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Intra-Ductal Glutamine Administration Reduces Oxidative Injury During Human Pancreatic Islet Isolation

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Oxidative stress during islet isolation induces a cascade of events injuring islets and hampering islet engraftment. This study evaluated islet isolation and transplantation outcomes after intra-ductal glutamine administration. Human pancreata deemed unsuitable for pancreas or islet transplantation were treated with either a 5 mM solution of L-glutamine (n = 6) or collagenase enzyme alone (n = 6) through the main pancreatic duct. Islet yield, viability, *in vitro* function; markers of oxidative stress [malondialdehyde (MDA) and Glutathione (GSH)] and apoptosis were assessed. Islet yields were significantly increased in the glutamine group compared to controls ($318,559 \pm 25,800$ vs. $165,582 \pm 39,944$ mean $\pm$ SEM, p < 0.01). The amount of apoptotic cells per islet was smaller in the glutamine group than the control. The percentage of nude mice rendered normoglycemic with glutamine-treated islets was higher than the controls (83% n = 10/12 vs. 26% n = 6/23; p < 0.01), and the time to reach normoglycemia was decreased in the glutamine group (1.83 ± 0.4 vs. 7.3 ± 3 days; p < 0.01). Glutamine administration increased GSH levels (7.6 ± 1.7 nmol/mg protein vs. 4.03 ± 0.5 in control, p < 0.05) and reduced lipid-peroxidation (MDA 2.45 ± 0.7 nmol/mg of protein vs. 6.54 ± 1.7 in control; p < 0.05). We conclude that intra-ductal administration of glutamine reduces oxidative injury and apoptosis and improves islet yield and islet graft function after transplantation.

Introduction

In the transplant setting, islets of Langerhans are faced with various types of stress related to the isolation and transplantation procedure, which trigger a cascade of cell signaling pathways that compromise their function and viability (1–5). Moreover, variable times of ischemia affect the number and function of islets recovered (6–8). Despite reports by groups achieving a critical beta cell mass and effective islet transplants into diabetic patients from one donor pancreas (9,10), the availability of pancreata and sufficient islets to achieve normoglycemia in patients continues to be the Achilles' heel of islet transplantation (11).

Oxidative stress plays a pivotal role in cell injury during islet isolation and transplantation procedures (12–14). The use of anti-oxidants has been shown to protect islets from oxidative injury during the isolation procedure and during the culture period (15–18). One of the most important nonenzymatic anti-oxidant defense compounds involved in this reaction is Glutathione (GSH) (19–21), a tripeptide (glutamate-glycine-cysteine) for which cell membranes are not readily permeable (22). Cells have the appropriate machinery to produce GSH if provided with its precursors. One way to increase GSH levels is to increase intra-cellular glutamate since this promotes ATP production and cystine uptake and alleviates the glutamate-induced inhibition of L-gamma-glutamyl-L-cysteine synthase (HCS) activity (23). Glutamine supports the intra-cellular pool of glutamate, avoiding its depletion and the depletion of GSH; glutamine can be considered a precursor molecule to GSH (24). The importance of glutamine for cell survival and proliferation *in vitro* was first described in detail by Eagle et al. in 1956 (25). A cytoprotective and anti-apoptotic effect of glutamine has been demonstrated in intestinal epithelial cells by Evans et al. (26), and also in pancreatic islets (27). Glutamine has also been shown to enhance islet cell function through increase in β-cell insulin secretion capacity (28–31).

In a rodent study (18), we demonstrated that intra-ductal administration of L-glutamine increases GSH levels in pancreata exposed to 30 min of warm ischemia. We found no difference between glutamine or control groups when there was no injury to the pancreas, but when pancreata were subjected to ischemia glutamine improved viability and islet yields after isolation. Similar results with increased

islet yields were reported in pigs when using intra-ductal glutamine administration before the islet isolation procedure (32).

The purpose of the present study was to evaluate islet isolation and transplantation outcomes after intra-ductal glutamine administration in human pancreata procured from marginal donors. We hypothesize that intra-ductal administration of L-glutamine replenishes intra-cellular GSH levels, consequently protects against oxidative injury and improves human islet isolation and transplantation outcomes by preventing lipid peroxidation and oxidative stress.

