was trypsinized, collected, and quantified using nuclei counting. As the initial OCR density increased, the fraction of original nuclei collected from culture as adherent tissue (FN_A) decreased while FN_{NA} increased (Figure 3). For most cultures above $0.5 \text{ nmol/min cm}^2$ trypsinization was not conducted since very little tissue was adherent. This was always confirmed by visual inspection under the microscope.

To further understand this phenomena we focused on low density culture (1.3 mm medium at densities $< 0.1 \text{ nmol/min cm}^2$) when tissue was in the absence of O_2 limitations. The data in Figure 4 indicate the fraction of original OCR, nuclei, and insulin positive cells collected from culture as adherent, non-adherent, and combined (adherent + non-adherent) tissue from these low density cultures. It should be noted that the human islets used in this particular set of experiments were cultured following isolation for 1-5 days before we received them for our experimental culture. When data from both adherent and non-adherent tissue fractions were combined the fraction of viable tissue collected from culture based on OCR measurements (FO_T) was nearly 1. This finding has been generally true with all our low density cultures ($< 0.1 \text{ nmol/min cm}^2$) that used previously cultured tissue. In contrast to complete recovery of viable tissue, only 0.68 ± 0.27 of all of the original nuclei and only 0.58 ± 0.32 of all original insulin positive cells were collected from these cultures (Figure 4). When performing similar cultures using tissue obtained immediately after isolation we observed a lower FO_T with a higher variation (0.72 ± 0.22) than that obtained when using precultured tissue (0.95 ± 0.09).

By combining the data for tissue recovery (Figure 3) with purity measurements we could determine the fraction of original insulin positive cells collected from culture as non-adherent (FI_{NA}) and adherent tissue (FI_A). As indicated in Figure 4, the non-adherent tissue collected from culture contained the majority of the insulin positive cells whereas the adherent tissue contained very few insulin positive cells (0.07 ± 0.04 of original insulin positive cells). FI_A was statistically lower than FI_{NA} .

Figure 5 shows the fraction of original viable tissue collected from culture based on OCR measurements (FO_T) and theoretical predictions as a function of the initial OCR density for culture in a 3 mm

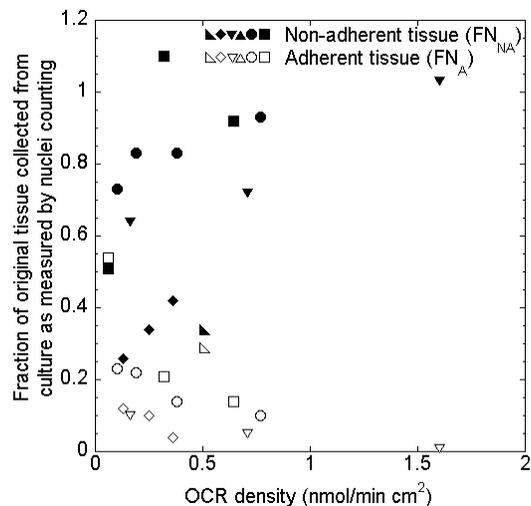


Figure 3. The fraction of original tissue collected from culture as non-adherent (FN_{NA} , filled) and adherent (FN_A , open) tissue plotted versus OCR density. Data are from 5 different islet isolations (represented by different symbols) cultured at varying densities in 3 mm medium for 34-60 hr at 37°C in a humidified environment on untreated polystyrene culture vessels.

