Optimization and Characterization of a Bioartificial Pancreas

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A novel method to treat type 1 diabetes is a bioartificial pancreas. The objective of this study was twofold: first determine an appropriate dose of a beta-cell poison, alloxan, to induce diabetes in the mouse model and then determine a cell concentration in alginate bead implants and alginate beads in macroconstructs to regulate blood glucose levels. The ability of the constructs to reverse the alloxan-induced diabetic state, as well as the design and material effects on the viability and function of entrapped βTC-tet cells, were studied *in vivo*. The data show that an alloxan dose of 75 mg/kg animal body mass is most appropriate to induce diabetes in the mouse model yet allow survival of the macroconstruct implantation surgery. Although alginate beads were successful in treating diabetes, they are not retrievable. The retrievable macroconstructs prolonged life; however, most mice survived at a hyperglycemic state. Future studies are needed to optimize materials, analyze serum protein levels, and add an NMR coil to noninvasively monitor and predict cell death in the construct.

INTRODUCTION

By the year 2030, over 438 million people globally will be affected by diabetes mellitus (Global Burden, 2009) and incidence continues to rise (Boyle et al., 2010). Type 1 diabetes (T1D) is caused by immune destruction of insulin-secreting β-cells, which are located in the Islets of Langerhans in the pancreas. At present, the standard treatment for T1D is strict dietary control, frequent blood glucose monitoring, and multiple daily insulin injections to regulate blood glucose levels. This therapeutic approach has improved the quality and lifespan for millions but is insufficient long-term; patients still may suffer from consequences of hyperglycemia such as blindness, limb loss, and cardiovascular disease. Treatments to replace insulin-secreting β-cells, through pancreatic or islet transplantation, show promise toward curing diabetes (Reach, 1992). However, the shortage of donor tissue and the need for continuous administration of immunosuppressive medication limit broad use of the transplantation approach. In response, we are constructing an implantable bioartificial pancreas that has the ability to sense glucose and secrete insulin appropriately, allowing for glucose regulation more similar to that afforded by an actual pancreas, and immunoprotected so as to avoid harsh lifelong immunosuppression.

Our flat-disk macroconstruct bioartificial pancreas design consists of insulin-secreting cells entrapped in alginate, a biocompatible biomaterial. Alginates are polysaccharides comprised of a linear chain of mannuronic and guluronic acid residues that gel upon interaction with divalent cations (Morch, Donati, Strand, & Skjak-Braek, 2006). The length and composition of the alginate influences its physical properties such as strength, durability, diffusion characteristics, and suitability as a matrix for cells (Skjak-Braek, Grasdale, and Smidsrod, 1989; Smidsrod, 1974). We believe our novel design can be integrated as an efficacious blood sugar regulator *in vivo*. In it, insulin-secreting βTC-tet cells are entrapped in alginate beads, mixed into an alginate plug (US and world patents: PCT/US2011/027921; WO2011/112822), and implanted into the peritoneal cavity of recipient diabetic mice.

Testing the bioartificial pancreas requires a predictable animal model. Inducing diabetes in animal models can be accomplished in a variety of ways, one of which is through chemical means (im Walde, Dohle, Schott-Ohly, & Gleichmann, 2002; Lenzen, 2008; Volland 2009; Szkudelski, 2001). To chemically induce diabetes in mice, beta-cell poisoning is necessary. Two diabetogenic chemicals are commonly used to destroy beta-cells: streptozotocin and alloxan. Its structure is composed of a nitrosourea moiety linked to carbon-2 of hexose (Lenzen, 2008; Szkudelski, 2001). Thus, part of the chemical resembles glucose, allowing it to be recognized by glucose transporter 2 (GLUT2) and to enter β-cells. Unpublished data from our laboratory show that STZ was inefficient at inducing diabetes in our mouse model. Moreover, STZ is harmful to the researcher and animal caregivers due to the toxicity of the compound and its excreted metabolites. Thus, it was not the optimal drug to chemically induce diabetes in our laboratory's mouse model.

