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# How Different Insulin Administration Routes Affect Hepatic Glycogen Levels after an Aerobic Exercise Bout in Male Rats with Type 1 Diabetes

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A thesis submitted in partial fulfillment of the requirements for the Master of Science degree in Kinesiology

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## **Abstract**

Type 1 diabetes mellitus (T1DM) is an autoimmune disease resulting in the destruction of pancreatic beta cells leading to deficient insulin production. Insulin plays a crucial role in regulating glucose metabolism, facilitating the utilization of dietary energy. Individuals with T1DM exhibit dysregulated blood glucose concentration and necessitate exogenous insulin administration. Intensive insulin therapy (IIT) is employed commonly, typically via subcutaneous (SQ) injections to treat individuals with T1DM. However, SQ insulin delivery fails to mimic endogenous insulin secretion, particularly its physiological distribution directly to the liver via the hepatic portal vein. Insulin concentration in the liver contributes significantly to glucose homeostasis, notably during aerobic bouts of exercise. Individuals with T1DM lack direct hepatic insulin exposure and exhibit depleted liver glycogen reserves. Lower liver glycogen results in a diminished ability to release glucose into the bloodstream, leading to greater susceptibility of exercise-induced hypoglycemia. This study examined an alternative route of insulin administration directly into the Omentum pouch (OP.). The OP is an anatomical structure located within the abdominal cavity, vascularly akin to the pancreas. This study aimed to evaluate the efficacy of OP insulin delivery in enhancing liver glycogen storage and mitigating post-aerobic exercise (AE) hypoglycemia. Contrary to expectations, OP insulin administration exhibited inferior glycemic control and impaired glycogen utilization compared to traditional SQ administration. This finding was attributed to abnormally increased insulin concentration in the hepatic portal vein.

## **Key Words**

Insulin Administration, Hepatic Glycogen, Aerobic Exercise, Type 1 Diabetes, Male Rats, Diabetes Mellitus, Glycemic Control, Exercise Physiology, Endocrinology, Animal Model, Insulin Therapy, Metabolism, Liver Function, Diabetes Research, Exercise-Induced Glycogen Changes

## **Summary for Lay Audience**

Type 1 diabetes mellitus (T1DM) arises when an individual's immune cells attack the pancreas resulting in the loss of insulin production in the body. This is a prominent issue as insulin is a hormone that is responsible for the utilization of energy stored in food. Consequently, people with T1DM tend to have increased, unregulated blood sugar (BG) concentration which requires external sources of insulin. To treat patients with T1DM, intensive insulin therapy (IIT) is used. IIT involves multiple SQ injections which keep BG within the normal physiological range. However, SQ insulin injections do not replicate how the body would physiologically produce insulin in individuals without diabetes. The body produces a specific quantity of insulin in the pancreas to match intestinal blood glucose, which in turn travels directly to the liver, via the hepatic portal vein. Among many other roles, the insulin in the liver is important for storage of glycogen (molecules of glucose). Liver glycogen is important for many physiological processes including regulating decreasing blood glucose during exercise via a process called hepatic glycogenolysis, where hepatic glycogen is converted to glucose, a usable energy source of the body. During exercise, in individuals with T1DM, the SQ insulin only reaches the liver after muscle, fat and other insulin sensitive tissues utilize it first, thus rendering hepatic glycogen stores low. This contributes to individuals with T1DM being more susceptible to entering a

hypoglycemic (low blood sugar) state while exercising. An alternative method for administering insulin via the Omentum pouch (OP) has been theorized to combat the indirect supply of insulin to the liver. The OP is a large two-fold organ located adjacent to the pancreas, with very similar vasculature to the pancreas. This study sought to determine whether insulin administration to the OP would result in better liver glycogen storage and as a result mitigate the drop in blood glucose post-aerobic exercise (AE). The current study found that OP insulin administration results in poorer blood insulin regulation and a decreased ability to utilize stored glycogen, suggesting too much insulin was being administered to the hepatic portal vein causing negative outcomes.

### **Co-Authorship Statement**

Dr. Jamie Melling of Western University, London, Ontario, Canada was involved in project organization and development, interpretation of findings, and thesis revision. Mitchell James Sammut was involved in animal care, data analysis, tissue preparation and tissue analysis. Benjamin Thorne was involved in tissue preparation and tissue analysis.

