

The Effects of Exenatide on the Autoimmune Development of Diabetes

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by

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Abstract

Autoimmune diabetes is a chronic disease that occurs when the pancreas does not produce the insulin required to properly control blood glucose levels. Also known as type 1 diabetes, this disease is caused by an autoimmune attack of a person's T-cells on islet β cells.

Exenatide, an incretin mimetic, is an analog to Glucagon-like peptide-1 (GLP-1). GLP-1 has many actions that improve glucose homeostasis such as potentiation of glucose-stimulated insulin secretions, promotion of β cell proliferation and survival, and inhibition of glucagon secretion. Some studies have shown that exenatide and related compounds can protect β cells from apoptosis as well as block the migration of CD4+ T-cells to islets. Therefore, we hypothesize that exenatide may prevent the autoimmune development of diabetes in the non-obese diabetic (NOD) mouse model.

Thirty NOD mice were split into two groups. Beginning at 6 weeks of age, Group 1 (n=15) was given a 100ng dose subcutaneous of exenatide once daily and Group 2 (n=15) was given a dose of saline once daily. Glucose levels were monitored twice per week and animals were considered diabetic when blood glucose levels exceeded 300mg/dl. Once animals were considered diabetic, the pancreas was removed for histological analysis by staining for CD4+ T-cells and insulin.

By 30 weeks of age, 76.9% in Group 1 (exenatide) remained free of diabetes compared to 13.3% of animals in Group 2. Immunohistochemical staining revealed higher concentrations of insulin producing cells and lower concentrations of CD3+ T-cell presence in treatment mice from Group 1.

Based on the results from this study, it appears that exenatide reduces the incidence of diabetes in NOD mice. Animals treated with exenatide showed reduced blood glucose levels compared to those receiving saline, while immunohistochemical staining revealed insight into the possible mechanisms by which exenatide may function. From this study, it can be concluded that exenatide is an effective preventative treatment for the autoimmune development of diabetes.

To further study the mechanisms by which exenatide works, a second study was proposed. In this study, we hypothesize that one mechanism by which exenatide delays or prevents the onset of type 1 diabetes is by modulating the migration of T-cells to the pancreas, thus preventing islet beta cell destruction and subsequent development of the disease.

Seventeen NOD mice were split into two groups. Group 1a (n=9) received a 100ng dose subcutaneous daily of exenatide while Group 2a (n=8) mice received a 100ul subcutaneous dose of saline daily. Glucose levels were monitored once per week and animals were considered diabetic when blood glucose levels exceeded 300mg/dl.

Mice expressing the luciferase gene have proven useful in research due to the ability to image their cells/tissue using an in vivo imaging system (IVIS) that detects the luminescence produced after administration with the enzyme substrate luciferin. Isolated splenocytes from NOD L2G85 luciferase positive mice were transferred to the study group mice at individual euthanization time points. Bioluminescence imaging was used 3 days following T cell transfer in order to visualize the location and/or pathway of the T cells.

Average photons in each experimental group were measured within a region of

interest (ROI) at the approximate location of the pancreas using IVIS equipment. The exenatide group was found to have an average of 1.190×10^5 photons whereas the saline group was found to have an average of 1.445×10^5 photons. Bioluminescence imaging did show some reduction in the presence of transferred T-cells at the pancreas of exenatide-treated compared to saline mice. Although the difference in measured photons is not significant between the two groups, we do not discredit our hypothesis. We believe that the experiment should be repeated with modifications to enable a better analysis of our results.

Introduction

Autoimmune diabetes, otherwise known as type 1 diabetes or juvenile diabetes, is a chronic disease that occurs when the pancreas does not produce the insulin required to properly control blood glucose levels. Autoimmune diabetes is commonly diagnosed in children, adolescents, and young adults after symptoms such as extreme fatigue, thirst, increased urination, and weight loss, arise. The exact cause of autoimmune diabetes is unknown, but is thought to be a result of an environmental trigger that causes an overactive immune response in combination with genetic susceptibility. Production of various autoantibodies, including islet cell antibody, islet cell protein tyrosine phosphatase, and glutamic acid decarboxylase, may contribute to an individual's risk for the disease. The overactive immune response attacks islet β cells, destroying them and rendering the individual incapable of producing insulin. Islets are clusters of cells that make up approximately 1-2% of the pancreas. About 70-80% of the cells within these clusters are beta (β) cells which produce insulin.

