Randomized, double-blinded pilot study: Nitrite Infusion in Islet Cell Transplantation: Test of Clinical Efficacy and Determination of Cytoprotective Mechanisms

Study Protocol & Statistical Analysis Plan

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Randomized, double-blinded pilot study: Nitrite Infusion in Islet Cell Transplantation: Test of Clinical Efficacy and Determination of Cytoprotective Mechanisms (8/9/16)

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1.0 OBJECTIVES

Diabetes is a major health care problem in our country and contributes to the development of severe co-morbidities, including coronary artery disease, heart failure, stroke, renal failure and many others. Whereas the etiology of diabetes is multifactorial, patients with chronic pancreatitis or recurrent acute pancreatitis may acquire insulin-dependent diabetes due to islet cell destruction. Islet cell autotransplantation is the optimal therapeutic approach for many of these patients.

Islet cell autotransplantation is typically done by excision of the pancreas, followed by isolation of the islet cells and then infusion of these cells into the sinusoids of the liver. Isolation of the islet cells occurs in an ischemic and hypoxic environment, resulting in ischemia reperfusion (IR) injury and destruction of islet cells following infusion into the sinusoids. Hence, strategies to prevent IR injury and subsequent islet cell destruction, such as the administration of inhaled nitric oxide (NO) or sodium nitrite ($NaNO_2$), could enhance islet cell survival following reperfusion, thereby decreasing long-term insulin requirement.

The anion nitrite (NO_2^-) releases NO in biological systems and has been demonstrated to inhibit IR injury (REFS) and improve outcomes (REFS) in patients undergoing Liver transplantation. The objective of this study is to determine whether the NO donor, nitrite will prevent I/R injury and reduce long-term insulin requirements in patients receiving islet cell autotransplantation.

The **central hypothesis** is that in patients undergoing islet cell autotransplantation, infusion of nitrite will inhibit islet cell destruction. The hypotheses of the specific aims are that nitrite will:

1) Increase diabetes cure rate - decrease the amount of and/or the need for long-term insulin requirement.
2) Decrease the ischemic injury during islet cell procurement,
3) Decrease IR injury following islet cell infusion, and

The purpose of this study is to act as a preliminary or "pilot" study. If the data obtained affirm the central hypotheses, further investigations will be conducted to determine mechanism and clinical efficacy.

2.0 BACKGROUND & RATIONALE

2.1 Scope of the Public Health Problem

Diabetes is a significant medical problem for patients, as it can lead to multiple other medical problems i.e. Coronary Artery Disease, Vascular Disease, and Kidney Disease. National statistics indicate that 25.8 million people have diabetes in the US. Approximately 18 million people have been diagnosed with an additional 7 million people yet to be diagnosed. Some 215, 000 people under the age of 20 have the disease. Among people over the age of 20, about 1.9 million were diagnosed in 2010. After the age of 65, 26.9% of people have diabetes. The estimated costs in 2007 were $174 Billion dollars with $58 Billion dollars being indirect costs i.e. disability, work loss, and premature mortality.

In type 2 diabetes, the body either doesn't produce enough insulin or is unable to utilize it. Without enough insulin, cells are unable to use glucose for energy production. Over time the high blood glucose levels cause microvascular and/or macrovascular damage. Type 2 diabetes complications include cardiovascular disease, cerebrovascular disease, kidney failure, retinopathy, and neuropathy. Diabetes is the leading cause of kidney failure, non-traumatic limb amputation, and new onset blindness in adults in the U.S. Diabetes is the seventh leading cause of death in the U.S. The risk of death is twice the non-diabetic patient at the same age.

In patients with chronic pancreatitis or recurrent acute pancreatitis, Islet Cell transplantation may lead to a cure of their Diabetes related to the resection of the Pancreas. In patients undergoing Islet Cell Auto Transplantation, the removal of the pancreas and the retrieval of the islet cells produces a hypoxic environment. This process leads to Ischemic Reperfusion (IR) injury of the islet cells. Hence, strategies to prevent IR injury, including inhaled Nitric Oxide (NO) and Sodium Nitrite ($NaNO_2$) are being tested. For example, replenishing NO via therapeutic administration may serve to abrogate reperfusion injury in these vulnerable patients.

