CALIFORNIA STATE UNIVERSITY, NORTHRIDGE

Effect of Human Progenitor Cells on an Animal Model of Type 1 Diabetes

A thesis submitted in partial fulfillment of the requirements

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Master of Science in Biology

By

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The thesis of Angelica Cruz is approved:

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Table of Contents

List of Tables

List of Figures

Abstract

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This project focuses on a novel progenitor cell therapy for type 1 diabetes, a chronic illness that is categorized by pancreatic beta cell death. The current advancements in diabetes research has brought attention to innovative cellular applications that can help increase insulin production. Here, human progenitor cells were used to mimic and replace beta cells in a diabetic rat model induced by a one-time intraperitoneal injection of streptozotocin that selectively damages beta cells. The effects of inducing diabetes were examined by testing blood glucose levels daily using Accu-Chek glucometers. After establishing diabetes in the rats (>400mg/dL glucose), preparation for transplantation began by immunosuppressing the diabetic rat using Alzet pumps that chronically administers cyclosporine over the course of 23 days. During this time, blood glucose and weight were monitored to ensure the rat's viability for successful transplant surgery and post-surgery outcomes. Next, live human progenitor cells, live differentiated pancreatic progenitor cells and dead human progenitor cells (control) were injected into different regions of the diabetic rat: the circulatory system, peritoneal cavity, pancreas, and kidney capsule. The last two treatments require an additional surgery of exposing either the pancreas or kidney capsule in order to transplant the human progenitor cells. Finally, immunohistochemistry of the rat's organs showed the presence of surviving human progenitor cells in the kidney capsule 37 days post-transplantation. One day after cell treatment, pancreatic progenitor cell treatment group via kidney capsule had significant decrease in blood glucose levels ($p<0.005$) compared to dead progenitor cells injected into the kidney capsule blood glucose levels. This experiment aimed to alleviate pancreatic beta cell death and possibly be one step closer to curing type 1 diabetes.

Introduction

According to the American Diabetes Association, type 1 diabetes affects 1.25 million Americans (American Diabetes Association, 2017). This disease is caused by an autoimmune reaction that leads to death of pancreatic beta cells responsible for insulin production in reaction to high glucose levels in the blood. Once these cells are destroyed they cannot be replaced within the pancreas; the body becomes stressed with little to no insulin to regulate glucose homeostasis. This problem eventually can lead to insulin dependence and constant monitoring of blood glucose levels in diabetic patients. Once a patient is diagnosed with type 1 diabetes, insulin treatment is a lifelong encumbrance.

Unfortunately, while insulin injections via syringe or pump can extend the lifespan of patients, there is no cure for type 1 diabetes. Thus, one current focus of regenerative medicine is to utilize functional insulin-producing cells to replace the missing beta cells. However, since regeneration of the patient's own beta cells is not possible, research has focused on beta cell replacement therapy using either islet tissue transplantation or human stem cells. (Baranovski et al., 2016; Szot et. al., 2015). Beta cell transplantation often results in multiple surgeries since the body's immune system will continue to damage the healthy replacement beta cells (Efrat, 2008). The major drawbacks from direct islet beta cell transplantation are the limited amount of beta cells that are available, the non-regenerative properties of islet beta cells result in multiple surgeries over a lifetime, and the lack of beta cell manipulation to serve each specific patient's needs (Efrat, 2008). Although direct cell or pancreas transplantation have been shown to be somewhat effective in clinical trials, most often islet cell and pancreas organ donors are only able to donate to one patient which reduces the chance for diabetic patients to receive multiple islet cell or

pancreas transplantations from that same donor (Sutherland et. al., 2004). Direct islet beta cell transplantation has also failed to fulfill the malleability and regenerative properties that are found in stem cells because islet cells are a non-renewable source (Baranovski et al., 2016).

Human stem cells (hSCs) have been shown to be effective in beta cell regeneration and protection from streptozotocin (STZ) induced diabetes in animal models (Kroon et al., 2008). In addition, hSCs can express specific cell markers via genetic modifications enabling them to differentiate into beta cells (Szot et al., 2015; Alipio et al., 2010; Rezania et al., 2012). Although this stem cell replacement approach has many challenges, the reversal of diabetes has been demonstrated in many animal models. One study transplanted human embryonic stem cells that were derived into pancreatic progenitor cells into the kidney capsule of diabetic rats and mice which resulted in functional pancreatic endoderm cells that had similar features of human fetal pancreas development (Renzania et al., 2012). Another study generated functional human pancreatic beta cells *in vitro* that were derived from human pluripotent stem cells (Pagliuca et al., 2014). This study observed that these human pancreatic progenitor cells successfully differentiated into insulin secreting cells that maintain similar functions as normal beta cells when transplanted under the kidney capsule in immunodeficient mice (Pagliuca et al., 2014). Additionally, human embryonic stem cell derived pancreatic endoderm was surgically implanted into the abdominal cavity as well as into the kidney capsule in diabetic mice, reversing STZ-induced hyperglycemia (Kroon et al., 2008). Finally, intrapancreatic injection of human bone marrow derived mesenchymal stem cells helped reduce hyperglycemia in STZ-induced diabetic mice

(Murai, et al., 2017). These studies demonstrate that stem cell transplantation into diabetic patients may provide relief to their symptoms and act as a potential cure.

The human neural progenitor cells (OK99-2; Celavie Biosciences, LLC) used in my study have shown positive outcomes between the graft and animal host environments. Celavie's human-derived cells have proven to be effective in both human and many animal models in a variety of different disorders. One study showed that the human neural progenitor cells were able to graft, differentiate and survive over six years in two Huntington's patients (Keene et al., 2007). Another study used these same human derived progenitor cells to improve tendonitis in injured race horses (Watts et al., 2011). This trial observed that injection of cells into the horse's leg tendon increased mobility (Watts et al., 2011). Finally, multiple cell lines of OK99 cells have been tested in ataxic Han Wistar rats (Uhlendorf et al., 2017). Shortly after intracranial transplantation, the ataxic symptoms normally observed in these rats were alleviated. Hence, the OK99-2 cells have multipotent abilities and show potential as transplanted grafts into diabetic models to help replace pancreatic beta cells restoring normal insulin production.

To further study the potential of Celavie's OK99-2 cells as treatment in diabetes, transplantation was used as a treatment for STZ-induced type 1 diabetes in rats. First, undifferentiated progenitor cells were injected through intraperitoneal (IP) and intravenous (IV) routes. This allows the cells to migrate easily throughout the body. Next, pre-differentiated cells that resemble pancreatic beta cells were surgically transplanted under the kidney capsule. Through these various approaches I was able to identify that kidney capsule transplantation was the best method of administering Celavie's cells to reduce (albeit temporarily) hyperglycemic symptoms in diabetic rats.

Materials and Methods

Animals: Han Wistar rats (mix of males and females) were utilized in this study and housed in the CSUN Vivarium. Rats have unlimited access to water and food (except during blood glucose testing days). They were housed in large plastic cages with wood shavings bedding experiencing a 12 hr/12 hr light/dark cycle. I used a mix of male and female rats consisting of a total of 89 rats in eleven groups. First, live undifferentiated OK99-2 cells treatment groups consisted of intraperitoneal injection (n=8), intravenous tail vein injection (n=8), intrapancreatic injection (n=9) and kidney capsule transplantation (n=8). Another group of rats received live pancreatic progenitor OK99-2 cells for kidney capsule transplantation (n=7). Next, four dead OK99-2 cell treatment groups consisted of intraperitoneal injection (n=10), intravenous tail vein injection (n=10), intrapancreatic injection (n=9) and kidney capsule transplantation (n=9). Finally, two untreated groups: diabetic (n=6) and normal rats (n=5) were used as control groups. Diabetic rats received a one-time injection of streptozotocin (65 mg/kg) and were determined diabetic or normal one week after injection. Diabetic and normal control rats did not receive additional immunosuppression. All procedures detailed in these experiments have been approved by CSUN's IACUC.

Streptozotocin injection (day 1): A one-time intraperitoneal injection of streptozotocin (STZ; 65 mg/kg; Thermo Fisher, Canoga Park, CA) dissolved in sodium-citrate buffer (14.71g sodium citrate/200mL DI water mixed with 20.1 g citric acid/200 mL DI water with a pH of 4.5) was administered at 60 days of age to healthy, normal Han Wistar rats. This dose was determined by preliminary studies using three different doses of STZ (55mg/kg, 60mg/kg, and 65mg/kg) with 65mg/kg being the most effective in producing

approximately 75% of injected rats which became diabetic by seven days (Figure 2). Other STZ doses yielded poorer numbers of diabetic animals. Injections of 50 mg/kg and 60 mg/kg of STZ resulted in inconsistent diabetic animals (and less than 50% effectiveness) over the course of three weeks.

