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Acute Ischemia Induced by High-Density Culture Increases Cytokine Expression and Diminishes the Function and Viability of Highly Purified Human Islets of Langerhans

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Background. Encapsulation devices have the potential to enable cell-based insulin replacement therapies (such as human islet or stem cell–derived β cell transplantation) without immunosuppression. However, reasonably sized encapsulation devices promote ischemia due to high β cell densities creating prohibitively large diffusional distances for nutrients. It is hypothesized that even acute ischemic exposure will compromise the therapeutic potential of cell-based insulin replacement. In this study, the acute effects of high-density ischemia were investigated in human islets to develop a detailed profile of early ischemia induced changes and targets for intervention. **Methods.** Human islets were exposed in a pairwise model simulating high-density encapsulation to normoxic or ischemic culture for 12 hours, after which viability and function were measured. RNA sequencing was conducted to assess transcriptome-wide changes in gene expression. **Results.** Islet viability after acute ischemic exposure was reduced compared to normoxic culture conditions ($P < 0.01$). Insulin secretion was also diminished, with ischemic β cells losing their insulin secretory response to stimulatory glucose levels ($P < 0.01$). RNA sequencing revealed 657 differentially expressed genes following ischemia, with many that are associated with increased inflammatory and hypoxia-response signaling and decreased nutrient transport and metabolism. **Conclusions.** In order for cell-based insulin replacement to be applied as a treatment for type 1 diabetes, oxygen and nutrient delivery to β cells will need to be maintained. We demonstrate that even brief ischemic exposure such as would be experienced in encapsulation devices damages islet viability and β cell function and leads to increased inflammatory signaling.

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Islet transplantation (ITx), a form of cell-based insulin replacement therapy, is an attractive approach for the treatment of uncontrolled or "brittle" type 1 diabetes. However, large scale application of ITx is limited by the need for lifelong immunosuppression and a shortage of human cadaveric pancreas donors, which is exacerbated by the need for islets from multiple pancreata per patient to achieve insulin independence.[1,2] Encapsulation (immunoisolation) devices have the potential to address these critical limitations. They have demonstrated efficacy in protecting transplanted islets even in the absence of immunosuppression, and may allow for alternative cell-based insulin replacement therapies for type 1 diabetes, for example, by enabling the use of stem cell-derived β cells (SC-βs) which can be sourced on a virtually unlimited scale.[3-10]

However, encapsulating therapeutic β cell doses into reasonably sized (postage stamp) immunoisolation devices creates a high-density environment in which the availability of nutrients (especially glucose and oxygen) is limited, leading to ischemia.[7] This has ultimately hindered the clinical application of macroencapsulation in β cell replacement therapies. Insufficient oxygen is a particular concern for the insulin secreting β cells within islets. β cells lack sufficient levels of the enzyme lactate dehydrogenase α to generate ATP using anaerobic glycolysis, and overexpression of lactate dehydrogenase α in β cells diminishes their glucose responsiveness.[11,12] Moreover, once separated from their native vasculature, islets depend solely on diffusion for the delivery of oxygen and energetic substrates. Deprivation of these critical nutrients and the accumulation of toxic metabolites lead to impaired insulin secretion and eventual cell death.[13-19] These effects are even more pronounced in islets with large diameters, and clinical ITx outcomes are improved for patients receiving preparations composed of smaller average diameter islets.[20-22]

Considering the recent surge of interest in immunoisolation devices and the benefits that their application can offer, it will be of critical importance to understand how these devices impact β cell physiology to facilitate successful clinical outcomes. Therefore, we investigated the viability, insulin secretion, and transcriptional adaptations of a highly purified human islets with an emphasis on β cell function, stress, and inflammation after acute ischemic exposure in high-density "pellet" model. This model has been characterized previously in the context of islet shipping, and is used here to simulate and study the effects of the high density environments created by delivery of therapeutic β cell doses in reasonably sized immunoisolation devices.[23,24]

