

Beneficial Effects of Desferrioxamine on Encapsulated Human Islets – *In Vitro* and *In Vivo* Study

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As many as 2000 IEQs (islet equivalent) of encapsulated human islets are required to normalize glucose levels in diabetic mice. To reduce this number, encapsulated islets were exposed to 100 μ M desferrioxamine (DFO) prior to transplantation. Cell viability, glucose-induced insulin secretion, VEGF (Vascular endothelial growth factor), HIF-1 α (Hypoxia inducible factor-1 alpha), caspase-3 and caspase-8 levels were assessed after exposure to DFO for 12, 24 or 72 h. Subsequently, 1000, 750 or 500 encapsulated IEQs were infused into peritoneal cavity of diabetic mice after 24 h exposure to DFO. Neither viability nor function *in vitro* was affected by DFO, and levels of caspase-3 and caspase-8 were unchanged. DFO significantly enhanced VEGF secretion by 1.6- and 2.5-fold at 24 and 72 h, respectively, with a concomitant increase in HIF-1 α levels. Euglycemia was achieved in 100% mice receiving 1000 preconditioned IEQs, as compared to only 36% receiving unconditioned IEQs ($p < 0.001$). Similarly, with 750 IEQ, euglycemia was achieved in 50% mice receiving preconditioned islets as compared to 10% receiving unconditioned islets ($p = 0.049$). Mice receiving preconditioned islets had lower glucose levels than those receiving unconditioned islets. In summary, DFO treatment enhances HIF-1 α and VEGF expression in encapsulated human islets and improves their ability to function when transplanted.

Key words: Desferrioxamine, encapsulation, islet transplantation, minimal islet mass, VEGF

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Introduction

Microencapsulating human islets is a means being explored to overcome the immune mediated rejection of the graft without toxic immunosuppression. Recently, phase 1 clinical trials using calcium alginate-poly-L-ornithine (1) and barium alginate microcapsules (2) showed that allografting of microencapsulated human islets is safe although only a minor clinical benefit was observed. Many factors influence the survival and function of encapsulated human islets, with hypoxia representing one of the major limiting factors for graft survival (3).

Native human islets reside in a microenvironment supplied with a dense network of capillaries thereby enjoying ample blood supply, oxygen (pO_2 of 40 mmHg) and abundant nutrients (4). However, the capillary networks are destroyed during the isolation process and the islets suffer from post-isolation hypoxic stress (5). Further, transplanted islets suffer from hypoxia in the immediate posttransplantation period (with mean pO_2 of 5–10 mmHg) until revascularization occurs during the next fortnight, leading to reperfusion injury and cell death (5). Furthermore, microcapsules prevent the revascularization process thereby subjecting the islets to further hypoxic stress. This hampers the ability of the microencapsulated islets to function. To improve their efficiency, novel strategies are required to protect the islets from hypoxia induced oxidative stress.

Several strategies have been explored to enhance vascularization and minimize hypoxic stress to prevent apoptosis in the immediate posttransplantation period with both encapsulated and nonencapsulated human islets. These include prevascularization (6,7), heat shock (8), ischaemic preconditioning (9), stimulation of Bcl-2/Bcl-xl complex (10,11), X-linked inhibitor of apoptosis protein (XIAP) overexpression (12), inhibition of cellular FLICE inhibitory protein (cFLIP) (13) and A20 activation (14,15). None of the above strategies has been tried with microencapsulated human islets. We hypothesized that a combination of the above strategies, particularly ischemic preconditioning and enhanced VEGF expression would be beneficial.

Desferrioxamine (DFO), an iron chelator, induces VEGF expression in β (16) and non- β cells, and protects them against oxidative stress and activated macrophages (17–19). Preconditioning with DFO stabilizes HIF-1 α and protects neuronal cells against cytokine mediated cell death

(20). *In vivo* DFO also has benefits and treatment of diabetic mice allografted with islets results in less graft rejection (21,22). Further, preconditioning of human islets with DFO reduces the number of islets needed to achieve normoglycemia in diabetic immunodeficient mice (23). However, there are no reports investigating the effect of DFO on encapsulated human islets. Therefore, the aim of this study was to investigate the effects of DFO on microencapsulated human islets both *in vitro* and *in vivo*.

Materials and Methods

Human islet isolation and shipment

Human islets were isolated from pancreases of brain dead cadaveric multiorgan donors ($n = 3$) at the Cell Isolation laboratory of the University of Illinois at Chicago, USA. Briefly, the human islets were isolated by digestion with SERVA Collagenase NB 1 (Nordmark, Germany) using the method described previously (24). The isolated islets were then cultured in supplemented CMRL-1066 media (Mediatech, Herndon, VA) containing 1.5% human albumin for 1–2 days before being shipped to Sydney using a commercial courier service. The islets were then cultured for a day before being encapsulated. All the procedures regarding obtaining human islets were approved by the Institution's Human Research Ethics Committee.

Encapsulation

The cultured human islets were pooled together and washed in 0.9% NaCl. The human islets were then suspended in highly purified 2.2% alginate (60:40 guluronic: mannuronic acid, UPMVG PRONOVA, FMC Biopolymer) solution in 1:8 ratio. The microcapsule formation was carried out in an air-driven droplet generator (Steinau, Berlin, Germany) as described previously (25). The microencapsulated human islets were then cultured for a day in CMRL-1066 media prior to *in vitro* or transplantation studies.

DFO treatment

For all the *in vitro* studies, 1000 IEQs of encapsulated human islets were cultured in CMRL-1066 media containing 100 μ M DFO for 12, 24 or 72 h. For the *in vivo* studies different IEQs of encapsulated human islets were preconditioned with 100 μ M DFO for 24 h and subsequently transplanted the following day into the peritoneal cavity of diabetic NOD/SCID (Nonobese Diabetic/Severe Combined Immunodeficiency) mice.

Viability

Viability of encapsulated human islets cultured in CMRL-1066 media with/without 100 μ M DFO was assessed using the fluorescent dyes 6-carboxyfluorescein diacetate (6-CFDA; Sigma, St. Louis, MO) and propidium iodide (PI; Sigma). The percentage of green (live cells) to red (dead cells) fluorescence was assessed to evaluate the viability ($n = 100$, for each time point and preparation). Samples were analyzed under a Zeiss-Axioskop 2 microscope using Axiovision LE software.

Insulin secretion and content

Aliquots of encapsulated human islets from each preparation treated with/without DFO for 24 and 72 h were exposed to either 2.8 mM glucose (basal, $n = 3$) or 20 mM glucose (stimulus, $n = 3$) for one hour at 37°C with gentle agitation. After 1 h, the supernatant was collected and human insulin measured by radioimmunoassay (RIA; Linco, St. Charles, MO). The remaining pellet was washed in Hanks Balanced Salt solution (HBSS) solution followed by addition of cold acid ethanol and vortexed vigorously to enhance cell lysis. The cell extract was kept at 4°C overnight and the supernatant collected the following day for measuring insulin content by RIA.

