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The Development of an Inquiry-Based Laboratory Module Exploring the Pathophysiology of Diabetes

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Abstract: Histotechnology is commonly used in medical research, pathological testing, and pharmaceutical development. We designed a three-week, inquiry-based laboratory module that helps prepare students for biomedical careers by teaching them tissue sampling, processing, and imaging. Rats were treated with streptozotocin (a known diabetogen) while control rats were injected with buffer solution. Rats were sacrificed one week following treatment. Pre- and post-injection weights were compared and blood samples were collected for glucose analysis and insulin determinations using an enzyme-linked immunosorbent assay (ELISA). Pancreatic tissue was collected, preserved in Bouin's fixative, embedded in paraffin, and sectioned using a microtome. Students then performed hematoxylin/phloxine staining. The number of islet beta cells were compared between control and treated rats. Blood glucose measurements demonstrated that treated rats had significantly higher blood glucose levels and lower beta cells numbers, while the ELISA tests indicated that treated rats had lower blood insulin concentrations. Following this module, students presented an individual poster with images and quantitative data analyses that included insulin concentrations, blood glucose levels, and histological images of pancreatic islets, in addition to beta cell quantification. Overall, students gained hands-on experience with hypothesis testing and an understanding of the pathology of diabetes.

KEYWORDS: Rat, diabetes, insulin, β cells, pathophysiology, enzyme-linked immunosorbent assay

Introduction

The National Science Foundation (2011) Vision and Change document calls on scientific educators to actively involve students in their learning process, rather than make them passive learners. Therefore, there is a need for an inquiry-based scientific education (IBSE) where students learn to acquire knowledge on their own, through hands-on experiences. This provides them with an increased understanding of the scientific method, increased scientific literacy, and direct practice in the processes of hypothesis testing (NSF 2014; Riga et al. 2017; Russell et al. 2007). Undergraduate students that participate in inquiry-based laboratory courses are more likely to be retained and prepared for graduate degrees and professional positions in biomedical sciences (Gregerman et al. 1998; Science 2011; Weaver et al. 2008). Further, participation in an inquiry-based laboratory experiences by women and underrepresented minorities demonstrably increases the likelihood that they will pursue a graduate or professional programs by 14-17% (Eagan et al. 2013; Gregerman et al. 1998). Including a research component in required coursework can also help increase diversity in scientific research careers, especially when independent research lab positions are not available (Bangera and Brownell 2014). Therefore, reform efforts in undergraduate STEM have focused on shifting to a learner-centered and applied, hands-on learning environment (NSF 2014; Woodin et al. 2010).

We developed an inquiry-based research experience that affords students the opportunity to follow the scientific method, formulate hypotheses, perform experiments and collect, interpret and present the scientific data they collected. We used a rat model and induced diabetes using streptozotocin (STZ; a known diabetogen). STZ destroys β cells in the pancreas and induces type 1 (insulin-dependent) diabetes (Szkudelski 2001). Using this diabetic model, students in our physiology laboratory course were involved in a research experience where they were able to collect both histological and physiological data. This three-week laboratory module allows students to link changes in tissue morphology, blood glucose and insulin levels and body weight with the destruction of pancreatic β cells. Specifically, students are able to visualize the pathophysiological effects of diabetes on the endocrine portions of the pancreas, specifically by examining islet morphology and measuring insulin levels. Additionally, students can also examine changes in weight and blood glucose levels. This lab affords them to opportunity to learn laboratory techniques while examining the clinical manifestations of type 1 diabetes.

Diabetes mellitus, type 1 diabetes, results from the inability of the pancreas to produce insulin and accounts for 5-10% of all cases of diabetes. It is characterized by a loss of pancreatic β cells, known to produce insulin and is classified as either immune-mediated or idiopathic (Atkinson et al. 2014).