MATERIALS AND METHODS

Islet Source and Maintenance

Human islets (n = 11 independent preparations) were obtained from the Integrated Islet Distribution Program, the University of Minnesota, the University of Arizona, the McGill University Health Centre, and the University of California-San Francisco. Islets were cultured in oxygen permeable silicone rubber membrane GRex vessels (Wilson Wolf, St Paul, MN). Islet culture media consisted of CMRL 1066 (Mediatech, Inc., Manassas, VA) supplemented with 0.5% human serum albumin (BioChemed Services, Winchester, VA), 1% heparin (SAGENT, Schaumburg, IL), 1% l-glutamine (Mediatech, Inc.), and 1% penicillin/streptomycin (GE Healthcare Life Sciences, Logan, UT). Before experiments, islets were maintained at 25°C and ambient pO_2 supplemented with 5% CO_2.

Induction of Pelletized Culture

After standard culture, islets from each donor were divided into normoxic and ischemic conditions. Ischemia was modeled through high-density "pelletized" culture in which 10 000 islet equivalents (IE) as quantified by DNA were allowed to settle in the bottom of a 1.5-mL centrifuge tube.[25] This condition was selected because it mimics both traditional islet shipping conditions as well as the high-density environments that can result from encapsulation. For the normoxic condition, an equal number of islets were cultured in a 10-cm^2 GRex vessel (Wilson Wolf). Normoxic and ischemic islets were cultured for 12 hours at 37°C, ambient O_2, and 5% CO_2.

Islet Encapsulation

To test the effects of high density encapsulation on human islets and verify comparable effects to pelletized culture, 8000 IE as quantified by DNA were loaded into 4.5 μL (0.34 cm^2) TheraCyte (TheraCyte, Laguna Hills, CA) devices using a

TABLE 1.

Donor metrics

Donor ID	Age, y	BMI	Cold ischemia time, h	Islet purity, %	Islet viability, %	Sex	RNASeq
D1	44	36.7	9.9	85	90	F	Yes
D2	39	26.9	13.6	90	93	F	N/A
D3	40	33.9	3.5	95	95	M	Yes
D4	44	23	12.2	80	90	F	Yes
D5	52	32.2	8.5	85	95	F	N/A
D6	61	31.1	5.6	90	95	F	Yes
D7	54	40.3	5.5	95	97	M	N/A
D8	45	27.6	6.3	95	86	M	N/A
D9	44	22.8	0.6	90	98	M	N/A
D10	44	26.5	9.5	80	85	M	N/A
D11	35	24.8	14.0	95	97	M	N/A
Average ± SD	45.6 ± 7.4	29.6 ± 5.7	8.1 ± 4.3	89.1 ± 5.8	92.8 ± 4.5	N/A	N/A

Shown above are characteristics of donors from which islets were isolated and included in the present study.

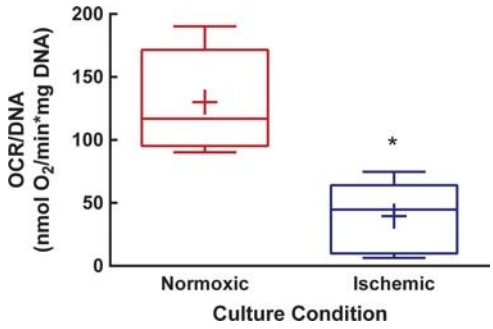

FIGURE 1. Human islet viability is reduced after acute ischemia. After 12 hours of control (normoxic) or ischemic exposure, islet viability was determined by OCR/DNA. Shown above are values for n = 8 paired experiments. *P = 0.01. Data mean are indicated by +, whiskers indicate minimum and maximum values. Box bounds indicate upper and lower quartiles, and the median value is indicated by the line within the box.

Hamilton syringe. Devices were then placed into oxygen permeable, 10 cm^2 GRex vessels (Wilson Wolf) filled with culture media and maintained for 12 hours at 37°C, ambient O_2, and 5% CO_2. Encapsulated islets were compared with matched pellets and controls prepared as described above.

Measurement of DNA

Islets were suspended in 1 mL of AT Buffer (1 M solution of ammonium hydroxide in nanopure water, supplemented with 0.2% Triton X-100) and sonicated using a Sonic Dismembrator Model 500 (Fisher Scientific, Waltham, MA) for 30 seconds at 11% amplitude. DNA was assessed using a Quant-iT PicoGreen dsDNA kit (Life Technologies, Carlsbad, CA) according to manufacturer instructions. 96 well plates were read using a SpectraMax M5 plate reader and SoftMax Pro software (Molecular Devices, Sunnyvale, CA).

