Improved Outcomes in Islet Isolation and Transplantation by the Use of a Novel Hemoglobin-based O$_2$ Carrier

J. G. Avila[a], Y. Wang[a], B. Barbaro[a], A. Gangemi[a], M. Qi[a], J. Kuechle[a], N. Doubleday[a], M. Doubleday[b], T. Churchill[c], P. Salehi[a], J. Shapiro[c], L. H. Philipson[d], E. Benedetti[a], J. R. T. Lakey[c] and J. Oberholzer[a,*]

[a]*Division of Transplantation, University of Illinois at Chicago, Illinois, USA*
[b]*Northfield Laboratories, Evanston, Illinois, USA*
[c]*Surgical-Medical Research Institute, University of Alberta, Edmonton, Canada*
[d]*Department of Medicine, University of Chicago, Illinois, USA*
*Corresponding author: Jose Oberholzer, jober@uic.edu

During isolation, islets are exposed to warm ischemia. In this study, intraductal administration of oxygenated polymerized, stroma-free hemoglobin-pyridoxalated (Poly SFH-P) was performed to improve O$_2$ delivery. Rat pancreata subjected to 30-min warm ischemia were perfused intraductally with collagenase in oxygenated Poly SFH-P/RPMI or RPMI (control). PO$_2$ was increased by Poly SFH-P (381.7 ± 35.3 mmHg vs. 202.3 ± 28.2, p = 0.01) and pH maintained within physiological range (7.4–7.2 vs. 7.1–6.6, p = 0.009). Islet viability (77% ± 4.6 vs. 63% ± 4.7, p = 0.04) was improved and apoptosis lower with Poly SFH-P (caspase-3: 34,714 ± 2167 vs. 45,985 ± 1382, respectively, p = 0.01). Poly SFH-P improved islet responsiveness to glucose as determined by increased intracellular Ca^{2+} levels and improved insulin secretion (SI 5.4 ± 0.1 vs. 3.1 ± 0.2, p = 0.03). Mitochondrial integrity was improved in Poly SFH-P-treated islets, which showed higher percentage change in membrane potential after glucose stimulation (14.7% ± 1.8 vs. 9.8 ± 1.4, respectively, p < 0.05). O$_2$ delivery by Poly SFH-P did not increase oxidative stress (GSH 7.1 ± 2.9 nm/mg protein for Poly SFH-P vs. 6.8 ± 2.4 control, p = 0.9) or oxidative injury (MDA 1.8 ± 0.9 nmol/mg protein vs. 6.2 ± 2.4, p = 0.19). Time to reach normoglycemia in transplanted diabetic nude mice was shorter (1.8 ± 0.4 vs. 7 ± 2.5 days, p = 0.02), and glucose tolerance improved in the Poly SFH-P group (AUC 8106 ± 590 vs. 10,863 ± 946, p = 0.03). Oxygenated Poly SFH-P improves islet isolation and transplantation outcomes by preserving mitochondrial integrity.

Introduction

Islet transplantation can reverse insulin-dependent diabetes (1–3), but the procedure is still hampered by inadequate supply of islets and gradual loss of islet function after transplantation (4). The inconsistency of islet isolation outcomes has been a major limitation to widespread clinical application of islet transplantation. Among the variety of factors influencing postisolation islet yield, viability and function, ischemic time is of particular importance. The length of ischemia is inversely correlated with islet isolation outcomes (5–8). Ischemia renders cells more susceptible to oxidative stress by impairing mitochondrial antioxidant defenses (9). Providing O$_2$ to ischemic tissue has been shown to be a 'double-edged sword' due to reperfusion injury (10–13). Reactive oxygen species (ROS) produced by mitochondria play a significant part in this type of injury. Oxidative stress to pancreatic islets during the isolation procedure has been well documented (14–16), and the use of antioxidants has been shown to protect islets from oxidative injury (17–20). Organ preservation solutions such as histidine-tryptophan-ketoglutarate (HTK) and University of Wisconsin (UW) solution (21–23) are designed to protect pancreatic tissue from the deleterious effects of ischemia, but do not prevent ischemia *per se*.

Maintaining an appropriate O$_2$ level would seem important to prevent ischemic damage and reperfusion injury during organ preservation and pancreatic islet isolation. Indeed, artificial O$_2$ carriers, such as perfluorocarbons (PFC), have a beneficial effect on islet isolation and transplantation outcomes when used during pancreas preservation with UW solution in the two layer method (TLM) (24–27).

Artificial O$_2$ carriers are synthetic solutions capable of binding, transporting and unloading O$_2$. They have been originally developed as blood substitutes, but none of the original PFC-based products have been approved for clinical use, and in clinical trials anaphylactic reactions were

observed (28). Moreover, PFCs have the inconvenience of being hydrophobic and difficult to keep in aqueous solution (29). Hemoglobin-based O_2 carriers (HBOC's) are hydrosoluble, and in phase I and II trials no anaphylactic reactions have been observed (30–32). In contrast to PFC, polymerized, stroma-free hemoglobin pyridoxalated (Poly SFH-P) presents with an O_2 saturation curve similar to red blood cells.

We hypothesize that intraductal pancreas perfusion with a HBOC provides sufficient O_2 during the islet isolation procedure to reduce ischemia and improve postisolation islet viability and function. The purpose of the present study was to test this hypothesis in a rodent model of islet isolation and transplantation.

Research Design and Methods

Preparation of Poly SFH-P solution

Polymerized human hemoglobin was manufactured and kindly donated by Northfield Industries, Evanston, Illinois. Poly SFH-P containing 10 g/dL of polymerized hemoglobin was custom prepared for our isolation model. We investigated the effect of collagenase on the stability of polymerized hemoglobin by HPLC analysis. Poly SFH-P was incubated with or without collagenase under different conditions, before and after oxygenation, at 4 and 37°C. HPLC analysis did not reveal any degradation of Poly SFH-P. In addition, we studied the formation of Methemoglobin (MetHb) and carboxyhemoglobin (COHb) after various oxygenation times and did not find any significant MetHb or COHb formation.

A quantity of 250 mL Poly SFH-P solution were packed in sterile bags (500 mL capacity) to allow for the introduction of 250 mL of O_2. In previous tests it was determined that Poly SFH-P had a shelf life of 12 months. Poly SFH-P was stored at 4°C for no more than 9 months.

Islet isolation and preservation treatment

All animals were housed at the Biologic Resources Laboratory, University of Illinois at Chicago (UIC) and procedures involving animals were performed in accordance with the guidelines of the National Institutes of Health and the Animal Care Committee (ACC) at UIC. Male Lewis rats (Harlan Industries, Indianapolis, IN), weighing between 175 and 200 g were used as pancreas donors for islets. Animals were anesthetized by isoflurane inhalation using a vaporizer and masks (Viking Medical, Medford Lakes, NJ). There were two experimental groups:

Poly SFH-P group: Poly SFH-P containing collagenase (10 isolations, 4 rats per group and isolation).