We, therefore, conducted a study where a group of human pancreata procured from donors deemed unsuitable for pancreas or islet transplantation were treated with glutamine through the main pancreatic duct, to determine if pancreatic glutamine perfusion improved not only islet recovery and function *in vitro*, but also *in vivo* after transplantation into diabetic athymic nude mice.

Research Design and Methods

Islet isolation and anti-oxidant treatment

Human pancreata refused by other centers for whole pancreas or islet transplantation were obtained from organ procurement organizations (Gift of Hope, Illinois; Gift of Life, Michigan; and Indiana Organ Procurement Organization). Pancreata were obtained using standard organ procurement techniques (pancreas recovered with spleen and duodenum attached) by the liver team responsible and sent on ice to the Cell Isolation Laboratory at the University of Illinois at Chicago (UIC). The pancreata were perfused at the same time of liver and kidney perfusion via abdominal aorta with cold preservation solution. Human pancreata from 12 donors were used. Donor characteristics are summarized in Table 1.

Islets of Langerhans were isolated using the semi-automated method described by Ricordi et al. (33). Pancreas digestion was performed using Liberase-HI (purified collagenase) (Roche, Indianapolis, IN). The enzyme

was dissolved with cold Hanks balanced salt solution (HBSS) for the control group. The enzyme for the treatment group was dissolved in HBSS containing a final concentration of 5 mM L-glutamine. Prior to digestion, pancreata were manually perfused (syringe loaded) through the main pancreatic duct with either the standard HBSS (control, n = 6) or with HBSS + 5mM L-Glutamine (treatment, n = 6), and left at least 15 min before its transfer to the digestion chamber. Pancreata were randomly allocated to treatment or control groups. After digestion, islets were purified in a COBE 2991 cell separator using continuous Ficoll gradients, and purity was assessed using dithizone staining as previously described (8).

Assessment of Isolation Outcomes

In vitro

Islet yield: The outcome of the islet isolation was assessed through the quantification of islet mass by dithizone staining and counts expressed in equivalent islet numbers (EIN). Two independent investigators assessed the quantity and purity of the preparations in accordance with the criteria established at the 1989 International Workshop on Islet Assessment (34).

Viability: Islet viability was assessed by fluorescent staining with Syto-Green/ethidium bromide (35), and the percentage of dead and live cells was estimated in both control and treatment groups as previously described (18). Briefly, double fluorescence was performed to assess the amount of live (green) versus dead (red) islet cells in a representative sample, where a minimum of 100 islets was counted per sample.

Function: *In vitro* islet function was performed by static glucose incubation and expressed in terms of stimulation index (SI) by calculating insulin secretion of islets challenged with Krebs-Ringer bicarbonate buffer (KRBB) (pH 7.35) containing 10 mM HEPES and 0.5% BSA (Sigma) with high glucose concentration (16.7 mM) and dividing it by insulin secretion under low glucose conditions

Table 1: Characteristics of donors

	Glutamine-treated donors	Control (HBSS) donors	p-value
Number	6	6	
Mean age (range)	51 (37–61)	53 (31–75)	p = 0.74
Gender (% female)	50 % Females	33 % Females	p = 0.55
Weight (kg)	90 (80–99)	82 (47–112)	p = 0.45
Mean BMI (kg/m^2) (range)	30 (26–32)	25.3 (18–30)	p = 0.06
Cause of death	50% CNS bleed or stroke, 50% trauma	50% CNS bleed or stroke; 50% trauma	p = 1.0
			p = 1.0
Nonheart beating donors	NONE	NONE	
Cold ischemia time	8.5 h	8 h	p = 0.68
Glycemia (admission)	202.3 (96–353)	172.3 (101–286)	p = 0.55
Maximum glycemia	226.8 (117–290)	276.0 (115–405)	p = 0.42
Insulin dose	22 IU (4–37)	21.1 IU (10–40)	p = 0.83
Donors other organ transplanted:			
Kidneys	(6/6)	(5/6)	
Liver	(2/6)	(2/6)	
Heart	(0/6)	(0/6)	
Lungs	(0/6)	(0/6)	