Measurement of Oxygen Consumption Rate Normalized to DNA

After 12 hours, islets and media were mixed to ensure homogenous sampling. Oxygen consumption rate (OCR) was conducted as previously described.[26] Briefly, islets were resuspended in Media 199 (Mediatech, Inc.) warmed to 37°C and divided evenly between 3 OCR chambers of known volumes, or conducted on whole devices as in the case of encapsulated islets (Instech Laboratories, Inc., Plymouth Meeting, PA). Measurements of pO_2 in each chamber over time were recorded using fiber optic sensors and NeoFox viewer software (Ocean Optics, Inc., Dunedin, FL). The oxygen consumption rate in nanomoles of O_2 per minute was then estimated from the slope of the decline in pO_2 over time. This value was normalized to the DNA content of each chamber (as described above) to give a final measurement of OCR/DNA (nmol O_2/min*mg DNA).

Measurement of % Viability by Fluorescein Diacetate/Propidium Iodide Staining

From each condition, 100-μL samples of islets were combined with 377.6 μL of dithizone (Sigma-Aldrich, St. Louis, MO). To this, 1.39 μL of fluorescein diacetate (Sigma-Aldrich) and 21.05 μL of propidium iodide (Sigma-Aldrich) were added for final concentrations of 0.067 and 4.0 μM, respectively. Islets were incubated in the dark for 20 minutes, after which they were allowed to settle and 100 μL of islets were placed on a slide for imaging. To determine the proportion of live versus dead cells, islets were imaged on a Zeiss Observer.Z1 (Zeiss, Oberkochen, Germany) using the 10× objective and an AxioCam MRm camera with ZEN 2012 (blue addition) software (Zeiss).

β cell Function

The Biorep Technologies Peri-4.2 Perifusion System (Biorep Techonologies, Inc., Miami Lakes, FL) was used to measure dynamic glucose-stimulated insulin secretion (GSIS). Triplicate measurements using 100 IE each underwent baseline stimulation with 2.8 mM glucose in oxygen-saturated (95% O_2) Krebs-Ringer Bicarbonate buffer for 20 minutes before and after a 40-minute stimulation period with 16.7-mM glucose. Perfusate was collected at a rate of 100 μL/min in a 96-well plate. Perfusate was tested for insulin content using an insulin enzyme linked immunosorbent assay (ELISA, Mercodia, Winston-Salem, NC). ELISA plates were read using Softmax Pro software and a SpectraMax M5 plate reader (Molecular Devices). Insulin content was normalized to DNA as described above.

RNA Library Preparation

Paired normoxic and ischemic islets from 4 donors were selected at random for high throughput RNA sequencing (RNAseq). For RNA samples, islets were collected in microcentrifuge tubes and washed twice 1× Dulbecco's phosphate buffered saline to remove serum. RNA was isolated using the RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. RNA quality was assessed by Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA), and a minimum RIN of 7.0 was required for inclusion in the study. Libraries were prepared using the Illumina TruSeq Stranded mRNA Sample Preparation Kit (Illumina, San Diego, CA). mRNA was sequenced using Illumina HiSeq 2000 sequencer and quantified into transcripts using EA-Quintiles mRNAv8 pipeline.

RNAseq Analysis

Briefly, 50 × 50 base, paired-end reads were checked for quality by comparison to intergenic and ribosomal sequences. Sequencing reads were aligned to the human University of

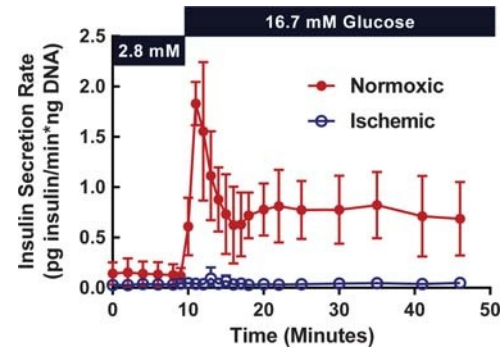

FIGURE 2. Human islet function is absent after acute ischemic exposure. To determine β cell function, GSIS was measured on a perifusion system. Shown above are the insulin secretion profiles for control and ischemic islets. The figure indicates mean ± SD values for n = 4 pairs of islets.