Insulin is released in response to increased levels of glucose in the blood due to ingestion of food. Insulin initiates a signal transduction, which increases glucose uptake and storage, therefore decreasing blood glucose levels. Individuals lacking those insulin producing β cells, type 1 diabetics, are unable to control their blood glucose levels internally and consequently require daily insulin therapy to survive. Type 1 diabetics also need to monitor their diet and exercise while monitoring blood glucose levels often.

Lack of diabetes control may lead to various complications that are both burdensome and life-threatening. Diabetics with poor control are at a higher risk for

cataracts, hypertension, high cholesterol, damaged blood vessels, nerve damage, kidney disease, heart disease, stroke, blindness, and even death. Although treatment for type 1 diabetes is currently available, a preventative treatment that would significantly lower the incidence of diabetes, and these previously mentioned risks resulting from diabetes, has yet to be discovered.

Exenatide is a synthetic version of exendin-4, a hormone found in the saliva of the Gila monster (*Heloderma suspectum*) that was first isolated by Dr. John Eng in 1992. Exenatide is an analog to Glucagon-like peptide-1 (GLP-1). GLP-1 improves glucose homeostasis by inducing glucose-dependent stimulation of insulin secretion while suppressing glucagon secretion (1,2).

Some studies have shown that exenatide and related compounds can protect β cells from apoptosis. *In vitro* analysis of INS-1 cells has shown complete abrogation of apoptosis following pre-treatment with exenatide. The same study also showed a decrease in the oxidative stress-inducing thioredoxin interacting protein (TXNIP) and the apoptotic factors caspase-3 and Bax after *in vivo* treatment with exenatide (2).

In vitro studies have also shown that exenatide reduces expression of JAK-1 and STAT-1, which mediate transcriptional effects of interferon- γ (IFN- γ) involved in beta cell apoptosis in the autoimmune development of type I diabetes. Microarray analysis and quantitative real-time PCR showed decreases in JAK-1 and STAT-1 expression in both INS-1 cells and isolated human islets after co-culture with exenatide (3).

Furthermore, studies have shown that exenatide and related compounds can stimulate the proliferation and differentiation of insulin-secreting β -cells (4,5). In addition, a compound related to exenatide, a dipeptidyl peptidase IV (DPPIV) inhibitor MK0431,

has been previously shown to modulate T-cell migration to islet cells (6).

Exenatide is currently an injectable prescription medicine manufactured by Amylin Pharmaceuticals and Eli Lilly and Company. It was approved by the FDA in April of 2005 for use by type II diabetics to better control blood sugar levels.

Methods

Exenatide, supplied by Amylin Pharmaceuticals and Eli Lilly and Company, was diluted in saline to obtain appropriate dosage of an injectable medication. Multiple dilutions were performed to result in a final concentration of 100ng exenatide/0.1mL saline. Exenatide solutions were then stored at -20°C.

The effects of exenatide were tested using the non-obese diabetic (NOD) mouse model. The NOD mouse is genetically predisposed to developing diabetes and is currently the only animal model for autoimmune diabetes. Female NOD mice develop diabetes earlier and at a higher incidence (90-100% by 30 weeks of age) than male NOD mice (40-60% by 30-40 weeks of age). Thirty female NOD mice were split into two groups: treatment (group 1) versus control (group 2). Beginning at 6 weeks of age, group 1 (n=15) was given a 100ng subcutaneous dose of exenatide once daily (0.1ml volume) while group 2 (n=15) was given a 0.1ml dose of saline daily. Glucose levels were monitored twice per week by snipping the animals' tails and obtaining approximately 10µl of blood to be read on a standard glucometer (Ascensia Elite®, Bayer). Animals were considered diabetic when two consecutive blood glucose levels exceeded 300mg/dl. Once animals were considered diabetic, the animal's pancreas was removed for immunohistochemical analysis by staining for insulin and CD3+ T-cells.

Results

Diabetes progression was measured from the primary study in order to analyze the effects of exenatide on the autoimmune development of diabetes. It was found that group 1 (treatment) had 76.9% of animals free of diabetes by 30 weeks of age whereas group 2 (control) had 13.3% of animals free of diabetes by 30 weeks of age (Figure 1).