(1.6 mM) (8). Briefly, groups of five handpicked islets identified under a stereoscopic microscope were placed in five different wells of a 12-well plate, then incubated with 1ml of KRBB-low glucose concentration (1.6 mM glucose final concentration) for 30 min, allowing them to stabilize insulin secretion, and then the supernatant was collected and discarded. Islets were then incubated for 1 h in low glucose KRBB (1.6 mM glucose final concentration) at 37°C and 5% CO_2, and supernatants were collected under a microscope taking care not to remove any islets from the well. The same step was repeated by addition of KRBB-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 human insulin (Mercodia, Uppsala, Sweden).

Oxidative Stress: GSH levels were measured using the monochlorobimane (mcbm) method (36) in islets at 12h post-isolation as previously described (18). Briefly, 1000 EIN were cultured in one well of a 6-well plate in 5 mL CMRL culture medium and 10 µL of mcbm (50 mM) (Molecular Probes; Eugene, OR) and were incubated for 30 min at 37°C. Islets were collected, washed with phosphate buffered saline (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. Durham, NC) 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). MDA is a product of lipid peroxidation. MDA levels were assessed by the thiobarbituric acid method (TBARS) (37). Briefly, a reaction mixture was prepared containing 0.1 M HCl, 0.67 % TBA, 10 % phosphotungstic acid and 7% sodium dodecylsulphate (SDS) (all from Sigma). 1000 EIN were sonicated in 700 µL PBS into a cell lysate. After centrifugation at 15 000 rpm, 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 and 8 mM).

Apoptosis

Immunohistochemical analysis [Detection of free 3-OH strand breaks resulting from DNA degradation by the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL)]: In order to assess the influence of glutamine on islet cell apoptosis, islets from six different pancreata were fixed, sections performed and apoptosis stained (ApopTag Plus Peroxidase In Situ Apoptosis Detection Kit, Chemicon International, USA). Five sections from each pancreas per day (1 and 3) were stained and counted; a minumum of 30 islets per section were randomly counted by field.

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, Indianapolis, IN). Animals were housed and surgeries performed under a laminar flow hood located in 'Barrier' rooms at the Biologic Resources Laboratory, UIC.

Mice were rendered diabetic by a single Intra-peritoneal (IP) injection of Streptozotocin (Sigma) 220 mg/kg body wt. Diabetes was considered after two or more nonfasting blood glucose levels of >300 mg/dL from the tail vein, which generally occurred after a maximum of 36h post-injection. Animals were anesthetized by inhalation with isoflurane using an isoflurane vaporizer with anesthetic system (Viking Medical; Medford Lakes, NJ). This procedure was performed in accordance with the guidelines of the National Institutes of Health and the Animal Care Committee (ACC) at UIC. Islets from 12 different pancreata (control and treatment) were cultured for a maximum of 36 h under standard culture conditions (CMRL-1066 supplemented with 10% human albumin, insulin-transferrin-selenium (ITS)), without L-Glutamine. This was performed while giving time for mice to become diabetic. Islet grafts of 1000 EIN from the control or glutamine-treated groups were transplanted into each mouse under the left kidney capsule using a method previously described (38). In previous experiments, we determined that 1000 human islet equivalents would render normoglycemic less than 50% of diabetic nude mice. Successful transplantation was defined by reduction of glycemia levels to <200 mg/dL after transplantation. After 5–7 weeks post-transplantation, normalized recipients were subjected to nephrectomy in order to remove the islet graft. 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 by IP glucose/arginine stimulation test (IPG/AST) 1 week after transplantation. Briefly, for IPG/AST, a solution containing 2 mg/kg body weight of glucose and 0.3 mg/kg of L-arginine was injected IP into randomly selected euglycemic animals. Blood glucose levels were detected by tail puncture at serial time-points (0, 5, 15, 30, 45 and 60 min).