2.2

Chronic pancreatitis is the progressive and irreversible destruction of the pancreas characterized by inflammation and fibrosis that initially results in exocrine deficiency, but can progress to endocrine dysfunction. This disease often follows a chronic relapsing pattern with patients experiencing intermittent anorexia, nausea, vomiting, and abdominal pain. As the disease becomes severe, many patients become malnourished and develop debilitating chronic abdominal pain.[1,2,3] The most common etiology of chronic pancreatitis in Western society is alcohol consumption, comprising 70% to 90% of cases.[4] Other causes include biliary obstruction, pancreatic division, trauma, hypertriglyceridemia, hereditary, idiopathic, and autoimmune pancreatitis.[5]

Total or completion pancreatectomy is the surgical treatment for patients with small duct disease, diffuse glandular involvement, or recurrent pain after a previous operation for chronic pancreatitis.[6] Due to the resulting apancreatic state, patients undergoing total pancreatectomy are rendered insulin dependent diabetic. Unlike Type I insulin dependent diabetics, apancreatic patients are deficient in insulin, glucagon, and pancreatic polypeptide. Peripheral insulin receptors are upregulated, enhancing insulin sensitivity[7] while the absence of glucagon causes patients to be prone to wide variations in serum glucose levels.[8,9] Pancreatic polypeptide deficiency may contribute to difficulty in glycemic control by increasing hepatic resistance to insulin. This difficult to control and often unstable metabolic state is commonly referred to as "brittle diabetes".[10,11]

Pancreatic islet cell autotransplantation at the time of total or completion pancreatectomy can be effective in preventing or minimizing pancreatogenic diabetes.[12,17] Sutherland et al. performed the first successful total pancreatectomy with islet cell autotransplantation in 1977.[13] Since that time, techniques have been refined and technology improved. Experience at the University of Minnesota demonstrated a rate of insulin independence of 71% after autotransplant in those

without a previous pancreatic or abdominal operation. Only 18%-20% of patients who had undergone a previous Puestow drainage or distal pancreatectomy were insulin independent.[14] Islet yield is a strong predictor of postoperative insulin independence, with best results obtained when greater than 300,000 islet cells[13] or >2500 Islet cells/kg[14] are transplanted. Timing of islet cell autotransplant becomes a factor since 3 of 4 patients with chronic pancreatitis will develop diabetes in less than 6 years after developing the disorder.[15] Some authors have recommended earlier total pancreatectomy with islet cell autotransplant for causes of pancreatitis including hereditary pancreatitis[16] which may increase islet cell yield and rate of postoperative insulin independence.

2.3 Nitrite Therapy: Novel Approaches for Treatment for Ischemic Diseases

Until recently, nitrite was thought to have minimal biological activity *in vivo*. However, it is now known that nitrite undergoes reductive conversion to NO by a number of mechanisms including the enzymatic actions of xanthine oxidoreductase, non-enzymatic disproportionation, and a hemoglobin reductase activity that is under allosteric control. These mechanisms of nitrite reduction favor bioconversion of nitrite to NO under the hypoxic and acidic conditions present during ischemia and have led to a series of experimental studies demonstrating that therapeutic administration of low dose nitrite in animal models of ischemia and reperfusion result in dramatic cytoprotection in the liver, heart, lungs, brain and kidney.

The mechanism of nitrite cytoprotection in ischemic diseases is not fully known but does depend on the release of NO. For example, there is a loss of cytoprotection when animals were treated with the NO scavenger carboxy-PTIO, suggesting the importance of NO in the mechanism of cytoprotection. Pretreatment of animals with an NO synthase inhibitor or use of eNOS knockout mice [42] did not inhibit cytoprotection, proving that the nitrite effect is NO synthase-independent. Reactive oxygen species (ROS) generation by the mitochondria has been determined to be a necessary component of mitochondrial signaling in cytoprotection. However, the large burst of oxidizing reactive oxygen species generated after reperfusion following ischemia can also contribute to cellular injury, necrosis and apoptosis. S-nitrosation of complex I of the electron transport chain by S-nitroso glutathione inhibits the activity of this complex and will decrease mitochondria-derived reactive oxygen species formation during reperfusion, an effect associated with cellular cytoprotection. Nitrite can similarly nitrosate complex I during ischemia and reperfusion. This modification limits complex I dependent reperfusion ROS formation, activation of the mitochondrial permeability transition pore, and cytochrome C release.