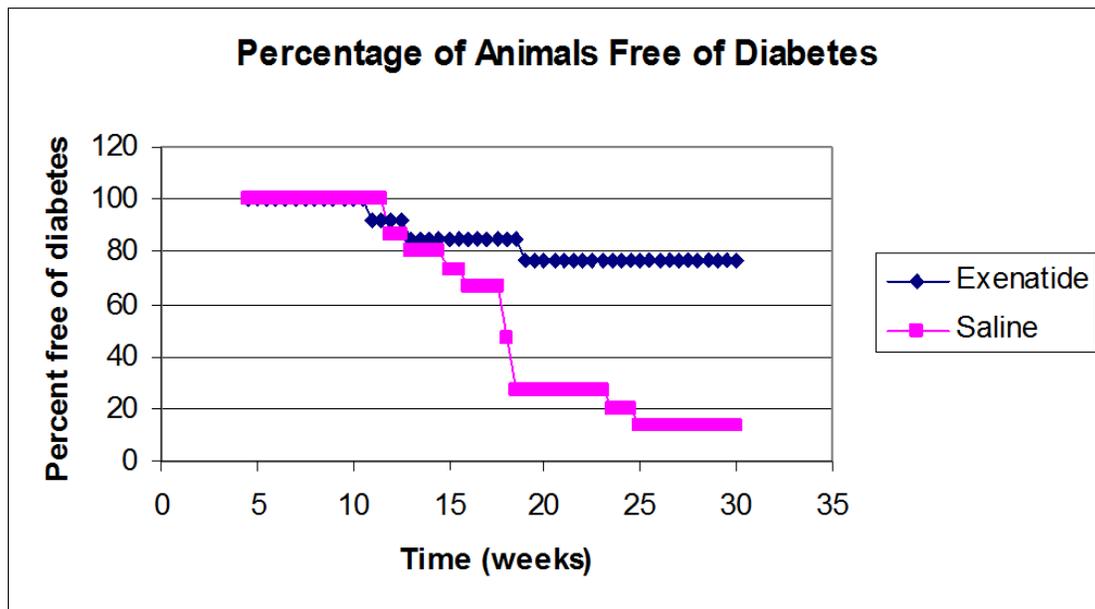


Figure 1. Diabetes progression, showing percent of animals free of diabetes. Animals were considered diabetic when blood glucose levels exceeded 300 mg/dl for two consecutive measurements. Group 1 (treated with exenatide) had 76.9% of animals free of diabetes at 30 weeks of age, with disease diagnosis at 3 time points between 11 and 19 weeks. Group 2 (treated with saline) had 13.3% of animals free of diabetes at 30 weeks of age, with disease diagnosis at various time points between 12 and 30 weeks.

Immunohistochemical staining on the pancreas was performed once animals were considered diabetic in order to gain insight into the mechanisms of exenatide. Although the data could not be quantified, it was observed that untreated animals (Figures 2a and 2b) had higher incidence of CD3+ stained islet cells than treated animals (Figure 2c).

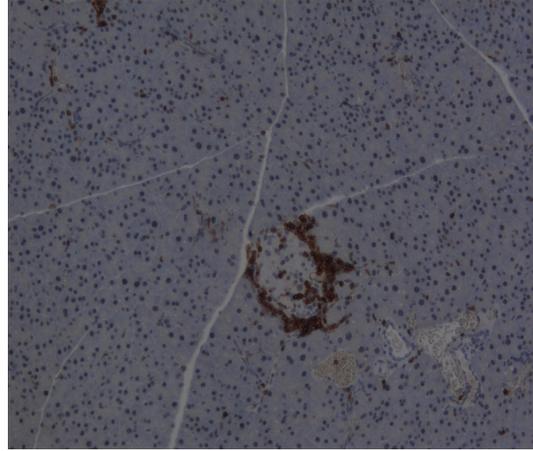
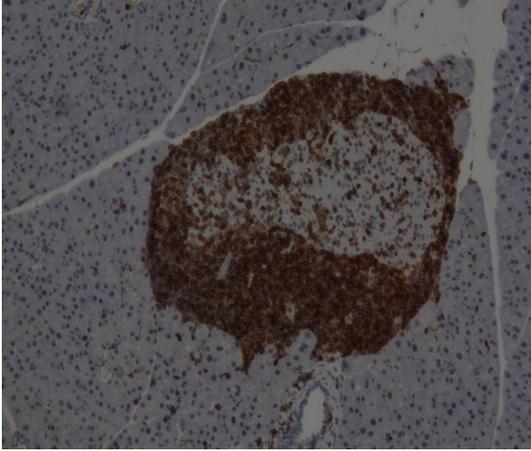


Figure 2. CD3 stained islet beta cells from the pancreata of diabetic mice. Figure 2a shows an islet beta cell from mouse 5.1 (saline control animal) and Figure 2b shows a beta islet cell from mouse 4.4 (saline control animal). Both cells are CD3+ illustrating attack of islet beta cells by CD3 T-cells. Each mouse developed autoimmune diabetes due to the destruction of insulin producing islet beta cells.