Statistical methods

Statistical analysis was carried out by Student's *t*-test and Pearson chi-square test. p-values of 0.05 or less were regarded as statistically significant.
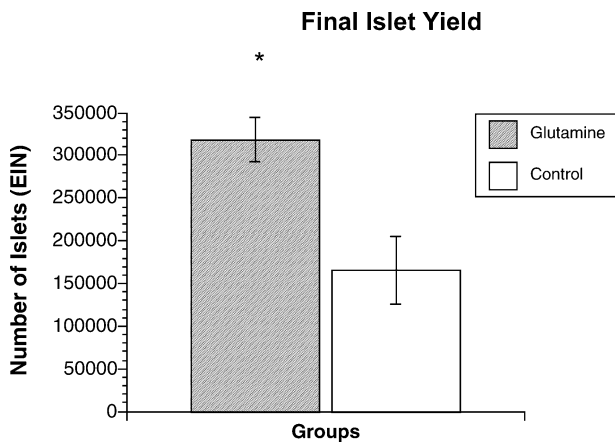
Final Islet Yield

Figure 1: **Final islet yield expressed in EIN, means ± SEM for both groups.** Glutamine n = 6 and control n = 6 human isolations. Differences between groups were considered statistically significant (*p < 0.01, by Student's *t*-test).
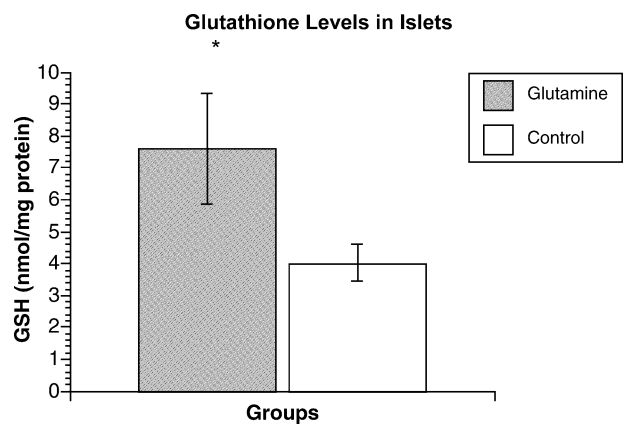
Glutathione Levels in Islets

Figure 2: **Levels of reduced glutathione (GSH) expressed in nmol/mg of total protein for both groups, mean ± SEM.** Glutamine n = 6 and control n = 6 human pancreata. Differences between glutamine treatment and control groups were statistically significant when measured at 24h post-isolation (*p ≤ 0.05, by Student's *t*-test).

Results

Twelve different donor pancreata were used to assess the influence of glutamine perfusion on islet isolation and transplantation outcomes. Donor characteristics in regard to age, weight and cold ischemia time were comparable (Table 1).

Islet isolation results

Islet yields from pancreata perfused with L-glutamine were significantly higher than in control pancreata (Figure 1). The islet purity was 83% ± 3.09 for the glutamine and 71.6% ± 5.02 for the control group (p = 0.097).

Islet viability assessed by Syto-Green/Ethidium Bromide immediately after the isolation procedure was similar between both groups (mean ± SEM: 87% ± 3 for glutamine group vs. 82% ± 4 for the control, p = 0.8. The viability between both groups at 24 h and 36 h after islet isolation showed no statistical difference either (data not shown). Assessment of *in vitro* islet function by static glucose incubations at 12–24 h after islet isolation show similar stimulation indices in both groups (mean ± SEM for S.I., 8.9 ± 2.8 for glutamine group vs. 6.91 ± 1.5 for control, p = 0.2). Stimulation indices at 48 h were also similar for both groups (data not shown).

Assessement of oxidative stress

Glutathione: After the isolation procedure, islets from pancreata treated with intra-ductal glutamine had higher GSH levels than the control islets (Figure 2).