Blood glucose monitoring: Throughout the experiment, blood glucose was tested twice per week using Accu-Chek Compact glucometers and test strips (Roche Diabetes Care Inc., Pleasanton, CA) by researchers who were blind to treatment. First, all rats were fasted 6 hours before glucose testing. Blood samples were taken by swabbing the tail area with 70% ethanol and pricking the tail vein of the rat with a lancet to create one drop of blood to test via the Accu-Chek test strips. Rats that exhibited a blood glucose level of over 400 mg/dL within seven days after STZ injection qualified as diabetic (Deeds et al., 2011) and were allowed to proceed to the cell treatment studies.

Immunosuppression (day 7): Seven days after inducing diabetes with STZ (and confirmed with glucose testing), diabetic rats were immunosuppressed using Alzet Model 2ML4 osmotic pumps (Alzet Osmotic Pumps, Cupertino, CA) that chronically administered cyclosporine (15 mg/kg/day). To insert the pumps, an inch-long incision was made between the scapulae of the rat. Sterilized pumps were activated by incubation in 0.9% saline solution at 37ºC for 24 hours prior and then inserted into the rat by forming a pocket in the scapulae using hemostats. Surgical staples were used to close the incision. In order to immunosuppress the rats over the course of the experiment (7 weeks), the

cyclosporine pumps were replaced after 23 days. Pumps were not inserted into normal and diabetic controls.

OK99-2 undifferentiated progenitor cell culture: The undifferentiated progenitor cells were cultured in Celavie Stem Cell Growth Medium which consists of Earle's balanced salt solution medium that allowed these cells to be cultured in a feeder free suspension. This growth medium was supplemented with growth factors at proprietary concentrations of epidermal growth factor (Peprotech, Rocky Hill, NJ, USA), leukemia inhibiting factor (LIF) (Millipore, Temecula, CA, USA), basic fibroblast growth factor (bFGF), insulin-like growth factor (IGF), transforming growth factor α (TGF-α), N2 (Invitrogen, Carlsbad, CA, USA), and Gem 21 (Gemini Bioscience, Sacramento, CA, USA). These cells were obtained by following the National Institute of Health Ethical Guidelines and have been fully characterized by in-house experiments at Celavie Biosciences, LLC. As a paracrine control for this experiment, dead undifferentiated progenitor cells were injected into a separate group of diabetic rats. These dead cells were originally live undifferentiated progenitor cells that were freeze-killed by placing them in a -20 degree Celsius freezer for 30 minutes and stored in a -80 freezer until needed.

Progenitor cell differentiation into Pancreatic Progenitor cells: Differentiation of the human progenitor cells into pancreatic progenitor cells follows Kroon, et al (2008) and D'Amore, et al. (2006) pancreatic differentiation protocol without new modifications (Table 1). Two weeks prior to differentiation, OK99-2 cells were thawed in a 37° C incubator with Celavie Stem Cell Growth Medium. OK99-2 cells were grown over the

course of 14 days. Growth medium was changed daily corresponding to the differentiation stage of the cells (Table 1). After this process, flow cytometry was used to evaluate beta cell differentiation using C-peptide antibody (0.2 mg/ml, BD Pharmigen, San Diego, CA). Lastly, OK99-2 pancreatic differentiated cells were counted and resuspended into a concentration of 1 million cells per 10 μL Stage 5 medium.

Stage 1		**Stage 2**	**Stage 3**	**Stage 4**	**Stage 5**
Day 1	**Day 2-3**	**Day 4-5**	**Day 6-8**	**Day 9-11**	**Day 12-14**
RPMI-1640	RPMI-1640	RPMI-1640	DMEM (High Glucose)	DMEM (High Glucose)	DMEM (High Glucose)
0.2% FBS	0.2% FBS	2% FBS	1X Glutamax	1X Glutamax	1X Glutamax
1X Glutamax	1X Glutamax	1X Glutamax	1% B27 Supplement	1% B27 Supplement	1% B27 Supplement
100ng/mL Activin A	100ng/mL Activin A	50ng/mL FGF7	2uM Retinoic Acid	1uM γ-Secretase Inhibitor	50ng/mL Exendin-4
25ng/mL Wnt-3a			0.25uM KAAD-Cyclopamine	50ng/mL Exendin-4	50ng/mL IGF-I
			50ng/mL Noggin		50ng/mL HGF

Table 1. Stages of Kroon's pancreatic differentiation protocol done by Celavie Biosciences.

Cell transplantation (day 17): After 10 days of immunosuppression by cyclosporine, OK99-2 cells (kindly provided by Celavie Biosciences) were transplanted using a sterile

Hamilton syringe into diabetic rats. These live progenitor cells (LPC), live pancreatic progenitor cells (DIFF LPPC), or dead progenitor cells (DPC) were injected into different regions of the diabetic rat: the circulatory system (tail vein injection of 1 million cells/200 μL, n=8 LPC, n=10 DPC), peritoneal cavity (intraperitoneal injection of 1 million cells/200 μL, n=8 LPC, n=10 DPC), pancreas (surgical intrapancreatic injection of 500,000 cells/3μL, n=9 LPC, n=9 DPC) or the kidney capsule (surgical injection of 1 million cells/10 μL, n=8 LPC, n=9 DPC, n=7 DIFF LPPC).

For intrapancreatic injection, rats required surgery to expose the pancreas. The animals were given deep anesthesia injection of chloral hydrate (350 mg/kg) about 10 minutes before surgery. A small incision was made on the left side of the abdominal area, and the spleen was located. This anatomical reference point served to determine the location of the distal end of the pancreas, closest to the spleen. A sterile Hamilton syringe was used to inject the cells into the tail end of the pancreas. The pancreas and spleen were gently placed back into the body cavity. Silk sutures and staples were used to close the muscle and outer skin layer, and antibiotic ointment was applied to the suture site.

The last treatment required an additional surgery of exposing the kidney capsule in order to transplant the OK99-2 cells. Here, rats were given a deep anesthetic injection of chloral hydrate (350 mg/kg) approximately 10 minutes prior to surgery. The kidney was exposed and kept hydrated with sterile mammalian saline (0.9% NaCl). A sterile Hamilton syringe was used to inject the cells (1,000,000 cells/10μL) under the kidney capsule. Either right or left kidney received treatment, depending on which side was less impeded by the Alzet pump. After infusion, the Hamilton syringe was removed, a sterile gel foam sponge was used to prevent excessive bleeding from the capsule injection site, and the kidney was

gently placed back into the body cavity. Silk sutures were used to stitch together the dorsal muscle layer, and surgical staples were used to close the outer skin layer. Again, antibiotic ointment was applied to the suture site.

For the remainder of the experiment, the health of all rats was monitored through bi-weekly blood glucose tests and weights.

Perfusion: Rats were trans-cardially perfused after seven weeks post-treatment. They received an intraperitoneal injection of a lethal dose of chloral hydrate (400 mg/kg). First, 0.9% saline flushed out the remaining blood in the rat, then 4% paraformaldehyde was flushed through the rat to fix the organs and remaining progenitor cells.

Histology: Histology of the kidneys, pancreas, and lungs were examined for the presence of human progenitor cells. These tissues were cryoprotected by placed in 30% sucrose in 1xPBS for at least 24 hours prior to slicing on a cryostat. Then, 30 μm sections were sectioned, adhered to glass slides and prepared for staining.

Cresyl violet staining: A nuclear stain was used to view cell morphology. First, tissue sections were hydrated by a sequence 100% ethanol, 95% ethanol, 70% ethanol, and DI water for two minutes each. Then, the tissue sections were stained in cresyl violet (Sigma Chemical, St. Louis, MO) for three minutes and was rinsed with DI water for two minutes. The stain was fixed with acetic formalin for five minutes and washed with DI water for two minutes. Then the tissue sections were dehydrated in a sequence of 95% ethanol, 100% ethanol, and 100% ethanol for two minutes each. Lastly, the tissues were washed with

xylene for 5 minutes. Then, the tissue slides were cover slipped and mounted with Permount (Fischer Scientific, Santa Clara, CA). After this process, the slides were left to dry overnight.