Recent studies indicate that circulating nitrite is the transducer of protective effects of inhaled nitric oxide in human liver transplantation in which therapy improved the rate of recovery of liver function post-transplantation and decreased hospital length of stays. Inhaled NO (iNO) was approved by the U.S. Food and Drug Administration in December, 1999, for the treatment of persistent pulmonary hypertension of the newborn. Over the last decade, the primary advantage of iNO was seen to be its ability to selectively decrease pulmonary vascular resistance with minimal effects on systemic blood pressure; however, there is currently much interest in exploring its other benefits, including its antioxidant properties and cytoprotective capabilities

(22). In animal studies, iNO decreased infarct size and left ventricular dysfunction after ischemia-reperfusion injury, increased coronary artery patency after thrombosis, increased blood flow in brain, kidney and peripheral vasculature, decreased leukocyte adhesion in bowel during ischemia reperfusion, and decreased platelet aggregation (23). Date *et al* reported the use of iNO in 15 out of 32 patients who suffered from immediate severe allograft dysfunction with iNO at 20-60 ppm following lung transplantation. The mortality was significantly lower in the iNO group (7% and 24%, respectively). The gross benefits reported were that iNO improves oxygenation, decreases pulmonary artery pressure, shortens the period of postoperative mechanical ventilation, and reduces airway complications and mortality (24). Likewise, a recent retrospective study also reported an improvement of overall respiratory functions. The authors encouraged the administration of iNO for the prevention and treatment of early graft failure in lung transplant recipients (25). Varadarajan *et al* were the first group to study the relationship between NO metabolism and IRI in human liver transplantation (26). From their study, they concluded that reduced eNOS expression contributed to IRI one hour after portal reperfusion. On the other hand, iNOS did not contribute to early IRI after human liver transplantation. Clinical and mechanistic reports on therapeutic use of iNO have demonstrated improved NO bioavailability in extrapulmonary tissues specifically in the setting of ischemic stress although how these effects are mediated remains unclear. Specifically, iNO improved human forearm blood flow in tourniquet induced ischemia and protects against IRI in a feline model of mesenteric ischemia. The general consensus is that iNO increases the circulating levels of a stable NO-donor and includes *S*-nitrosothiol (SNOs) and nitrite. We recently conducted a double-blinded randomized controlled trial whereby, 80 ppm of iNO was administered to patients undergoing orthotropic liver transplantation (16). Many advantages were reported in the iNO group, including reduced platelet transfusion, an improvement in the rate at which liver function was restored post-transplantation, and a decrease in the length of hospital stay. Most interesting was the finding of an approximated 75% reduction of hepatocellular apoptosis in patients treated with iNO. Moreover, of the possible biochemical intermediates formed from iNO in the plasma (nitrate, nitrite, S-nitrosothiols, C- or N-nitroso compounds), the most likely candidate transducer of iNO on liver IRI was concluded to be nitrite.

Webb, A., et al showed that nitric oxide derived from the reduction of nitrite protected against myocardial ischemia/reperfusion damage. Duranski et al. showed cytoprotective effects of nitrite in heart and liver. Jung et al showed protection of the brain in ischemia-reperfusion injury in the brain. These findings suggest an opportunity for nitrite therapy for human diseases associated with ischemia-reperfusion. The goal of this application is to test nitrite therapy in human islet cell autotransplantation and to test the hypothesis (related to the bedside component of this funding mechanism) that nitrite will protect the transplanted islet cells from ischemic injury during procurement and reperfusion injury following islet cell infusion into the liver sinusoids. Furthermore, if the data support the central hypothesis, nitrite infusion may lead to marked reduction in the amount of long term insulin requirement and perhaps, a higher rate of cure.