Malondialdehyde (MDA): Islet levels of MDA were measured as a marker of lipid peroxidation (oxidative injury) at 24 h post-isolation. MDA levels were found to be significantly lower in the glutamine group (Figure 3).
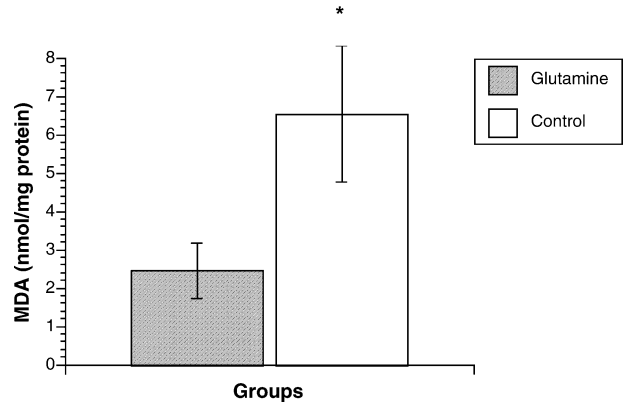
MDA levels in Human Islets

Figure 3: **Levels of malondialdehyde (MDA) expressed in nmol/mg of total protein for both groups, mean ± SEM.** Glutamine n = 6 and control n = 6 human pancreata. Islets from glutamine-treated pancreata were found to have lower amounts of MDA than islets from the control group. Differences between glutamine treatment and control groups were statistically significant when measured at 24h post-isolation (*p ≤ 0.05, by Student's *t*-test).

Identification of apoptosis by TUNEL staining: Human islets from glutamine-treated pancreata showed less apoptotic cells per islet than the control group at day 1 and day 3 post-isolation:0.63 ± 0.04 apoptotic cells per islet for glutamine at day 1 vs. 1.88 ± 0.14 apoptotic cells per islet for control, p ≤ 0.01 (Figures 4A–D); and 0.44 ± 0.02 apoptotic cells per islet at day 3 for glutamine vs. 1.82 ± 0.14 for control, p ≤ 0.01 (Figures 5A–D).

In vivo *islet function after transplantation into diabetic nude mice*: Islet function *in vivo* was assessed through transplantation of glutamine-treated and control islets into
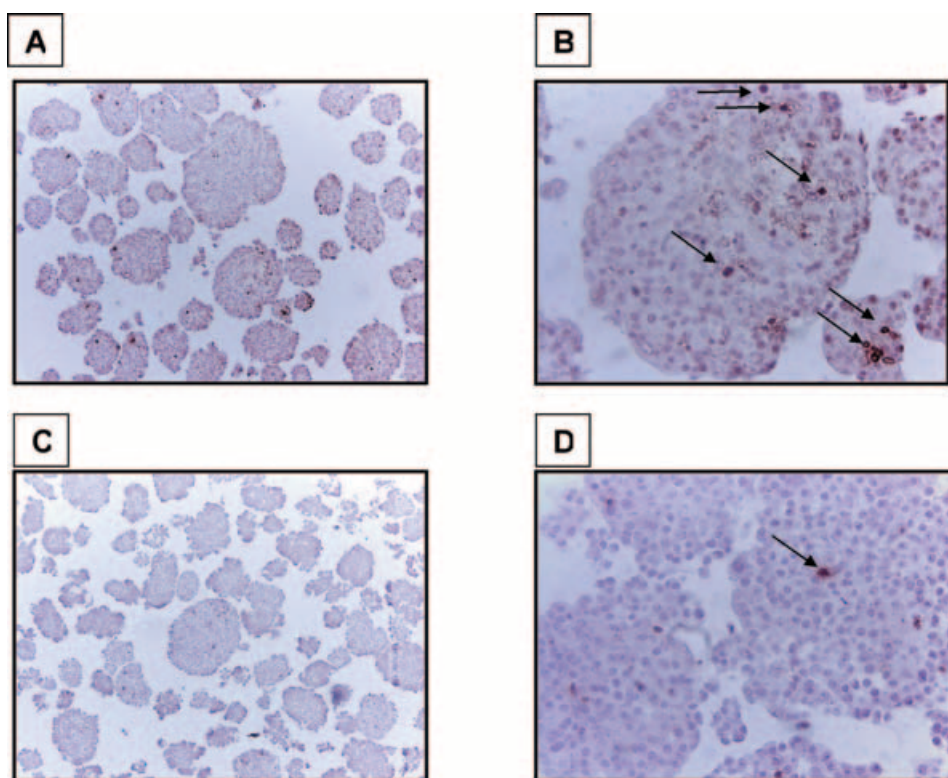
Figure 4: Day 1 post-isolation: (A) Control islet section at 10× magnification showing dark-brown dots corresponding to apoptotic islet cells. (B) Control islet section at 40× magnification showing apoptotic islet cells (black arrows). (C) Glutamine-treated islet section at 10× magnification showing fewer dark-brown dots, corresponding to apoptotic islet cells. (D) Glutamine-treated islet section at 40× magnification showing fewer apoptotic islet cells (black arrow).