Control group: RPMI-1640 containing collagenase (10 isolations, 4 rats per group and isolation).

Rat islet isolation was performed following the standard method previously described (33), modified by using the warm ischemia model that we have developed and described (20). Briefly, after the animal was anesthetized, a laparotomy incision was performed followed by incision into the thoracic cavity and section of the heart for euthanasia by exsanguination. The abdominal cavity was closed, covered with gauze and left for 30 min before pancreas perfusion. Collagenase type XI (Sigma) 1 mg/mL was reconstituted either with Poly SFH-P/RPMI (Treatment) or RPMI 1640 (Control), and both, treatment and control were oxygenated by bubbling the solutions with 100% O_2 for 15 min. The oxygenated enzyme solutions were injected via the bile duct and into the main pancreatic duct for distention of the pan-

creas. After excision, each pancreas was placed in a 50 mL conical tube with 7.5 mL of its respective perfusion solution followed by incubation in a 37°C water bath (digestion phase) for 18 min. After this step, each pancreas was gently shaken in the tubes, washed with cold RPMI and transferred into a 500 mL beaker. After wash, islets were purified from the exocrine tissue by discontinuous Ficoll density gradients (Mediatech Inc. Herndon, VA). Islets were centrifuged for 15 min at 640g and then handpicked from the middle layer of the Ficoll gradient, followed by washing and culture in RPMI containing 10% fetal calf serum (FBS), 10% penicillin/streptomycin (Invitrogen) without glutamine, for 24 h culture at 37°C.

Oxymetry assessment

O_2 tension and pH were measured in the pancreas perfusion medium (Poly SFH-P and control) before and after digestion phase using a blood gas analyzer (ABL/700 Radiometer, Copenhagen, Denmark) (n = 3 experiments per group).

In vitro *evaluation of islet yield, viability and function*

Islet yield: Dithizone stained islets from a representative sample were counted under a stereoscopic microscope (Leica Microsystems, Bannockburn, IL).

Vital staining: Islet viability was assessed by staining with trypan blue dye (Sigma). Islets stained more than 25% of its surface were considered dead. Live versus dead islets were assessed in a representative sample, where a minimum of 50 islets were counted per sample.

Apoptosis: The level of apoptotic cell death was measured using a living cell fluorescein active caspase-3 staining kit (Biovision, CA). Briefly, an aliquot of 1200 islets per group was counted and divided into four eppendorf tubes with 300 μL of media (RPMI 1640 with 10% FBS and 10% Pen/Strep). One microliter of FITC-DEVD-FMK (fluorescent dye for Caspase-3) was added into two of the tubes of each group (the other two were left without the probe as controls for the assay) and incubated for 1 h at 37°C and 5% CO_2. Cells were centrifuged at 344g for 1 min and supernatant removed. Cells were then resuspended with the wash buffer from the kit, repeating this step twice, followed by resuspension of the cells in 100 μL of the wash buffer and transferred into each well of a black microtiter plate. Measurement of fluorescence intensity was performed with excitation of 485 nm and emission of 535 nm in a fluorescent plate reader (GENios, Tecan US Inc., Durham, NC).

Intracellular Ca^{2+} measurement: Intracellular Ca^{2+} during glucose stimulation was measured for functional evaluation of isolated islets, using standard wide-field fluorescence imaging with dual-wavelength excitation fluorescent microscopy (34,35). Islets were loaded with Fura-2 through exposure to a 25-min incubation at 37°C in Krebs solution with 2 mM glucose (KRB2), containing 5 μM Fura-2/AM (Molecular Probes Inc., Eugene, OR). The islets were then placed into a temperature-controlled perfusion chamber (Medical Systems Inc., Paola, KS) mounted on an inverted epifluorescence microscope (TE-2000U, Nikon Inc., Melville, NY) and perfused by a continuous flow (rate 2.5 mL/min) of 5% CO_2-bubbled KRB2 buffer at 37°C (pH 7.4). Krebs containing different glucose concentrations (5, 8 and 14 mM) was administered to the islets and followed for 15 min each, rinsing with KRB2 in between. Multiple islets were imaged with 10×–20× objectives. Fura-2 dual-wavelength excitation was set at 340 and 380 nm, and detection of fluorescence emission set at 510 nm. The reading was performed using Metafluor/Metamorph imaging acquisition and analysis software (Universal Imaging Corporation); images were collected with a high-speed, high-resolution charge-coupled device (Roper Cascade CCD). Estimation of the level of Ca^{2+} was accomplished by using an *in vivo* calibration method. The percentage change of intracellular Ca^{2+} between both groups was calculated by the maximum increase after glucose stimulation, minus the basal (2 mM glucose) Ca^{2+} level for each group.

Addition of tolbutamide: Tolbutamide, an inhibitor of K$^+$-ATP channels (100 μM concentration) was added after 5 min to the perfusion medium containing islets in Krebs (2-mM glucose). The measurement of intracellular Ca^{2+} levels after the addition of tolbutamide was performed in islets as described above, but without glucose stimulation.

Static glucose incubation: Static glucose incubation was used to compare glucose induced insulin secretion (stimulation index, SI) between the groups. SI was defined by the ratio of stimulated versus basal insulin secretion (36). Briefly, for each experiment, groups of five handpicked islets with similar size (approximately 100 μm) were placed in five different wells of a 12-well plate (5 replicates); then preincubated with 1 mL of Krebs-low glucose concentration (1.6 mM glucose final concentration) for 30 min, then the supernatant was collected and discarded. Islets were then incubated for 1 h in low glucose Krebs (1.6 mM glucose final concentration) at 37°C and 5% CO$_2$, and supernatants were collected under a microscope taking care of not removing any islets from the well. The same step was repeated by addition of Krebs-high glucose solution (16.7 mM glucose final concentration) and incubation for 90 min. Supernatants were collected and frozen at −20°C for later measurement using an ELISA kit for rat insulin (Mercodia, Uppsala, Sweden). All samples are measured in duplicates in the kit.

Mitochondrial membrane potential and morphology: The fluorescent dye Rhodamine 123 (Rh123) is a probe of the transmembrane potential and is accumulated within the inner mitochondrial membrane (Rh123 is a lipophilic cation that integrates selectively into the negatively charged mitochondrial membranes). In cells preloaded with Rh123, when membrane potential increases (hyperpolarization) as seen after glucose stimulation, more Rh123 is concentrated into the mitochondrial membrane, leading to aggregation of dye molecules and decrease (quenching) of the fluorescence signal. Rh123 was used as previously described (37). Briefly, islets were incubated in Krebs solution with glucose (2 mM), supplemented with 10 μg/mL Rh123 (Molecular Probes) for 20 min at 37°C, then placed into a temperature-controlled perfusion chamber (Medical Systems Inc) mounted on an inverted epifluorescence microscope (TE-2000U, Nikon Inc.). The islets were perfused by a continuous flow (rate 2.5 mL/min) of 5% CO$_2$-bubbled Krebs buffer at 37°C (pH 7.4). Islets were then stimulated with 14-mM glucose and the changes in fluorescence were measured for 15 min after stimulation. Rh123 fluorescence was excited at 540 nm and emission measured at 590 nm. Images were collected with a charged coupled device camera (Roper Cascade CCD). Data were normalized to the average fluorescence intensity recorded during a 5-min period prior to glucose stimulation. All experiments were performed at 37°C. The percentage change in fluorescence intensity between both groups was calculated by the maximum reduction in fluorescence intensity after 14-mM glucose stimulation, minus the basal fluorescence intensity for each group.