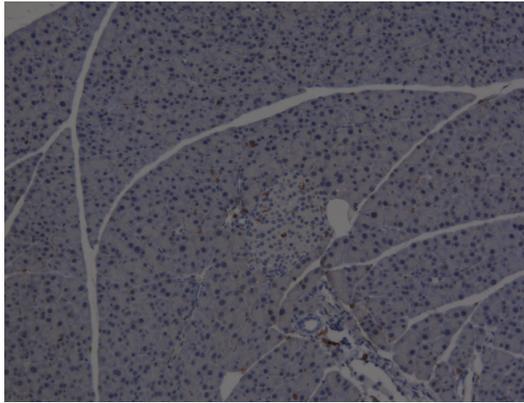


Figure 2. Figure 2c shows an islet beta cell from mouse 1.4 (exenatide-treated animal). This islet beta cell is CD3- illustrating no attack of by CD3 T-cells. This mouse did not develop diabetes presumably due to the protection of islet beta cells by treatment with exenatide.

Methods continued

Upon successful completion of the primary study in addition to the validation of our hypothesis, another study was proposed. The mechanism of action of exenatide was tested also using the non-obese diabetic (NOD) mouse model. Seventeen female NOD mice were split into two groups: treatment (group 1a) versus control (group 2a). Beginning at 6 weeks of age, group 1 (n=9) was given a 100ng subcutaneous dose of exenatide once daily while group 2 (n=8) was given a 0.1ml dose of saline daily. Glucose levels were monitored once per week to track diabetes progression and animals were considered diabetic when two consecutive blood glucose levels exceeded 300mg/dl.

We chose a euthanization and imaging time point of 15 weeks, due to the fact that most NOD mice develop diabetes anywhere from 12 to 30 weeks of age. At 15 weeks of age, splenocytes were isolated from luciferase-positive NOD L2G85 mice. Isolated spleens were mechanically digested to a single cell suspension. Viable splenocytes were counted using trypan blue, and approximately 25 million isolated T-cells were transferred to the experimental groups by tail vein injection. Three days post transfer, each mouse was individually anesthetized with isoflurane, given a 150 μ l dose of RediJect D-Luciferin (Caliper Life Sciences) intraperitoneal., and imaged ex vivo using the IVIS equipment. The abdomen of each mouse was opened for imaging and the pancreas located so that we could visualize the specific location of the transferred cells. Once images were obtained, the mice were euthanized.

Images were analyzed by creating a region of interest (ROI) at the approximate location of the pancreas in each mouse image. The photons were then measured from this ROI using a standardized scale for luminescence and recorded. An average was obtained

for both treatment and control groups for the photons measured within the ROI as well as the glucose levels obtained immediately prior to imaging.

Results continued

In the secondary study, the presence of T-cells at the pancreas of treated versus untreated animals was quantified by using bioluminescent imaging. An average number of photons from transferred luciferase-positive splenocytes at the pancreas was measured in each group as well as average blood glucose levels obtained immediately prior to imaging for comparison. The exenatide group was found to have an average of 1.190×10^5 photons whereas the saline group was found to have an average of 1.445×10^5 photons (Figure 3). The p value for this data was found to be $p=0.05$. Average blood glucose measurements obtained immediately prior to imaging show an average blood glucose level of 197mg/dl for the treated group and 287mg/dl for the untreated group (Figure 4). The p value for this data was found to be $p=0.2$. Of important note, the p values that were obtained for the data represented in Figures 3 and 4 exclude one exenatide-treated animal that had a low blood glucose and high number of photons. The inclusion of this animal in the analyses skewed the data, giving a p value of $p=0.4$ for the blood glucose measurement comparison and a p value of $p=0.2$ for the comparison of photons measured. Figures 5 and 6 show representative bioluminescent images obtained from a treated and control animal respectively.