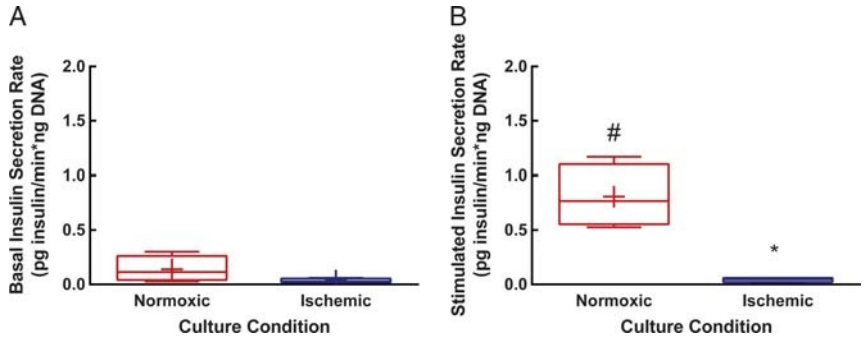

FIGURE 3. Basal and stimulated insulin secretion in normoxic versus ischemic human islets. Area under the curve was calculated and divided by the corresponding time interval to give the average insulin secretion rate for basal and stimulated portions of the curves shown in Figure 2. A, there is no significant difference in the rate of insulin secretion under basal conditions. B, ischemic islets show significantly lower insulin secretion than do normoxic islets under stimulated conditions. $^{*}p = 0.006$ versus stimulated normoxic condition. Similarly, ischemic islets do not have a significant increase in insulin secretion under stimulatory glucose conditions, whereas normoxic islets do show a significant increase. $^{\#}P = 0.009$ versus basal rate. Means for n = 4 samples are indicated by +, whiskers indicate minimum and maximum values. Box bounds indicate upper and lower quartiles, and the median value is indicated by the line within the box.

California Santa Cruz known gene transcriptome using RNA-Seq by Expectation Maximization v1.2.0 and transcript abundance was quantified. An average of 97.4 ± 0.4% of total reads mapped to reference genomes. The aligned reads for each gene were summarized as described in Li and Dewey.[27] All genes and isoforms have been assigned a normalized coverage rate in fragments per kilobase per million mapped reads (FPKM). Differential gene expression was performed using the edgeR R package scripts (bioconductor.org) using paired sample statistical procedures.[28,29] A paired-sample (generalized paired t test) analysis was conducted to investigate the effects of ischemia while adjusting for baseline differences between patients. Genes with a *P* value less than 0.05 following Benjamini-Hochberg correction were considered to be statistically significant.

Functional Analysis of Differentially Expressed Genes

Significantly upregulated or downregulated genes were submitted to KOBAS 2.0 to determine which canonical pathways were enriched by ischemia, as well as to determine associated gene ontology (GO) terms.[30] Significant enrichments were defined as a corrected *P* value less than 0.05 following a Fisher exact test with Benjamini Hochberg correction in KOBAS 2.0. To summarize GO enrichment and reduce redundant terms, ReviGO was used to cluster similar GO terms using small (0.5) SimRel similarity and the whole Uniprot as the database for categorical GO term sizes.[31] Significant pathways were manually curated to remove pathways containing redundant or nonspecific gene findings. Preferential association between the lists of upregulated and downregulated genes was also evaluated.