Intracellular ATP measurement

Aliquots of encapsulated human islets from each preparation treated with/without DFO for 24 h were exposed to either 2.8 mM glucose (basal, $n = 3$) or 20 mM glucose (stimulus, $n = 3$) for one hour at 37°C with gentle agitation. Thereafter the encapsulated islets were decapsulated (50 mM EDTA and 10 mM HEPES in PBS) and immediately placed on ice. The cells were then washed twice with ice-cold PBS and lysed. ATP was measured using the luminescence ATP detection assay system (ATPlite, PerkinElmer, Norwalk, CT) following the manufacturer's protocol.

Gene expression

For the gene expression studies 1000 IEQs of encapsulated human islets were cultured with/without DFO for 12, 24 and 72 h. At each time point, the encapsulated islets were decapsulated and RNA was extracted using the RNeasy mini kits (Qiagen, Hilden, Germany). The cDNA was prepared using the SuperScript III First-Strand Synthesis System and random hexamers (Invitrogen Corporation). Gene expression was determined by real time PCR as described previously (26). Briefly, for each reaction 2 μ L of diluted cDNA, 10 μ L of SYBR green master mix, 0.15 μ L of 10 μ M forward and reverse primers and 7.7 μ L of nuclease-free water was used making a total volume of 20 μ L. Q-PCR was carried out using the Mx3500P Real-Time PCR system (Stratagene, NSW, Australia). The relative expression levels of HIF-1 α and VEGF were calculated using a mathematical model (27) based on the individual Q-PCR primer efficiencies and the quantified values were normalized against the housekeeping gene 18S. The primer sequences were:

18S: 5'-GTTCCGACCATAAACGATGC-3' (forward),

5'-AACCAGACAATCGCTCCAC-3' (reverse),

HIF-1 α : 5'-TCCAGTTACGTTCCCTTCGATCA-3' (forward)

5'-TTTGAGGACTTGCCTTTCA-3' (reverse),

VEGF: 5'-GCCTTGCTGCTACTCCTCA-3' (forward),

5'-CAAGGCCACAGGGATTTT-3' (reverse)

Enzyme linked immunosorbent assay (ELISA)

1000 IEQs of encapsulated human islets treated with/without DFO were cultured for 12, 24 and 72 h. VEGF ELISA was performed on culture supernatant in duplicates using a Quantikine VEGF ELSIA kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. Briefly, 200 μ L of the culture supernatants was added to the wells and incubated for 2 h at 37°C. The plate was washed with 400 μ L wash buffer twice and 200 μ L VEGF conjugate added followed by incubation for 2 h. After the addition of substrate and stop solution, the optical density was determined using a microplate reader (Bio Rad 680 XR, Australia) at 450 nm with wavelength correction of 570 nm.

Western blots

2000 IEQs of encapsulated human islets treated with/without DFO for 12, 24 and 72 h were decapsulated and the human islets lysed using the cold lysis buffer (10 mM Tris, 10 mM NaH₂PO₄/Na₂HPO₄, 130 mM NaCl, 1% Triton-X100, and 10 mM sodium pyrophosphate) supplemented with protease inhibitors. The protein concentrations were determined using the Bradford method (Bio-Rad, Alfred Nobel Drive, Hercules, CA). Equal amount of protein were then loaded on 10% Ready Gel TrisHCl gels (Bio-Rad, Australia), separated and then transferred to a nitrocellulose membrane (Bio-Rad). The membrane was then blocked for 1 h and then incubated with primary antibodies overnight at 4°C. The membranes were then washed with Tris-buffered saline containing tween [10 mmol/L Tris, 140 mmol/L NaCl, 0.02% Tween 20 (pH 7.6)] and probed with corresponding secondary antibody (1:500) for 1 h at room temperature. Proteins were detected using an ECL Plus western blotting detection system (Amersham Biosciences, Piscataway, NJ). The primary antibodies used in this study were the rabbit

anti-HIF-1 α antibody (1:1000, Novus biologicals, USA), rabbit anti-Caspase-8 (1:2000, Millipore, Billerica, MA), mouse anti-Caspase-3 (1:1000, Cell Signaling, Danvers, MA) and rabbit anti-human actin (1:5000, Sigma Aldrich). The secondary antibodies were polyclonal goat anti-rabbit Ig/HRP and polyclonal goat anti-mouse Ig/HRP (1:5000, both from Dako).

Transplantation of microencapsulated human islets

Male NOD/SCID mice (6–8 weeks) were made diabetic by three consecutive intraperitoneal injections of streptozotocin (70 mg/kg) (Alexis Biochemicals, Australia). Animals with three consecutive blood glucose levels >12 mmol/L were considered diabetic and used for the study. Briefly, the mice were anaesthetized with pentobarbitone (65 mg/kg) and the encapsulated human islets preconditioned with/without 100 μ M DFO were injected into the peritoneal cavity using a 20-gauge catheter. Mice were transplanted with 10 000, 5000, 3000, 2000, 1000, 750 or 500 encapsulated IEQs. All the procedures involving the mice were approved by the Institution's Animal Care and Ethics Committee.

Oral glucose tolerance test

Animals were considered normoglycemic if three consecutive random BGLs (blood glucose levels) were <6 mmol/L. Once normoglycemia was achieved, an oral glucose tolerance test (OGTT) was performed. For this, the mice were fasted overnight followed by an oral glucose gavage (3 mg/g of 300 mg/mL glucose solution) and BGLs were measured at 0, 20, 40, 60 and 120 min after glucose administration. OGTTs also were carried out on diabetic and nondiabetic control NOD/SCID mice.

Immunohistochemistry

The encapsulated human islets pretreated with/without DFO were retrieved from diabetic NOD/SCID mice, which became normoglycemic at 60 days posttransplantation. The retrieved capsules were washed twice in PBS, fixed in buffered formalin and embedded in paraffin blocks. The blocks were sectioned at 5 μ m intervals and examined by hematoxylin-eosin (H&E) staining to assess graft vascularization.

Statistical analysis

All values were expressed as mean \pm SEM. The statistical software NCSS97 was used to perform the analysis of data. One-way analysis of variance and Duncan's multiple comparison tests were used to compare data among groups, and Student's *t*-test between the groups. Kaplan–Meier analysis was used to compare the survival curves between the treated and non-treated groups. The results were considered significant when *p*-values were <0.05.

Results

DFO did not affect the viability of encapsulated islets

To study the effect of DFO on islet survival, human islets were treated with DFO and the viability assessed. Treatment with 100 μ M DFO for 12, 24 and 72 h did not affect the viability of encapsulated human islets. As seen in Figure 1 the islet viability in the DFO treatment and control groups was similar at 12, 24 and 72 h. On the other hand, treatment with a higher concentration of DFO, 1000 μ M, was toxic to the islets with a significant decrease in the viability by 24 h (data not shown).