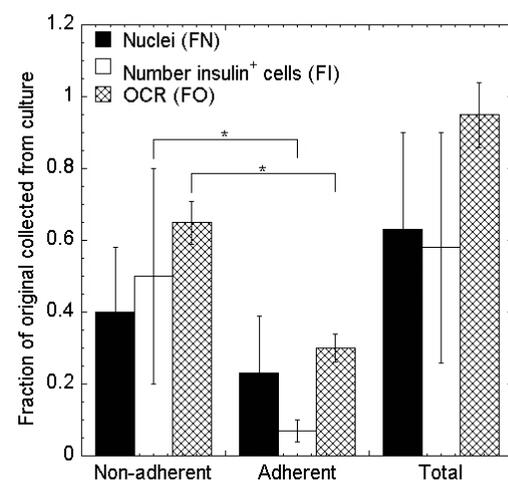


Figure 4. The fraction of original nuclei (black bars), insulin positive cells (white bars), and OCR (checkered bars) collected as adherent, non-adherent, and combined (adherent + non-adherent) tissue for islet culture (using precultured human islets) at densities below $0.1 \text{ nmol/min cm}^2$ in 1.3 mm medium on untreated polystyrene culture vessels. The error bars are \pm the standard deviation from $n=4-6$ islet preparations. * indicates statistical significance ($p < 0.05$) for a two-tailed paired t-test.

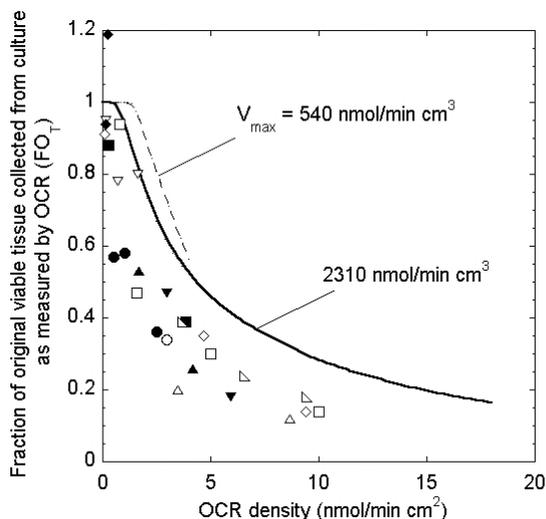


Figure 5. Experimental results and theoretical predictions for the fraction of original viable tissue collected from culture vs. OCR density for culture in 3 mm medium on untreated polystyrene culture vessels. The different symbols represent different islet preparations.

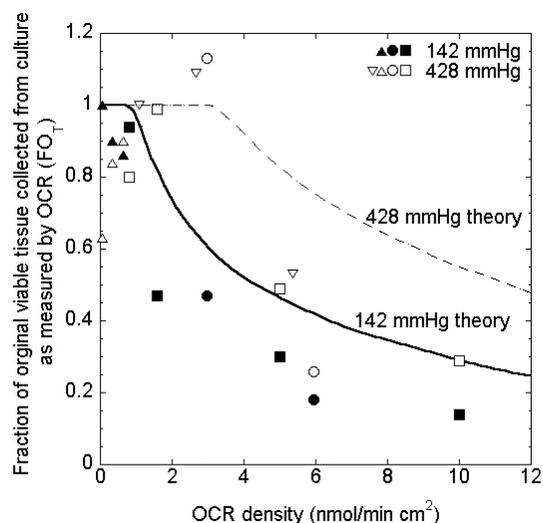


Figure 6. FO_T (symbols) and theoretical predictions (lines) as a function of OCR density for culture on polystyrene with a 3 mm medium depth in either 142 mmHg (filled symbols, solid line) or 428 mmHg O_2 (open symbols, dashed line). The differently shaped symbols represent different islet preparations. The V_{max} used in the theoretical calculations was 2210 $nmol/min\ cm^3$.

This is contrary to that for culture in 3 mm of medium as indicated above. For culture on silicone rubber the data does not follow the theoretical prediction. The theoretical prediction indicates no drop in FO_T for the range of densities tested using a 500 μm silicone rubber membrane ($V_{max} = 2210\ nmol/min\ cm^2$) while a decrease in FO_T is observed empirically. Additionally the FO_T resulting from culture on 100- μm silicone rubber was similar to that for culture on 500- μm silicone rubber (Figure 7).

medium depth. The V_{max} values used in the theoretical predictions correspond to the largest and smallest V_{max} measured for the islet preparations used in the culture experiments as determined by OCR measurements of the islet tissue prior to culture. Using conventional culture on a polystyrene dish there was a rapid decrease in the measured fraction of original viable tissue collected from culture as the density increased. The theoretical predictions follow the same trend as the experimental data, but over predict the fraction of original viable tissue collected from culture. Recall that at low density in the absence of oxygen limitations nearly 100% of the original viable tissue can be recovered from culture. These control cultures are not indicated in Figure 5.