RT qPCR Array

Results from RNAseq were compared with quantitative reverse transcriptase polymerase chain reaction results for a single islet preparation. Purified islet total RNA was run on RT^2 Profiler PCR Array Human Hypoxia Signaling Pathway plates (Qiagen) using iQ5 Real-Time PCR Detection System (Bio-Rad Laboratories, Irvine, CA). Fold changes were determined

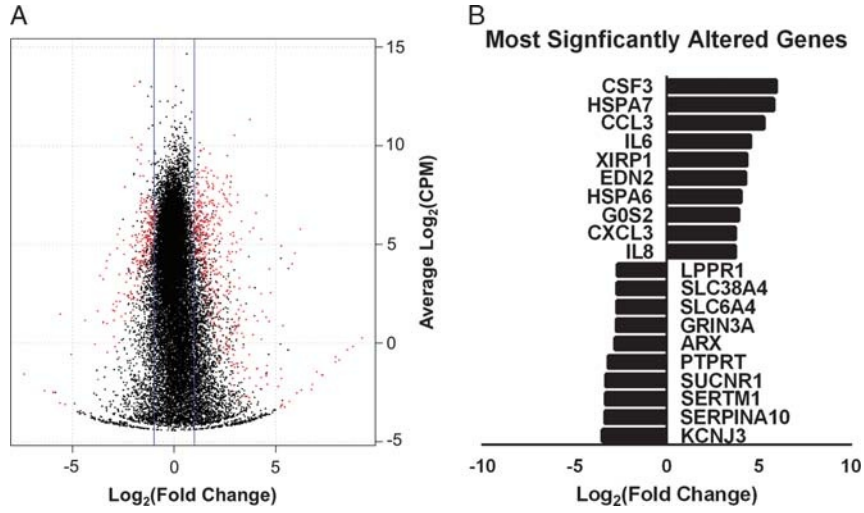

FIGURE 4. RNAseq summary. Shown above is a summary of differential expression for RNAseq data. A, A volcano plot indicating the distribution of genes detected by log2 (fold change) and log2 (CPM). Differentially expressed genes are shown in red, and nondifferentially expressed genes are indicated in black. B, The most upregulated and downregulated genes in ischemic islets as determined by fold change are shown. Genes had a minimum CPM cutoff value of 1. CPM, counts per million.

according to manufactures instructions. Gene expression was normalized to housekeeping genes *RPLP0*, *B2M*, and *HPRT1*.

Statistics

Statistical analysis of physiological parameters was conducted in SAS 9.4 (SAS Institute Inc., Cary, NC). OCR/DNA data were analyzed using Wilcoxon Signed Rank Test. Matched fluorescein diacetate/propidium iodide and OCR/DNA data were analyzed using a Student *t* test. Perifusion data were analyzed using a generalized linear mixed model for repeated measurements. Fold changes from the RT qPCR array were correlated to fold changes from RNAseq using linear regression analysis in GraphPad Prism version 6.07 (GraphPad Software, Inc., La Jolla, CA). Unless otherwise indicated, data represent mean ± standard deviation.

RESULTS

Donor Metrics

Individual donor characteristics and islet quality parameters (% viability measured by membrane integrity staining, purity, and cold ischemia time) as reported by the distributing isolation center are presented in Table 1.

Pellet Model Characterization and Comparison to Encapsulation

The viability of islets from n = 2 human islet preparations was compared under matched normoxic, pelletized, and encapsulated conditions to confirm that the ischemic damage caused by the pellet condition is similar to what is caused in devices loaded at high densities.[7] Pelletized islets and encapsulated islets showed similar % reductions in OCR/DNA values versus control (57.7 ± 19.5% and 80.3 ± 21.1%, respectively).

Islet Viability

Islet viability was determined in n = 8 independent islet preparations. Average OCR/DNA values for normoxic islets align closely with clinical averages observed for both islet auto and allotransplantation.[32,33] Islet OCR/DNA values are reduced after warm ischemic exposure ($P < 0.01$, Figure 1), which correlates to lower, although not entirely diminished, viability. In a subcohort of n = 4 independent islet preparations, OCR/DNA results were compared with fluorescein diacetate/propidium iodide staining. Staining revealed a significant loss of viability for control versus pelletized islets (% 87.3 ± 1.0% vs 8.8 ± 5.5%, respectively, $P < 0.01$). OCR/DNA measurements for matched samples were similarly reduced for control vs. pelletized islets (122.9 ± 53.3 and 53.4 ± 12.0 nmol O_2/min*mg DNA, respectively, $P < 0.05$).