DFO treatment did not activate the apoptotic cascade

Caspases or cysteine-aspartic proteases (cysteine proteases) are essential in cells for apoptosis or programmed

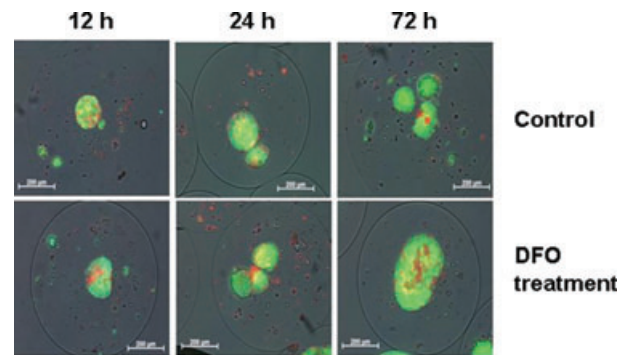


Figure 1: Viability of encapsulated human islets was not affected by treatment with DFO as measured by CFDA and PI staining; DFO treatment vs. Control: after 12 h (72.8 \pm 1.1 vs. 74.1 \pm 0.8%), 24 h (72.9 \pm 1.1 vs. 73.3 \pm 0.8%) and 72 h (71.9 \pm 1.2 vs. 69.9 \pm 1.1%), Values are mean \pm SEM, *p* > 0.05, (*n* = 3).

cell death, necrosis and inflammation. The caspases are regulated at the posttranslational level and are synthesized as inactive procaspases or initiator caspases which then activate the effector caspases thereby triggering the apoptotic process (28). To investigate whether DFO (100 μ M) treatment triggered the apoptotic pathway in encapsulated human islets; we measured the levels of initiator caspase (Caspase-8) and also the levels of effector caspase (Caspase-3) by Western blot at 12, 24 and 72 h. As seen from Figure 2A and B, there was no significant up-regulation in the levels of either caspase-8 or caspase-3 in the DFO treated groups compared to the controls. This

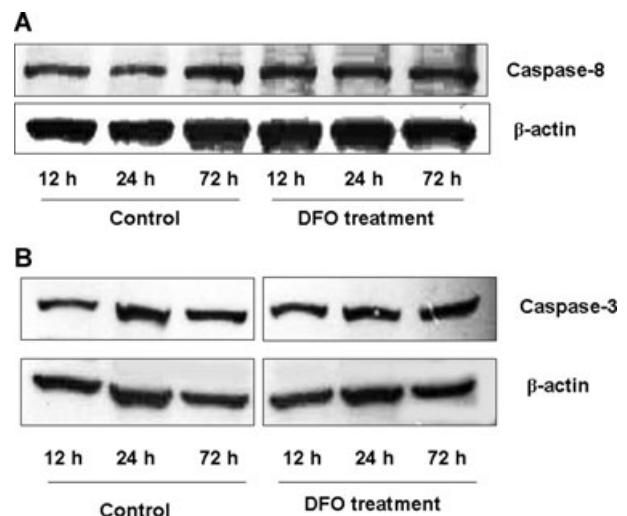


Figure 2: DFO treatment did not activate the apoptotic cascade. DFO treatment of encapsulated islets for 12, 24 and 72 h increased neither the proapoptotic nor apoptotic markers caspase-8 (A) and caspase-3 (B) levels, respectively as measured by Western blots. Representative of three independent experiments (*n* = 3).

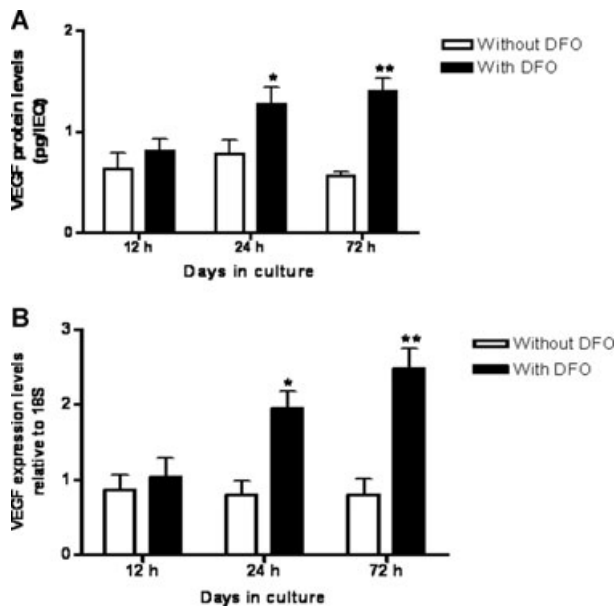


Figure 3: DFO treatment increased VEGF expression. (A) DFO treatment significantly enhanced VEGF protein expression at 24 h (1.6-fold, * $p < 0.05$) and 72 h (2.5-fold, ** $p < 0.01$) and gene expression at 24 h (2.4-fold, * $p < 0.05$) and 72 h (3-fold, ** $p < 0.01$); but not at 12 h. Protein expression was measured by ELISA, and gene expression by real-time PCR. Values are mean \pm SEM (n = 3).

suggests that DFO treatment was not toxic to the islets at the dosage used in this study.

DFO treatment increased VEGF expression

DFO treatment enhances VEGF secretion in the Rim5F cell line and in rat pancreatic islets (16), but there are no reports on its effect on human islets. Accordingly, encapsulated human islets were treated with DFO for 12, 24 and 72 h and the VEGF gene and protein expression measured. As shown in Figure 3A, DFO treatment significantly enhanced VEGF secretion at 24 h by 1.6-fold: DFO 1.28 ± 0.16 versus control 0.78 ± 0.14 pg/IEQ ($p < 0.05$) and at 72 h by 2.5-fold: DFO 1.40 ± 0.13 versus control 0.57 ± 0.04 pg/IEQ ($p < 0.01$) respectively. However there was no significant increase in VEGF expression after 12 h of treatment: DFO 0.81 ± 0.11 versus control 0.63 ± 0.16 pg/IEQ ($p > 0.05$). Consistent with the increase in levels of protein there was a significant increase in VEGF mRNA levels at 24 h and 72 h after DFO treatment by 2.4-fold (DFO 1.95 ± 0.22 vs. control 0.8 ± 0.18 , $p < 0.05$) and 3-fold (DFO 2.45 ± 0.28 vs. control 0.81 ± 0.2 , $p < 0.01$), respectively but not at 12 h, Figure 3B.