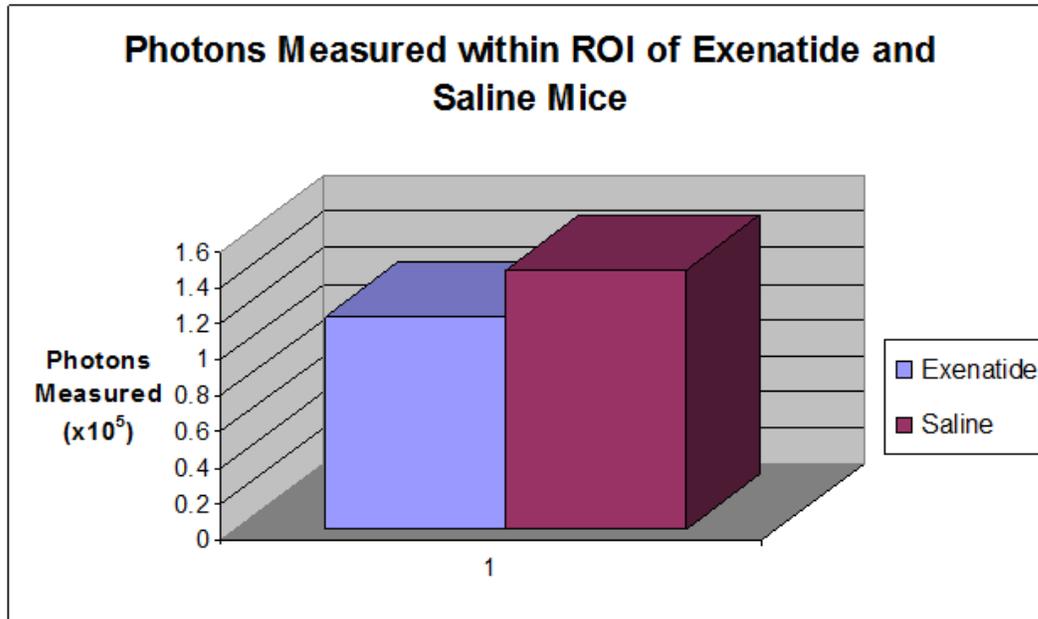


Figure 3. Average photons measured within region of interest (ROI) of exenatide (treated) and saline (control) mice. Images were taken by IVIS equipment 3 days following transfer of approximately 25 million luciferase-positive splenocytes. Data analysis yielded a p value of $p=0.05$

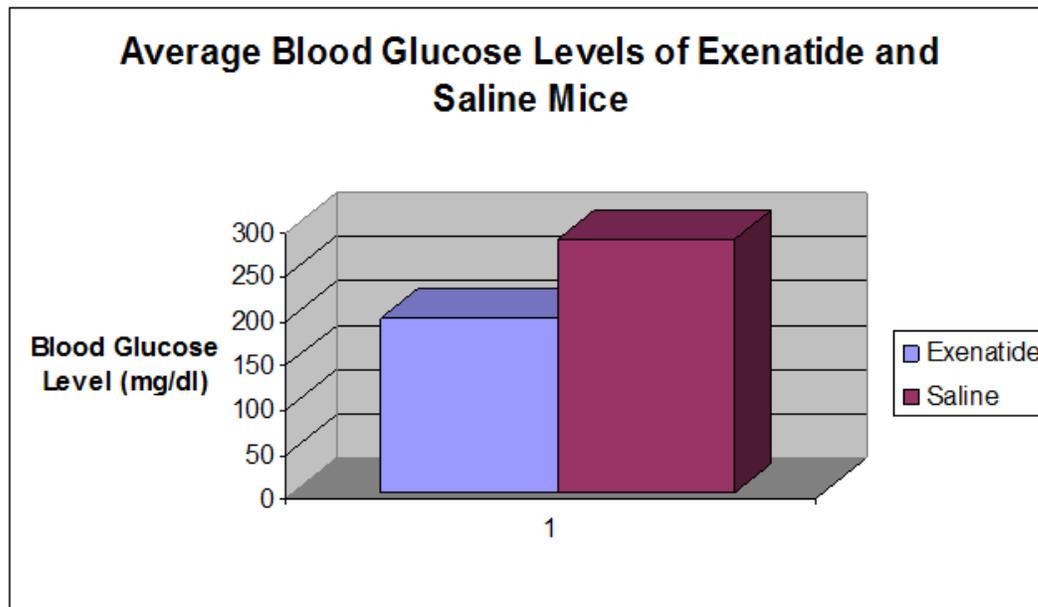


Figure 4. Average blood glucose levels of exenatide (treated) and saline (control) mice. Blood glucose levels were measured immediately prior to luminescence imaging via IVIS equipment. Data analysis yielded a p value of $p=0.2$