Becuase there is a loss of pancreatic β cells, there is a decline in insulin production and release. Insulin is a peptide hormone that is one of the main anabolic hormones of the body. Due to the lack of insulin production during type 1 diabetes, there is a subsequent hyperglycemia as insulin does not activate 'insulin sensitive' Glut 4 receptors and allow for the uptake of glucose into the cell. This results in increases in thirst and poly- and glucosuria. A decrease in glucose uptake by the cells leads to ketosis and lipolysis and subsequent weight loss (Sonksen and Sonksen 2000). Clinical manifestations of diabetes can thus be investigated by students in lab. This allows students to link the changes they see with the destruction of pancreatic β cells.

The rat serves as an excellent model organism to study the pathophysiology of diabetes. One week following STZ injections, blood and tissues can be collected for analysis by students in the lab. Specifically, blood glucose levels can be analyzed and treated and control animals can be compared. Blood plasma can also be collected and an enzyme-linked immunosorbent assay (ELISA) can be used to determine blood insulin levels. Lastly, pancreatic tissues can be preserved in Bouin's fixative, embedded in paraffin, sectioned, and stained with hematoxylin-phloxin to identify and count β cells in pancreatic islets. This laboratory module allows students to make hypotheses based on what is known about the pathology of type 1 diabetes. They can then test these hypotheses using blood and tissues collected from the rat. As a final exercise in scientific data interpretation, students generate research posters in order to provide the opportunity to solidify background knowledge, use primary research references, and relate collected data to the pathophysiology of type 1 diabetes. Overall, this laboratory research activity provides exposure to biological practices such as tissue fixation, histology, tissue staining, performing an ELISA, hands-on hypothesis testing, statistical analyses, and the interpretation and scientific presentation of data.

Materials & Methods

Animal Treatments

Male Sprague Dawley CD rats (*Rattus norvegigicus*) weighing mean (± standard deviation) 235.2 ± 7.7 grams were obtained from Charles Rivers Laboratories International, Inc. (Wilmington, Massachusetts). The rats were housed in groups of four in the animal care facility at the University of Detroit Mercy, and were fed rodent chow, given water *ad lib*, and cages were cleaned every three days or more frequently as needed (IACUC approved by the University of Detroit Mercy Institutional Animal Care and Use Committee; June 2017). All treated animals were weighed and given an intraperitoneal (IP) injection of 60 mg/kg of STZ. A stock solution of STZ was prepared by dissolving 15 mg/mL of STZ in citrate buffer (pH 4.5). Control animals were given an IP injection of the buffer only (Ahmed et al. 1998). All animals were given distinctive tail markings using a marker. These were refreshed as needed.

Six days following the STZ or control treatment, rats were fasted overnight and each rat was euthanized the following morning using an overdose of CO_2 (AVMA 2013). The rats were then weighed and blood was collected from cardiac puncture and stored in vacutainers containing EDTA in order to preserve blood and collect blood plasma for the insulin analysis. Blood glucose readings may be obtained immediately by placing one drop of blood into a blood glucose monitoring system (e.g. ONETOUCH Ultra2, Lifescan). Blood, collected in the EDTA vacutainers, can also be preserved for future blood glucose recordings by transferring the blood to microcentrifuge tubes, flash freezing it in liquid nitrogen and storing in a -20ºC freezer. Plasma required for performing the insulin analysis was obtained by centrifuging the vacutainers for 10-15 minutes (1,000-2,000 x g) in a refrigerated centrifuge. Lastly, the pancreas of both control and STZ-treated rats was removed by the course instructors, cut into small pieces (~5 mm x 15 mm) and placed in Bouin's fixative for at least 24 hours before embedding the tissue.