DFO enhanced HIF-1 α expression

DFO has been shown to increase HIF-1 α levels (23) and reduce free radical mediated cell injury in insulin producing cells (17–19). This beneficial effect of DFO can be attributed to its ischemic preconditioning properties as a

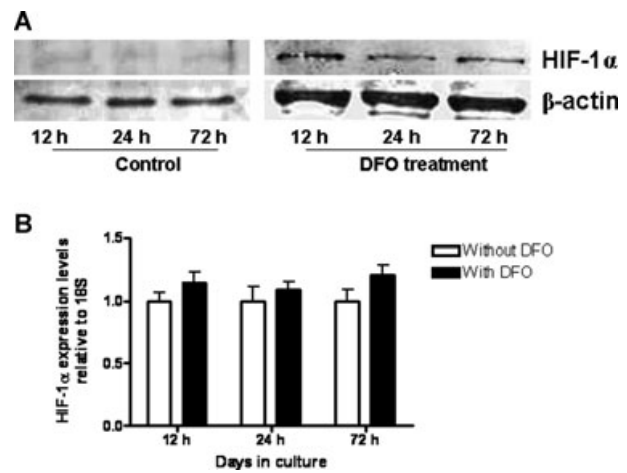


Figure 4: DFO enhanced HIF-1 α expression. (A) Nuclear extracts of encapsulated human islets treated with/without DFO for 12, 24 and 72 h. DFO treatment significantly enhanced HIF-1 α protein at all time points as measured by Western blots. Representative of three independent experiments (n = 3). (B) DFO treatment for 12, 24 and 72 h did not enhance the expression of the HIF-1 α gene levels as quantified by real-time PCR. Values are mean \pm SEM, $p > 0.05$, (n = 3).

hypoxic mimetic pharmacological agent. So, to determine whether DFO treatment affected HIF-1 α expression in encapsulated human islets, they were treated with 100 μ M DFO for 12, 24 and 72 h and the HIF-1 α gene and protein levels measured by real-time PCR and western blots respectively. As shown in Figure 4A, DFO treatment for 12, 24 and 72 h significantly induced HIF-1 α expression in the encapsulated human islets. However, there was no corresponding increase in gene expression levels (Figure 4B), a phenomenon described previously (29). DFO is acting to stabilize expression of HIF-1 α , rather than increasing its transcription or translation.

DFO enhanced insulin secretion

Based on the above data, it seemed DFO pretreatment began to exert its full effect on encapsulated islets only from 24 h. Accordingly, glucose-induced insulin secretion was examined from this time. At 24 h but not 72 h, DFO pretreatment significantly enhanced both basal and glucose-induced insulin secretion compared to controls by 1.3- and 1.4-fold respectively (Figure 5). At both times, the treated islets remained responsive to glucose. The increase in both the basal and glucose-induced insulin secretion at 24 h was accompanied by a corresponding increase in the intracellular ATP concentration by 1.7- and 2.1-fold, respectively (Figure 6).

Minimal number of encapsulated islets required to achieve normoglycemia

Transplantation of large number of encapsulated untreated human islets was performed initially to determine the

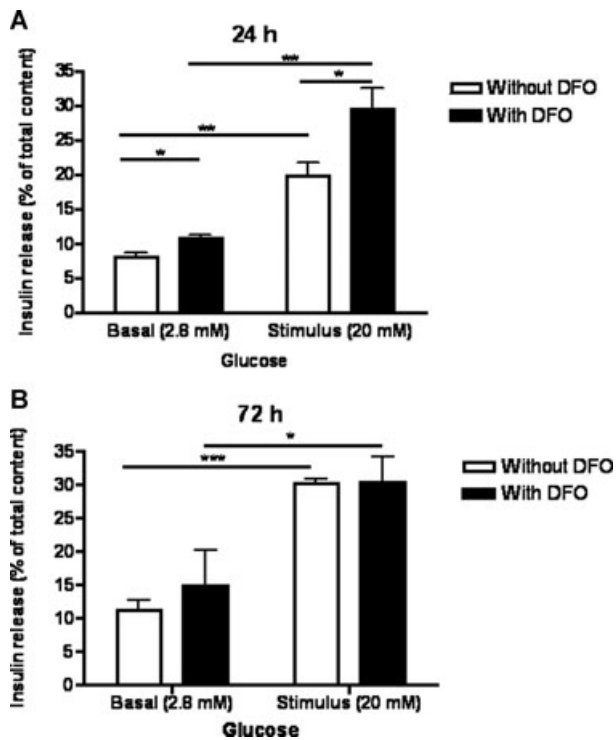


Figure 5: DFO treatment enhanced insulin secretion. Effect of DFO treatment for 24 (A) and 72 h (B) on basal (2.8 mM) and glucose-stimulated (20 mM) insulin secretion as measured by radioimmunoassay. Values are mean \pm SEM, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ($n = 3$).

minimum number of islets required to normalize blood glucose levels. Accordingly diabetic mice were transplanted with 10 000, 5000, 3000, 2000 and 1000 IEQs, respectively. All the mice transplanted with 2000–10 000, but not 1000 IEQs became normoglycemic 3.6 ± 0.8 days posttransplantation (range 2–5 days). Blood glucose levels

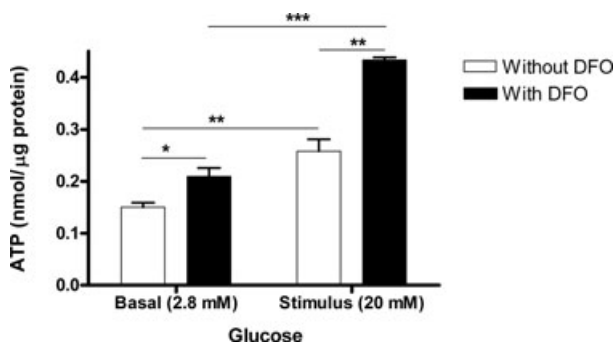


Figure 6: DFO treatment increased intracellular ATP content. DFO treatment for 24 h increased the intracellular ATP concentration at both low (2.8 mM) and high glucose (20 mM) concentrations, as measured by luminescence. Values are mean \pm SEM, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ($n = 3$).

declined from 21.3 ± 0.6 to 4.2 ± 0.5 mmol/L. All these animals remained euglycemic till the end of the study at 60 days posttransplantation (Figure 7A). However, transplantation of 1000 IEQ resulted in normoglycemia only in 1 of 7 mice, and that was 27 days postsurgery. To assess graft function further, an OGTT was carried out at 30 days posttransplantation (Figure 7B). All the mice transplanted with ≥ 2000 IEQs handled glucose normally; whereas those that received 1000 IEQs did not. From these data, we concluded that the minimal number of IEQs required to consistently normalize blood glucose levels in diabetic NOD/SCID mice was 2000. So, to investigate the effect of DFO, further studies were carried out using fewer than this number, namely 1000, 750 and 500 IEQs.