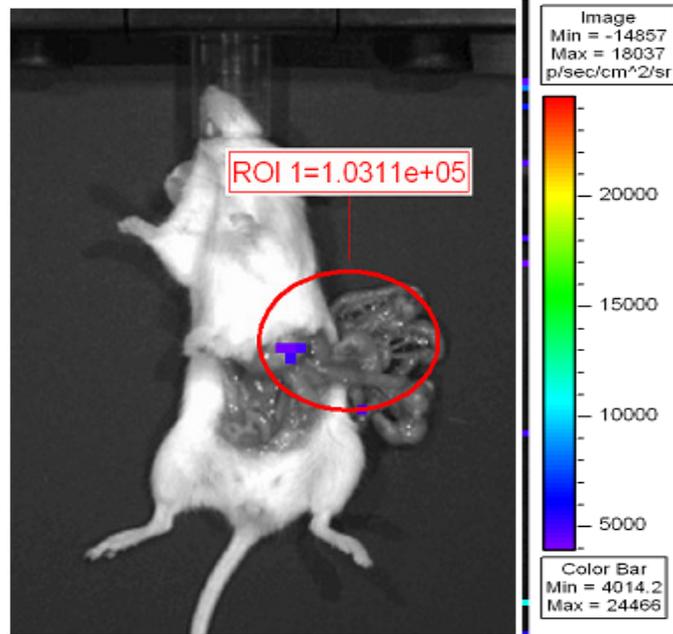


Figure 5. IVIS image taken from exenatide cage # 1 mouse #1. Photons measured in the ROI were observed to be 1.0311×10^5

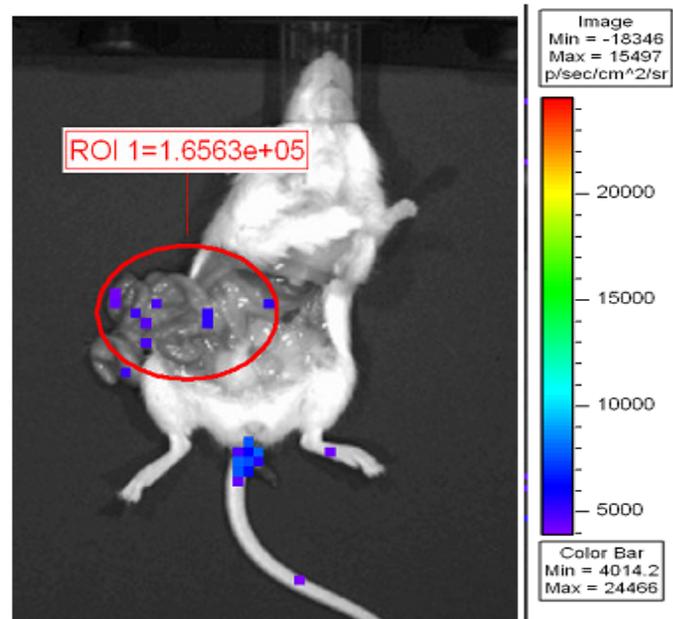


Figure 6. IVIS image taken from saline cage #3 mouse #3. Photons measured in the ROI were observed to be 1.6563×10^5

For saline mice, regression analysis showed a positive linear relationship with an R-squared of 41.7% (Figure 7). One exenatide-treated mouse had a low blood glucose and high number of photons, and this skewed the data from this group. With the inclusion of

this animal in the regression analysis, the R-squared value was only 6.6%. However, when this outlier was removed from analysis, there also appeared to be a positive linear relationship between blood glucose levels and number of photons in the exenatide group as well with an R-squared of 48.8% (Figure 8). These correlations are relatively weak, but there still seems to be a relationship between the data.