Immunohistochemistry: Immunostaining with anti-human nuclei antibody (Millipore MAB4383, Burlington, CA) and Vectastain ABC HRP staining kit (Vector Laboratories, Burlingame, CA) were used to identify surviving human progenitor cells. First, the tissue was washed in 10x wash buffer (Diagnostic Biosystems, Pleasanton, CA) for 5 minutes. Next, the tissues were washed in endogenous peroxidase blocking solution [0.36% β-D-Glucose (Fisher Scientific, Waltham, MA), 0.01% Glucose Oxidase (MP Biomedicals, Solon, OH) and 0.013% sodium azide (Fischer Scientific) in 1X PBS (Fisher Scientific)] for 60 minutes followed by wash with buffer for 5 minutes. Then, the tissue was washed with permeabilizing solution containing 0.1% Triton X-100 (Fisher Scientific) in 1X PBS for 30 minutes followed by wash buffer treatment for 5 minutes. After, 5% normal horse serum (Vector Laboratories) was applied for 20 minutes. Afterwards, the tissue was rinsed with avidin blocking solution (Vector Laboratories) for 15 minutes followed by wash buffer for 1 minute. Next, the tissue was rinsed with Biotin Blocking solution (Vector Laboratories) for 15 minutes followed by wash buffer for 1 minute. Next, the tissue slides were incubated for 60 minutes at room temperature with human nuclei primary antibody (Millipore MAB4383) prepared in antibody diluent (Diagnostic Biosystems) followed by wash buffer for 5 minutes. The anti-mouse biotinylated secondary antibody (Vector Laboratories) was applied for 45 minutes followed by wash buffer for 5 minutes. Then, the immPACT DAB Substrate (Vector Laboratories) was applied for 5 minutes and then rinsed

with wash buffer for 5 minutes. Methyl Green counterstain (Vector Laboratories) was applied for 1 minute at 60° C. Immediately after, the tissue was washed with deionized water (Thermo Scientific) for 2 minutes. The tissues were dehydrated by placing them in 95% ethanol for 5 minutes, 100% Ethanol (Arcos Organics, Waltham, MA) for 5 minutes, 100% ethanol again for 5 minutes, xylene (Fisher Scientific) for 5 minutes, and xylene for 5 minutes. Then, the slides with tissue were cover slipped and mounted with xylene-based mounting medium (Poly Sciences, Warrington, PA). After this process, the slides were left to dry overnight. Tissue sections were inspected for human progenitor cell survival using an Olympus BX60 fluorescent microscope with ToupView version 7.3 software.

Results

Diabetic rats were treated with human undifferentiated progenitor cells or human pancreatic progenitor cells, and the effects of these various cell treatments on blood glucose levels and weight gain were observed. I also determined if there were any surviving human cells after 7 weeks of the experiment in the kidney capsules of diabetic rats.

Pretreatment determination of STZ dosage

In this study I produced an induced diabetic rat model using streptozotocin (STZ). Prior to testing progenitor cells on diabetic rats, three different doses were pretested to determine the most effective one for our progenitor cell treatment. The most effective dose for producing rats that remained diabetic with a blood glucose level over 400mg/dL was the 65mg/kg dose of STZ (Figures 1, 2 and 3). A general comparison was made between the diabetic pancreas and healthy pancreas. During perfusions there was a visible difference between the size and heft of the pancreas (Figure 1). This led me to believe that the STZ injections are working sufficiently in creating a diabetic rat model.

A small pilot study with few rats was performed to narrow down the most effective dose of STZ to be effective by being administered-one time to induce diabetes within a timely manner (Figure 2 and 3). Blood glucose levels were inconsistent and irregular in producing a true diabetic rat that could be identified 7 days after STZ injection for 55mg/kg and 60 mg/kg doses. However, when the 65 mg/kg dose was administered, consistent results in blood glucose was seen after 7 days of either over 400mg/dl or around 100-200 mg/dl (Figure 2). This made it easy to determine which rats would remain diabetic for the

remainder of the experiment. Weights and water intake of the rats were also measured to monitor the intensity of the diabetic profile (Figure 3 and 4).

One week after STZ injection, diabetic rats illustrated symptoms which included increased thirst, increased urine output and slightly lower weights than normal healthy rats (Figure 3 and 4). The 65 mg/kg STZ dose injections was the most effective in producing consistent diabetic rats. (Figure 2A) This treatment was effective in creating 75% of the experimental cohort diabetic. Other doses tested did not produce consistent results. An STZ treatments of 60mg/kg (Figure 2B) had about a 28.6% success rate for producing diabetic animals that had blood glucose levels that remained above 400mg/dL. Another dosage tested was 50mg/kg STZ injection that produced 1 out of 3 (33%) diabetic but had varying levels of blood glucose levels that would make it difficult to determine the true diabetic and non-diabetic animal (Figure 2C). These diabetic animals also had slightly decreased weights compared to non-diabetic rats (Figure 3). Water intake was also measured and there is a distinct difference between rats who received STZ treatment but did not become diabetic (non-diabetic) and diabetic animals, correlating with the traits of clinical diabetic patients (Figure 4).

Streptozotocin morphological effects on the pancreas

First, STZ treatment had a detrimental effect on the anatomy of the pancreas. The diabetic pancreas was shown to be thin and insubstantial compared to the normal rat (Figure 1). The normal pancreas with healthy beta cells unaffected by STZ treatment. The diabetic pancreas is no longer serving its purpose. The beta cells located in the islets of Langerhans have been destroyed which made the pancreas look smaller and thinner. This fragile state

of the diabetic pancreas confirmed that the 65 mg/kg dose of STZ was effective on destroying the pancreatic beta cells, producing a true rodent model for type 1 diabetes (Figure 1).

Progenitor cells effect on blood glucose levels

One week after injecting rats with STZ, diabetic rat blood glucose becomes very high (Figure 5), range between 400-600 mg/dl. All rats were tested twice a week until sacrifice. In summary, I found statistical significance among all treatment groups when comparing blood glucose levels (Between groups: $F=63.497$, $p<0.001$; Within groups: $F=11.422$, $p<0.001$). After investigating further using Tukeys post-hoc test starting at week 0.5 there was significance but it was due to the animals becoming diabetic after their initial STZ injection. On week 2.5 after cell treatment, pancreatic progenitor cell treatment group via kidney capsule (DIFF LPPC KC) had significance compared to DSC KC (0.042, Tukeys), DSC TV (0.005, Tukeys), normal (0.000, Tukeys). On week 4, LSC KC undifferentiated cell treatment had significance against diabetic control (0.012, Tukeys), DSC IP (0.038, Tukeys), DSC TV (0.020, Tukeys). On week 6, the end of the experiment, LSC TV undifferentiated had significance against diabetic control (0.003, Tukeys). The significance found in the live undifferentiated progenitor cells in kidney capsule and tail vein injections was between rats that were diabetic and severely diabetic rats. These results are not favorable. However, differentiated live pancreatic progenitor cells in the kidney capsule showed a decrease in blood glucose levels. This could be due to the cells taking effect for a short time period after injection. These animals did not become “cured” and remained diabetic for the remainder of the experiment.

Progenitor cell effect on weight

Rats were weighed twice weekly after their blood glucose was tested (Figure 6). The ANOVA analysis for weights showed significance within groups ($F=38.8$; $p<0.001$) but not between groups ($F=2.02$; $p>0.05$). After further analysis using Tukeys post-hoc test, on week 0.5 there was significance between DSC TV and diabetic control ($p<0.05$) groups but this time point was before they received treatment. The immunosuppressed diabetic rats that received cell treatment had lower weights. The untreated diabetic rat had the highest weight in this study. This could be due to not receiving immunosuppression.

Anti-Human Nuclei Immunostaining

Throughout the study, there were no rats that had lowered blood glucose levels close to normal (around 100-200 mg/dl). Immunohistochemistry revealed that the transplanted cells did not graft to the kidney or pancreas and most cells appeared to be lost most likely due to the perfusion process or 4% PFA treatment afterwards. Human cells were identified near the kidney capsule that remained intact after cresyl violet staining and this was verified by anti-human nuclear antibody (Millipore Sigma MAB4383) staining confirming that the human pancreatic progenitor cells remained (Figure 7). Some cells were lost during the immunohistochemistry process due to the fact that the cells did not graft onto the kidney or pancreas. The cells were able to survive in the kidney capsule for 37 days after injection.