3.0 ELIGIBILITY CRITERIA

All patients will be enrolled into the study at the University of Alabama at Birmingham Medical Center (UAB). Approximately 18 islet cell autotransplantations are conducted at UAB annually by one surgeon, thereby eliminating the need to include surgical technique as a possible confounding variable.

Based on our prior experience conducting similar studies, we anticipate that approximately 80% of patients undergoing islet cell autotransplantation meet enrollment criteria and agree to the written informed consent. Accordingly, we conservatively estimate that it will take approximately two years to complete enrollment for the study, followed by a third year for data analysis and study closeout.

3.1 Inclusion criteria

The study sample will include 30 adult (≥ 19 years of age) patients of either gender with acquired insulin dependent diabetes mellitus who provide informed written consent and:

- Patients admitted to UAB for islet cell autotransplantation
- >18 years old

3.2 Exclusion Criteria

Any patient with liver disease or unsuitable for surgery (as determined by surgeon).

4.0 RECRUITMENT PROCEDURES

4.1 Recruitment

Thirty (30) patients will be enrolled – 10 patients in each arm of the study. Patients will be screened and recruited by the patient coordinator in Dr. Christein's clinic. The coordinator will confirm eligibility and absence of any exclusionary criteria. Details of the study (including risks) will be explained to prospective participants and an informed, signed consent form will be obtained from participants.

4.2 Randomization Procedure

This is a single institution, blinded, placebo-controlled clinical trial. Patients will be randomized to one of three (3) arms of the study by the research pharmacist:

- Control group (saline infusion will be administered during the pre-isolation and post-isolation phases)

- Pre-isolation infusion (nitrite infusion will be administered during the pre-isolation phase and saline will be administered during the post-isolation phase)

- Post-isolation (saline will be administered during the pre-isolation phase and nitrite infusion will be ad mistered during the reperfusion phase)

Continuous infusion of 32 mg sodium nitrite in 100 mL normal saline at a rate of 10 mL per min over for the duration of the surgery, which are calculated to achieve steady-state plasma concentrations of approximately 10 μM, concentrations shown to have therapeutic efficacy in animal models of IRI.

5.0 EXPERIMENTAL PROTOCOL

Protocol Design:

This is a single institution, randomized, placebo-controlled clinical study. There will be 3 cohorts: control group, pre-isolation nitrite infusion, and a post-isolation (reinfusion) cohort.

After induction of general anesthesia with propofol (1-1.5 mg/Kg), tracheal intubation, the establishment of central venous access, invasive monitors and maintenance anesthesia with sevoflurane (1-1.5 minimum alveolar concentration) in 50:50 oxygen: air, infusions will be administered based on the randomization assignment.

I. Control Group A placebo (saline infusion) will be administered during the pre-isolation phase and during the reinfusion phase.