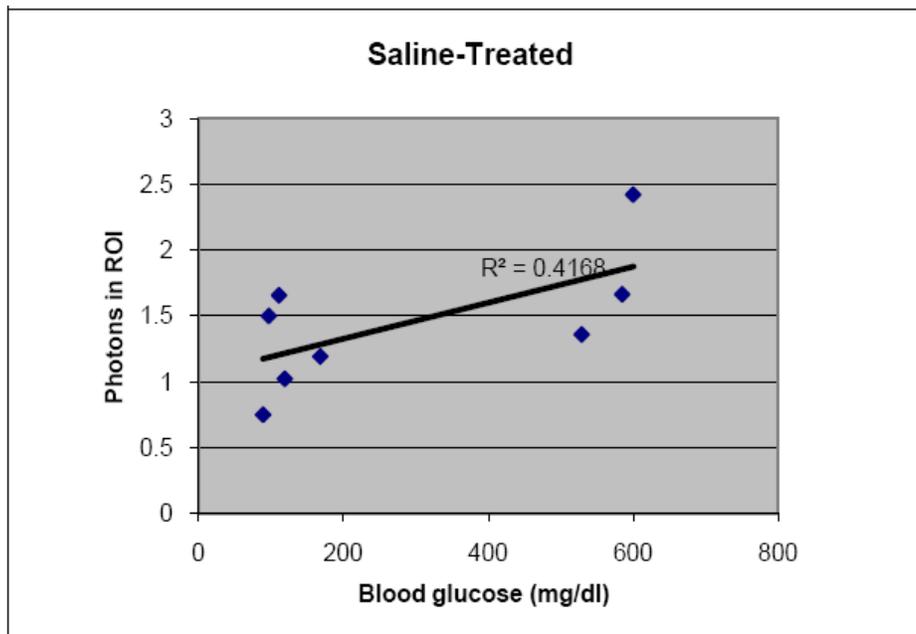


Figure 7. Regression analysis of saline-treated mice showed a positive linear relationship of photons within the ROI and blood glucose level with an R-squared of 41.7%

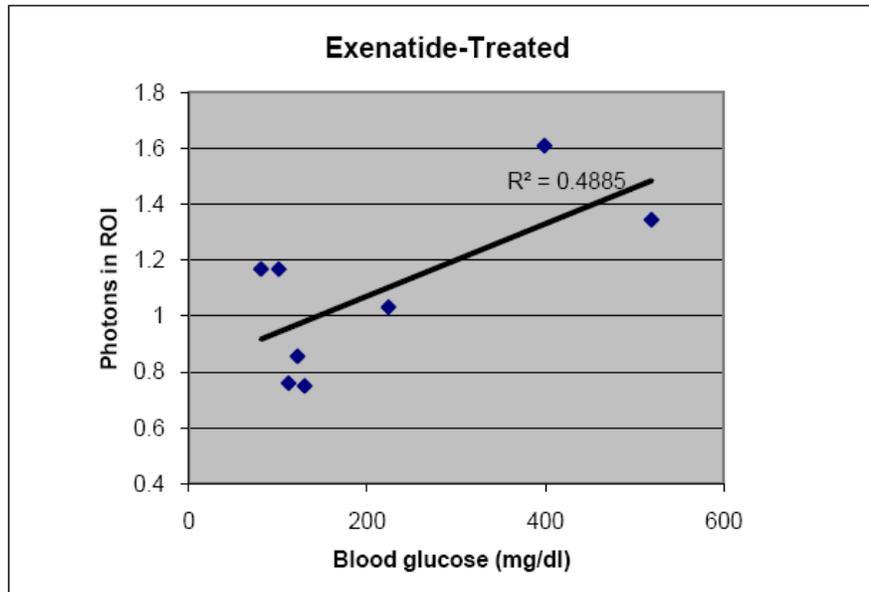


Figure 8. Regression analysis of exenatide-treated mice, with removal of the outlier, showed a positive linear relationship of photons within the ROI and blood glucose level with an R-squared of 48.8%