streptozotocin-induced, diabetic, athymic nude mice. We found a higher effectiveness of glutamine-treated islets to render normoglycemic diabetic nude mice than the control islets. Reversal of diabetes was achieved more frequently and the lag period to reach normoglycemia was shorter in animals transplanted with islets from the glutamine group (Figures 6 and 7). Mean glycemia in the mice transplanted with islets from the glutamine-treated pancreata was significantly lower than in the mice transplanted with the control group (Table 2). IP glucose/arginine stimulation test was performed in randomly selected animals reaching normoglycemia from both groups. Mice transplanted with islets from pancreata treated with glutamine exhibited a normal response to the test when compared to the mice transplanted with control islets, represented by a significantly smaller area under the curve for the glutamine group than the control, mean ± SEM (7153 ± 381 vs. 12360 ± 1587, respectively), $p = 0.03$ (Figure 8).

Discussion

The pre-requisite for successful islet transplantation is a large number of viable and functional pancreatic islets, ideally originating from a single donor. The organ donation, islet isolation and transplantation procedures expose pancreatic cells to various degrees of oxidative stress, compromising islet yields and cell viability and function. Accumulating evidence indicates that GSH has a central role in redox regulation (39). Our study indicates that intra-cellular GSH

levels can be increased by the simple means of intra-ductal glutamine administration prior to the isolation procedure. Consequently, we found lower MDA levels in islets isolated from glutamine-pre-treated pancreata, indicating less oxidative damage. Although we could not detect any differences in cell viability and islet function *in vitro*, the islets isolated from glutamine-pre-treated pancreata performed significantly better than the controls after transplantation in diabetic nude mice.