Alloxan provides an acceptable alternative method to induce diabetes. Alloxan is structurally similar to glucose and hydrophilic and has a half-life of 1.5 minutes at 37°C, which can be extended with lower temperatures (Lenzen, 2008). Alloxan induces insulin dependent diabetes mellitus in animals through destruction of the islets' response to glucose (Lenzen, 2008; Szkudelski, 2001; Volland, 2009). Once injected, β-cells quickly metabolize the poison,

leading first to rapidly induced insulin secretion, followed by total suppression of β-cell response to glucose, just as in T1D (Lenzen, 2008; Szkudelski, 2001).

The purpose of this research was twofold: first determine an appropriate dose of alloxan to induce diabetes in our mouse model; second, through the use of alginate bead implants as well as alginate beads in macroconstructs, determine a cell concentration that best improves glycemic control in recipient diabetic animals.

METHOD

Cell Culture

Murine insulinoma βTC-tet cells (Fleischer et al., 1998) were provided by the laboratory of Shimon Efrat, Albert Einstein College of Medicine, Bronx, NY. Cells were cultured as monolayers in T-175 flasks and fed with fresh medium consisting of Dulbecco's Modified Eagle's Medium (DMEM) with 20mM glucose and supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin, and L-glutamine to a final concentration of 6mM (SIGMA, St. Louis, MO). Cultures were maintained at 37°C under humidified (5% CO_2 / 95% air) conditions, and appropriate media were completely replaced every 2–3 days (as dictated by the metabolic activity of the cultures). Cell passages 31–49 were used for these studies.

Mice

Female C3H/HeN mice were purchased from Charles River (Willmington, MA) for these studies. Experiments were performed according to the guidelines of the IACUC and ACS at the University of Florida. The mice were divided into experimental and control groups.

Beta-Cell Poisoning to Induce Diabetes

Mice were given one i.v. injection of alloxan (Sigma, St. Louis, MO) via the tail vein following an overnight fast to terminate β-cells. Doses tested were 50, 62.5, 75, and 100 mg/kg b.w. To determine the effect of β-cell destruction on glucose regulation, fasting (4 h) blood samples were taken daily from the tail vein, and glucose levels were measured using a OneTouch Ultra2 (LifeScan, Milpitas, CA) blood glucose monitoring system.

Pancreatic Construct Assembly

The macroconstruct is composed of various sized polydimethylsiloxane (PDMS) rings attached to one another (Fig. 1). Its cavity is filled with 2% low-viscosity high guluronic acid content alginate (LVG) to house βTC-tet insulinoma cells encapsulated in 3% low-viscosity high mannuronic acid content alginate (LVM) beads (Fig. 2). Ultrapure alginates form the gelled beads. Filling material was purchased from NovaMatrix (Oslo, Norway). PDMS constructs were deproteinized in a 10 M nitric acid solution bath for 30 minutes, and sterilized by autoclaving prior to use. Prior to *in vivo* studies, the completed macroconstruct was washed in phosphate buffered saline, soaked in Hank's buffered saline, and implanted into the recipient mouse less than 2 hours after assembly completion.

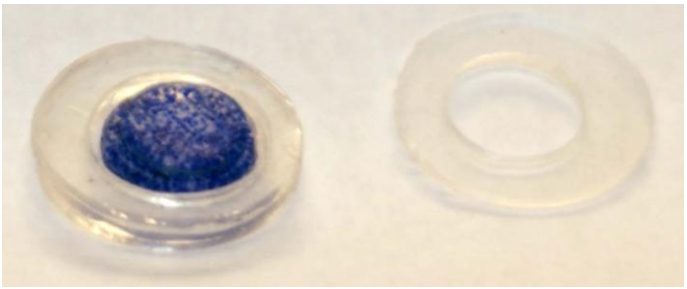

Figure 1. A PDMS/alginate bioartificial pancreas (top PDMS layer removed). Alginate beads that can contain insulin-secreting cells are stained blue.