Figures

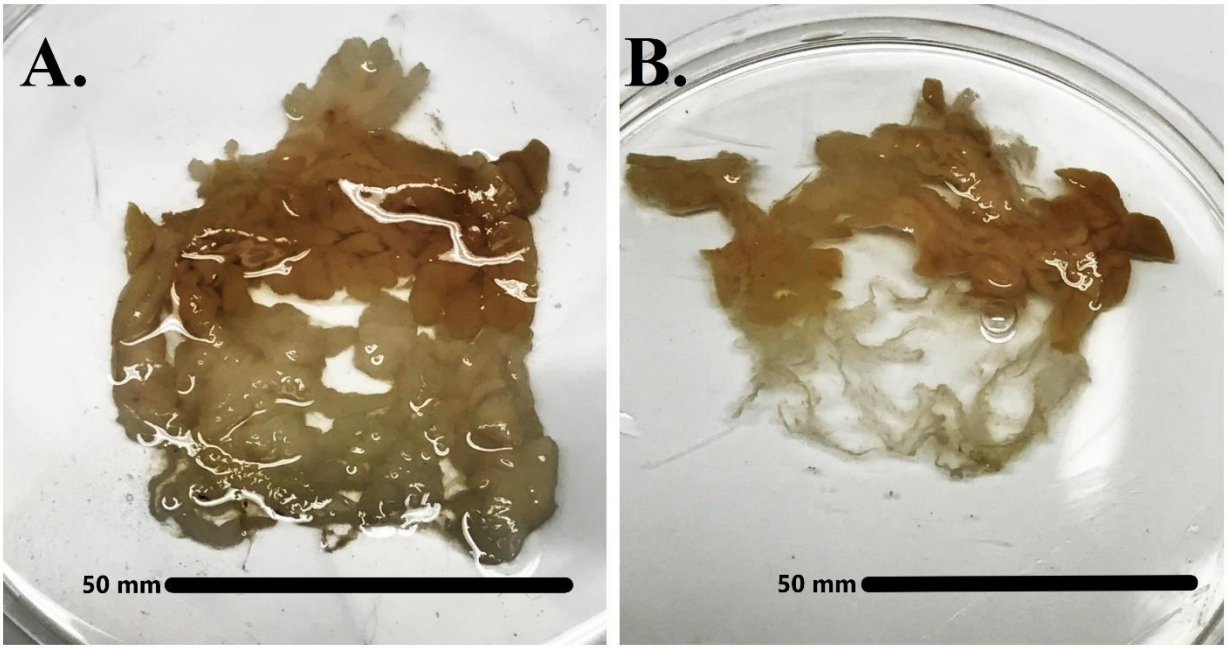

Figure 1. Comparison of a normal rat pancreas (A) with a diabetic rat pancreas (B) seven weeks after STZ injection (65 mg/kg). Note the size and obvious deterioration of the diabetic pancreas shown on the right (Figure 1B). Images were taken with a standard camera after a full body perfusion with 0.9% saline solution followed by 4% paraformaldehyde.

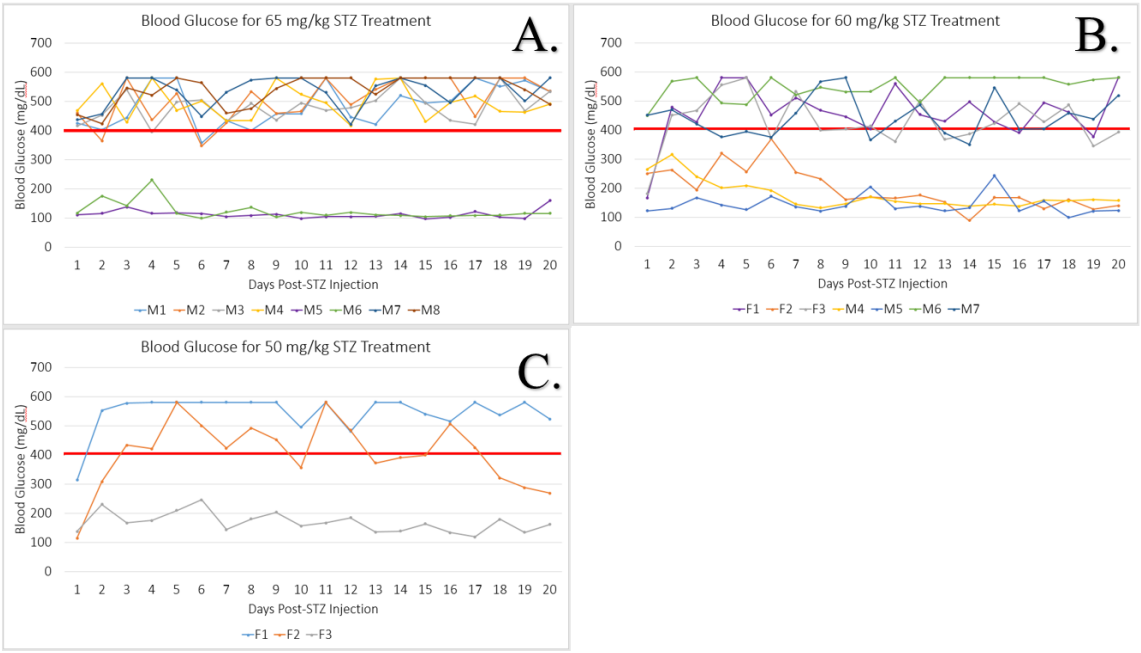

Figure 2A-C. Rats injected with 65 mg/kg (Fig. 2A), 60 mg/kg (Fig. 2B), 50 mg/kg (Fig. 2C) of STZ were observed. Diabetic rats were determined by their ability to develop an increased blood glucose level of over 400 mg/dl seven days after injection. Normal rats received STZ treatment but failed to become diabetic. M1 refers to Male rat with randomly assigned number 1, M2 refers to Male rat with randomly assigned number 2, F1 refers to Female rat with randomly assigned number 1, etc. Each animal is exclusive to each defined treatment in the graph. Solid red line represents the diabetic blood glucose cutoff criteria limit of 400 mg/dL.

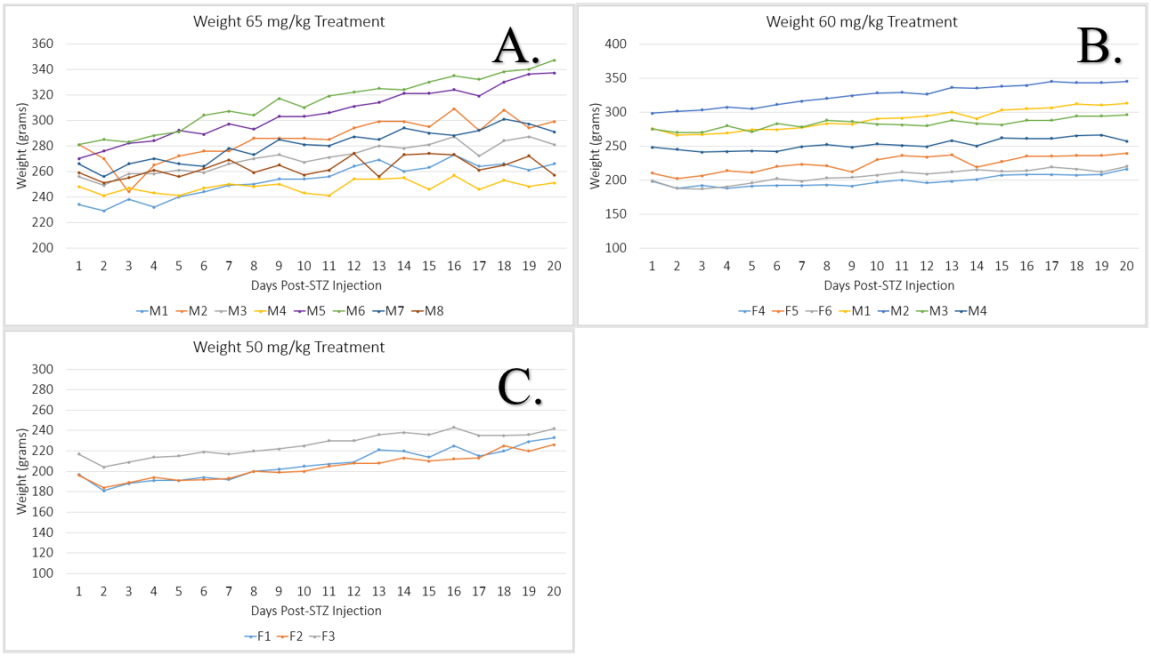

Figure 3A-C. Weight was observed over 20 days after STZ injection. Rats that became diabetic had slightly lower weights than rats that did not show a diabetic response to STZ.