For mitochondrial morphology assessment islets from Poly SFH-P and control groups were incubated with Rhodamine 123 in Krebs buffer at 2.5 μM for 15 min and visualized using a Carl Zeiss LSM 510 confocal microscopy equipped with 60× water immersion objective. The 488-nm line from an argon-krypton laser used for excitation and the emission from Rh 123 were detected through an LP 505 filter. The intensity and the distribution of fluorescence were used to morphologically characterize the mitochondrial integrity.

Fractional beta cell viability: For assessment of fractional beta cell viability the method of Ichii et al. was applied (38). This involves dissociating the islets and staining the cells with the dyes: 7-aminoactinomycin D (7aad), teramethylrhodamine ethyl ester (TMRE) and Newport Green (NG). A single cell suspension was created by incubating 1000 islets per condition in 2 mL Accutase (Innovative Cell Technologies Inc., San Diego, CA) for 7 min at 37°C followed by gentle pipetting. Then cells were incubated with 1 μM

NG PDX; (Invitrogen, Molecular Probes) and 100 ng/mL TMRE (Invitrogen, Molecular Probes) in phosphate buffered saline (PBS) for 30 min at 37°C. After washing, cells were stained with 5 μg/mL 7AAD (Invitrogen, Molecular Probes). The cells were analyzed using Cell Quest software and the LSR by Becton Dickinson (Mountainview, CA). Gating for NG was performed by side scatter and FL1.

Oxidative stress: GSH levels were measured as a marker of oxidative stress by using the monochlorobimane (mcbm) method (39) in islets at 12 h postisolation as previously described (20). Briefly, 500 islets were cultured in one well of a 12-well plate in 5 mL CMRL culture medium and 10 μL of mcbm (50 mM) (Molecular Probes) and were incubated for 30 min at 37°C. Islets were collected, washed with PBS at a pH of 7.5, resuspended in 500 μL of 50 mM TRIS buffer containing 1 mM EDTA (1 mL), then sonicated. After centrifugation the supernatant was read in a fluorescence plate reader (GENios, Tecan US Inc) with excitation set at 380 nm and emission set at 470 nm.

Oxidative injury: Lipid peroxidation was used as a marker of oxidative injury, determined by measurement of malondialdehyde (MDA), a product of lipid peroxidation. MDA levels were assessed by the thiobarbituric acid method (TBARS) (40). Briefly, a reaction mixture was prepared containing 0.1 M HCl, 0.67% TBA, 10% phosphotungstic acid and 7% sodium dodecylsulphate (SDS) (all Sigma). Five hundred islets were sonicated in 700 μL PBS into a cell lysate. After centrifugation at 20 142g, 500 μL of the supernatant were extracted and mixed with 875 μL of the reaction mixture, then boiled at 95–98°C for 1 h. After this process, samples were cooled down and mixed with 750 μL of N-butanol in order to extract the MDA and avoid interference of other compounds. After a short spin, 100 μL of supernatant were extracted and read in duplicates on a 96-well plate with a fluorometer (GENios, Tecan US Inc.) set at Excitation: 530/25, Emission: 575/15. Samples were read against MDA standards (Sigma) prepared at different concentrations (2, 4, 8 mM).

In vivo *assessment*

Islet transplantation into athymic 'nude' diabetic mice: Islet function was assessed *in vivo* by transplantation under the kidney capsule of diabetic athymic nude mice (Harlan Industries). Animals were housed and surgeries performed under a laminar flow hood located in 'barrier' rooms at the Biologic Resources Laboratory, University of Illinois at Chicago (UIC).

Diabetes was induced by a single intraperitoneal (IP) injection of Streptozotocin (Sigma) 220 mg/kg body weight. Diabetes was considered after three or more nonfasting blood glucose levels of >300 mg/dL from the tail vein, which generally occurred after a maximum of 72 h postinjection. Animals were anesthetized by isoflurane inhalation using a vaporizer and masks (Viking Medical). Rat islets were transplanted fresh immediately after isolation. Two hundred fifty islets from Poly SFH-P or control-treated pancreata were transplanted into each mouse under the left kidney capsule as previously described (41). The ischemia model was established and we determined that transplantation of 250 ischemic rat islets would reverse diabetes in less than 50% of recipients. Successful transplantation was defined by reduction of glycemia to below 200 mg/dL. Five to seven weeks posttransplantation, normoglycemic recipients underwent graft-bearing nephrectomy. Return to hyperglycemia was interpreted as indirect proof of islet graft function rather than spontaneous recovery of the native pancreas.

Graft function was also assessed by the lag period to achieve normoglycemia, and Intraperitoneal Glucose/Arginine Tolerance Test (IPG/ATT) 1 week posttransplantation. Briefly, for IPG/ATT, 2 mg/kg body weight of glucose and 3 mg/kg arginine in 0.5 cc was injected IP into a representative sample of randomly selected euglycemic animals (n = 5 for Poly SFH-P

Table 1: Oxymetry values (O$_2$ and pH) for perfusion media (Poly SFH-P and Control) before and after digestion

	O$_2$ tension (mmHg) predigestion	O$_2$ tension (mmHg) postdigestion	pH initial (without O$_2$)	pH initial (with O$_2$)	pH predigestion	pH postdigestion
Poly SFH-P	381.7 ± 35.3*	184.3 ± 39.8	7.4 ± 0.04**	7.4 ± 0.03[†]	7.4 ± 0.03[††]	7.2 ± 0.06[f]
Control	202.3 ± 28.2	128.3 ± 27.8	7.1 ± 0.03	7.8 ± 0.01	6.9 ± 0.04	6.6 ± 0.11