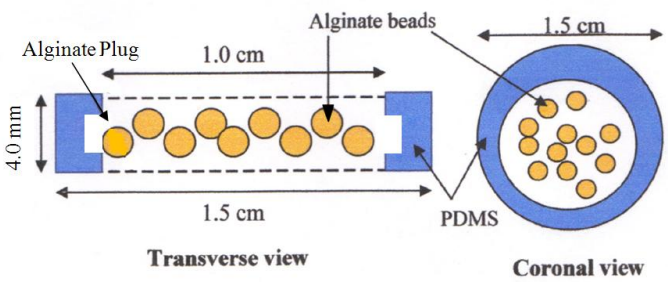

Figure 2. Schematic representation of a bioartificial pancreas. Insulin-secreting cells are entrapped inside the alginate beads, and the beads are entrapped in an outer layer of alginate (termed a plug here).

Animal Implantations

Implantation of constructs into recipient mice was performed via a midline celiotomy once mouse blood glucose levels exceeded 300 mg/dL for three consecutive days. Anesthesia was induced with 2% isoflurane (Minrad, Bethlehem, PA) inhalation (in oxygen), and maintained throughout the surgical procedure at 1.5% (in oxygen). Subcutaneous buprenorphine injections (0.05 mg/kg) were given prior to surgery and 24 h later to alleviate post-surgical pain. The constructs were placed into the peritoneal cavity and washed with Hank's saline containing 1% penicillin-streptomycin prior to closing. Additionally, diabetic mice not implanted with constructs were injected with an equal volume (160 µL) of βTC-tet cell-containing alginate beads or βTC-tet cells in Hank's solution for comparative analysis. Following treatment, animal mass and fasting blood glucose measurements were taken daily to monitor implant effectiveness and animal health. When

mice appeared distressed or weak, or lost 20% of initial body mass, they were euthanized and the construct was removed for histological analysis.

Alginate Bead Entrapment and Culture Propagation

Cells were entrapped in 3% LVM alginate beads. LVM has a low viscosity (325 mPa) and high (62%) mannuronic acid content. Alginate solutions were prepared by dissolving powdered LVM alginate into physiological saline (0.85% NaCl) at a 3% w/v concentration, then sterile filtering through a 0.22 micron membrane filter. Presence of sodium generates a more homogeneous gelled bead (Skjak-Braek et al., 1989). Cells were entrapped into 400–600 µm diameter alginate beads with an electrostatic bead generator (Nisco, Zurich, Switzerland). The cell density at the time of encapsulation was between $2x10^6$ and $7x10^7$ cells/ml alginate.

To reduce potential immune response to bovine sera in media, alginate beads were placed in Dulbecco's phosphate buffered saline (DPBS; Mediatech, Herndon, VA) supplemented with 20 mM HEPES, 20 mM glucose, 1% penicillin-streptomycin, and 100 nM dexamethasone. Beads were either used to manufacture the macroconstruct, or directly injected into the peritoneal cavity of diabetic mice less than 2 hours after bead creation. Recipient animal blood sugar and mass were monitored daily, as described above.

RESULTS

Alloxan Concentration for Diabetes Induction

Figure 3 demonstrates that alloxan selectively kills β-cells in a dose-dependent manner (Fig. 3A). A stable diabetic state in our mouse is defined as maintaining three consecutive days with a blood sugar above 300 mg/dl. According to this guideline, the alloxan injection of 50 mg/kg animal body mass did not induce frank diabetes in this mouse model. The 62.5 mg/kg alloxan dosage induces diabetes, but diabetes is not evident until day 2. In addition, mice receiving this dose survived the injection longer than mice injected with 75 mg/kg alloxan (Fig. 3B). Both the 75 mg/kg and 100 mg/kg alloxan dosages quickly induced diabetes in the mice; in addition to hyperglycemia, the mice became lethargic, urinated frequently, and lost body mass. However, the 100 mg/kg alloxan mice succumbed to the disease more than the 75 mg/kg mice (data not shown). Their considerable weight loss also did not make them suitable for the macroconstruct surgical implantation therapy. These results led us to use the 62.5 and 75 mg/kg doses in subsequent implantation studies.