The fact that islet viability *in vitro* did not differ between both groups, at least at 0, 24 and 48 h following the isolation, suggests that the changes that occur at these times between the treated and nontreated islets are subtle and not detected by the vital stain combination of syto-green and ethidium bromide used. Nevertheless, islets from glutamine treated pancreata reversed diabetes not only in a larger amount of mice, but also with a shorter lag period than the control group. The improved outcome in terms of percentage of mice achieving normoglycemia may be related to higher islet cell survival after transplantation consequent to higher intra-cellular GSH levels and reduced oxidative pre-damage after the isolation procedure. This is supported by a fewer amount of apoptotic cells per islet observed in the glutamine treated islets than in the control. The longer lag period to reach normoglycemia and the diabetic response to the IPG/AST observed in the control group may in addition be the consequence of lower insulin secretory capacity induced by oxidative stress (40). We suggest that glutamine increases survival and β-cell mass
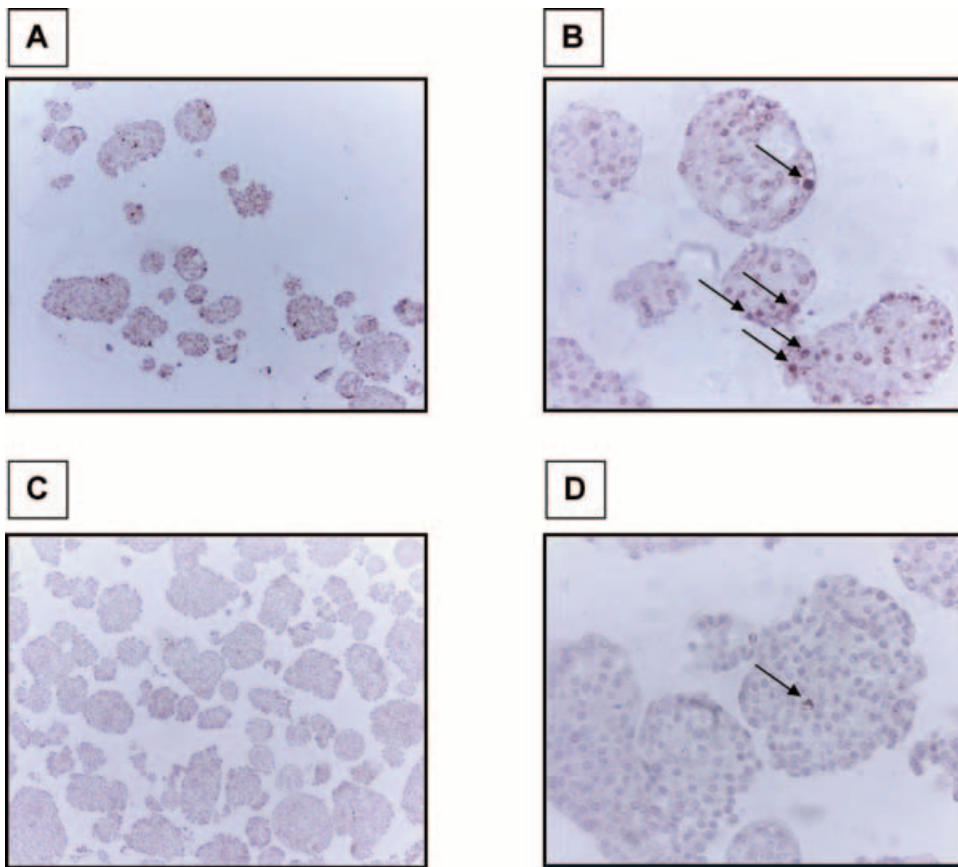
Figure 5: Day 3 post-isolation: (A) Control islet section at 10× magnification showing dark-brown dots corresponding to apoptotic islet cells. (B) Control islet section at 40× magnification showing apoptotic islet cells (black arrows). (C) Glutamine-treated islet section at 10× magnification showing fewer dark-brown dots, corresponding to apoptotic islet cells. (D) Glutamine-treated islet section at 40× magnification showing fewer apoptotic islet cells (black arrow).

by protecting islets from oxidative injury, which would ultimately lead to apoptosis and cell death. This finding correlates with previous studies observing a reduction in apoptotic cell death in pancreatic islets in the presence of glutamine (27,41). Studies by Evans et al. (26,42) suggest an anti-apoptotic protection of cells distinct of GSH, however, glutamine may have additional effects which our experiments and current literature reports have not addressed so far and therefore remain speculative.

A number of studies have investigated anti-oxidative and cytoprotective measurements to prevent islet injury during pancreatic islet isolation and transplantation. Bottino et al. demonstrated that islet isolation outcomes can be improved by the catalytic anti-oxidant probe AEOL10150 (manganese [III] 5,10,15,20-tetrakis (1,3,-diethyl-2imidazoyl)-manganese-porphyrin pentachloride [TDE-2,5-IP]) and observed reduced Nfκ-B binding to DNA, reduced release of cytokines and chemokines and reduced Poly(ADP-ribose) polymerase (PARP) activation in islet cells, resulting in higher survival and better insulin release. Other groups have demonstrated that intra-ductal administration of enzymes with preservation solutions improves islet yield and ischemic tolerance of isolated rodent and porcine islets assessed by functional success *in vitro* and *in vivo* (43–45).
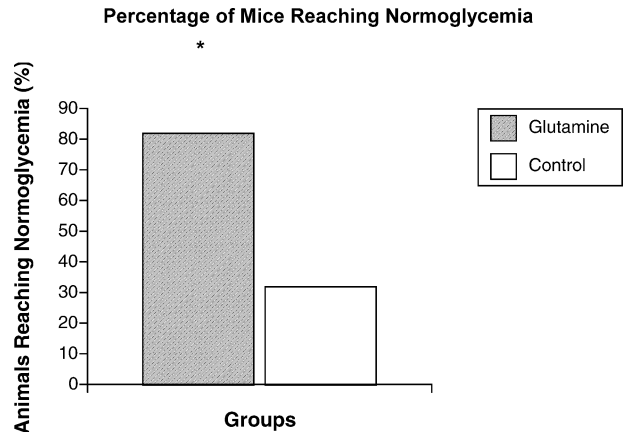

Figure 6: Number of mice reaching normoglycemia after transplantation of islets from both groups. Glutamine n = 10/12 mice and control n = 6/23 mice. Differences between groups were statistically significant (*p < 0.01, by Pearson chi-square test).