Student Exercise:

Students are initially given a lecture on the endocrine system anatomy and physiology. This includes information on the pancreas with specific information on the role of insulin in glucose metabolism. We also introduced students to the use of appropriate experimental or "sham" controls. Students were expected to formulate hypotheses and expectations based on background information from both textbooks and primary literature. Given the appropriate background information, students are then required to generate a working hypothesis on what anatomical and physiological changes they expect following the delivery of STZ, a known diabetogen that is selectively toxic to pancreatic β cells (Szkudelski 2001). While the instructors of the course perform the IP injections and euthanasia, students are informed of the procedure and husbandry in addition to pre- and post-injection weights. The physiological data collection and histological analyses take three to four laboratory sessions (or weeks) depending on the histology preparation. To conserve time, the teaching assistants and professors may complete the tissue embedding and sectioning. Tissues were prepared for paraffin embedding by treating tissues for 15 minutes with 50% ethanol, 75 % ethanol, 95% ethanol, 100% ethanol, 100% xylene, and paraffin (three replicates of each). Pancreatic tissue was embedded in a paraffin mold and allow to

cool. The molds were place in the freezer overnight to fully solidify the paraffin (Bancroft and Stevens 1990; Carson and Cappellano 2015).

Student Lab Week 1

Students formed groups of four and were provided with paraffin embedded tissue blocks from control and STZ-treated rats. They were also provided with a microtome, water bath and slide warmer. After being instructed on how to cut a ribbon of paraffin embedded tissue, students were shown how to float the 5 µm sections on the water bath (~50ºC) and collect them on slides. Following the collection, the slides were dried on a slide warmer (~60ºC). Following the instruction, student groups collected sections from control and STZ-treated rats on labelled microscope slides, floated on the water bath, and allowed to dry on the slide warmer for at least 30 minutes.

For the hematoxylin/phloxin, a standard procedure (Bancroft and Stevens 1990) was followed where slides with pancreatic sections were put through a descending xylene and ethanol series (100% xylene, 100% ethanol, 95% ethanol, 75% ethanol, 50% ethanol, water; 2 of each for 1 minute each in coplin jars). Slides were treated for 1 minute with 0.3% potassium permanganate/0.3% sulphuric acid mixture, decolorized with a 5% solution of sodium bisulphite, and washed with running tap water. Slides were then placed in a coplin jar containing chrome hematoxylin solution for 10-15 minutes until microscopic evaluation shows β cells to be deep blue. The slides were then rinsed with water and differentiated in 1% acid alcohol for 1 minutes to remove background staining. Then, they were washed in tap water until section is clear blue and stained with 0.5% aqueous phloxine for 5 minutes. Slides were rinsed again in water, treated with 5% phosphotungstic acid for 1 minute, washed in running tap water for 5 minutes when the section should regain its red color. Tissues were differentiated in 95% ethanol. If the sections were too red and the α cells are not clear, the sldies were rinsed for 10-20 seconds in 80% ethanol. Lastly, the slides were dehydrated with 100% ethanol, cleared in xylene and mounted in DPX (with coverslip). The slides were allowed to dry (~20 minutes).

Student Lab Week 2

In week 2, students completed their staining and viewed and imaged their sections using a microscope (Nikon Eclipse E200 with a DAGE-MTI colored camera). Images were collected using the Magic App program (400x magnification). Students also captured images of stage micrometers so that they could provide scale bars on the images they presented. Additionally, they counted the total number of cells present in each islet and determined the percentage of β cells present for both control (N= 3) and treated rats (N =3). They reported and compared the average (mean) and standard error (S.E.) for control and STZ-treated animals using an unpaired t-test.