B Cell Function

GSIS was measured in a subset of control and ischemic islet pairs (n = 4 independent preparations, Figure 2). GSIS of ischemic islets is virtually absent compared with control islets. Basal secretion was not different between control and ischemic islets (Figure 3A). However, under stimulatory glucose concentrations (Figure 3B), ischemic islets secrete less insulin than control islets ($P < 0.01$). Normoxic islets show elevated ($P < 0.01$) insulin secretion in response to stimulatory glucose concentrations, whereas ischemic islets are unresponsive.

Global Gene Expression Changes After Ischemia

An average of 19 932 ± 138 genes were detected with RNAseq. Of these, 657 genes were differentially expressed in ischemic versus control islets ($P < 0.05$) (Figure 4A). Genes most upregulated include immune molecules interleukins 6 and 8 (*IL6*, *IL8*), chemokine 3 (*CCL3*), and chemokine 3 like (*CXCL3*). Genes most downregulated include the inward rectifier K^+ channel K_{ir} 3.1 (*KCNJ3*) and Na^+-coupled neutral amino acid transporter 4 (*SLC38A4*) (Figure 4B).

Fold changes calculated from RNAseq correlated ($P < 0.0001$, $r^2 = 0.75$) to fold changes calculated by RT qPCR array (**Figure S1, SDC,** http://links.lww.com/TP/B421). The high correlation between independent measures of gene expression corroborates findings from RNAseq model.

Functional Analysis of Differentially Expressed Genes

Canonical pathways significantly associated with differentially expressed genes were identified (Table 2). Specific differentially expressed genes associated with inflammation and cytokine signaling, and also associated with pathways

TABLE 2.

Signaling pathways enriched following acute ischemia in human islets

Canonical pathway	Database	DE genes	Corrected *P* value
TNF signaling pathway	KEGG PATHWAY	23	4.1×10^{-8}
Cytokine-cytokine receptor interaction	KEGG PATHWAY	25	4.1×10^{-4}
HIF-1-alpha transcription factor network	PID	17	4.3×10^{-4}
Cellular Senescence	Reactome	16	9.8×10^{-4}
Chemokine receptors bind chemokines	Reactome	9	9.8×10^{-4}
Extracellular matrix organization	Reactome	20	1.6×10^{-4}
NOD-like receptor signaling pathway	KEGG PATHWAY	10	2.7×10^{-3}
Cellular responses to stress	Reactome	18	3.3×10^{-3}
AP-1 transcription factor network	PID	15	4.8×10^{-3}
ATF-2 transcription factor network	PID	13	8.7×10^{-3}

Shown above is a summary of the most enriched pathways in ischemic human islets. Top pathways were determined for upregulated genes using KOBAS 2.0, drawing from KEGG, PID, and Reactome databases. Significance was defined as a $P < 0.05$ following Fisher exact test with Benjamini-Hochberg correction. The number of genes differentially expressed in islets and annotated in these pathways are presented in DE genes column and compared to the total number of genes annotated to that pathway in the databases to generate frequency. Note that although Malaria and Rheumatoid Arthritis appeared in the top 10 pathways shown above, they were excluded from the list due to appearance from nondisease specific inflammatory genes including CCL2, CCL20, CCL3L3, CSF2, CSF3, CXCL1, CXCL5, CXCL8, FLT1, FOS, HBA2, HBB, HGF, ICAM1, IL6, JUN, SELE, and VCAM1.

FIGURE 5. Heatmaps for significantly enriched pathways. Genes in enriched pathways as well as related DE genes of interest were plotted as heatmaps. A, A heatmap detailing changes in inflammatory genes. B, Changes in genes involved in the hypoxia response, cell death, ion transport, and cell damage. FPKM values were converted to FPKM + 1 before log transformation. Values in heat maps represent $\log_2$ (fold change).

for the HIF-1α response, AP-1 (FOS) signaling, and ion transport are detailed in Figure 5. Enriched GO terms were in agreement with pathway findings, showing enhancement of cytokine signaling and inflammation as well as downregulation of cellular ion transport (Table 3). Genes associated with hypoxia and nutrient deprivation (including *SLC2A1*, *HK1*, *HMOX1*, *ARG1*, and *UPP1*) were also upregulated ($P < 0.05$).