Discussion

Our primary study shows that exenatide significantly reduces the incidence of diabetes in NOD mice (Figure 1). Animals treated with exenatide showed reduced blood glucose levels and reduced levels of T-cells in immunohistochemical staining of the pancreas compared to those receiving saline (Figure 2). This led our laboratory group to perform a secondary study to evaluate the possible mechanism of action of exenatide in reducing the incidence of diabetes. A study published in 2009 by another laboratory showed that the DPPIV inhibitor MK0431 was able to modulate the migration of T-cells to islets in transplanted NOD mice (6). DPPIV inhibitors are a class of compounds that are related to the GLP-1 agonists (including exenatide). DPPIV is a natural enzyme in the body that degrades GLP-1. Thus, DPPIV inhibitors result in increased levels of GLP-1. Treatment with exenatide and other GLP-1 agonists results in high levels of exogenous GLP-1. We hypothesized that, like the related DPPIV inhibitor, exenatide may also modulate T-cell migration to the pancreas and therefore be able to reduce destruction of islets by T-cells. To test this, we transferred luciferase-positive NOD T-cells into treated or control NOD mice and used bioluminescent imaging to track the migration of these cells.

Our secondary study shows that bioluminescent imaging did show some reduction in the presence of transferred T-cells at the pancreas of exenatide-treated animals compared to controls (Figure 3). However, the difference was not significant. To test the correlation between presence of T-cells at the pancreas and blood glucose levels, blood glucose measurements were taken immediately prior to imaging. Higher average blood glucose levels in saline mice correlated with a higher average number of photons at the

region of interest (ROI), while lower average blood glucose levels in exenatide-treated mice correlated with a lower average number of photons at the region of interest (ROI).

Although our secondary study did not produce significant results, we do not discredit our hypothesis. Many difficulties arose throughout the duration of the experiment that may have negatively affected our data. Given that diabetes diagnosis time points are varied, and therefore unknown for any specific mouse, visualization of T-cells in the ROI is extremely difficult to obtain. By only imaging our mice at 15 weeks of age, we were extremely limited with our analysis of the hypothesis. In addition, the experimental groups used for this study were small. In order to address these concerns, experiments should be repeated with modifications for a better analysis of our hypothesis.

We suggest that our next steps should be to repeat the secondary experiments using larger experimental group sizes as well as incorporating additional time points of imaging and euthanization. We also suggest obtaining in vivo images until the presence of T-cells is observed, in which we would then proceed to obtain ex vivo images. By adding additional imaging and euthanization time points and/or primarily obtaining in vivo images before proceeding to ex vivo images, we would allow a larger window of time to obtain appropriate images at various points of diabetes progression. We strongly believe that these modifications would yield significant results.

The effects of exenatide on the autoimmune development of diabetes has multiple positive consequences for many individuals in our world today. While effective treatment for autoimmune diabetes is well-known, no preventative measures exist. Exenatide may prove to be a prospect for this preventative treatment. Though many issues have been revealed for the application of exenatide in a clinical setting, we strongly believe that this

preventative treatment will substantially change how type I diabetes is approached in the future.

Type I diabetes is generally diagnosed once the patient is already in critical condition, when approximately 90% of the islet beta cells are already destroyed. The first weeks of a diabetes diagnosis, typically known as the “honeymoon phase”, is a primary target for successful application of exenatide. When islet beta cells are still present, we believe that rapid treatment with exenatide may protect the remaining islets and subsequently prevent the development of autoimmune diabetes. If this application were going to be used, further studies should be done to determine appropriate time points for effective treatment. A potential study could include the use of NOD mice, in which blood glucose levels would need to be measured twice per week, and treatment with exenatide or control treatment with saline would need to be initiated at the time of initial diabetes diagnosis. Results from a study such as this would produce a better understanding of this potential application of exenatide.

In addition, we believe that the possibility for genetic testing for specific markers or autoantibodies may assist with identifying those at risk for development of the disease. This in addition to a family history of diabetes, or any autoimmune condition, may present substantial reason to believe that the particular individual is largely at risk for developing the disease. Again, this may prove to be a target for the clinical application of exenatide.

As previously mentioned, treatment for type 1 diabetes is currently available, but a preventative treatment that would significantly lower the incidence of diabetes, and the risks associated with diabetes, has yet to be discovered. Our studies outlined in this thesis show that exenatide significantly reduces the incidence of diabetes in NOD mice by a

mechanism that reduces the presence of transferred T-cells at the pancreas of exenatide-treated animals compared to controls. This shows that exenatide is involved in protection of islet beta cells from an autoimmune attack of T-cells, subsequently preventing the development of type I diabetes. Therefore, we believe that exenatide may prove to be useful in future clinical applications involving preventative treatment of type I diabetes.

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