Pretreatment with DFO reduced number of IEQs required for normoglycemia

Normalization of blood glucose levels was achieved in all 14 mice receiving 1000 DFO preconditioned islets. Euglycemia was achieved in 86% ($n = 12$) of the mice by 5.3 ± 0.6 days (range 3–7 days) posttransplantation, with the remaining mice achieving this at 27 days. In contrast, only 36% ($n = 5/14$) of mice receiving unconditioned encapsulated islets became normoglycemic. The time required to achieve this was longer than in the DFO group, 16.2 ± 1.9 days (range 13–21 days). To be certain that these results were not due to a difference in the batch of islets used, other mice were transplanted with 2000 unconditioned encapsulated islets prepared from the same batch used for the 1000 IEQ experiment. All six control mice given 2000 IEQs became normoglycemic within 3 days of being transplanted (Figure 8A).

To analyze graft function further, an OGTT was carried out at day 30 posttransplantation. Animals transplanted with DFO preconditioned islets had lower blood glucose levels compared to mice that received unconditioned islets (Figure 8B).

Transplantation with 750 IEQs of DFO treated encapsulated islets resulted in normoglycemia only in 50% of mice ($n = 5/10$) and by 9.8 ± 2.1 days (range 3–13 days) posttransplantation. In contrast, only 10% ($n = 1/10$) of mice receiving unconditioned islets became normoglycemic (Figure 9A). OGTTs of normalized mice receiving 750 IEQs treated islets exhibited lower blood glucose levels similar to nondiabetic controls compared to diabetic controls (Figure 9B).

No diabetic mouse grafted with 500 encapsulated IEQs became normoglycemic. However, DFO pretreatment was beneficial, with mice receiving treated islets as compared to untreated islets having lower blood glucose levels (data not shown).

In all mice transplanted with encapsulated islets pretreated with/without DFO, almost all capsules were freely floating,

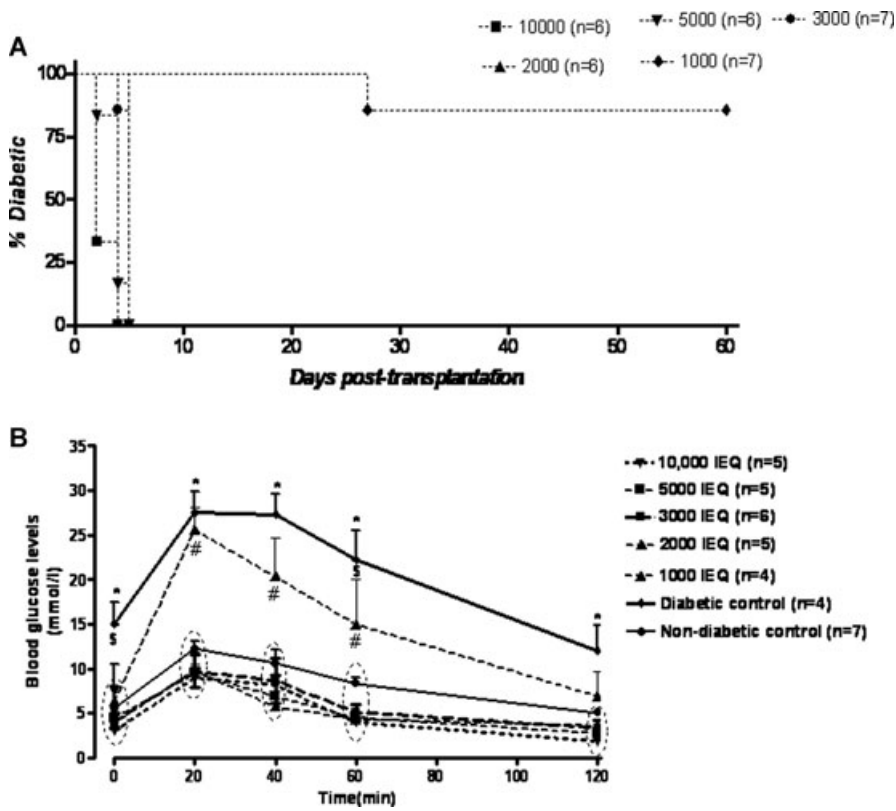


Figure 7: Minimal number of encapsulated human islets required to normalize blood glucose levels. 1000–10 000 IEQs of encapsulated human islets were transplanted into peritoneal cavity of male NOD/SCID mice rendered diabetic by streptozotocin (70 mg/kg). (A) Normoglycemia was achieved in 100% mice receiving 2000–10 000 IEQs by 3.6 ± 0.8 days post-transplantation. However, normoglycemia was achieved in only 1 of 7 mice receiving 1000 IEQs, at day 27 posttransplantation. (B) Oral glucose tolerance test at 30 days post-transplantation. Values are mean \pm SEM. 2000–10 000 IEQs and non-diabetic control <1000 IEQs #; and < diabetic control \$ (ANOVA and Duncan’s multiple comparison test).

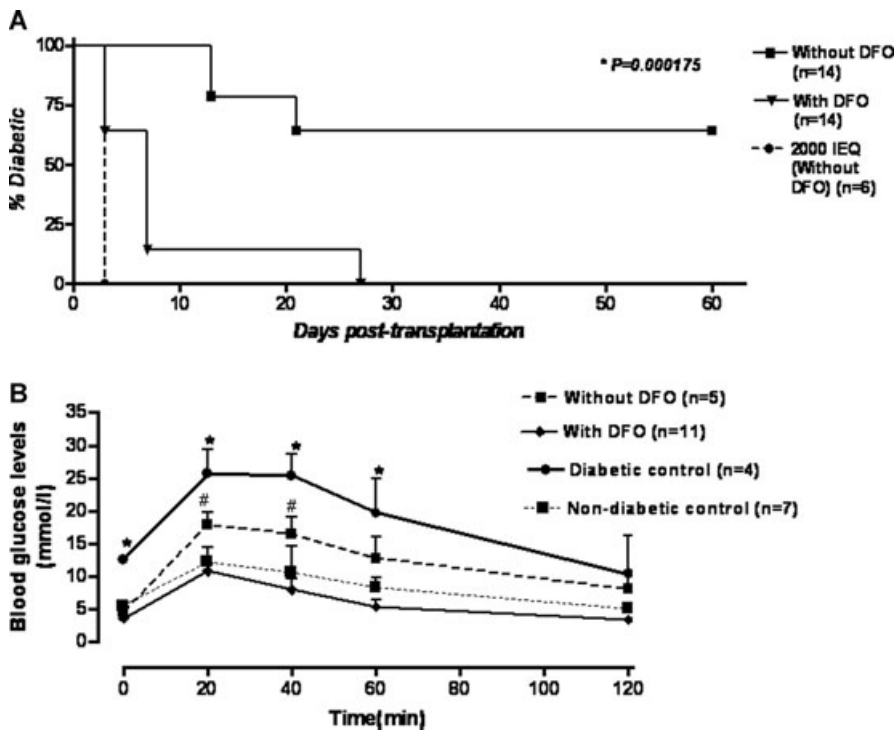
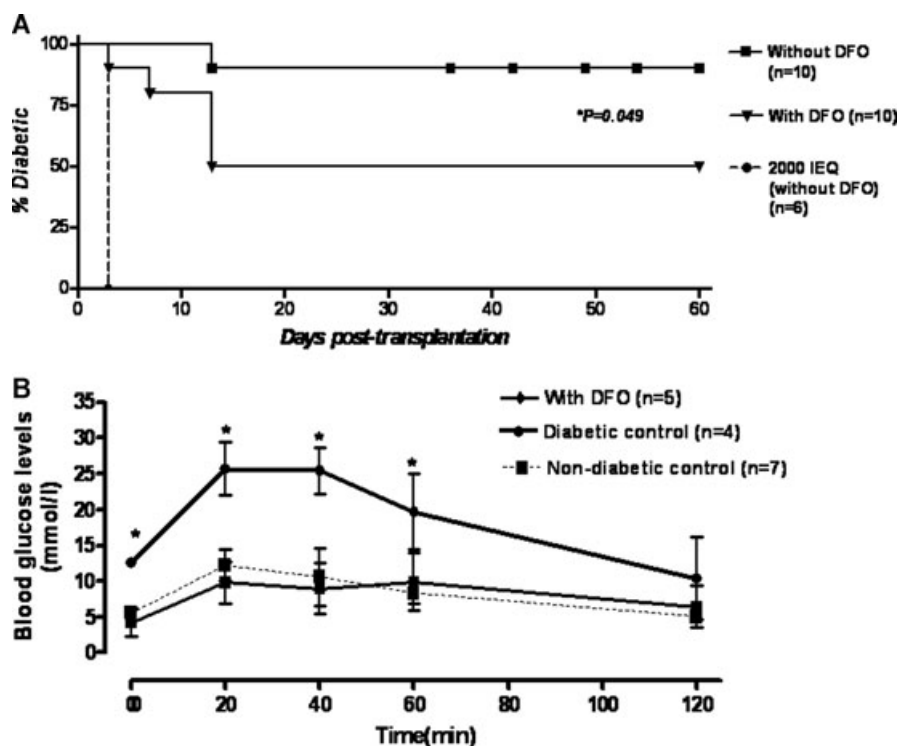