II. Pre-Isolation Infusion Group

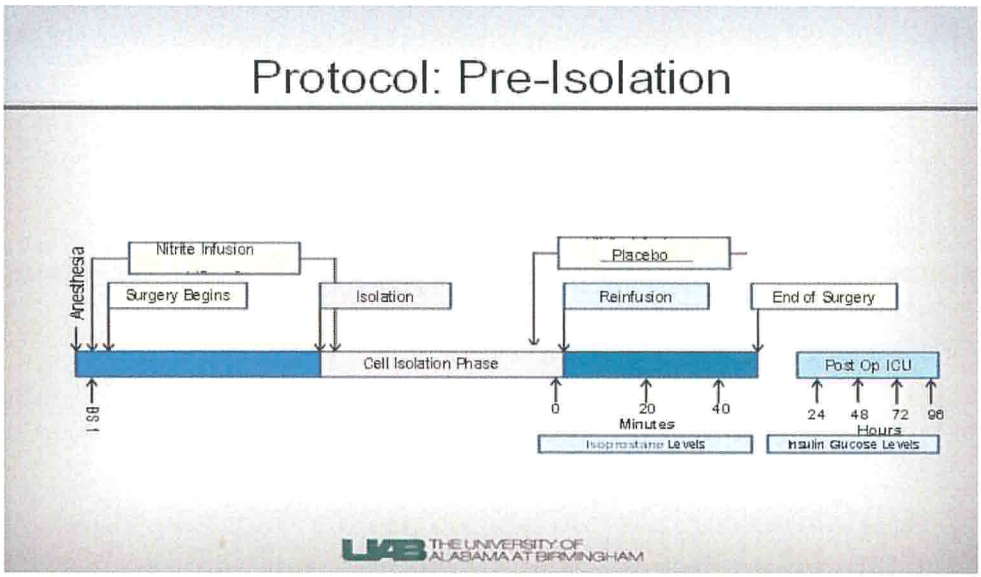

Figure 1: Pre-isolation group schematic: Sodium nitrite will be initiated (pre-isolation infusion group) during the pre-isolation phase. Saline will be administered during the reperfusion period.

III. Post-Isolation (Reinfusion) Group

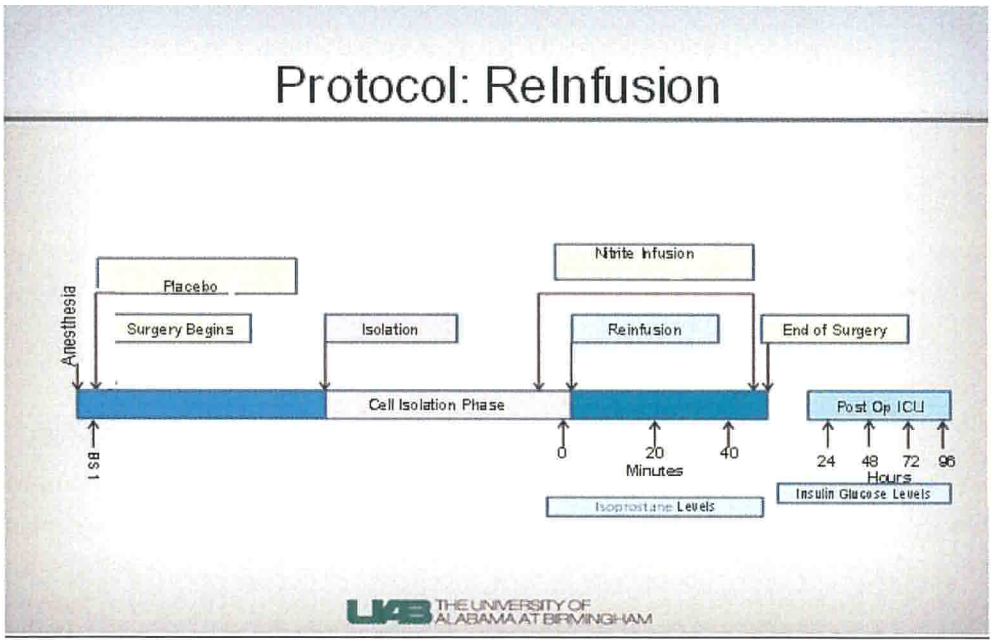

Figure 2: Reinfusion group schematic: Saline will be administered during the pre-isolation period. Sodium nitrite infusion will be initiated just prior to the reinfusion.

For all 3 groups:

Intraoperative arterial and venous blood samples (BS) will be drawn immediately prior to initiation of the infusion (BS1), 5 min prior to infusion of Islet Cells (BS2), 20 min after infusion of Islet Cells (BS3), and 40 min after infusion of Islet Cells (BS4).

Postoperative blood samples will be obtained 24, 48, 72 and 96 hours after the completion of surgery.

Results of routine lab work performed at the routine 3 month follow-up visit will be collected.