Others have successfully protected islets from oxidative injury by viral transduction to upregulate anti-oxidant and anti-apoptotic genes (46–48). However, the applicability of viral transduction in clinical islet transplantation is currently limited. Our study is unique in that the anti-oxidant
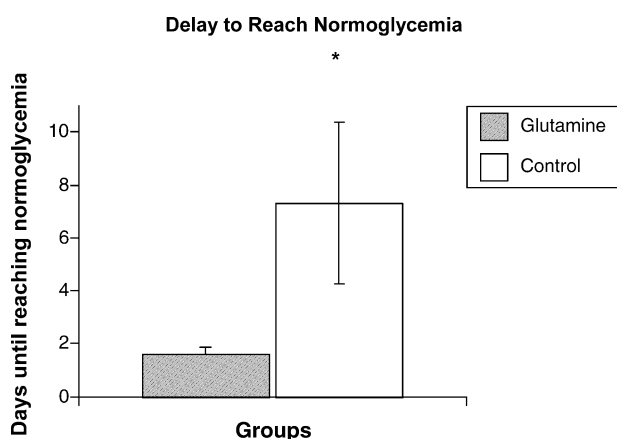
Delay to Reach Normoglycemia

Figure 7: Number of days (lag time) to reach normal glycemia values in cured mice after transplantation of islets from both groups. Glutamine n = 10 mice and control n = 6 mice. Differences between groups were statistically significant (*p < 0.01, by Student's t-test).

Table 2: Blood glucose in mice during the first 5 weeks after transplantation

Weeks	Glutamine Glycemia (mg/dL)	Control Glycemia (mg/dL)	p-value
0	351.58 ± 26.44	422.7 ± 15.53	0.10
1	146.09 ± 23.68*	300.9 ± 27.26	<0.01
2	151.38 ± 38.76*	319.9 ± 32.37	<0.01
3	137.79 ± 29.07*	343 ± 39.25	<0.01
4	140.93 ± 33.47*	333.2 ± 39.25	<0.01
5	140.26 ± 31.87*	322.2 ± 40.08	<0.01

Results of weekly blood glucose values (mg/dL) of mice transplanted with islets from both groups (glutamine and control). Values are means ± SEM. p-values calculated by Student's t-test. *p < 0.01.

strategy used could be easily applied in clinical practice, as glutamine is a nontoxic, simple amino-acid.

We conclude from the work presented that early oxidative events contribute to loss in islet mass and function, which can be prevented by the use of anti-oxidants. Glutamine exerts anti-oxidant properties by replenishing the pools of GSH in islets and reducing lipid peroxidation (MDA) and apoptosis. Intra-ductal injection of glutamine is a simple and clinical applicable means to improve human islet isolation and transplantation outcomes.

Acknowledgments

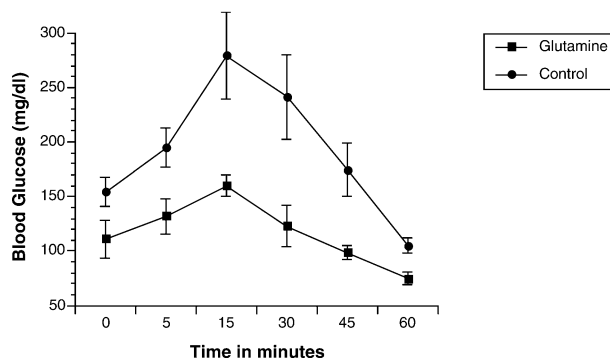## Intraperitoneal Glucose/Arginine Stimulation Test

Figure 8: Intra-peritoneal glucose/arginine stimulation test (IPG/AST) in mice that reached normoglycemia transplanted with islets corresponding to glutamine (n = 4) and control (n = 4) groups.

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