Values are means ± SEM, n = 12 rats per group. *p = 0.01; **p = 0.009; [†]p = 0.006; [††]p = 0.001; [f]p = 0.009.
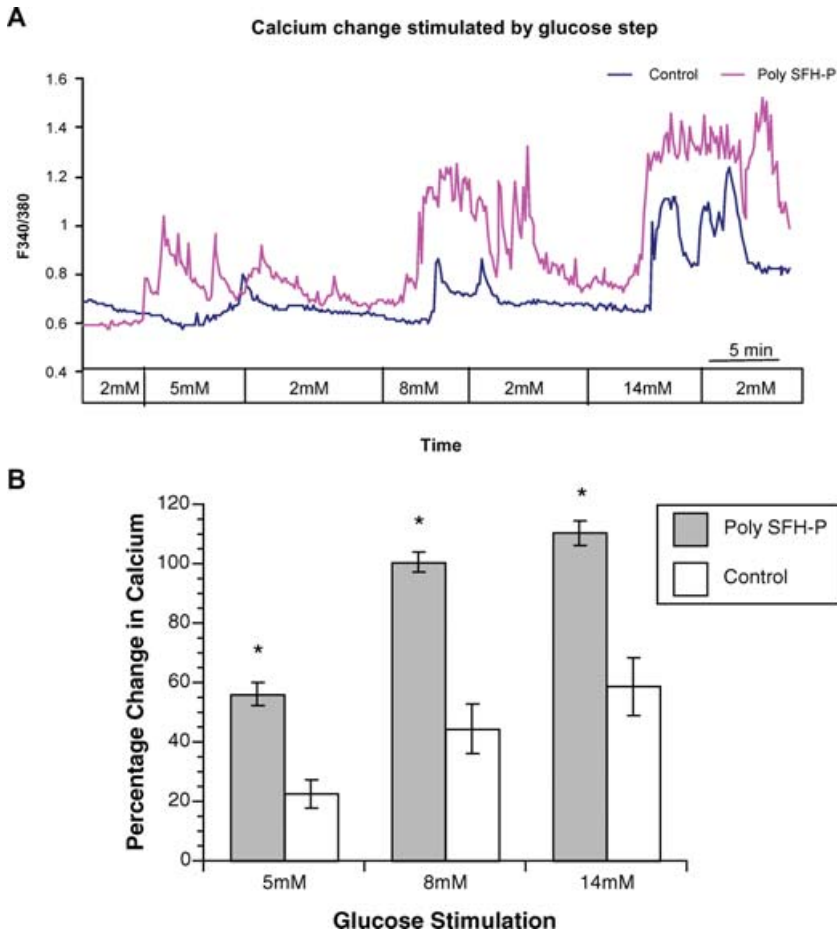
Figure 1: (A) Changes in ratio-metric values (Fura 2/AM) as a measurement of intracellular calcium levels in two representative islets under basal (2 mM) and stimulated (5, 8 and 14 mM) glucose conditions. **(B)** Percentage of intracellular calcium change in response to glucose stimulation (5, 8 and 14 mM glucose concentrations) in islets from Poly SFH-P and control groups. n = 3 isolations, 25 islets per group/isolation, mean ± SEM. *p < 0.05.

and n = 3 for control; in the control group only four animals achieved normoglycemia). Blood glucose levels were detected by tail puncture at serial time-points (0, 5, 15, 30, 45 and 60 min). p Value for IPGTT was obtained by calculating the Area Under the Curve under the mentioned time points for each individual in both groups and compared with the Student's t-test.

Statistical methods: Statistical analysis was carried out by Student's t-test and Pearson chi-square test. p values <0.05 were regarded as statistically significant.

Results

Oxymetry and pH assessment
Oxygen tension and pH were measured before and after the digestion phase. After oxygenation and perfusion of the pancreas, O$_2$ tension was higher in Poly SFH-P

containing perfusion solution (Table 1). During the digestion phase, Poly SFH-P maintained the pH in physiological range, whereas in the control group the pH fell significantly (Table 1). In order to exclude that maintenance of pH during the digestion phase was due to higher buffer capacity of Poly SFH-P as compared to RPMI alone, we measured the buffering capacities for Poly SFH-P and RPMI and did not find a significant difference (data not shown).

In vitro *evaluation*
Islet isolation outcomes in Poly SFH-P and control pancreata were analyzed by the following parameters.

Islet yield and viability
Perfusion of rat pancreata with Poly SFH-P did not have a significant impact on postisolation islet yields when

A

Calcium influx stimulated by Tolbutamide
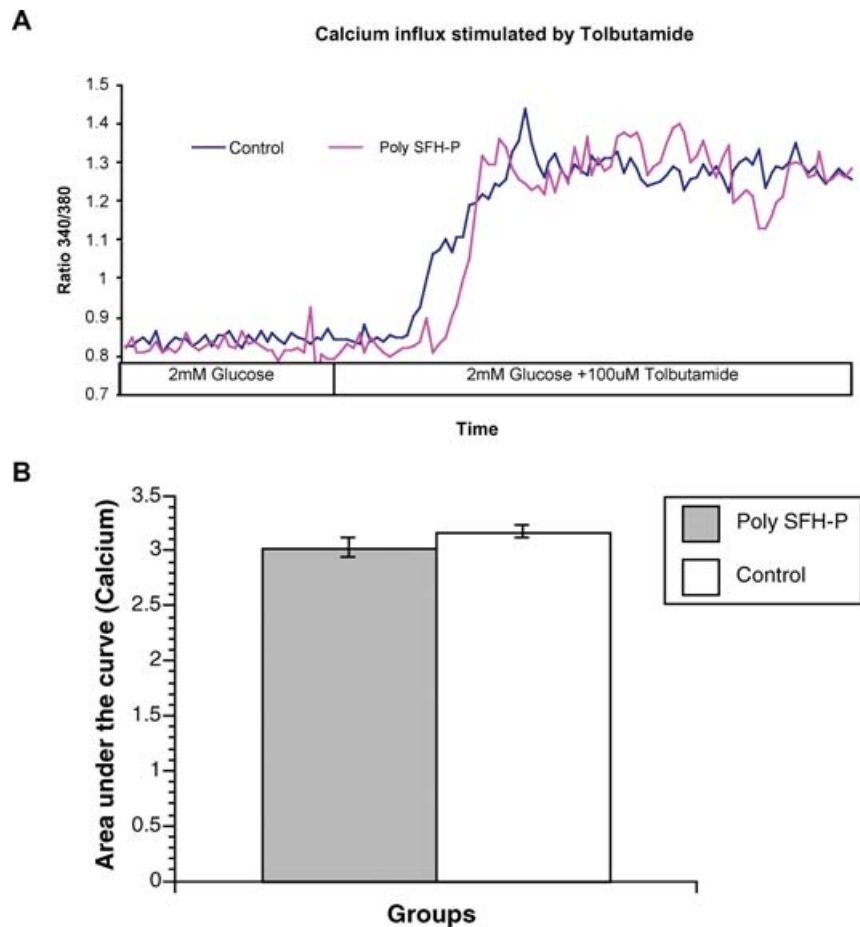
Figure 2: (A) Changes in ratio-metric values (Fura 2/AM) as a measurement of intracellular calcium levels in two representative islets under basal glucose (2 mM) conditions after the addition of Tolbutamide (100 µM). (B) Area under the curve (AUC) for intracellular calcium levels under basal glucose concentration (2 mM) in islets from both groups after the addition of Tolbutamide (100 µM). n = 3 isolations, 25 islets per group/isolation, mean ± SEM. p = 0.183.

compared to the control group (207 ± 33 vs. 172 ± 32 islets/rat respectively, p = 0.46. However, islet viability, as assessed by vital staining, was significantly improved in the Poly SFH-P group compared to the control (77% ± 4.6 vs. 63% ± 4.7 respectively, p = 0.04).