Figure 8: DFO preconditioned islets normalized blood glucose levels with minimal islet mass of 1000 IEQs. 1000 IEQs of encapsulated human islets were preconditioned with/without 100 EM DFO for 24 h and subsequently transplanted into the peritoneal cavity of diabetic male NOD/SCID mice. (A) Normoglycemia was achieved in 100% (n = 14/14) mice receiving 1000 DFO preconditioned islets as compared to only 36% (n = 5/14) mice receiving unconditioned islets. All controls mice, which were transplanted with 2000 IEQ unconditioned islet, became normoglycaemic. *DFO treated islets > untreated islets (1000 IEQs) p < 0.001 (Log-Rank test). (B) OGTTs performed at 30 days posttransplantation. Values are mean \pm SEM. DFO treated, untreated and nondiabetic control < diabetic control *, DFO treated < untreated # (ANOVA and Duncan’s multiple comparison test).

Figure 9: DFO preconditioned islets normalized blood glucose levels with minimal islet mass of 750 IEQs.

750 IEQs of encapsulated human islets were preconditioned with/without 100 EM DFO for 24 h and subsequently transplanted into the peritoneal cavity of diabetic male NOD/SCID mice. (A) Normoglycemia was achieved in 50% ($n = 5/10$) mice receiving 750 DFO preconditioned islets against only 10% ($n = 1/10$) mice receiving unconditioned islets by day 60. Controls were mice transplanted with 2000 IEQ unconditioned islets (100% normoglycemic by day 3). DFO treated islets $>$ untreated islets (750 IEQs) $*p = 0.049$ (Log-Rank test). (B) OGTTs performed at 30 days posttransplantation. Values are mean \pm SEM. DFO treated and nondiabetic controls $<$ diabetic controls $*$ (ANOVA and Duncan's multiple comparison test).



with $<1\%$ attached to abdominal organs. Histological examination of the capsules showed them to be smooth with no cells or blood vessels attached to their surface (Figure 10).

Discussion

Human islets are subjected to both immunological and nonimmunological insults in the immediate posttransplantation period leading to islet cell death and graft failure (30–33). VEGF is an angiogenic factor that is known to enhance survival of both endothelial and other cell types.

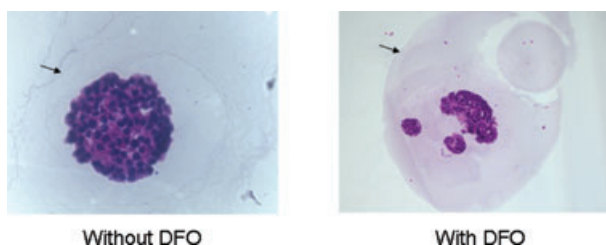


Figure 10: H&E staining of retrieved encapsulated human islets transplanted into diabetic NOD/SCID mice. Examination of encapsulated islets from transplanted mice showed that most of the capsules were free floating with $<1\%$ attached to abdominal organs. There was no evidence of vascularization of the floating capsules, regardless of whether the islets had been pretreated with DFO. Arrows indicate the smooth capsule surface.

It can act in an autocrine manner (34,35) and exerting its effects by inducing PI3K/Akt signalling and expression of antiapoptotic proteins (36,37). Recent studies have shown that isolated human islets express VEGF and its receptors suggesting that VEGF may play a role in enhancing both the survival and function of β cells (38,39). We hypothesized that upregulating VEGF expression would be beneficial to both the survival and functioning of encapsulated human islets that were transplanted into diabetic recipients. In this study we used a clinically recommended iron-chelating agent DFO to induce VEGF secretion in microencapsulated human islets. We have shown that treating encapsulated human islets for 24 h with DFO increased VEGF secretion 1.6-fold. We also showed that VEGF induction by DFO preconditioning improved the outcome of microencapsulated human islets and normoglycemia could be achieved completely with a minimal islet mass of 1000 IEQs and partially with marginal islet mass of 750 IEQs. Glycemic control improved as early as 3 days posttransplantation in mice receiving DFO preconditioned islets, suggesting that VEGF may have a protective effect on the islet cell viability other than by enhancing vascularization, which takes up to 2 weeks to occur (40). Further support for accepting that vascularization was not the means for the beneficial effect of VEGF was the lack of blood vessels on the surface of the grafted capsules, almost all of which were freely floating in the peritoneal cavity (Figure 10). Others have previously shown a beneficial effect of VEGF on human (39,41), mouse (42), rat islets (43) and beta-cell lines (44), but without the use of DFO. As might be expected, the lack of VEGF, as occurs in VEGF-A null mice, results in

impaired insulin secretion (45,46). In conclusion, our study is the first to show a beneficial effect of enhancing VEGF in encapsulated human islets; it supplements a previous study showing a similar effect with encapsulated rat islets (43).