Student Lab Week 3

Students were provided with a sample of recently thawed blood from control (N=3) and STZ-treated (N=3) rats. They placed one drop of blood onto a blood glucose test strip and monitoring system to obtain blood glucose results. The mean (± S.E.) was calculated and compared using an unpaired t-test. Additionally an insulin ELISA (Invitrogen Rat Insulin ELISA kit, ThermoScientific, Frederick, MD) was performed following the instructions provided (Scientific 2015). This ELISA was started by the professor before the lab period began as it takes >5 hours to perform and each lab period is only three hours in length. For the assay, initially, the plate and solution were brought to room temperature. Then, standards and samples were prepared, loaded into the appropriate wells, and allowed to incubate for 2.5 hours at room temperature with gentle shaking. The solution was then discarded and the wells were washed 4 times with 300 µL of wash buffer using a multi-channel pipette. The plate was then inverted and blotted against a clean paper towels. Biotinylated antibody (100 µL) was added to each well and incubate for 1 hour at room temperature with gentle shaking. The solution was then discarded and washed again. Streptavidin-HRP solution (100 µL) was added to each well and incubated for 45 minutes at room temperature with gentle shaking. The solution was discarded and the plate was washed again. Lastly, TMB substrate (100 µL) was added to each well and incubate for 30 minutes at room temperature in the dark with gentle shaking. The plate was then evaluated after stopping the reaction. The mean absorbance was determined for standards and unknowns using an ELISA plate reader (Versa max microplate reader with Softmax Pro 5 software) set at 450 nm and 550 nm. The readings from 550 nm values were subtracted from the values obtained at 450 nm values to correct for optical imperfections in the microplate. Instructors performed all initial steps in the procedure, but the students were able to add the Streptavidin-HRP solution and continue to the end of the assay.

Each student generated a standard curve by plotting the average absorbance (450 nm minus 550 nm) obtained for each standard concentration on the vertical (Y) axis versus the corresponding Insulin concentration on the horizontal (X) axis. Microsoft Excel was used to generate a graph and determine a line of best fit. The equation of the line was used to determine the insulin values of the control (N=3) and STZ-treated rats (N=3). Students obtained and average (± standard error) and compared the control and treated groups using an unpaired t-test.

Assessment

Formative assessment of learning outcomes were evaluated by means of a research poster. Each student submitted and presented a poster with an introduction, methods, results, discussion, and reference section. They also provided an appendix where they included all of the raw data they collected in addition to the micrographs and standard curve. The poster afforded students the ability to use primary research references and relate collected data to the pathophysiology of type 1 diabetes. Instructors can choose to have students present their posters in a research-style symposium or have them individually present material to their laboratory class or instructor.

Results

Initial results exposed students to early indicators of the clinical manifestations of diabetes mellitus, those being weight loss and hyperglycemia. One week after STZ exposure, students noted that control rats gained significantly more weight than STZ-treated rats (Fig. 1, paired t-test, $p = 0.005$, $n = 3$ per treatment). On average control rats gained 39.7 ± 5.7 (mean ± S.E) more grams that the STZ-treated rats, which gained only an average of 1.0 ± 4.4 grams. After weight determinations and prior to preparation of blood samples for the ELISA, plasma glucose concentrations were determined. Students observed a four-fold increase in blood glucose levels in the STZ-treated rats (Fig. 2, unpaired t-test, $p = 0.002$, $n = 3$ per treatment), with mean (± S.E) glucose concentrations of 499.7 ± 58.0 mg/dL compared to 99.00 ± 9.0 mg/dL in control rats.

Hematoxylin/phloxine histochemical staining of prepared pancreatic tissue by students allowed them to visualize islets of Langerhan and identify pancreatic β cells that stain a deep magenta within the islets (Fig. 3). Analysis of images allowed the students to quantify the number of pancreatic β cells and contrast differences between control and STZ-treated rats (Fig. 4). Using an unpaired t-test, STZ-treated rats had significantly lower percentages of pancreatic β cells ($p < 0.0001$, $n = 6$ per treatment). When compared to control rats, the STZ-treated rats had 51.8 % lower pancreatic β cells (mean ± S.E. was 72.3 ± 6.6 % and 20.5 ± 15.6 % for control and STZ-treated rats, respectfully). Although not quantified, qualitative observations of STZ-treated rats by students noted these rats to have smaller islets, and remaining pancreatic β cells to appear "vacuolated", most likely due to the effects of STZ.

Quantified pancreatic β cells comparisons allowed students to link histologic data with physiologic data resulting from the insulin determinations from the ELISA (Fig. 5). ELISA results suggest an average decrease of 8.2 µIU/mL of plasma insulin levels in STZ-treated rats from that of control rats (mean ± S.E. was 11.4 ± 5.4 µIU/mL and 3.2 ± 2.6 µIU/mL for control and STZ-treated rats, respectfully).