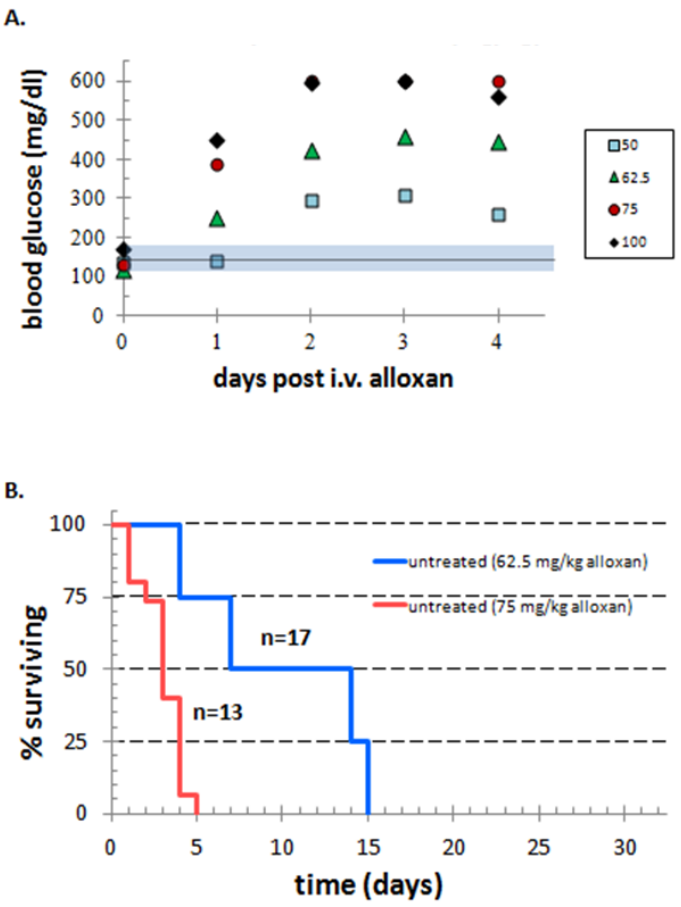

Figure 3. Induction of diabetes by alloxan injection. **3A.** The average blood glucose levels of mice rendered diabetic by i.v. alloxan injection in tail vein. Only surviving animals are considered in these calculations. Average and standard deviation blood sugar levels of non-diabetic mice are represented by the gray line and shaded region, respectively. **3B.** Kaplan-Meier survival curve of diabetic mice given either a 62.5 mg/kg or 75 mg/kg alloxan dose without any intervention.

Effect of βTC-tet Cell Injection in Diabetic Mice

To determine if insulin-secreting cells alone could reverse diabetes, alloxan-induced (75 mg/kg) diabetic C3H/HeN female mice were injected with a total of either $2x10^6$ cells (n=3) or $1.5x10^6$ cells (n=4) suspended in a Hank's buffered solution. None of the mice responded positively to either βTC-tet cell injection treatment, and all remained at a hyperglycemic state (400–600 mg/dl, data not shown). All mice that received the $1.5x10^6$ cell density died by day 11, except one which died at day 10 (Fig. 4). Mice with the greater cell density survived longer (50% dead by day 14 and all dead by day 21), but all remained in a diabetic state for the duration of the trial.