The beneficial effect of DFO on encapsulated human islets also might be attributed to an increase in expression of HIF-1 α , independent of the stimulation of VEGF by this protein. Adaptive responses to stress including hypoxia-induced tolerance to ischemia are mediated by HIF-1 α , thereby enhancing cell survival (47–50). It is quite feasible, therefore, that the enhanced expression of this protein which we and others (23) observed in encapsulated and nonencapsulated human islets respectively, after exposure to DFO may have conditioned the islets to better survive the hypoxic conditions of the peritoneal cavity where they were transplanted. The beneficial effects of enhancing expression of HIF-1 α in β cells have been shown previously. Insulin secretion in MIN6 cells has been enhanced (51) and the number of human islets needed for normalization of blood glucose levels in recipient immunodeficient diabetic mice reduced (23) by increasing levels of HIF-1 α . Conversely, reduction in expression of HIF-1 α in β cells results in an impaired insulin secretion and HIF-1 α knockout mice have abnormal glucose tolerance (52). Also islets isolated from patients with type 2 diabetes had reduced levels of HIF-1 α (52), suggesting a possible role of this protein in β -cell survival and function. The mechanism for the beneficial effect of enhancing expression of HIF-1 α appears to be through stimulation of the classical pathway for insulin secretion. In a β cell, glucose causes an increase in levels of ATP, which initiate the signal cascade that results in insulin secretion. In DFO-treated islets, levels of ATP increased both in the basal state and when exposed to high concentrations of glucose (Figure 6).

In summary our data show that treating microencapsulated human islets with DFO for 24 h induces HIF-1 α protein and increased VEGF expression, one of the downstream genes regulated by HIF-1 α . The data also show that preconditioning microencapsulated human islets with DFO prior to transplantation improves their functional efficiency, reducing the number of islets needed to normalize blood glucose levels to as few as 750 IEQs. The benefit obtained *in vivo* can be explained by the effects observed *in vitro*. Our results support the use of DFO in preparing encapsulated insulin-producing cells for transplantation into humans.

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References

1. Calafiore R, Basta G, Luca G et al. Microencapsulated pancreatic islet allografts into nonimmunosuppressed patients with type 1 diabetes: First two cases. *Diabetes Care* 2006; 29: 137–138.
2. Tuch BE, Keogh GW, Williams LJ et al. Safety and viability of microencapsulated human islets transplanted into diabetic humans. *Diabetes Care* 2009; 32: 1887–1889.
3. Van Schilfgaarde R, de Vos P. Factors influencing the properties and performance of microcapsules for immunoprotection of pancreatic islets. *J Mol Med* 1999; 77: 199–205.
4. Carlsson PO, Palm F, Andersson A, Liss P. Markedly decreased oxygen tension in transplanted rat pancreatic islets irrespective of the implantation site. *Diabetes* 2001; 50: 489–495.
5. Pileggi A, Ricordi C, Alessiani M, Inverardi L. Factors influencing Islet of Langerhans graft function and monitoring. *Clin Chim Acta* 2001; 310: 3–16.
6. Wang W, Gu Y, Tabata Y et al. Reversal of diabetes in mice by xenotransplantation of a bioartificial pancreas in a prevascularized subcutaneous site. *Transplantation* 2002; 73: 122–129.
7. Sörenby AK, Kumagai-Braesch M, Sharma A, Hultenby KR, Wernerson AM, Tibell AB. Preimplantation of an immunoprotective device can lower the curative dose of islets to that of free islet transplantation: Studies in a rodent model. *Transplantation* 2008; 86: 364–366.
8. Bellmann K, Wenz A, Radons J, Burkart V, Kleemann R, Kolb H. Heat shock induces resistance in rat pancreatic islet cells against nitric oxide, oxygen radicals and streptozotocin toxicity in vitro. *J Clin Invest* 1995; 95: 2840–2845.
9. Hagerkvist R, Sandler S, Mokhtari D, Welsh N. Amelioration of diabetes by imatinib mesylate (Gleevec®): Role of β -cell NF- κ B activation and anti-apoptotic preconditioning. *FASEB J* 2007; 21: 618–628.
10. Dupraz P, Rinsch C, Pralong WF, et al. Lentivirus-mediated Bcl-2 expression in betaTC-tet cells improves resistance to hypoxia and cytokine-induced apoptosis while preserving in vitro and in vivo control of insulin secretion. *Gene Ther* 1999; 6: 1160–1169.
11. Klein D, Ribeiro MM, Mendoza V et al. Delivery of Bcl-XL or its BH4 domain by protein transduction inhibits apoptosis in human islets. *Biochem Biophys Res Commun* 2004; 323: 473–478.
12. Emamaullee JA, Rajotte RV, Liston P et al. XIAP overexpression in human islets prevents early posttransplant apoptosis and reduces the islet mass needed to treat diabetes. *Diabetes* 2005; 54: 2541–2548.
13. Cottet S, Dupraz P, Hamburger F, Dolci W, Jaquet M, Thorens B. cFLIP protein prevents tumor necrosis factor- α mediated induction of caspase-8-dependent apoptosis in insulin-secreting β Tc-Tet cells. *Diabetes* 2002; 51: 1805–1814.
14. Grey ST, Arvelo MB, Hasenkamp W, Bach FH, Ferran C. A20 inhibits cytokine-induced apoptosis and nuclear factor kappaB-dependent gene activation in islets. *J Exp Med* 1999; 190: 1135–1146.
15. Grey ST, Longo C, Shukri T et al. Genetic engineering of a suboptimal islet graft with A20 preserves beta cell mass and function. *J Immunol* 2003; 170: 6250–6256.
16. Langlois A, Bietiger W, Mandes K et al. Overexpression of vascular endothelial growth factor in vitro using deferoxamine: A new drug to increase islet vascularization during transplantation. *Transplant Proc* 2008; 40: 473–476.