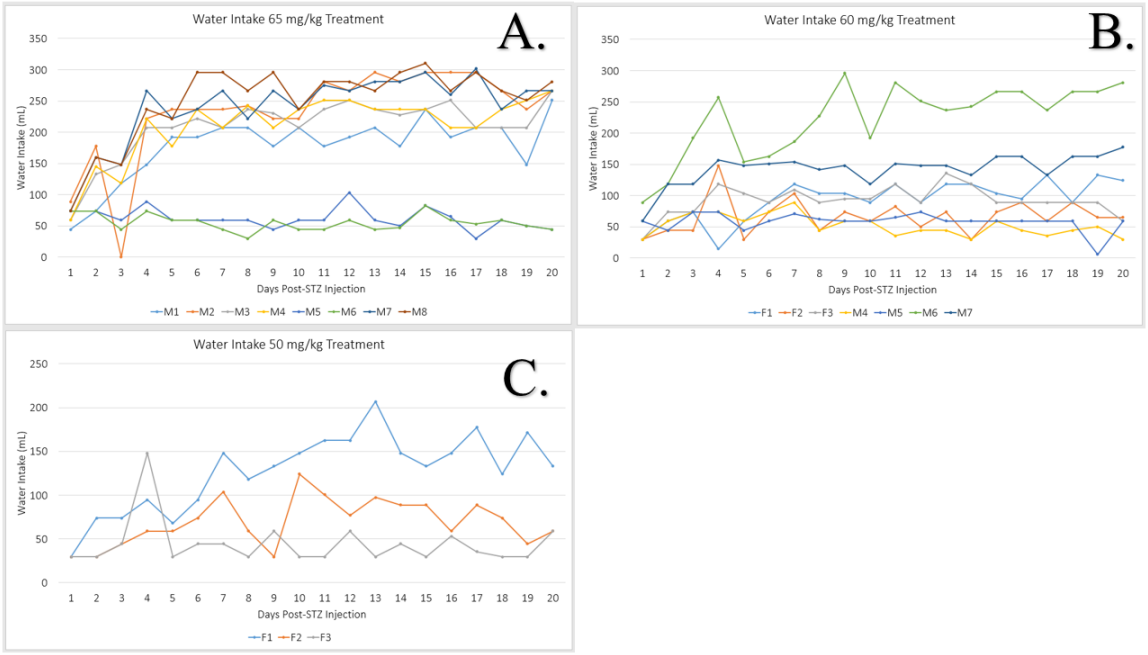

Figure 4A-C. Water intake was measured daily. Rats that showed elevated blood glucose levels (Figure 2) had elevated water consumption during the 20 day trial.

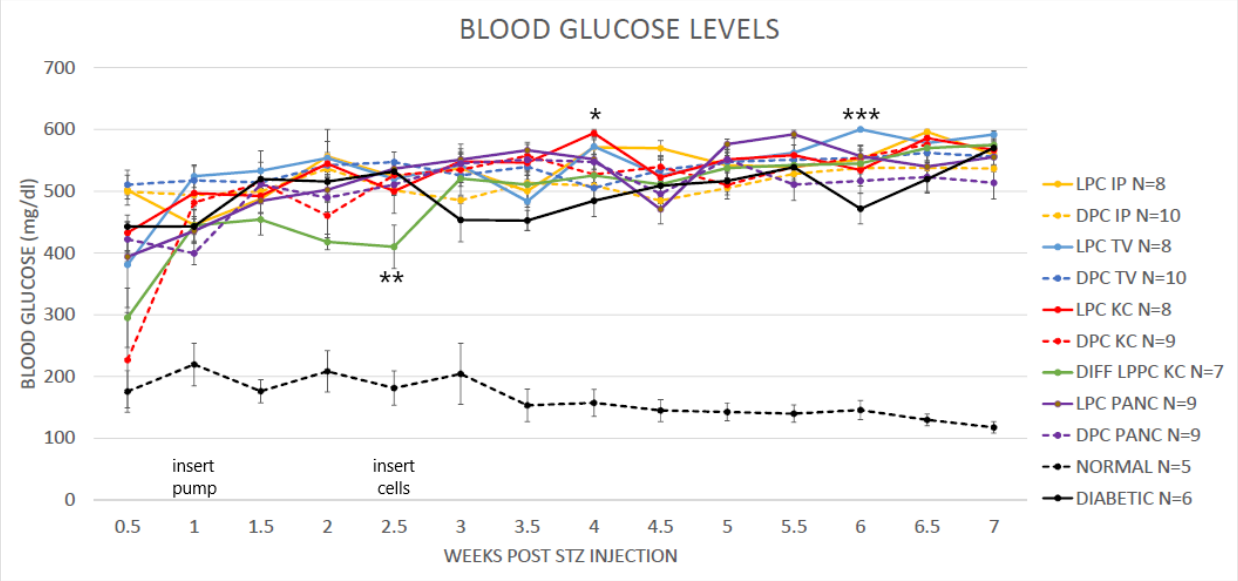

Figure 5. Mean blood glucose levels for all rats over a seven week period of time. All diabetic animals, regardless of treatment remained diabetic within the blood glucose range of 400 mg/dl-600mg/dl. Blood glucose levels were tested on rats after six hours of fasting. The normal rats with no treatment had the lowest blood glucose levels overall compared to the diabetic rats regardless of treatment. Statistical significance was only found at week 2.5 ($p<0.005$), 4 ($p<0.03$), and 6 ($p<0.001$). Treatments: LPC = Live Undifferentiated Progenitor Cells, DPC = Dead Undifferentiated Progenitor Cells, DIFF LPPC = Differentiated Live Pancreatic Progenitor Cells, IP = Intraperitoneal injection, TV = Tail Vein injection, KC = Kidney Capsule treatment, PANC = Pancreatic Injection, Normal = no STZ treatment, Diabetic = STZ but no additional treatment. All values are means ± SEM.

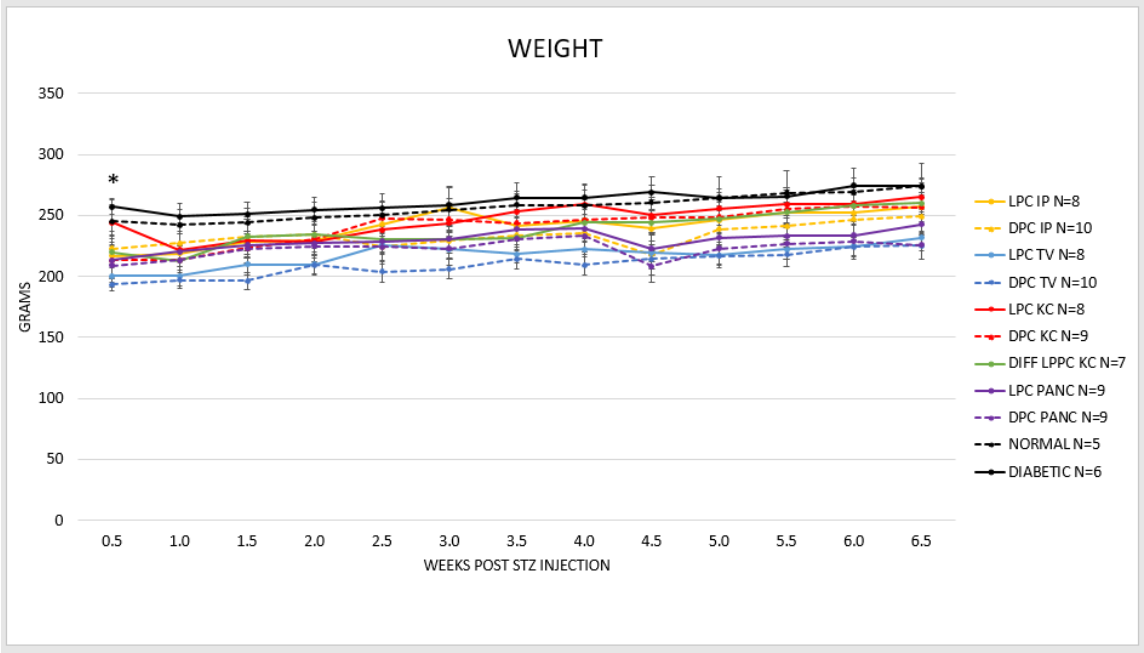

Figure 6. Mean weight for all rats over a 6.5 week period of time. All diabetic animals receiving cell treatment had a lower weight than untreated animals. The normal rats had similar weight over time compared to the untreated diabetic rats. Statistical significance was found at week 0.5 ($p<0.005$) which may be due to the varying levels of diabetes in the beginning. Treatments: LPC = Live Undifferentiated Progenitor Cells, DPC = Dead Undifferentiated Progenitor Cells, DIFF LPPC = Differentiated Live Pancreatic Progenitor Cells, IP = Intraperitoneal injection, TV = Tail Vein injection, KC = Kidney Capsule treatment, PANC = Pancreatic Injection, Normal = no STZ treatment, Diabetic = STZ but no additional treatment. All values are means ± SEM.