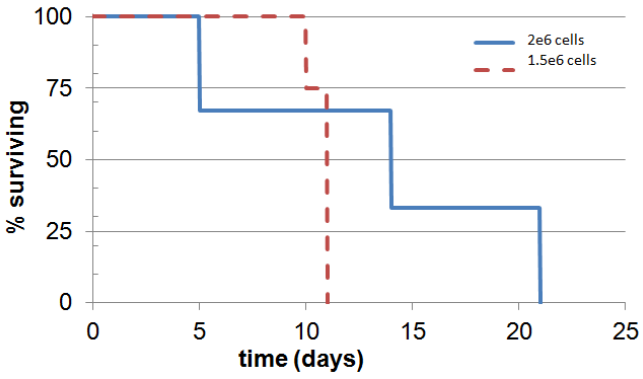

Figure 4. Kaplan-Meier survival curve of diabetic mice given different numbers of insulin-secreting βTC-tet cells, either $2x10^6$ total cells (solid blue line, n=3) or $1.5x10^6$ cells (dashed red line, n=4), in a Hank's solution.

Effect of βTC-tet 3% LVM Alginate Bead Injection in Diabetic Mice.

The efficacy of β-cell containing microbeads has demonstrated successful reversal of T1D in animal models (Soon-Shiong, 1992; Stabler, 2001; Stabler, 2002). To study this cell line and alginate combination, four different cell concentrations were encapsulated into 3% LVM alginate beads. The same number of cellularized microbeads were then injected into the peritoneal cavity of alloxan induced (75 mg/kg) diabetic mice (Fig. 5). Animals receiving $2x10^5$ (n=5) or $2x10^6$ total cells (n=7) had a higher *in vivo* survival rate than acellular controlled mice, but a lower *in vivo* survival rate than animals receiving concentration total of $3.5x10^6$ (n=8) or $7x10^6$ total cells (n=5). Additionally, fasting blood glucose was lowered by the injection of more encapsulated cells. The overall average fasting blood glucose level for mice given the $7x10^6$ total cells was 297 mg/dl. Animals receiving $3.5x10^6$ total cells responded similarly, with an average blood glucose of 299.2 mg/dl. Animals receiving $2x10^6$ total cells had a fasting blood glucose average of 396.5 mg/dl.

Effect of βTC-tet Concentration in Bioartificial Pancreatic Construct in Diabetic Mice

Bioartificial pancreatic constructs, or macroconstructs, were manufactured to contain a 2% LVG alginate plug that housed βTC-tet cells encapsulated in 3% LVM alginate beads (Figs. 1 & 2). Figure 6 demonstrates that diabetic mice implanted with constructs containing a total of $7x10^6$ βTC-tet cells (n=12) survived longer than those receiving constructs with a total of $3.5x10^6$ βTC-tet cells (n=4). Half of the animals receiving the lower cell number died by 5 days post implantation, and all had died by day 14. The higher cell concentration macroconstruct had a 50% survival of around 32 days, with the last succumbing at day 70. Fasting blood glucose levels in mice ranged from normal to hyperglycemia, but mice appeared healthy and free of diabetic symptoms (polyuria, weight loss, etc.) for extended periods.

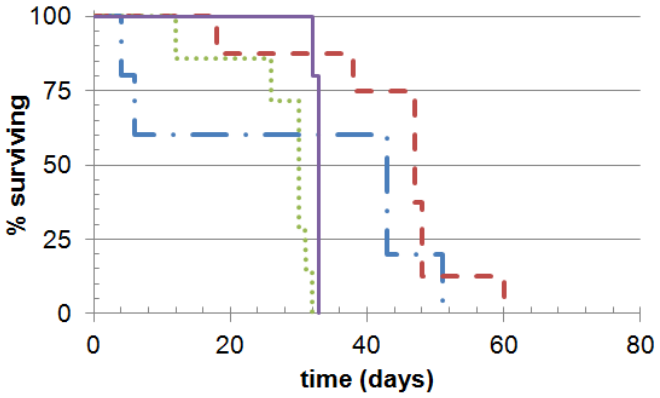

Figure 5. Kaplan-Meier survival curve of diabetic mice treated with different numbers of βTC-tet cells encapsulated in alginate beads. Total encapsulated cells delivered were: $2x10^5$ (solid purple line, n=5), $2x10^6$ (dotted green line, n=7), $3.5x10^6$ (dashed red line, n=8), and $7x10^6$ total cells (large dashed blue line, n=5).