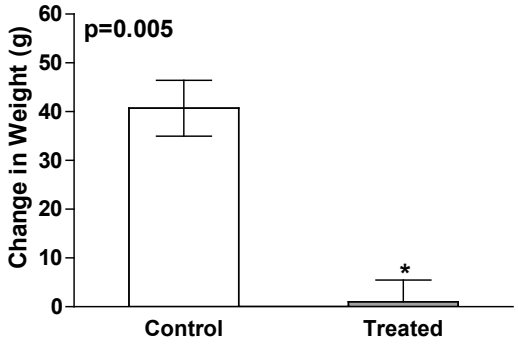

Fig. 1: One week after exposure to STZ, pre- and post-treated weights were compared. The STZ-treated rats gained significantly less weight than the control rats (unpaired t-test, $p = 0.005$, $n = 3$ per treatment).

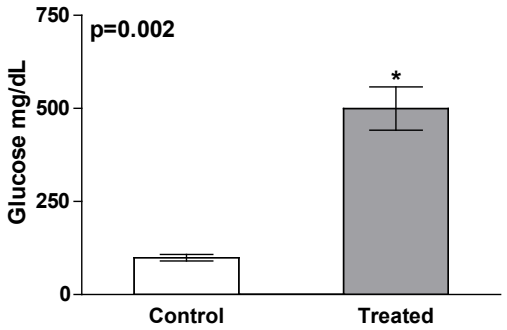

Fig. 2: Following treatment with STZ, STZ-treated rats demonstrated a significantly higher plasma glucose concentration than the control rats using the ONETOUCH glucometer (unpaired t-test, $p = 0.002$, $n = 3$ per treatment).

Discussion

We have developed a three week, hands-on, inquiry-based laboratory module that allows students to gain insights into the cause and effects of type 1 diabetes. Overall this module provided BIO 4640 (Physiology Laboratory) students with a deeper understanding of the physiology of the pancreas, as an essential organ in the abdominal pelvic cavity with both exocrine and endocrine functions. The exocrine functions of pancreas are performed by glands that secrete enzymes which aid in the process of. digestion, while its endocrine functions are a result of release of hormones that regulate the levels of blood glucose (Atkinson et al. 2014). The endocrine portions of the pancreas are composed of group of cells called islets of Langerhans or just islets. Pancreatic islets are composed of three types of

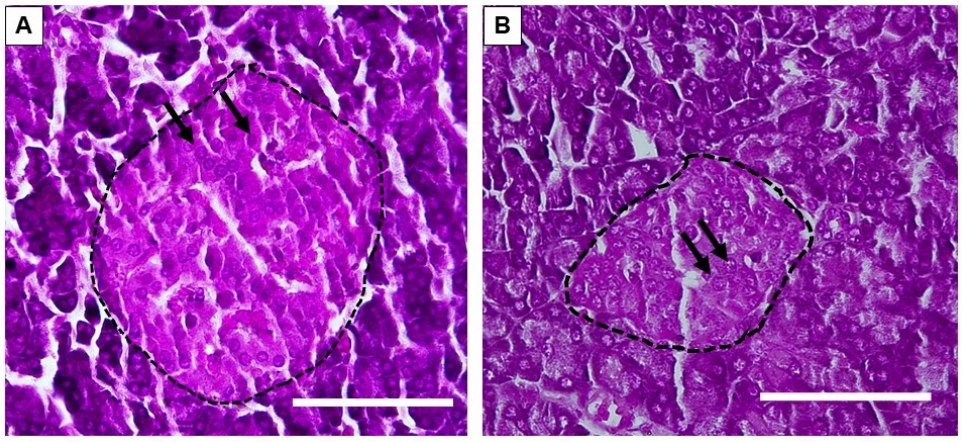

Fig. 3: Following sectioning students stained and imaged pancreatic islets (dashed outlined) from control (A) and STZ-treated (B) rats. Arrows indicate pancreatic β cells (mag. 400x, scale bar = 100 µm).