Procedure for Sample collection (email from Rakesh Patel 6/28/16) – to be performed by lab tech in BMR

1) *Transferring blood to Eppendorf tubes,*
2) *Centrifuging (using a table top min-fuge which we can provide),*
3) *Transferring plasma to 4 other vials (1 containing a pre-made solution, which we can provide) and flash freezing all vials in liquid N2*
4) *Taking some of the red cell pellet and adding to vials (containing pre-made solution that we can provide) and flash freezing all vials in liquid N2*
5) *All samples need to be stored at -80C.*

Primary Endpoints:

- Markers of pancreatic function:
    - Blood glucose levels
    - HbA1c levels pre-operatively and at the 3 month post-operative visit
    - Exogenous insulin use
        - Measured from blood samples 24, 48, 72, 96 hours post op & at 3 months post op
- Decrease in reperfusion injury
- Incidence of diabetes post-operatively

Intraoperative blood samples will be analyzed for serum and methemoglobin levels and biochemical markers of oxidative and nitrosative stress, including plasma and hepatic 3-nitrotyrosine, oxidized lipids (isoprostanes, 8-epi-PGF2α), protein carbonyls and the NO-metabolites, nitrite, nitrate, S-nitrosothiols, C-N-nitroso compounds and heme nitrosyls. If the above processing is not part of the costs, and assuming 30 patients, each with 6 blood draws then running assays for plasma and red cell NOx, C-peptide levels, circulating pro-inflammatory chemokines/cytokines and perform immunophenotyping of monocytes and neutrophils by flow cytometry and mRNA accumulation.

	Sample Name	Blood Glucose	HbA1c	Insulin level (exogenous)	RBC NOX	C-peptide	Circ pro-inflam chemokines/ cytokines	Immunophen monocytes and neutrophils	mRNA accum			
Intraoperative												
Prior to infusion	BSI	X	X			X	X	X	X			
5 min prior to Islet cell infusion	BS2	X										
20 mins after infusion of Islet cells	BS3	X										
40 mins after infusion of Islet cells	BS4	X										
Post-op												
24 hours	BS5	X		X		X	X	X	X			
48 hours	BS6	X		X		X	X	X	X			
72 hours	BS7	X		X		X	X	X	X			
96 hours	BS8	X		X		X	X	X	X			
Follow up												
Clinic visit 3 months post-op	BS9	X	X	X		X	X	X	X			

6.0 EXPERIMENTAL TECHNIQUES

Not applicable

7.0 STATISTICAL CONSIDERATIONS

Primary Study Variables include: Demographics (age, gender, pre-operative weight, pre-operative BMI,

General Data Analysis: All anthropometric data will be compared using unpaired Student's t-test. Length of stay data will be compared using Cox proportional hazard

analysis. All other data will be compared using one-way repeated measures ANOVA with post-hoc analysis by unpaired Student's t-test or Chi Square analysis as appropriated. For all comparisons, a value of $p< 0.05$ will be considered significant.

This is a pilot study. We plan to enroll 10 patients in each of the 3 arms of this study (total N=30).

Study Population Availability: Approximately 8-10 patients present to UAB for this procedure annually. Potential study subjects will be identified by Dr. Christein and his clinic team.

8.0 SAFETY MONITORING

The intraoperative anesthetic and postoperative intensive care management of these patients will be the responsibility of the anesthesiologist or critical care physician, respectively.

Possible Adverse Events: Primary clinical concerns include the formation of methemoglobin and inhibition of platelet function. Phase I studies of sodium nitrite infusion have been completed in more than 80 normal human volunteers at the doses planned for this trial with no observed toxicity (e.g., no clinically significant increases in methemoglobin or hypotension). Nevertheless, plasma methemoglobin will be measured throughout the intraoperative and immediate postoperative periods. The study drug will be discontinued should methemoglobin levels increase to 5% or greater. Parameters related to coagulation and bleeding will be monitored per standard of care. Adverse coagulation or excessive bleeding will lead to immediate cessation of study drug. Based on our prior experience with inhaled NO **(Lang REF)**, we do not anticipate increased bleeding relative to placebo and anticipate that metHb levels will remain <5% for the duration of nitrite administration

Assessment of Level of Risk: Very low (numerous studies have established safety of nitrite infusion).

Oversight of this investigation will be provided by the PI, surgeon, and co-investigators. They will be responsible for all aspects of the conduct of the trial.

Safety oversight will be provided by the Data and Safety Monitoring Board (DSMB).

9.0 Adverse Events Reporting: This study poses minimal risk.

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