FO_T at 428 mmHg O_2 was higher than that for conventional culture at densities $> 1\ nmol/min\ cm^2$ as indicated in Figure 6 and predicted by theory. However, the theoretical predictions significantly overestimate the empirical data at densities $> 3\ nmol/min\ cm^2$. At densities $< 1\ nmol/min\ cm^2$ there was no consistent difference for tissue cultured at 428 mmHg O_2 than that at 142 mmHg. Additionally, the data for the lowest density tested at 428 mmHg O_2 resulted in a significantly lower FO_T than 142 mmHg O_2 . Though this may have been a random occurrence, additional experiments using low density culture ($0.1\ nmol/min\ cm^2$) at 570 mmHg O_2 for 48 hr also resulted in a lower FO_T than 142 mmHg O_2 (results not shown). A likely cause of this decrease is oxygen toxicity, which in other experiments in our laboratory has been shown to decrease $\beta TC3$ cell viability in a dose and time dependence manner (results not shown).

Theoretical and experimental data for culture on polystyrene and silicone rubber with a medium depth of 22 mm are shown in Figure 7. Both theoretical and empirical data demonstrate that culture on silicone rubber greatly increases FO_T . The theoretical predictions for polystyrene culture with a medium depth of 22 mm underestimated the empirically determined FO_T though the trends were similar.

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Discussion

In this study we used a theoretical mass transport model to explore O_2 transfer in order to predict the loss of viable tissue at a variety of tissue densities during steady state conventional islet culture. We demonstrated empirically that viable tissue surface density as measured by OCR has a significant impact on tissue recovery, adherence, and viability. We investigated culture in elevated pO_2 and on a silicone rubber membrane to improve O_2 transport during culture. Then we compared these techniques to conventional culture on a polystyrene dish at 37°C in 142 mmHg O_2 through empirical measurements and a theoretical O_2 transport model.

The fraction of original tissue collected from culture as non-adherent tissue (FN_{NA}) increased as initial OCR density increased due to tissue adherence to the untreated culture vessels at low densities. The fraction of original tissue collected from culture as adherent tissue (FN_A) decreased as OCR density increased and contained a significantly lower fraction of original insulin positive cells collected from culture than the non-adherent tissue at densities < 0.1 $\text{nmol}/\text{min cm}^2$ indicating that most of the tissue that adheres is non-islet tissue. When considering culture densities < 0.1 $\text{nmol}/\text{min cm}^2$ we observed that the combined non-adherent and adherent tissue collected from culture accounted for about 0.68 of original nuclei and only 0.58 of the original insulin positive cells. Based on this information there is a preferential clearance of insulin positive tissue compared to non-insulin positive tissue. This is a bit concerning because it suggests that during culture we lose on average about 40% of the original β cells even when both adherent and non-adherent tissue fractions are combined. Though this is a large amount of β cells, we must remember that we assume these cells are already dead or dying when culture is initiated since nearly all of the original viable tissue as measured by OCR is collected from the same low density cultures.

When the non-adherent and adherent tissue is combined nearly all of the original viable tissue is collected when precultured human islets are cultured in the absence of O_2 limitations. This is very important because it indicates that any loss of viable tissue from high-density culture is caused by the change in OCR density and/or medium height both of which effect nutrient transport.

Culture of freshly isolated human islets results in a smaller and more variable FO_T than precultured tissue. We believe this is due to the stress applied to the tissue during the isolation process. It has been shown that the isolation process causes a variety of changes in and around β cells which can lead to apoptosis [19]. The cell goes through a series of events once the cell death process is initiated. Depending how far through the cell death process a cell is determines whether it will be measured as viable by a particular assay. Each assay does not necessarily give the same viability measurement [3]. If the cell death process has been initiated, but has not proceeded to the point where respiration stops, then even though a portion of the sample is destined to die it would initially be thought of as viable tissue in our analysis. Precultured tissue has allowed damaged cells to advance further through the cell