DISCUSSION

Ischemia remains a pervasive issue that directly influences the outcomes of cell-based insulin replacement therapies, such as ITx. In this study, we implement an ischemic model that has previously been used to study islet shipping and captures the conditions seen in high density islet encapsulation, including a significant reduction of islet viability which is associated with oxygen and nutrient deprivation, and consistent with results from the transcriptome analysis.[19,23,24] Furthermore, we demonstrate that the viable population of islets following acute ischemia exhibits significant β cell dysfunction corresponding to global alterations in gene expression. Inflammatory responses were evident in the gene signatures of islets after ischemia, with concurrent downregulation of genes involved in nutrient and ion transport. The persistence of β cell dysfunction due to ischemic stress has been previously demonstrated, whether due to cellular reprogramming or ischemia-reperfusion injury, and the endurance of a concurrent proinflammatory signature is likely, given the present work.[19,34,35] Interestingly, kidney capsule islet transplants represent a commonly used in vivo correlate to our high-density in vitro model in which islets are transplanted as a "pellet" below the renal capsule.[36] Although this method induces less ischemic stress than what might be experienced in an immunoisolation device, when these islets are exposed to nerve growth factor (which has an anti-apoptotic effect), graft function is improved.[37-39] This supports our finding that even acute ischemia is detrimental to islet viability and function and indicates that early mitigation of these factors is beneficial to transplant outcomes.

The differential gene expression profile caused by acute ischemia was predominantly increased inflammatory and stress response signaling. More specifically, there was increased expression of key inflammatory genes (including *TNF* and *IL6*), which are known to contribute to islet death in the peritransplant period by the instant blood mediated inflammatory reaction.[40] The link between hypoxia and this proinflammatory profile has been demonstrated previously in rat islets.[41] This increased inflammatory signaling may be important not only from an immunological standpoint, but also in the context of β cell function. TNFα has previously been shown to inhibit GSIS in β cell lines as well as downstream insulin signaling in adipocytes.[42-44] The potential damage to insulin signaling is compelling, as autocrine insulin signaling in β cells has been demonstrated to influence insulin expression.[45] Key genes involved in the coupling of glucose sensing to insulin secretion (for example *KCNJ11*, which encodes the major subunit of the ATP-sensitive K^+ channel) are also significantly downregulated. Together with the effects of inflammatory factors, these changes may underlie the significant loss of β cell function. By mitigating these damaging adaptations, loss of islets in the peritransplant period may be minimized and ultimately help to facilitate lower curative doses of islets for diabetes reversal.[46]

Lifelong immunosuppression remains a critical barrier which limits the application of β cell replacement therapies. Implantable macroencapsulation devices are a possible solution and have been demonstrated to be alloprotective in animal models as well as in humans.[3,47] However, packing therapeutic β cell doses into practically sized devices results in a high-density environment in which diffusion of oxygen and nutrients is severely limited. This study demonstrates

TABLE 3.

GO terms associated with significantly upregulated or downregulated genes after acute ischemia in human islets

GO category	UP after acute ischemia	DOWN after acute ischemia
Molecular function	• RNA polymerase II core promoter proximal region sequence-specific DNA binding transcription factor activity • Serine-type endopeptidase inhibitor activity • Cytokine receptor binding	• Ion transmembrane transporter activity
Biological process	• Blood vessel development • Immune system process • Inflammatory response	• Homophilic cell adhesion via plasma membrane adhesion molecules • Cell-cell signaling

GO terms were summarized using the REVIGO tool.
Above are the most affected GO terms categorized by molecular function and biological process.

that such high-density environments are damaging to β cell function and may ultimately affect transplant outcomes.[5,48] Addressing the effects if ischemia by ensuring adequate nutrient delivery will be critical to the success of cell-based insulin replacement therapies going forward. With respect to maintaining oxygenation, numerous advances have been made with regard to supplying oxygen to the pancreas during the preservation period as well as to isolated islets during culture.[49,50] Attempts have also been made to supply islets in encapsulation devices with oxygen, and this remains an active area of research although a permanent solution has yet to be achieved.[3]

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