Apoptosis

Isolated islets from Poly SFH-P perfused pancreata showed lower activity of caspase-3 (relative caspase-3 activity: 34,714 ± 2167 vs. 45,985 ± 1382 respectively, p = 0.01), indicating a lower incidence of apoptosis in the Poly SFH-P group. (n = 3 isolations, 1500 islets/group/isolation).

Intracellular Ca^{2+} levels

Poly SFH-P also improved islet responsiveness to glucose as determined by increased intracellular Ca^{2+} levels in islets after stimulation with different glucose concentrations (Figure 1A). In all three concentrations of glucose (5, 8 and 14 mM) the Poly SFH-P islets demonstrated in a dose–response manner significantly higher intracellular Ca^{2+} values than the control (Figure 1B). The addition of Tolbutamide (inhibitor of ATP-dependent K$^+$ channels) showed that when the mitochondrial ATP regulation in these chan-

nels was bypassed, there was no significant difference between both groups in intracellular Ca^{2+} levels (Figure 2A and B).

Static glucose incubation

The pretreatment of rat pancreata with Poly SFH-P increased significantly insulin secretion of isolated islets in response to glucose compared to the control group (Figure 3).

Mitochondrial membrane potential and morphology

Measurements of mitochondrial membrane potential indicated a better functional integrity of Poly SFH-P islets than in the control group assessed by an increased percentage of change in fluorescence, (Rh123) representative of electrochemical potential as a response to glucose stimulation (14 mM), (Figure 4A and B). In addition, the results of morphology assessment showed mitochondria in islets from the control group appearing swollen and fragmented, assessed by decreased staining with Rh123 around the nuclei with loss of the continuity of the staining. In the Poly SFH-P group islet mitochondrial morphology was improved with reduced swelling and fragmentation with increased staining around the nuclei (Figure 5).
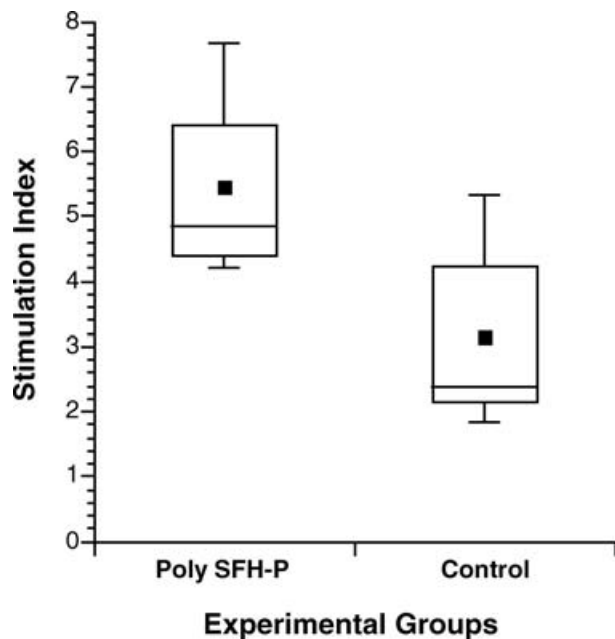
Figure 3: In vitro function: insulin secretion of islets in response to glucose challenge, expressed as a stimulation index (SI), mean±SEM. Poly SFH-P n = 5 isolations, control n = 5 isolations. *p = 0.03. The top, bottom, and line trough the middle of the box correspond to the 75th percentile, 25th percentile and 50th percentile, respectively. The whiskers on the bottom extend from the 10th percentile and top 90th percentile. ν represents the arithmetic mean.

Assessment of fractional beta cell viability

In order to determine the effect of Poly SFH-P perfusion specifically on the beta cell population and support the previous assessments we applied the method presented by Ichii et al. (38). This allows for assessing cell membrane stability (7aaD) and mitochondrial membrane stability (TMRE) of beta and nonbeta cells (gating the NG high vs. NG low population). The beta and nonbeta cell viability was improved in the Poly SFH-P group (Figure 6).

Oxidative stress and oxidative injury

To evaluate whether O_2 delivery by Poly SFH-P would lead to increased oxidative stress or injury we measured GSH and MDA levels.

Oxygenated Poly SFH-P did not decrease glutathione levels (7.1 ± 2.9 nmol/mg protein for Poly SFH-P and 6.8 ± 2.4 for control; p = 0.93). Lipid peroxidation as measured by MDA levels was not increased in the Poly SFH-P group as compared to the control (1.8 ± 0.9 nmol/mg protein vs. 6.2 ± 2.4, respectively; p = 0.19) indicating that there was no increased oxidative stress or injury by the presence of higher O_2 levels. (n = 8 isolations, 1000 islets/group/isolation for MDA and GSH).

In vivo *islet function after transplantation into diabetic nude mice*

Islet function *in vivo* was assessed through transplantation into streptozotocin induced, diabetic, athymic nude mice. The percentage of cured mice transplanted with Poly SFH-P or control islets was similar (6 out of 10 and 4 out of 9 respectively, p = 0.4). Mice transplanted with islets treated with Poly SFH-P achieved normoglycemia and reversed diabetes in a significantly shorter time than the mice transplanted with islets from the control group (Figure 7). Moreover, the mice receiving Poly SFH-P islets showed better graft function with lower glucose levels during IPG/ATT (Figure 8).

Discussion

Our study shows that intraductal perfusion of ischemic pancreata with Poly SFH-P improved islet viability and function through the maintenance of mitochondrial integrity and did not lead to increased oxidative stress in isolated islets.

Intact mitochondria are the 'powerhouse' of the cell, but if damaged, turn into a major contributor to apoptotic cell death via release of cytochrome c. Improvement of mitochondrial function and integrity should lead to both improved glucose-stimulated insulin secretion and decreased cell death. O_2 is an essential substrate for the production of energy by the cell. However, the provision of O_2 in an already damaged mitochondrial system may increase the production of ROS or free radicals producing further damage by oxidative stress, which is part of entity known as reperfusion injury (9). Islets are exposed to significant oxidative stress during the islet isolation and transplantation procedure (14–16).

In our study, higher O_2 availability to Poly SFH-P-treated islets was shown by higher O_2 tensions in the perfusion media compared to the control. The availability of substrate for mitochondria may be responsible for the improved viability observed in islets from the Poly SFH-P group.