## **Dedication**

To my family, thank you for supporting me and encouraging me to pursue both my academic and extra-curricular goals. Your feedback, conversations and love were instrumental in getting to this final product.

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## **List of Abbreviations**

DM - Diabetes Mellitus

T1DM - Type 1 Diabetes Mellitus

T2DM - Type 2 Diabetes Mellitus

BM - Body Mass

Gly - Glycogen

Gast - Gastrocnemius

IR - Insulin Resistance

IIT - Intensive Insulin Therapy

HLA - Human Leukocyte Antigen Complex

ER - Endoplasmic Reticulum

FA - Fatty Acid

LA - Lactic Acidosis

ROS - Reactive Oxygen Species

DKA - Diabetic Ketoacidosis

TG - Triglycerides

BGL - Blood Glucose Concentration

A1c - Glycated Hemoglobin

Hb - Hemoglobin

GC - Glycemic Control

HPS - Hepatic Portal System

GCRS - Glucose Counter Regulatory System

CVD - Cardiovascular disease

CIT - Conventional insulin therapy

CS - Control sedentary

CT - Control trained

OP - Omentum pouch

AE - Aerobic exercise

STZ - Streptozotocin

BSA - Bovine serum albumin

ELISA - Enzyme-linked immunosorbent assay

ANOVA - Analysis of variance

## Chapter 1

### 1.1 Overview of Type 1 Diabetes Mellitus

Diabetes Mellitus (DM) is a metabolic disease, best characterized by elevated blood glucose (1). DM has several different but the two major forms that are most commonly seen in the population are type 1 and type 2 diabetes mellitus. Type 2 diabetes mellitus (T2DM) develops when the body does not respond normally to insulin, resulting in insulin resistance (IR) (2). To combat the rising blood glucose (hyperglycemia), the pancreas produces more insulin. Due to the IR development in several of the body's insulin sensitive tissues, blood sugar concentrations rise irrespective of the insulin quantity, leading to several metabolic complications including cardiovascular disease, vision loss and kidney disease (2). T2DM is the most common form of diabetes accounting for 90% of diagnosis (3). In contrast, type 1 diabetes mellitus (T1DM) is a chronic autoimmune disease where the beta cells in the Islets of Langerhans located in the pancreas are destroyed by T-cells and macrophages in the body resulting in insulin deficiency (4). The current treatment methods for T1DM include the use of intensive insulin therapy which involves multiple injection of exogenous insulin to regulate blood glucose within euglycemia, 3.9-10.0 mmol/L (5).

### 1.2 Etiology and Pathophysiology

While the etiology of T1DM is unclear, evidence supports that both genetics and environmental factors contribute the development of this disease. The possession of certain genes may make individuals more likely to develop T1DM. The risk of developing T1DM is increased by variants of the human leukocyte antigen complex (HLA) DQA1, HLA-DQB1, and HLA-DRB1 genes



(6), (7), (4). These genes belong to a cohort called the HLA which play a critical role in immune function, specifically distinguishing the body's own proteins from proteins made from foreign invaders like bacteria and viruses. Other risk factors include putative environmental triggers such as microbial infections, neonatal nutrition, and exposure to certain toxins such as nitrates (8). Age and family history also contribute to the development of T1DM (9). DM is a very prevalent disorder; T2DM is thought to account for the majority of the global increase; affecting up to 10.9% of the population by 2045 (10). Likewise, the prevalence of T1DM is also expected to increase by 60-100% by 2040 (11). The development of effective therapeutic approaches to better treat and prevent T1DM development are increasingly vital given the predicted rise in the prevalence of this condition rises among the population.

T1DM develops when immune effector cells in genetically susceptible individuals infiltrate the pancreas and activate inflammatory pathways which destroy insulin producing beta cells (12). Under homeostatic conditions, insulin production is tightly coupled with protein synthesis in the endoplasmic reticulum (ER). When insulin secretory granule proteins are in excess, they are broken down creating a proinflammatory environment around the ER which in turn leads to elevated oxidative stress in the beta cells of the pancreas. This is followed by chemokines release which attract white blood cells such as macrophages and T-cells. T-cell interactions can directly and indirectly damage the Islet of Langerhan cells, cells which produce not only insulin, but also glucagon, a hormone that plays an important role in releasing glucose into the bloodstream. Cellular damage exacerbates ER and oxidative stress, perpetuating this cycle, ultimately causing beta cell death (12).