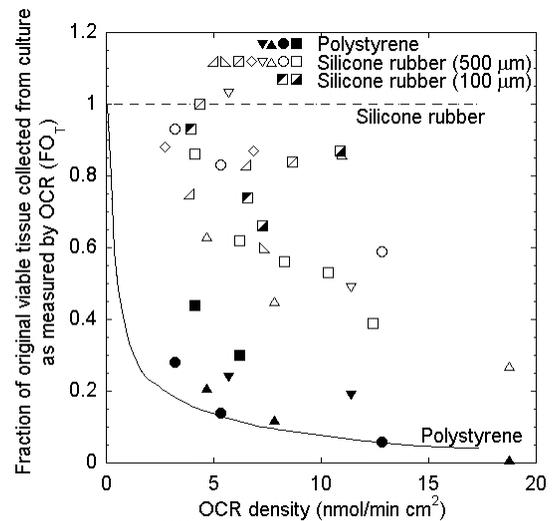


Figure 7. FO_T (symbols) and theoretical predictions (lines) as a function of OCR density for culture in 142 mmHg with 22 mm medium on either polystyrene (filled symbols, solid line), 500 μm silicone rubber (open symbols, dashed line), or 100 μm silicone rubber (half filled symbols). The differently shaped symbols represent different islet preparations. The V_{max} used in the theoretical calculations was $2210 \text{ nmol}/\text{min cm}^3$.

death process resulting in an accurate measurement of healthy non-damaged tissue by OCR. This is reflected by an FO_T that is nearly one and has a low variability.

The theoretical model for O_2 transport during islet culture predicted that culture in elevated O_2 or on a silicone rubber membrane improved O_2 transport significantly. Necrotic cores develop at about $0.9 \text{ nmol/min cm}^2$ for culture on polystyrene in $142 \text{ mmHg } O_2$ at 37°C with a medium depth of 3 mm . Culture on polystyrene in $428 \text{ mmHg } O_2$ at 37°C with a 3 mm medium depth supplies sufficient O_2 until a density of about 3 nmol/min cm^2 at which a necrotic core begins to develop. Thus by culturing at 428 mmHg we can increase the culture density over 3 fold without sacrificing tissue quality. Culture on a $500 \text{ }\mu\text{m}$ silicone rubber membrane removes all O_2 limitations for densities up to at least 15 nmol/min cm^2 indicating over a 16 fold increase in tissue density before a necrotic core would develop.

OCR density has perhaps its largest impact on tissue viability. The fraction of original OCR collected from culture, what we believe represents the fraction of original viable tissue collected from culture, decreased drastically as OCR density increased. This demonstrated that small increases in the OCR density reduce the fraction of original viable tissue that can be collected from culture significantly emphasizing the importance of culture density.

Empirical measurements indicate that elevated pO_2 improves FO_T as compared to conventional culture for islet tissue cultured at densities $> 1 \text{ nmol/min cm}^2$. Since the only difference between conventional culture and culture at elevated pO_2 was the O_2 level, the increase in FO_T has to be attributed to the increase in O_2 . This confirms that O_2 limitations are partly responsible for the decrease in FO_T at high culture densities during conventional culture. Though culture at elevated pO_2 is promising we must remember that tissue exposure to too high of a pO_2 can result in O_2 toxicity.

Culture on silicone rubber membranes had an even larger impact on tissue characteristics. FO_T was significantly higher for culture on a $500 \text{ }\mu\text{m}$ silicone rubber membrane as compared to polystyrene at the same medium height (22 mm). This was predicted by the theoretical O_2 transport model, though theory overestimated viability. Decreasing membrane thickness to $100 \text{ }\mu\text{m}$ had no significant effect on FO_T indicating that the improved O_2 transport gained by decreasing membrane thickness did not increase the amount of viable tissue collected from culture. This demonstrates that O_2 is not limiting during high density culture on silicone rubber and therefore another limitation is responsible for the decrease in FO_T . This is confirmed by the theoretical prediction which indicated that no loss in viability should result from O_2 limitations in islet tissue on a $500\text{-}\mu\text{m}$ silicone rubber membrane.