In our study, increased O_2 availability protected Poly SFH-P islets from apoptosis determined by lower caspase-3 activity than in the control group. This result was not unexpected, since hypoxia has been shown to initiate apoptosis, mainly through the release of mitochondrial mediators into the cytosol (42). An event in mitochondrial respiration is the transmembrane transfer of protons, creating an electrochemical proton gradient, which can be used as an indicator of mitochondrial viability and function. In this context, mitochondrial functional integrity was shown to be superior in Poly SFH-P islets with improved membrane electrochemical potential in response to glucose stimulation. Functional integrity was complemented by the conservation of mitochondrial structure in the Poly SFH-P islets, determined by less swelling and more elongated mitochondria. We also showed enhanced

A

Mitochondrial Membrane Potential

B
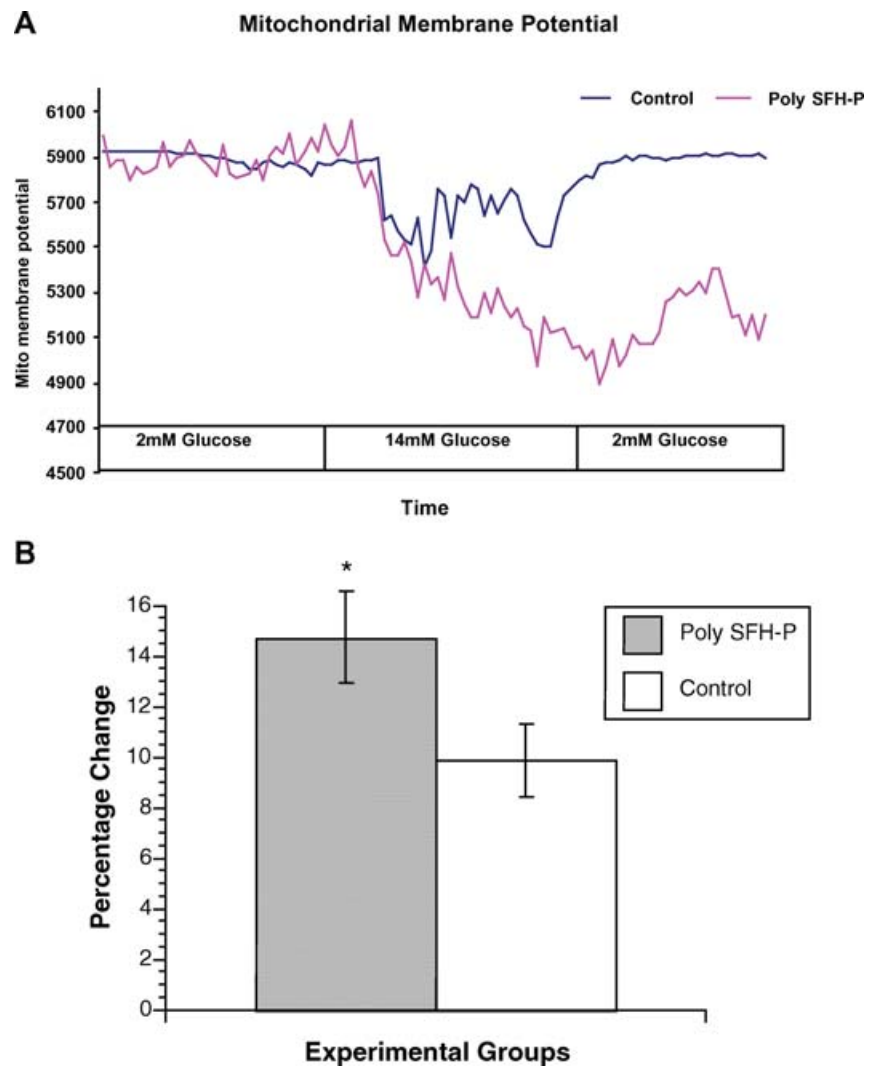
Figure 4: (A) Levels of Rh123-fluorescence outside the mitochondrial inner membrane in two representative islets under basal (2 mM) and glucose-stimulated conditions (14 mM). A gradual decrease in fluorescence represents the incorporation of Rh123 into the membrane as an indirect measurement of membrane potential. **(B) Percentage change in mitochondrial potential in islets from Poly SFH-P and control groups.** n = 3 isolations, 25 islets per group/isolation, mean ± SEM. *$p < 0.05$.

mitochondrial staining representative of improved perinuclear localization in the Poly SFH-P islets. Similar mitochondrial morphology was observed by Bindokas et al. comparing islets from Zucker lean and diabetic fatty rats (43). In their study, structural damage was shown in the islets from the fatty rats.

Our study also indicates that *in vitro* function of isolated islets was improved by intraductal administration of Poly SFH-P to the ischemic pancreas. Higher stimulation indices were obtained in Poly SFH-P-treated islets compared to the control in response to a static glucose challenge. The enhanced function for Poly SFH-P treated islets was supported by higher intracellular Ca^{2+} levels in response to glucose. This test represents the efficiency of the mitochondrial pathway in increasing cytosolic Ca^{2+}, necessary for insulin secretion in beta cells. After stimulation of pancreatic islets with glucose, it is uptaken into the cytosol by membrane transporters and converted into pyruvate, which enters mitochondria. Healthy mitochondria further

oxidize pyruvate in order to produce energy. An increase in (ATP/ADP) ratio closes ATP-dependent potassium channels in the cell membrane. This produces depolarization, opening the Ca^{2+} channels and promoting the fusion of granules with the cell membrane during exocytosis. To further dissect the involvement of the mitochondrial pathway on insulin secretion in response to glucose we used tolbutamide (a K^+-ATP channel inhibitor). Blocking K^+-ATP channels with tolbutamide allows cells to depolarize and raise calcium levels, directly promoting insulin secretion. After the addition of tolbutamide, intracellular Ca^{2+} response to glucose was similar between both groups. These results suggest that the provision of O_2 by Poly SFH-P protected the mitochondria allowing for preserving insulin secretion in response to glucose and explaining the functional differences observed between Poly SFH-P and control groups.

Long periods of ischemia can significantly alter the electron transport complexes in mitochondria (9). Damage to the electron transport chain complex results in leaking of
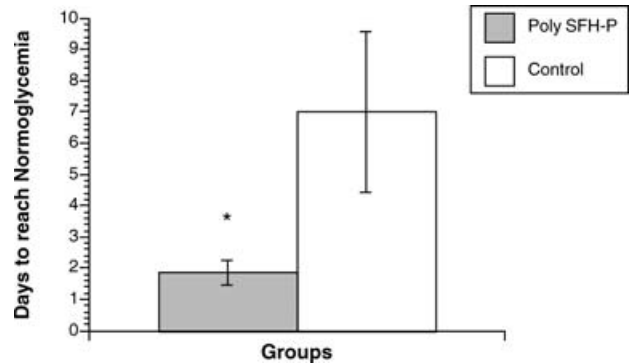
Poly SFH-P