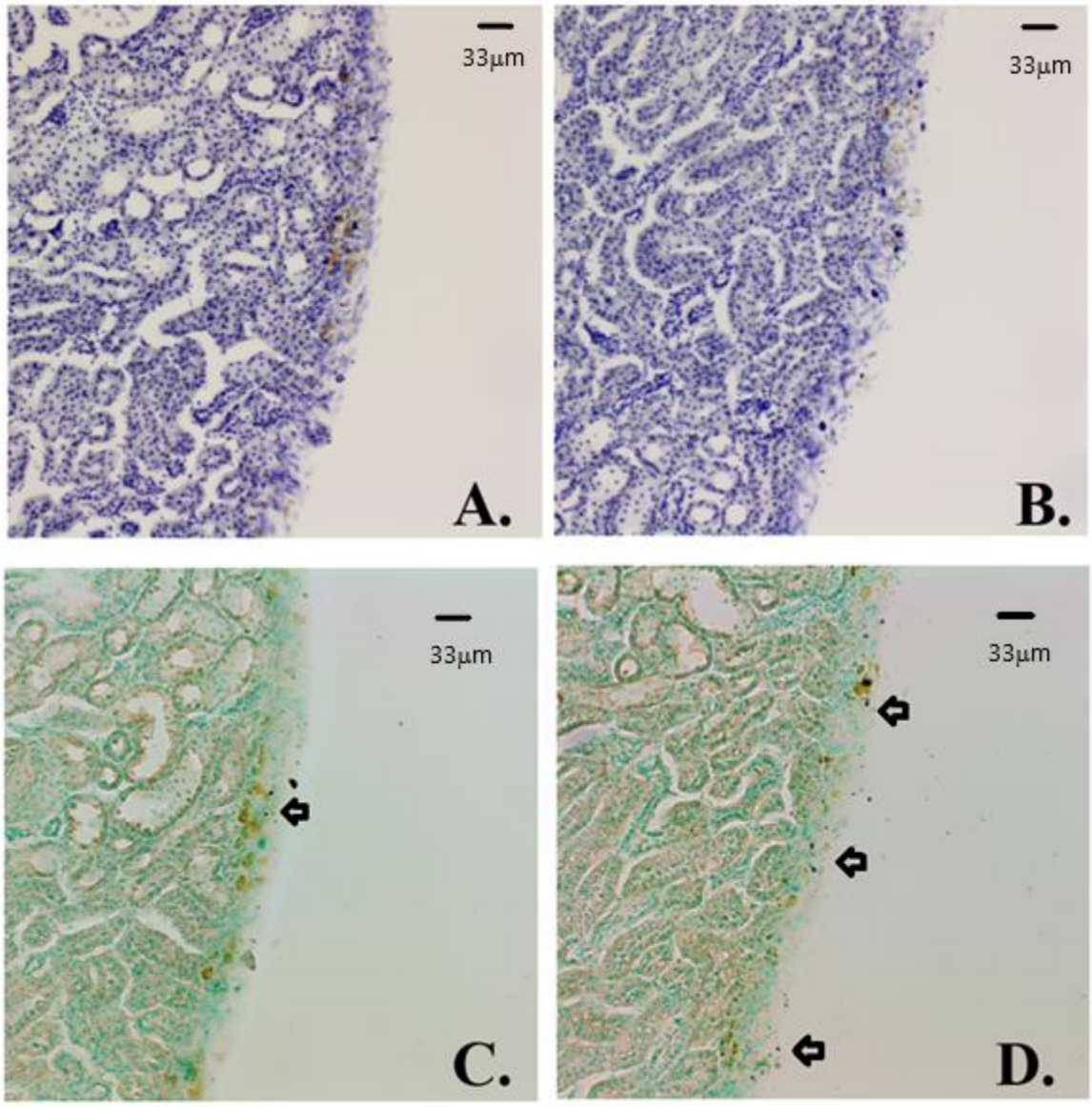

Figure 7A-D. Histologic photomicrographs of rat kidney capsule with transplanted pancreatic progenitor cells still present after 37 days. Human pancreatic progenitor cells were made visible by using anti-human nuclear staining and DAB staining, a dark brown dye. Methyl green stain was used as a nuclear counterstain). There is some background staining of blood shown as a light brown/yellow color confirmed by cresyl violet. These pictures A-D were taken from the same kidney that received pancreatic progenitor cell

transplant into the kidney capsule using an Olympus BX60 fluorescent microscope with ToupView version 7.3 software displayed in the kidney capsule at 100x magnification.

Discussion

Many stem cell lines have been manipulated in specific culture conditions to be differentiated into insulin producing beta-like cells of the pancreas. Each cell line is unique in its own way, and scientists continue to produce cell lines that are similar to beta cells (Cowan et al., 2004). Some stem cell transplantation studies have shown remarkable progress, such as bone-marrow derived stem cells implanted into STZ-induced diabetic mice lowered blood glucose levels due to the cells differentiating into a mix of endothelial cells and insulin-secreting cells (Hess et al., 2003). Also, human fetal live cells differentiated into insulin secreting cells reversed hyperglycemia by activating beta cell genes during cell culture (Zalzman et al., 2003). These studies have provided a foundation for future stem cells lines to be created.

However, the scientific community has yet to create a perfect pancreatic progenitor beta cell as there have been just as many failures. A study that characterizes pancreatic progenitor cells found that their cells secreted a low amount of insulin compared with human islet cells, and noted that further optimization was needed to increase insulin output before transplanting in a diabetic model (Noguchi et al., 2010). Another study determined their stem cells had differentiated into cells that closely resemble fetal-like beta cells instead of adult beta cells (Hrvatin et al., 2014). These researchers also lamented that even the most perfected protocol and cell line will produce mixed populations of pancreatic cells that are difficult to purify and analyze due to the lack of appropriate cell surface markers. One review discussed the difficulties in differentiating pluripotent stem cells into

multipotent pancreas progenitor cells since specific cell surface markers have not been determined (Jiang and Morahan, 2015).

In my thesis project, I created a diabetic rat model for type 1 diabetes. The standard approach to producing an animal model of diabetes is to inject streptozotocin in order to destroy insulin producing beta cells. Many studies use 350-400 mg/dL blood glucose range to define hyperglycemia in diabetic rats (Akbarzadeh et al., 2007; Lee et al., 2003) close to the diabetic blood glucose level that I chose (400mg/dL). A small group of animals were tested with three different doses of STZ (55 mg/ml, 60 mg/ml, 65 mg/ml), and I discovered that the most effective dose was 65 mg/ml because it provided the most consistent diabetic rats after one week post-injection. These diabetic rats remained in a diabetic state for three weeks, and were the perfect model to inject cells through four different routes, IP, IV, kidney capsule and pancreas.

However, in summary, all diabetic rats that received various progenitor cell treatments did not significantly lower their blood glucose concentration compared to any of the negative control groups (dead progenitor cells or untreated STZ rats); their blood glucose levels remained in the diabetic range of over 400 mg/dl (Figure 5). After testing the diabetic rats, there was little to no significant decrease in blood glucose levels (Figure 5). Obviously, the live undifferentiated cells that were transplanted in the blood, IP or kidney capsule were ineffective. These diabetic rats had incredibly high blood glucose and continued to show signs of diabetes after cell transplantation such as thirst and frequent urination which required bedding to be changed every day to ensure cleanliness and to prevent infection to surgical or injection site. Even the rats with suppressed immune systems (via chronic dosing with cyclosporine) were not statistically significant from

untreated diabetic control which did not receive immunosuppression from cyclosporine (Figure 6). After finding no cell survival in the undifferentiated progenitor cell transplant recipients, it is not surprising to see that there was no improvement in blood glucose levels taken throughout the course of the study.