17. Markel TA, Crisostomo PR, Wang M et al. The struggle for iron: Gastrointestinal microbes modulate the host immune response during infection. *J Leukoc Biol* 2007; 81: 393–400.
18. Olejnicka BT, Ollinger K, Brunk UT. A short exposure to a high-glucose milieu stabilizes the acidic vacuolar apparatus of insulinoma cells in culture to ensuing oxidative stress. *APMIS* 1997; 105: 689–698.
19. Mendola J, Wright JR, Lacy PE. Oxygen free-radical scavengers and immune destruction of murine islets in allograft rejection and multiple low-dose streptozocin-induced insulinitis. *Diabetes* 1989; 38: 379–385.
20. Yao S, Soutto M, Sriram S. Preconditioning with cobalt chloride or desferrioxamine protects oligodendrocyte cell line (MO3.13) from tumor necrosis factor- α -mediated cell death. *J Neurosci Res* 2008; 86: 2403–2413.
21. Bradley B, Prowse SJ, Bauling P, Lafferty KJ. Desferrioxamine treatment prevents chronic islet allograft damage. *Diabetes* 1986; 35: 550–555.
22. Nomikos IN, Prowse SJ, Carotenuto P, Lafferty KJ. Combined treatment with nicotinamide and desferrioxamine prevents islet allograft destruction in NOD mice. *Diabetes* 1986; 35: 1302–1304.
23. Stokes R, Cheng K, Scott C et al. Hypoxia inducible factor 1 α (HIF-1 α) is required for successful transplantation and increasing HIF-1 α with desferrioxamine markedly improves outcomes [abstract]. *Transplantation* 2008; 86(2S): 141–142.
24. Qi M, Strand BL, Mørch Y et al. Encapsulation of human islets in novel inhomogeneous alginate-Ca²⁺/Ba²⁺ microbeads: In vitro and in vivo function. *Artif Cells Blood Substit Immobil* 2008; 36: 403–420.
25. Foster JL, Williams G, Williams LJ, Tuch BE. Differentiation of transplanted microencapsulated fetal pancreatic cells. *Transplantation* 2007; 83: 1440–1448.
26. Gao SY, Lees JG, Wong JC et al. Modeling the adhesion of human embryonic stem cells to poly(lactic-co-glycolic acid) surfaces in a 3D environment. *J Biomed Mater Res A* 2010; 92: 683–692.
27. Pfaffl M. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* 2001; 29: 2002–2007.
28. Fesik SW, Shi Y. Controlling the caspases. *Science* 2001; 294: 1477–1478.
29. Nakamura M, Bodily JM, Beglin M, Kyo S, Inoue M, Laimins LA. Hypoxia-specific stabilization of HIF-1 α by human papillomaviruses. *Virology* 2009; 387: 442–448.
30. Vaithilingam V, Sundaram G, Tuch BE. Islet cell transplantation. *Curr Opin Organ Transplant* 2008; 13: 1–6.
31. Kaufman DB, Platt JL, Rabe FL, Dunn DL, Bach FH, Sutherland DE. Differential roles of Mac-1⁺ cells, and CD4⁺ and CD8⁺ T lymphocytes in primary nonfunction and classic rejection of islet allografts. *J Exp Med* 1990; 172: 291–302.
32. Liu X, Hering BJ, Mellert J et al. Prevention of primary nonfunction after porcine islet allotransplantation. *Transplant Proc* 1997; 29: 2701–2072.
33. Cui W, Wilson JT, Wen J et al. Thrombomodulin improves early outcomes after intraportal islet transplantation. *Am J Transplant* 2009; 9: 1308–1316.
34. Gerber HP, Malik AK, Solar GP et al. VEGF regulates haematopoietic stem cell survival by an internal autocrine loop mechanism. *Nature* 2002; 417: 954–958.
35. Bachelder RE, Crago A, Chung J et al. Vascular endothelial growth factor is an autocrine survival factor for neuropilin-expressing breast carcinoma cells. *Cancer Res* 2001; 61: 5736–5740.
36. Gerber HP, Dixit V, Ferrara N. Vascular endothelial growth factor induces expression of the antiapoptotic proteins Bcl-2 and A1 in vascular endothelial cells. *J Biol Chem* 1998; 273: 13313–13316.
37. Gerber HP, McMurtrey A, Kowalski J et al. Vascular endothelial growth factor regulates endothelial cell survival through the phosphatidylinositol 3'-kinase/Akt signal transduction pathway. Requirement for Flk-1/KDR activation. *J Biol Chem* 1998; 273: 30336–30343.
38. Menger MD, Jaeger S, Walter P, Feifel G, Hammersen F, Messmer K. Angiogenesis and hemodynamics of microvasculature of transplanted islets of Langerhans. *Diabetes* 1989; 38(S1): 199–201.
39. Cross SE, Richards SK, Clark A et al. Vascular endothelial growth factor as a survival factor for human islets: Effect of immunosuppressive drugs. *Diabetologia* 2007; 50: 1423–1432.
40. Mattsson G, Jansson L, Carlsson PO. Decreased vascular density in mouse pancreatic islets after transplantation. *Diabetes* 2002; 51: 1362–1366.
41. Narang AS, Sabek O, Gaber AO, Mahato RI. Coexpression of vascular endothelial growth factor and interleukin-1 receptor antagonist improves human islet survival and function. *Pharm Res* 2006; 23: 1970–1982.
42. Zhang N, Richter A, Suriawinata J et al. Elevated vascular endothelial growth factor production in islets improves islet graft vascularization. *Diabetes* 2004; 53: 963–970.
43. Sigrist S, Mechine-Neuville A, Mandes K et al. Induction of angiogenesis in omentum with vascular endothelial growth factor: Influence on the viability of encapsulated rat pancreatic islets during transplantation. *J Vasc Res* 2003; 40: 359–367.
44. Langlois A, Bietiger W, Mandes K et al. Adenoviral infection or deferoxamine? Two approaches to overexpress VEGF in beta-cell lines. *J Drug Target* 2009; 17: 415–422.
45. Iwashita N, Uchida T, Choi JB et al. Impaired insulin secretion in vivo but enhanced insulin secretion from isolated islets in pancreatic beta cell-specific vascular endothelial growth factor-A knockout mice. *Diabetologia* 2007; 50: 380–389.
46. Jabs N, Franklin I, Brenner MB et al. Reduced insulin secretion and content in VEGF-A deficient mouse pancreatic islets. *Exp Clin Endocrinol Diabetes* 2008; 116(S): 46–49.
47. Bergeron M, Yu AY, Solway KE, Semenza GL, Sharp FR. Induction of hypoxia-inducible factor-1 (HIF-1) and its target genes following focal ischaemia in rat brain. *Eur J Neurosci* 1999; 11: 4159–4170.
48. Bergeron M, Gidday JM, Yu AY, Semenza GL, Ferriero DM, Sharp FR. Role of hypoxia-inducible factor-1 in hypoxia-induced ischemic tolerance in neonatal rat brain. *Ann Neurol* 2000; 48: 285–296.
49. Bernaudin M, Nedelec AS, Divoux D, MacKenzie ET, Petit E, Schumann-Bard P. Normobaric hypoxia induces tolerance to focal permanent cerebral ischemia in association with an increased expression of hypoxia-inducible factor-1 and its target genes, erythropoietin and VEGF, in the adult mouse brain. *J Cereb Blood Flow Metab* 2002; 22: 393–403.
50. Jones NM, Bergeron M. Hypoxic preconditioning induces changes in HIF-1 target genes in neonatal rat brain. *J Cereb Blood Flow Metab* 2001; 21: 1105–1114.
51. Gunton JE, Kulkarni RN, Yim S et al. Loss of ARNT/HIF1 β mediates altered gene expression and pancreatic-islet dysfunction in human type 2 diabetes. *Cell* 2005; 122: 337–349.
52. Cheng K, Ho K, Stokes R et al. Hypoxia-inducible factor-1 α regulates β cell function in mouse and human islets. *J Clin Invest* 2010; May 3. pii: 35846. doi: 10.1172/JCI35846.