Control
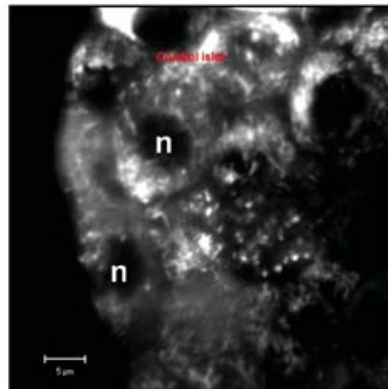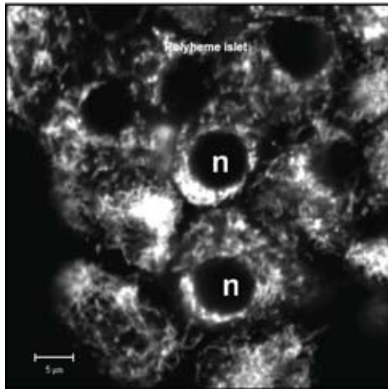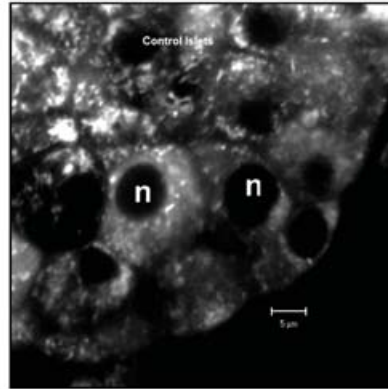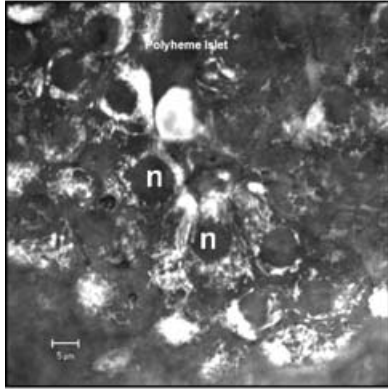
Figure 5: Mitochondrial Morphology: Mitochondria were stained with rhodamine 123 dye. Two representative images (confocal reconstructions) from individual islets from Poly SFH-P and control groups are shown. Images are maximum intensity projections, 1 μm slice thickness. Cell nuclei in the islets are identified with the letter 'n'. Mitochondrial morphology and distribution around the nuclei appear superior in the Poly SFH-P group than the control. Contrast has been balanced to reveal details of mitochondrial morphology. Scale bar is 5 μm. n = 3 isolations, 20 islets/group/isolation.
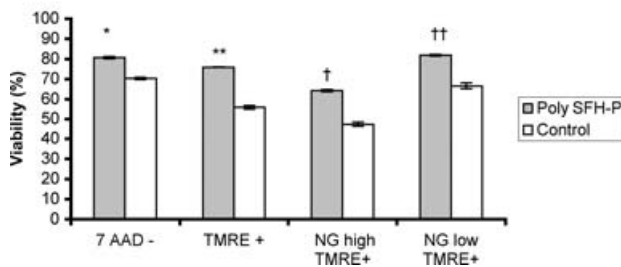
Figure 6: Viability staining specifically on the beta and non-beta cell population: Viability assessment for cell membrane stability (7aaD), mitochondrial membrane stability (TMRE), in beta cells (gating the NG high population) versus nonbeta cell (NG low). n = 3 per group. *p < 0.001; **p < 0.001; †p < 0.001; ††p < 0.001.


Figure 7: Number of days (lag time) to reach normoglycemia after islet transplantation. Poly SFH-P n = 6 mice and control. n = 4 mice. *p = 0.02.

electrons. Under certain conditions, oxygen can react with the leaking electrons to generate ROS leading to oxidative stress and tissue damage (9). In our study, the introduction of O_2 by Poly SFH-P did not increase oxidative stress or damage to the islets, determined by similar levels of GSH and MDA in both groups. The amount of O_2 provided by Poly SFH-P was more important in terms of structural and

functional recovery of mitochondria, and did not produce significant amount of oxidative stress.

Poly SFH-P improved integrity of both beta and nonbeta cells. Fractional islet cell viability assessment indicated that beta cells were more vulnerable to ischemic damage than nonbeta cells in the islets.
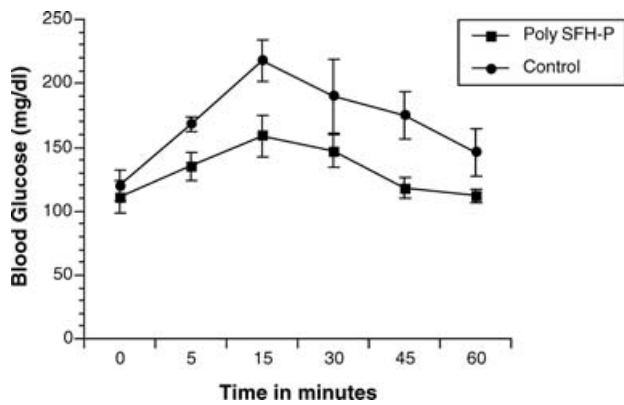
Figure 8: Intraperitoneal Glucose/Arginine Tolerance Test (IPG/ATT) in a representative sample of mice that reached normoglycemia after transplantation with islets from Poly SFH-P. n = 5 and control n = 3. Values indicate mean blood glucose levels ± SEM. p = 0.03.

Poly SFH-P perfusion of the ischemic rat pancreas improved islet graft function *in vivo* as shown by a shortened lag time to reach normoglycemia after transplantation and a better response to IPG/AT stress test. Improvement in human transplantation outcomes through O$_2$ delivery to islets have been previously shown (36). These *in vivo* results confirmed the improved function of Poly SFH-P treated islets observed *in vitro*.

In conclusion, we have shown that intraductal perfusion with Poly SFH-P can improve islet isolation and transplantation outcomes through the maintenance of mitochondrial integrity, and does not lead to increased oxidative stress.