Additionally, three injection methods (tail vein, intraperitoneal, and pancreas) were probably not the most effective route of ensuring cell survival. Rats who were given IP injections of undifferentiated progenitor cells remained diabetic and continued to maintain a low weight. Tail vein injections of undifferentiated progenitor cells also did not show survival after they were administered. Intrapancreatic transplantation of cells did not reduce blood glucose levels. Several studies have shown that these methods were very effective in reducing blood glucose levels and alleviating hyperglycemia in diabetic rodents.

Researchers have found previous success using these three methods. Intraperitoneal placenta-derived mesenchymal stem cell injection was administered to diabetic mice and resulted in a potential treatment for diabetic gastropathy, a side effect of diabetes (Park et al., 2018). Another study was able to gain control of type 1 diabetes in mice by intraperitoneal injection of a combination of islets and adipose derived mesenchymal stem cells from non-diabetic mice, canines and humans (Westenfelder et al., 2017). Tail vein injection of mouse adipose derived mesenchymal cells into type 2 diabetic mice inhibited hyperglycemia and insulin resistance and alleviated liver fibrosis (Liao et al., 2017). Another study used tail vein injection on day 17 and day 24 of their study using allogenic bone marrow derived mesenchymal stem cells into type 1 diabetic Han Wistar rats and found that it reduced blood glucose levels significantly and increase islet-neogenesis

(Bhansali et al., 2015). Intrapancreatic injection of human bone marrow–derived mesenchymal stem cells into diabetic mice was the most effective treatment for type 1 diabetes by increasing insulin production in a study that delivered cell injections on day 7 and day 28 of the experiment (Murai et al., 2017). Lastly, kidney capsule transplantation of human embryonic stem cell–derived pancreatic endoderm cells restored glycemic control by in vivo differentiation into glucose responsive insulin secreting cells (Pepper et al., 2017).

Perhaps the most promising treatment method was the kidney capsule transplantation due to the enclosed nature. In this method the cells were ensured to remain inside the kidney capsule with the potential of migrating within the kidney. While no undifferentiated progenitor cells were found in the immunohistochemistry analysis, pancreatic progenitors were recovered in one of seven of the rats that received the cell transplant into the kidney capsule (Figure 7A-D).

Thus, there be a glimmer of hope-one treatment group that received live differentiated pancreatic progenitor cells in the kidney capsule showed significantly lowered levels of blood glucose compared to other groups on week 2.5 post-STZ injection, one day after live pancreatic progenitor cells were injected. This was the only experimental group that exhibited these results which is a promising starting point to improve the experimental methods.

In addition, after I transplanted pancreatic progenitors into the diabetic rats, there was a momentary, yet significant decrease in blood glucose levels (Figure 5). Unfortunately, by week three, the blood glucose levels began to rise in these transplanted rats. This results could be due to short term survival of the transplanted pancreatic

progenitors temporarily alleviating hyperglycemia in the diabetic rats. In the immunohistochemistry there are a few surviving cells that appear to have survived 37 days after kidney capsule post-transplantation (Figure 7A-D). Although these cells survived, they did not provide long-term relief to the diabetic rats.

Previous researchers have transplanted stem cells or pancreatic endoderm into the kidney capsule and have found success in alleviating diabetic hyperglycemia (Kroon et al., 2008; Szot et al., 2015; Szot et al. 2007; Zhu et al., 2014). In Kroon et al. (2008), a slurry of cell aggregates was prepared by being absorbed on a gel foam disk and then coated with a layer of Matrigel. This type of adhesive preparation allowed the cells to engraft easily onto the kidney. The researchers delivered the cells with a PE-50 catheter. In Szot et al. 2007 and 2015, these researchers used PE50 catheter tubing attached to a Hamilton syringe to inject islet pellets or a slurry of cell aggregates under the kidney capsule. They also made an air pocket in the kidney capsule for the cells to be delivered into. In Zhu et al. (2014), a modified method for kidney capsule transplantation was described using a micro capillary pipette that was pulled on flame to create a fine but not sharp tip to administer cells. With this micro capillary pipette, the cells were sucked into it by mouth pipetting and blown into the kidney capsule. They also used very blunt tools such as a fire pulled glass Pasteur pipette that is blunt and slightly hooked to move kidney in and out of the body cavity. Although the methods used in my current study were fairly standard procedures, there may be optimizations that can be made to improve results in future studies. The use of a catheter could be used to deliver cells safely and effectively. Another improvement could be to use cell aggregates or cell slurry for effective cell delivery and survival.

Another factor to consider is the use of immunosuppressant drugs in transplant studies. Cyclosporine is a commonly used immunosuppressant drug for organ transplantation in humans. It is also used in research to immunosuppress rodents or other experimental animals receiving cell, tissue or organ transplants. Cyclosporine's main use is to reduce rejection during and after transplanting foreign cellular bodies. One study indicates that cyclosporine enhances the survival of transplanted neural progenitor cells (Hunt et al., 2010). Cyclosporine should not have played a negative role in this study.

This rat model of type 1 diabetes proves to be diabetic and mimic the autoimmune response shown in this debilitating disease. The innovative idea of progenitor cell transplantation as a therapy for type 1 diabetes should be continued to be studied. This research shows that there are many options for cell therapy for type 1 diabetes. The most promising result we found was a slight, temporary decrease of blood glucose when pancreatic progenitor cells were transplanted into the kidney capsule.

There are alternative methods that could be undertaken in future studies to successfully view the proliferation of the transplanted progenitor cells. In our study adult rats were utilized but the onset of the disease is usually found in younger patients. It could be theorized that this treatment could be used in a younger animal model. For future studies, more cells or a slurry of cell aggregates could be utilized to increase the potential amount of surviving cells (Kroon et al., 2008) (Szot et al., 2015) (Bruin et al., 2015). Supplementing the cellular treatment with insulin to lower blood glucose throughout the study could also help the cells survive and graft into the kidney much more effectively (Rezania et al., 2012). There could also be the possibility of poor survival of these specific human progenitor cells unable to thrive in this particular animal model.

Future research into stem cell treatment of type 1 diabetes is still quite promising. There is a phase one clinical trial on-going with Viacyte that aims to use pancreatic endoderm cells derived from their proprietary embryonic stem cells via an encapsulating device aimed to mimic a pancreas by providing insulin and hormone secretion. Viacyte's preliminary study using differentiated human induced pluripotent stem cells with mice is the basis of other studies as well, but with different insulin producing cell lines (Haller et al, 2019). As discussed before, researchers have not created a product of true beta cell derived from stem cells. These stem cell lines all have great potential to become a possible cure for type 1 diabetes.

References

1. Alipio, Z., Liao, W., Roemer, E. J., Waner, M., Fink, L. M., Ward, D. C., & Ma, Y. (2010) Reversal of hyperglycemia in diabetic mouse models using induced-pluripotent stem (iPS)-derived pancreatic β-like cells. *Proceedings of the National Academy of Sciences of the United States of America*, *107*(30), 13426–13431.
2. Akbarzadeh, A., Norouzian, D., Mehrabi, M. R., Jamshidi, S. h., Farhangi, A., Verdi, A. A., Mofidian, S. M., … Rad, B. L. (2007). Induction of diabetes by Streptozotocin in rats. *Indian journal of clinical biochemistry : IJCB, 22*(2), 60-4.
3. American Diabetes Association. (January 30, 2017) Investments in Type 1 Research. Retrieved from http://www.diabetes.org/research-and-practice/we-are-research-leaders/type-1-research-highlights/investments-in-type-1-diabetes.html
4. Baranovski, B. M., Ozeri, E., Shahaf, G., Ochayon, D. E., Schuster, R., Bahar, N., Kalay, N., Cal, P., Mizrahi, M. I., Nisim, O., Strauss, P., Schenker, E., and Lewis, E. C. (2016) Optimizing α1-Antitrypsin Protocol for Islet Transplantation. *Journal of Pharmacology and Experimental Therapeutics*, 359 (3) 482-490.
5. Bruin, J. E., Asadi, A., Fox, J. K., Erener, S., Rezania, A., & Kieffer, T. J. (2015). Accelerated Maturation of Human Stem Cell-Derived Pancreatic Progenitor Cells into Insulin-Secreting Cells in Immunodeficient Rats Relative to Mice. *Stem Cell Reports, 5(6), 1081–1096.*
6. Cowan C.A., Klimanskaya I., McMahon J., Atienza, J., Witmyer J., Zucker J.P., Wang S., Morton C.C., McMahon A.P., Powers D., Melton D.A. (2004) Derivation of embryonic stem-cell lines from human blastocysts. *N Engl J Med*; 350: 1353–56.