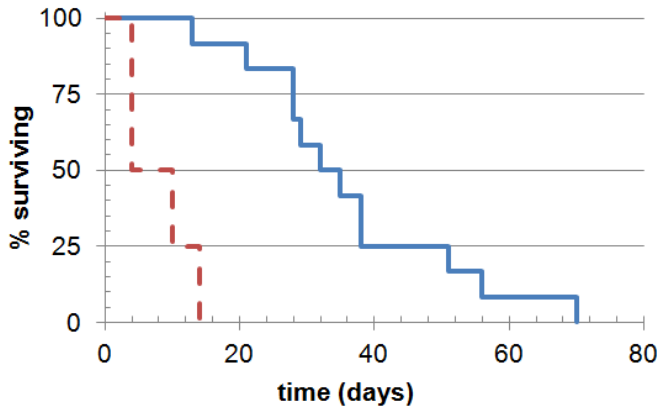

Figure 6. Kaplan-Meier survival curve of diabetic mice given macroconstructs containing either $7x10^6$ total cells (solid blue line, n=12) or $3.5x10^6$ total cells (dashed red line, n=4). All mice were induced with diabetes with a single i.v. injection of 75 mg/kg alloxan and implanted with the macroconstruct three days later.

DISCUSSION

We set out to find a dosing model for inducing stable T1D in mice and to test a bioartificial pancreas in said mice. The alloxan injection successfully induced diabetes in a dose dependent manner. As the dosage increased, the mice experienced a higher degree of diabetes. The 3% LVM-alginate beads in the 2% LVG macroconstruct design successfully extended life in the diabetic mouse.

In most mouse models, the alloxan dosage required to induce frank diabetes is less than what we required with the C3H/HeN animal model (im Walde et al., 2002). Reasons for this difference are likely due to strain differences in GLUT2 cellular levels in β-cells or enhanced oxygen scavenging machinery in the less sensitive strains. Mice

that received the lowest dose (50 mg/kg) did not become diabetic, and mice that received the highest dose (100 mg/kg) lost too much weight to survive the surgical treatment. The 75 mg/kg dosage was used for diabetes induction because it destroyed enough β-cells, as shown by the short survival rate of 5 days in untreated mice, and also left mice physically able to survive the surgery.

Injecting βTC-tet cells in Hank's solution did not reverse diabetes in mice. This demonstrates that the cells alone are not sufficient to reverse frank hyperglycemia. When $1.5x10^6$ cells were injected into the peritoneal cavity, all of the mice succumbed to the disease by day 11 (Fig. 4). More cells did allow for greater longevity, but glucose control was not observed. In this animal model, the cells are syngeneic to the recipient, so no immune issues will occur. If the insulin-secreting cell system was allogeneic, the exposed cells would be open for immune attack. In addition, the cells are not retrievable. Moreover, they are fragile, and may not withstand the rigors of implantation.

Encapsulating the syngeneic βTC-tet cells in alginate beads and injecting these beads into the peritoneal cavity confers the necessary protection for cells (Reach, 1992). The alginate bead allows for excellent diffusion, so the βTC-tet cells detect when glucose is present and produce the appropriate amount of insulin for the body, just as a physiologically normal β-cell is programmed to do. In addition, the alginate bead protects the cell from being detected, circumventing an immunological response, which would cause the therapy to fail. In fact, allogeneic implantations of alginate encapsulated pig islets have been successful in rodent and monkey animal models (Cui et al., 2009; Dufrane, Goebbels, and Gianello, 2010).

Beads that had $3.5x10^7$ total cells arguably survived longer than beads with $7x10^7$ total cells. This difference may be attributed to cell density—when beads have a higher cell density, cells closer to the surface of the sphere will receive more delivery of nutrients and oxygenation than cells closer to the center of the bead. These inner cells will not be as viable and will eventually die. Apoptosis may signal nearby βTC-tet cells to die, and once enough cells in the bead die, the immune system may respond to beads, leading to therapeutic failure. The less dense bead ($3.5x10^7$ cells) had a longer lasting effect, showing that this is a more appropriate concentration for alginate beads to balance βTC-tet cell survival and correct diabetes in the mouse. The cell density issue may be more important in allogeneic models.