Acknowledgments

References

1. Scharp DW, Lacy PE, Santiago JV et al. Results of our first nine intraportal islet allografts in type 1, insulin-dependent diabetic patients. Transplantation 1991; 51: 76–85.
2. Shapiro AM, Lakey JR, Ryan EA et al. Islet transplantation in seven patients with type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive regimen. N Engl J Med 2000; 343: 230–238.
3. Froud T, Ricordi C, Baidal DA et al. Islet transplantation in type 1 diabetes mellitus using cultured islets and steroid-free immunosuppression: Miami experience. Am J Transplant 2005; 5: 2037–2046.
4. Ryan EA, Paty BW, Senior PA et al. Five-year follow-up after clinical islet transplantation. Diabetes 2005; 54: 2060–2069.
5. Toso C, Oberholzer J, Ris F et al. Factors affecting human islet of Langerhans isolation yields. Transplant Proc 2002; 34: 826–827.
6. Hering BJ, Brendel M, Schultz AO, Schultz B, Bretzel RG. International islet transplant registry. Justus-Liebig-University of Giessen; 1996.
7. Lee TC, Barshes NR, Brunicardi FC et al. Procurement of the human pancreas for pancreatic islet transplantation. Transplantation 2004; 78: 481–483.
8. Powner DJ. Donor care before pancreatic tissue transplantation. Prog Transplant 2005; 15: 129–136; quiz 137.
9. Jassem W, Fuggle SV, Rela M, Koo DD, Heaton ND. The role of mitochondria in ischemia/reperfusion injury. Transplantation 2002; 73: 493–499.
10. Obermaier R, Von Dobschuetz E, Benthues A et al. Exogenous and endogenous nitric oxide donors improve post-ischemic tissue oxygenation in early pancreatic ischemia/reperfusion injury in the rat. Eur Surg Res 2004; 36: 219–225.
11. Her C, Mandy S. Acute respiratory distress syndrome of the contralateral lung after reexpansion pulmonary edema of a collapsed lung. J Clin Anesth 2004; 16: 244–250.
12. Bright R, Raval AP, Dembner JM et al. Protein kinase C delta mediates cerebral reperfusion injury in vivo. J Neurosci 2004; 24: 6880–6888.
13. Bonder CS, Knight D, Hernandez-Saavedra D, McCord JM, Kubes P. Chimeric SOD2/3 inhibits at the endothelial-neutrophil interface to limit vascular dysfunction in ischemia-reperfusion. Am J Physiol Gastrointest Liver Physiol 2004; 287: G676–G684.
14. Hennige AM, Lembert N, Wahl MA, Ammon HP. Oxidative stress increases potassium efflux from pancreatic islets by depletion of intracellular calcium stores. Free Radic Res 2000; 33: 507–516.
15. Wolff SP. Diabetes mellitus and free radicals. Free radicals, transition metals and oxidative stress in the aetiology of diabetes mellitus and complications. Br Med Bull 1993; 49: 642–652.
16. Miwa I, Ichimura N, Sugiura M, Hamada Y, Taniguchi S. Inhibition of glucose-induced insulin secretion by 4-hydroxy-2-nonenal and other lipid peroxidation products. Endocrinology 2000; 141: 2767–2772.
17. Bottino R, Balamurugan AN, Tse H et al. Response of human islets to isolation stress and the effect of antioxidant treatment. Diabetes 2004; 53: 2559–2568.
18. Bottino R, Balamurugan AN, Bertera S, Pietropaolo M, Trucco M, Piganelli JD. Preservation of human islet cell functional mass by anti-oxidative action of a novel SOD mimic compound. Diabetes 2002; 51: 2561–2567.
19. Ribeiro MM, Klein D, Pileggi A et al. Heme oxygenase-1 fused to a TAT peptide transduces and protects pancreatic beta-cells. Biochem Biophys Res Commun 2003; 305: 876–881.
20. Avila JG, Tsujimura T, Oberholzer J et al. Improvement of pancreatic islet isolation outcomes using glutamine perfusion during isolation procedure. Cell Transplant 2003; 12: 877–881.
21. Potdar S, Malek S, Eghtesad B et al. Initial experience using histidine-tryptophan-ketoglutarate solution in clinical pancreas transplantation. Clin Transplant 2004; 18: 661–665.
22. Fridell JA, Agarwal A, Milgrom ML, Goggins WC, Murdock P, Pescovitz MD. Comparison of histidine-tryptophan-ketoglutarate solution and University of Wisconsin solution for organ preservation in clinical pancreas transplantation. Transplantation 2004; 77: 1304–1306.
23. Canelo R, Hakim NS, Ringe B. Experience with hystidine tryptophan ketoglutarate versus University Wisconsin preservation solutions in transplantation. Int Surg 2003; 88: 145–151.

24. Kakinoki K, Fujino Y, Suzuki Y et al. Superiority of the two-layer method before islet isolation confirmed by in vivo viability assessment. Transplantation 2005; 79: 1516–1521.

25. Tsujimura T, Kuroda Y, Avila JG et al. Influence of pancreas preservation on human islet isolation outcomes: Impact of the two-layer method. Transplantation 2004; 78: 96–100.

26. Tsujimura T, Kuroda Y, Churchill TA et al. Short-term storage of the ischemically damaged human pancreas by the two-layer method prior to islet isolation. Cell Transplant 2004; 13: 67–73.

27. Tsujimura T, Kuroda Y, Avila JG et al. Resuscitation of the ischemically damaged human pancreas by the two-layer method prior to islet isolation. Transplant Proc 2003; 35: 2461–2462.

28. Fukushima K, Nakamura M, Hinuma K. Influence of Fluosol-DA infusion on the complement activation and on the blood level of histamine in man. Prog Clin Biol Res 1983; 122: 291–298.

29. Farrar D, Grocott M. Intravenous artificial oxygen carriers. Hosp Med 2003; 64: 352–356.

30. Gould SA, Moore EE, Hoyt DB et al. The life-sustaining capacity of human polymerized hemoglobin when red cells might be unavailable. J Am Coll Surg 2002; 195: 445–452; discussion 452–445.

31. Gould SA, Moore EE, Hoyt DB et al. The first randomized trial of human polymerized hemoglobin as a blood substitute in acute trauma and emergent surgery. J Am Coll Surg 1998; 187: 113–120; discussion 120–112.

32. Lanzkron S, Moliterno AR, Norris EJ et al. Polymerized human Hb use in acute chest syndrome: A case report. Transfusion 2002; 42: 1422–1427.

33. Lacy PE, Kostianovsky M. Method for the isolation of intact islets of Langerhans from the rat pancreas. Diabetes 1967; 16: 35–39.

34. Tamarina NA, Wang Y, Mariotto L et al. Small-conductance calcium-activated K+ channels are expressed in pancreatic islets and regulate glucose responses. Diabetes 2003; 52: 2000–2006.

35. Roe MW, Worley JF, 3rd, Mittal AA et al. Expression and function of pancreatic beta-cell delayed rectifier K+ channels. Role in stimulus-secretion coupling. J Biol Chem 1996; 271: 32241–32246.

36. Tsujimura T, Kuroda Y, Kin T et al. Human islet transplantation from pancreases with prolonged cold ischemia using additional preservation by the two-layer (UW solution/perfluorochemical) cold-storage method. Transplantation 2002; 74: 1687–1691.

37. Zhou YP, Pena JC, Roe MW et al. Overexpression of Bcl-x(L) in beta-cells prevents cell death but impairs mitochondrial signal for insulin secretion. Am J Physiol Endocrinol Metab 2000; 278: E340–E351.

38. Ichii H, Inverardi L, Pileggi A et al. A novel method for the assessment of cellular composition and beta-cell viability in human islet preparations. Am J Transplant 2005; 5: 1635–1645.

39. Fernandez-Checa JC, Kaplowitz N. The use of monochlorobimane to determine hepatic GSH levels and synthesis. Anal Biochem 1990; 190: 212–219.

40. Yagi K. Simple assay for the level of total lipid peroxides in serum or plasma. Methods Mol Biol 1998; 108: 101–106.

41. Oberholzer J, Yu D, Triponez F et al. Decomplementation with cobra venom factor prolongs survival of xenografted islets in a rat to mouse model. Immunology 1999; 97: 173–180.

42. Li P, Nijhawan D, Budihardjo I et al. Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. Cell 1997; 91: 479–489.

43. Bindokas VP, Kuznetsov A, Sreenan S, Polonsky KS, Roe MW, Philipson LH. Visualizing superoxide production in normal and diabetic rat islets of Langerhans. J Biol Chem 2003; 278: 9796–9801.