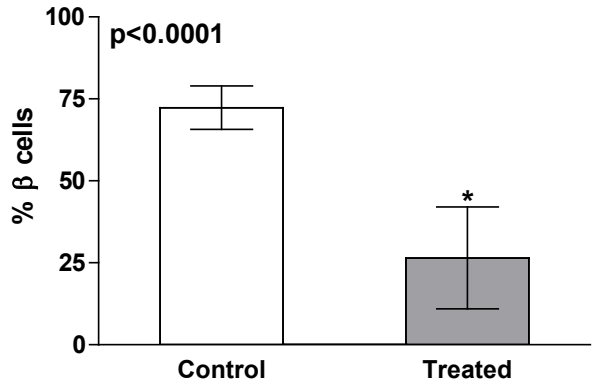

Fig. 4: Students analyzed images of pancreatic islets in control and STZ-treated rats and calculated the percentage of pancreatic β cells. There were significantly more β cells in control rats (unpaired t-test, $p < 0.0001$, $n = 6$ islets counted per treatment).

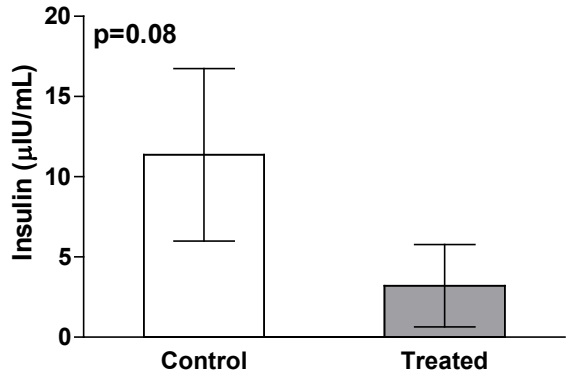

Fig. 5: Student ELISA results indicate control rats have slightly higher insulin concentrations than their STZ-treated counterparts (unpaired t-test, $p = 0.08$, $n = 3$ per treatment).

hormone secreting cells including alpha (α), beta (β) and delta (δ) cells. The hormone somatostatin is produced and secreted by δ cells and is involved in regulating growth hormone (Brereton et al. 2015). Hypoglycemic conditions stimulate α cells secrete glucagon leading to gluconeogenesis and increased blood glucose levels while hyperglycemic conditions lead to the release of insulin from β cells (Taborsky Jr 2010). Insulin-secreting β cells make up the majority of the cells found in pancreatic islets (~80% of islet cells). Insulin is a hormone that lowers the levels of blood glucose by activating GLUT4 receptors, subsequently increasing absorption of blood glucose for use in cellular respiration and storage in tissues (Saltiel and Kahn 2001). Type 1 diabetes and its subsequent pathologies are manifested after an immune system attack and destruction of insulin-producing pancreatic β cells (Atkinson et al. 2014).

ingIn this lab, we induced type 1 diabetes in a rat model by exposing treated rats to the known diabetogen STZ for one week. STZ has been successfully used to induce diabetes in rat models as it selectively targets and destroys pancreatic β cells. STZ enters β cells using a glucose transporter (GLUT2), leading to subsequent alkylation and damage of β cell DNA and production of free radicals (e.g. hydrogen peroxide and hydroxyl radicals) and by liberating toxic amounts nitric oxide. Overall, this causes β cell necrosis and a subsequent decrease in insulin production and release (Szkudelski 2001). The use of a STZ-injected rats allowed our students to investigate the physiological manifestations of type 1 diabetes. They were able to gain a deeper understanding of the physiology of pancreatic islets by using STZ-induced diabetic rat models and comparing the results with control rats. Students examined and linked various endpoints of diabetes with one another. Initially, students observed that

weight loss and hyperglycemia in STZ-treated rats when compared to the control rats. The weight gain in control rats is directly attributed to the normal functioning of the insulin hormone, secreted by pancreatic β cells as insulin is known to stimulate glycogen synthesis by stimulating a pathway that activates protein phosphatase 1 (Berg et al. 2002). However, the weight loss observed in our STZ-treated rats was attributed to hyperglycemia, which was quantified using a glucometer. Students then hypothesized that pancreatic β cells may not producing and secreting insulin.