Though the theoretical model generally predicts the same trend as the experimental data, there are some consistent discrepancies. The model over predicts the fraction of viable tissue collected for all cultures except culture on polystyrene with a 22 mm medium depth where it underestimates FO_T . There are several possible explanations why these discrepancies between theoretical predictions and empirical data may occur.

The slightly higher measured FO_T than predicted for the 22 mm medium depth may be related to observations of our collaborators which indicate that islets up-regulate LDH when exposed to a hypoxic environment allowing them to produce energy by glycolysis in the absence of O_2 (personal communication from Gordon Wier). Normally islet β cells have a low level of LDH expression [20]. If islets can up-regulate LDH fast enough, a larger fraction would be able to survive than predicted by the theoretical model.

The overestimation of FO_T during conventional culture (3 mm medium), elevated pO_2 culture, and culture on silicone rubber may result from inaccuracies to parameter values used in the model. Each parameter has some uncertainty and if inaccurate could introduce error into the prediction. The affects of variations in each parameter are discussed elsewhere [13].

Another possible cause for the discrepancies between empirical data and the theoretical predictions are the assumptions within the model. Our model assumes we have 150- μm diameter spheres of tissue arranged in a perfect square array. This assumes that all the islet and non-islet tissue are in these spheres. In reality we have a mixture of islets and exocrine tissue that can vary significantly in shape, size, and spatial distribution. Additionally, during high-density culture tissue tended to clump which can exacerbate local O_2 limitations. If clumping does occur, our model predictions of a uniform array of spheres will over predict viability because it does not take into account the extreme local hypoxia in the large clumps that will cause a large decrease in viability. Though this could explain the discrepancy for conventional culture and culture at an elevated pO_2 , it is unlikely the cause for the discrepancies observed for culture on a silicone rubber membrane. Decreasing membrane thickness, which enhances O_2 transport, had no significant effect on FO_T , so it is unlikely that the experimental departure from theory is due to O_2 limitations.

The overestimation of FO_T could result from limitations other than O_2 . Since medium was not changed during culture nutrients may have been depleted. Tissue at high density will deplete nutrients (i.e. glucose) at a faster rate than at low density causing limitations other than O_2 coming into play. One may say this could be true for culture in a medium depth of 3 mm, but is unlikely to be true at a medium depth of 22 mm, which was used for silicone rubber culture. However, even though silicone rubber culture included this large medium depth, the nutrients may have been consumed faster than they could be transported to the tissue surface from the bulk medium. Thus there could be a nutrient shortage even if the bulk concentration of the nutrient indicates sufficient supply. We are continuing to investigate whether other nutrient limitations are occurring during culture.

Waste buildup could also cause limitations during culture. Tissue at high density will produce waste (i.e. lactate) at a faster rate possibly exposing tissue to high concentrations of waste products which may be damaging to tissue. A large change in ammonia or lactate could cause local pH to change that in turn could cause cell death. Necrosis, which is what our model predicts, involves leakage of cell contents [21]. A high rate of necrosis may also result in significant waste production as cells break apart. Further investigation into waste buildup and pH changes are also being investigated.

To summarize we have demonstrated that viable tissue density is a key factor in determining the fraction of original viable tissue that can be collected from islet culture. At low viable tissue densities a fraction of the original tissue adheres to the culture vessel. This fraction of original tissue collected as adherent tissue decreased with increasing OCR density and was less pure than the non-adherent tissue collected from the same cultures. Conversely the fraction of original tissue collected as non-adherent tissue increased with increasing OCR densities. In the absence of O_2 limitations the non-adherent and adherent tissue account for nearly 100% of the original viable tissue placed into culture though smaller fractions of the original nuclei and the original insulin positive cells are collected from conventional cultures of precultured tissue. As OCR density increased the fraction of original viable tissue collected from conventional culture decreased drastically as predicted by our O_2 transport theory. Culture in 428 mmHg O_2 increased FO_T at culture densities $> 1 \text{ nmol/min cm}^2$ as predicted by theory and culture on silicone rubber removed all oxygen limitations resulting in an even higher increase in FO_T . In conclusion we have demonstrated that culture at an elevated pO_2 and on a silicone rubber membrane are techniques that can be used to significantly increase the fraction of original viable tissue collected from culture by removing O_2 limitations.

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