7. D'Amour, K. A., Bang, A. G., Eliazer, S., Kelly, O. G., Agulnick, A. D., Smart, N. G., Moorman, M. A., Kroon, E., Carpenter, M. K., Baetge, E. E. (2006). Production of pancreatic hormone–expressing endocrine cells from human embryonic stem cells. Nature Biotechnology, 24(11), 1392–1401.
8. Deeds, M., Anderson, J., Armstrong, A., Gastineau, D., Hiddinga, H., Jahangir, A., Eberhardt, N., Kudva, Y. (2011). Single Dose Streptozotocin Induced Diabetes: Considerations for Study Design in Islet Transplantation Models. *Laboratory Animals*, 45(3), 131–140.
9. Efrat, S. (2008) Beta-cell replacement for insulin-dependent diabetes mellitus. *Advanced Drug Delivery Reviews,* (60) 114-123.
10. Ferber, S., Halkin, A., Cohen, H., Ber, I., Einav, Y., Goldberg, I., Barshack, I., Seijffers, R., Kopolovic, J., Kaiser, N., Karasik, A. (2000). Pancreatic and duodenal homeobox gene 1 induces expression of insulin genes in liver and ameliorates streptozotocin-induced hyperglycemia. *Nature Medicine,* 6 (5): 568-572.
11. Haller, C., Piccand, J., De Franceschi, F., Ohi, Y., Bhoumik, A., Boss, C., De Marchi, U., Jacot, G., Metairon, S., Descombes P., Wiederkehr, A., Palini A., Bouche, N., Kely, O. G., R.-C. Kraus, M. (2019). Macroencapsulated Human iPSC-Derived Pancreatic Progenitors Protect against STZ-Induced Hyperglycemia in Mice. *Stem Cell Reports*, 12 (4), 787-800.
12. Hess D, Li L, Martin M, Sakano S, Hill D, Strutt B, Thyssen S, Gray DA, Bhatia M. (2003). Bone marrow-derived stem cells initiate pancreatic regeneration. *Nature Biotechnology*; 21: 763–70.

13. Hunt, J., Cheng, A., Hoyles, A., Jervis, E., & Morshead, C. M. (2010). Cyclosporine A Has Direct Effects on Adult Neural Precursor Cells. *Journal of Neuroscience, 30(8), 2888–2896.*
14. Jiang, F. X., & Morahan, G. (2015). Multipotent pancreas progenitors: Inconclusive but pivotal topic. *World journal of stem cells*, *7*(11), 1251–1261.
15. Kaur, G., Padiya, R., Adela, R., Putcha, U. K., Reddy, G. S., Reddy, B. R., Kumar, K. P., Chakravarty, S., Banerjee, S. K. (2016) Garlic and Resveratrol Attenuate Diabetic Complications, Loss of β-Cells, Pancreatic and Hepatic Oxidative Stress in Streptozotocin-Induced Diabetic Rats. *Frontiers in Pharmacology*, *7*, 360.
16. Keene C.D., Sonnen J.A., Swanson P.D., Kopyov O., Leverenz J.B., Bird T.D., Montine T.J. (2007) Neural transplantation in Huntington's disease: long-term grafts in two patients. *Neurology*. 68:2093–2098.
17. Keene, C. D., Chang, R. C., Leverenz, J. B., Kopyov, O., Perlman, S., Hevner, R. F., Montine, T. J. (2009) A patient with Huntington's disease and long-surviving fetal neural transplants that developed mass lesions. *Acta Neuropathologica*, *117*(3), 329–338.
18. Kroon, E., Martinson, L. A., Kadoya, K., Bang, A. G., Kelly, O. G., Eliazer, S., Young, E., Richardson, M., Smart, N. G., Cunningham, J., Agulnick, A. D., D'Amour, K. A., Carpenter, M. K., Baetge, E. E. (2008) Pancreatic endoderm derived from human embryonic stem cells generates glucose-responsive insulin-secreting cells in vivo. *Nature Biotechnology*, 4, 443-452.
19. Lee, J.-J., Yi, H.Y., Yang, J.-W., Shin, J.-S., Kwon, J.-H., & Kim, C.-W. (2003). Characterization of Streptozotocin-induced Diabetic Rats and

Pharmacodynamics of Insulin Formulations. *Bioscience, Biotechnology, and Biochemistry, 67(11), 2396–2401.*

20. Liao, N., Zheng, Y., Xie, H., Zhao, B., Zeng, Y., Liu, X., & Liu, J. (2017). Adipose tissue-derived stem cells ameliorate hyperglycemia, insulin resistance and liver fibrosis in the type 2 diabetic rats. *Stem cell research & therapy*, *8*(1), 286.
21. Murai N, Ohtaki H, Watanabe J, Xu Z, Sasaki S, Yagura K, Shioda S, Nagasaka S, Honda K, Izumizaki M. (2017) Intrapancreatic injection of human bone marrow-derived mesenchymal stem/stromal cells alleviates hyperglycemia and modulates the macrophage state in streptozotocin-induced type 1 diabetic mice. *PLOS ONE* 12(10): e0186637.
22. Noguchi, H., Naziruddin, B., Jackson, A., Shimoda, M., Ikemoto, T., Fujita, Y., Chujo, D., Takita, M., Kobayashi, N., Onaca, N., Hayashi, S., Levy, M. F., Matsumoto, S. (2010). Characterization of Human Pancreatic Progenitor Cells. *Cell Transplantation, 19(6-7), 879–886.*doi:10.3727/096368910x509004
23. Park, J. M., Lee, S. H., Kim, K. I., Kim, W. H., Cho, J. Y., Hahm, K. B., & Hong, S. P. (2018). Feasibility of intraperitoneal placental-derived mesenchymal stem cell injection in stomachs of diabetic mice. *Journal of Gastroenterology and Hepatology,* 33(6), 1242–1247. doi:10.1111/jgh.14045
24. Pepper, A. R., Pawlick, R., Bruni, A., Wink, J., Rafiei, Y., O'Gorman, D., Yan-do, R., Gala-Lopez, B., Kin, T., MacDonald, P. E., Shapiro, A. M. J. (2017). Transplantation of Human Pancreatic Endoderm Cells Reverses Diabetes Post Transplantation in a Prevascularized Subcutaneous Site. *Stem Cell Reports,* 8(6), 1689–1700.

25. Rezania A., Bruin J.E., Riedel M.J., Mojibian M., Asadi A., Xu J., Gauvin R., Narayan K., Karanu F., O'Neil J.J. (2012) Maturation of human embryonic stem cell-derived pancreatic progenitors into functional islets capable of treating pre-existing diabetes in mice. *Diabetes*, 61, 2016–2029.
26. Sutherland, D.E., Gruessner, R., Kandswamy, R., Humar, A., Hering, B., Gruessner, A. (2004) Beta-cell replacement therapy (pancreas and islet transplantation) for treatment of diabetes mellitus: an integrated approach. *Transplant. Proc*. 36 1697–1699.
27. Szot, G.L., Koudria, P., Bluestone, J.A. (2007) Transplantation of Pancreatic Islets Into the Kidney Capsule of Diabetic Mice. *J. Vis. Exp.* (9), e404.
28. Szot, G.L., Yadav, M., Lang, J., Kroon, E., Kerr, J., Kandoya, K., Brandon, E.P., Baetge, E.E., Bour-Jordan, H., Bluestone, J.A. (2015) Tolerance Induction and Reversal of Diabetes in Mice Transplanted with Human Embryonic Stem Cell-Derived Pancreatic Endoderm. *Cell Stem Cell*. 16(2), 148-157.
29. Uhlendorf, T.L., Nuryyev, R., Kopyov, A.O., Prieto, J., Younesi, S., Cohen, R.W., Kopyov, O. (2017) Efficacy of Two Delivery Routes for Transplanting Human Neural Progenitor Cells (NPCs) Into the Spastic Han–Wistar Rat, a Model of Ataxia. *Cell Transplantation*, 26, 259–269.
30. Watts, A. E., Yeager, A. E., Kopyov, O. V., & Nixon, A. J. (2011) Fetal derived embryonic-like stem cells improve healing in a large animal flexor tendonitis model. *Stem Cell Research & Therapy*, *2*(1), 4.

31. Yang, Y.-P., Thorel, F., Boyer, D. F., Herrera, P. L., & Wright, C. V. E. (2011) Context-specific α-to-β-cell reprogramming by forced Pdx1 expression. *Genes & Development*, *25*(16), 1680–1685.