Although the alginate bead injection therapy successfully cures diabetes, at least temporarily, beads cannot be retrieved from the body. At the clinical level, it would be ideal to have a long-lasting therapy for patients with diabetes that can be implanted and later replaced by simple surgical removal. This is not possible with beads, but a macroconstruct is easily retrievable. βTC-tet cells are encapsulated in 3% LVM alginate beads because this material has been shown to maximize viability by allowing cellular growth, nutrient exchange, and oxygenation (Simpson, 2005). The beads are then mixed into a 2% LVG alginate plug to inhibit cellular overgrowth if they grow out of the LVM beads. The LVG provides a stronger structure for the implant due to the high-guluronic composition (Draget & Taylor, 2011). It also provides potential protection from the immune system (Simpson, 2004; Simpson, 2005).

Cell density plays a role in function of the construct. Mice that received a construct with $7x10^7$ cells/ml ($7x10^6$ total cells) survived longer than mice that received a construct with a density of $3.5x10^7$ cells/ml (Fig. 6). In the construct, beads do not travel freely throughout the body and are kept tightly together in the LVG alginate plug. The embedded beads experienced fewer nutrients and oxygen delivery, so the therapy needed more cells to compensate, a reason why the higher cell concentration and number works in macroconstructs. Although the lifespan was greatly increased compared to the untreated alloxan-induced mice, only some mice had blood glucose levels in the normal glycemic range; clearly, this approach needs refining. Still, mice surviving the hyperglycemic range did not experience any other diabetic symptoms.

It was noted that animals given constructs made with cells of earlier passage numbers survived more frequently than animals given cells of later passage numbers. This study was not of sufficient statistical power to address this outcome. Although this aspect was not fully studied and is only an observation, one key investigation for the future would be to compare early passage number cell lines to later passage cell lines to determine which are more appropriate for inclusion. One problem with cell lines being passaged for long periods is that when under *in vitro* culture, genetic drift can occur. These changes can be insignificant to the function of the cell, or critical to the performance of the implant into which they are embedded. Cell passage studies are needed to examine how an earlier or later cell line will affect βTC-tet cell viability *in vivo*.

The 3% LVM-alginate beads in the 2% LVG macroconstruct design successfully extended life in the diabetic mouse. Glucose levels were controlled in some mice, but the approach has not been perfected. Work to be done includes improving the construct, optimizing the alginate material (e.g., variety and concentration of alginate), and improving the alginate gelling condition. Indeed, studies looking at effects of alginate type and gelling solutions have found these aspects influence the strength and longevity of the alginate (Skjak-Braek et al., 1989). This, in turn, can affect the growth of the embedded cell (Simpson et al., 2004).

To correlate success or failure of the implant with physiological changes, a number of assays testing the physiological state of the animal are needed in addition to the blood glucose monitoring. For example, if a blood serum assay shows presence of insulin or C-peptide, but the mouse is alive and appears healthy at a hyperglycemic

level of 500 mg/dl, we can determine that the mouse has developed type 2 diabetes, or a resistance to insulin released by the implant, and our implant is not the cause of glycemic dysregulation. Additionally, blood taken from an animal at the time of euthanasia may indicate that kidney or liver failure was the reason for the failure.

Future studies also will work towards inclusion of an NMR coil to monitor and predict cell failure so the construct can be non-invasively monitored *in vivo*, and replaced in a timely manner if the implant is showing signs of structural instability, loss of cellular viability, or immune attack. Due to our novel macroconstruct design, the NMR coil can be easily integrated into the PDMS ring surrounding the alginate plug (Volland, 2009). The importance of monitoring tissue-engineered approaches to treat disease cannot be overstated (Simpson & Sambanis, 2012).

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