To further investigate these clinical symptoms and determine if hyperglycemia observed in the STZ-treated rats was due to disrupted insulin production or function, student's sectioned paraffin embedded pancreatic tissues from control and STZ-treated rats. The histological analysis using a chrome alum hematoxylin-phloxin staining protocol on pancreatic tissue allowed students to easily visualize the β cells, located within the pancreatic islets. β cells appeared vacuolated in the STZ-treated rats when compared to the normal deep magenta β cells in the control pancreatic islets (Fig. 3). Further the percentage of β cells occupying the islets was determined by the students. They did this by quantifying the number of β cells and expressing them as a percentage of total cells found in the pancreatic islets. By using a t-test, students were able to show that islets of STZ-treated rats contained significantly less β cells than islets of control rats (Fig. 4). Further, students were able to link the morphological characteristics of the β cells and the reduction in the number of β cells in STZ-treated pancreatic islet tissue with the hyperglycemia and subsequent weight loss. The results obtained in this laboratory module are supported by several studies that have shown that disruption of β cells leads to increased blood glucose levels (e.g. Honka et al. 2014; Meier et al. 2008; Bonner-Weir 2000). In week three of this lab, students were able to directly link the histological data with the physiological function of β cells by performing an ELISA. After performing the ELISA, obtaining the data using a plate reader, preparation of a standard curve and calculation of a line of best fit using Excel, students were able to convert optical density for unknown control and STZ-treated rats to insulin concentrations. Students were able to show that control rats had more insulin than the STZ-treated rats, though this result was not significant (Fig. 5).

In summary, students who completed this three-week laboratory module were able to experimentally investigate the clinical symptoms of type 1 diabetes which include weight loss, increased blood glucose and decreased insulin levels and relate them to the underlying physiological cause, the destruction of pancreatic β cells. Here the students were able to apply their knowledge from preliminary coursework through a guided approach to a specific biological topic. Students had a greater understanding of the scientific process of developing a hypothesis, designing experiments, and collecting and performing rigorous statistical analyses of data to become well-informed, rather than passive readers. Students were required to write a comprehensive laboratory report based on their data indicating a deeper knowledge of the endocrine physiology, the scientific methodology. This led to an improvement in scientific presentation skills. After completing the laboratory module, students reported feeling confident with histological and analytical techniques. Students stated "I was also able to not only learn histological techniques, but also learned how to identify different structures within the pancreas, as well as their respective functions" and "In being a part of the histology lab, I was able to directly learn about the effects of diabetes on the pancreas using several different techniques".

Overall, this project stimulated and enhanced the cognitive skills of students in understanding type 1 diabetes and its underlying physiological changes. It also provided a great platform to learn and hone histo-technological skills like tissue preparation, sectioning, staining and imaging. Additionally, students became proficient in performing an ELISA, hands-on hypothesis testing, statistical analyses, and the interpretation and presentation of scientific data associated with it. It should be noted that this laboratory module could be modified by excluding the ELISA in order to shorten the laboratory to two weeks. Further, the student experience may be enriched by including paraffin embedding which will extend the duration of this module. Overall, we have developed an inquiry-based laboratory module that directly involves students in their learning process as recommended by the NSF. We believe that inquiry-based scientific experimentation and analytical skills, like those obtained by completing this laboratory module, are crucial for advanced biology courses for STEM majors and strongly recommend incorporating it in the coursework to improve scientific literacy.

Acknowledgements

The authors thank the National Institutes of Health (NIH) for a pilot award to RMB and GMG and the NIH ReBUILDetroit grant for equipment. We would also like to that the students of BIO 4640 (winter 2018 term) for data collection, tissue preparation and staining. We are also grateful for the assistance of Daniel Tuttle for image editing. Lastly, we are grateful for materials support from the University of Detroit Mercy.

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