### 1.3 Presentation and Diagnosis

T1DM usually presents through one of three typical patterns: classic new onset, silent diabetes or, diabetic ketoacidosis (DKA). Classic new onset T1DM involves polydipsia (excessive thirst), polyuria (excessive urine), polyphagia (excessive hunger), weight loss and lethargy. Silent diabetes does not typically display any drastic signs; however, it is diagnosed after monitoring individuals with suspected diabetes development or based on family history of T1DM. Finally, DKA is characterized by complete lack of insulin production such that triglycerides (TG) must be broken down to provide the body with energy. When the liver resorts to breaking down fat for fuel instead of carbohydrates (beta oxidation), acids called ketones are produced. The elevation of ketones quickly in the blood can cause dehydration, deficient blood perfusion through the liver and often lactic acidosis (LA) (13).

Plasma glucose monitoring tests are the most common type of measurement for blood glucose concentration (BG) at a single point in time. This value is expressed in milligrams per deciliter (mg/dL) or millimoles per liter (mmol/L) whereby a reading over 200 mg/dL (11.1 mmol/L) meets the diagnostic criteria for diabetes. Euglycemia, aka the physiological normal blood sugar range falls between 70-180 mg/dL (3.9-7.2 mmol/L) (5). A glycated hemoglobin (A1c) blood test can also be used to diagnose T1DM and is often representative of the average blood glucose over the past three months. Specifically, this test measures what percent of hemoglobin (Hb) proteins in the blood are glycated (coated with sugar). A reading of 6.5% or greater confirms the diagnosis of DM (14). Blood tests are completed to detect specific autoantibodies to confirm the type of diabetes (15).

## 1.4 Current Treatments

Numerous methods of insulin delivery currently exist to regulate BG. The primary goal of these strategies is to maintain BG as close to euglycemia as possible (3.9-7.2 mmol/L). SQ insulin injections are among the most popular methods to deliver exogenous insulin into the body (16). Insulin pens and syringes work in similar ways to provide consistent and gradual absorption of insulin into the bloodstream (17), (18). Insulin pumps, another SQ insulin administration method, involves the use of a small programmable device that delivers a continuous and controlled supply of insulin into the SQ tissue (19). Beyond SQ insulin administration, oral and transdermal methods also exist. Oral insulin delivery is an emerging method that is attractive due to its less invasive nature. This method is administered through a pill or capsule, absorbed in the gastrointestinal tract and enters the bloodstream (20). Finally, transdermal insulin delivery refers to the administration of insulin through the skin allowing the hormone to be absorbed through either the use of a patch or topical formulation directly into the bloodstream (21). The method used often comes down to personal preferences, lifestyle, and the specific requirements of the individual.

Although vast literature outlining the effects of subcutaneous, oral and transdermal insulin delivery methods on glycemic control exists (GC) (16, (17,18, (19, (20,(21), a common issue arises among these methods. Even advanced insulin delivery systems fail to resemble accurately the physiologic production and secretion of insulin. Current methods of exogenous insulin delivery prevent the storage of hepatic glycogen, which has important implications, especially in the context of exercise. In non-diabetic individuals, when b-cells of the pancreas secrete insulin, glucagon secretion is inhibited from neighboring islet alpha cells which create a specific insulin:

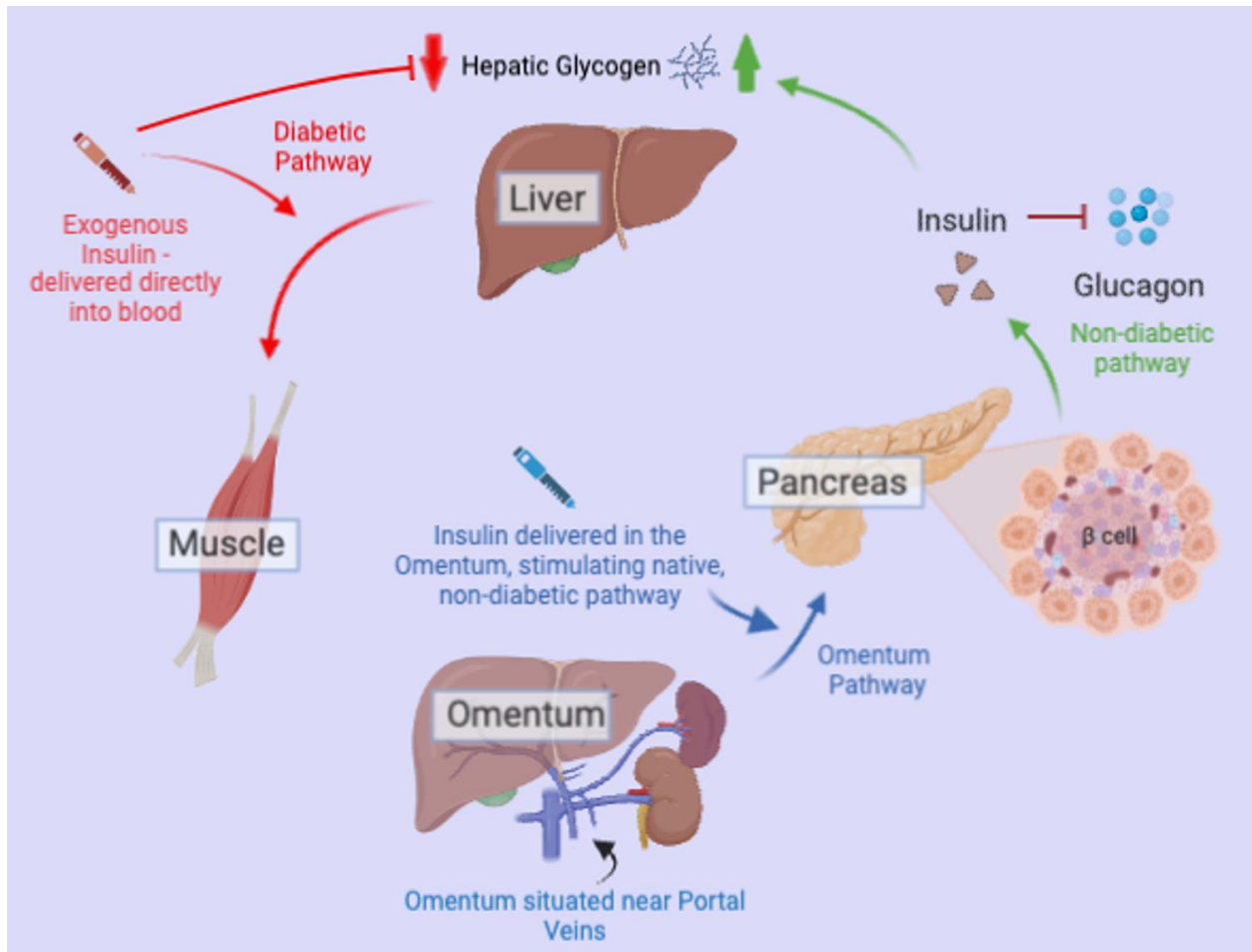
glucagon ratio. (22). Glucagon is responsible for raising blood glucose through releasing glucose into the blood. Anatomically, the liver is located directly downstream of pancreatic blood flow whereby the greatest concentration of pancreatic hormones are attained via the hepatic portal vein. The ratio of pancreatic insulin: glucagon traveling via the hepatic portal vein results in the optimal ratio of insulin: glucagon in the hepatocytes (cells of the liver) compared to the rest of the body (approximately 3:1). This specific ratio in the hepatic portal vein is lost when insulin is administered exogenously through means like SQ or transdermal injection, causing impaired regulation of hepatic glucose production (23). Specifically, exogenous insulin secretion diminishes the liver's ability to store glycogen as the insulin is released into the bloodstream, bypassing the liver (24), (25). Although hepatic gluconeogenesis is activated by diminished insulin, the glucose yield through this process is far less than what would be produced from the adequate storage of hepatic glycogen and subsequent glycogenolysis (23), (24), (25). Hepatic glycogen storage breakdown and gluconeogenesis are responsible for maintaining glucose concentration during the onset of exercise; hence exercise ability is also impacted. (26), (27).

### **1.5 Omentum Pouch as a Site for Insulin Delivery**

The omentum pouch (OP) is a double-layered fold of peritoneum (a smooth tissue membrane) that hangs down from the greater curvature of the stomach and extends over the intestines within the abdominal cavity. It has two main components, the greater and lesser omentum (28). The greater omentum is the larger of the two parts. It covers the anterior surface of the intestines and contains tissue, blood vessels, lymphatics, and immune cells. The OP plays important roles in the body's immune system; housing essential adipose tissue, blood vessels, lymphatics, and immune cells which allow it to create protective barriers and help facilitate healing processes (29).

Located right under the abdominal wall, the OP can be accessed with relative ease through a basic intra-abdominal incision. What makes the OP particularly interesting and a suitable candidate for exploratory insulin delivery research is the blood flow through this organ. OP blood flow moves via branches of the gastroepiploic arteries and veins, continuations of the splenic artery and vein, respectively (29). Moreover, blood exits the OP through the hepatic portal vein and ultimately enters the portal vein system. This is integral as this route of exogenous insulin delivery may better resemble how insulin is endogenously released.

Moreover, through the hepatic portal system (HPS), insulin and glucose will better filter through the hepatic portal system, theoretically leading to optimized storage and utilization of glycogen in the liver. While insulin administration via the OP remains a completely novel approach, a study investigating islet transplantation in diabetes induced rats provided further justification for the use of the OP as a route for insulin administration. Omer et al. (2004), studied the kinetics of islet secretion transplanted in various locations of a rodent. Of particular importance was the attempt to transplant Islets deep within the peritoneal cavity, housing the OP. This cavity was chosen because it has direct portal venous connection, mimicking how the vasculature of non-diabetic individuals. Importantly, rats receiving the islet transplants into the peritoneal cavity were able to normalize blood glucose within seven days, while rats receiving alternative islet transplantations remained hyperglycemic and required a second transplantation one week post initial transplant (54). This study further supported the idea that delivering insulin to the OP more closely resembles how a non-diabetic individual would make insulin, and lead to better glycemic control post-exercise compared to alternative delivery methods.



**Figure 1.1** Depiction of non-diabetic vs. SQ vs. OP insulin delivery. Image created using Bio-Rad.

Depicted in green text in the image above is the non-diabetic pathway of insulin production. Insulin is produced in the pancreas by the beta cells of the Islets of Langerhans. From there it travels downstream via the hepatic portal vein (not depicted) to the liver. Once at the liver, glycogen stores are created to be used during aerobic exercise. Depicted in the red pathway is the traditional SQ insulin administration pathway. Insulin is delivered directly into the bloodstream and travels directly to the working tissue, bypassing the liver entirely. This results in decreased glycogen storage in the liver and greater susceptibility for hypoglycemia during exercise. Finally, depicted in blue is the novel OP insulin delivery method. By administering insulin in the OP, the insulin will travel via the hepatic portal vein to the liver, stimulating glycogen storage. This glycogen can then be used to combat decreasing BG during exercise, mitigating the risk of hypoglycemia.

## 1.6 Aerobic Exercise and Type 1 Diabetes

Regular aerobic exercise (AE) has been established to provide both acute and long-term benefits for individuals with T1DM, including increases in cardiorespiratory fitness, decreased IR, and improved lipid blood concentration and endothelial function (30), (31), (32), (33). While many benefits exist, AE also presents potential acute adverse effects related to hypoglycemia in patients with T1DM (below 3.0 mmol/L) (34), (35), (36), (37). In non-diabetic individuals, the reduction in blood glucose below 4 mmol/L leads to a reduction insulin secretion and glucose-counter regulatory systems (GCRS) are activated. (38). Among many GCRS hormones, glucagon, stimulates glucose production in the liver, by promoting hepatic conversion of glycogen to glucose (glycogenolysis), stimulating de novo glucose synthesis (gluconeogenesis) and inhibiting glucose breakdown (glycolysis) and glycogen formation (glycogenesis) (39). However, in individuals with T1DM, the action of glucagon is also impaired. Insulin directly impacts the release and regulation of glucagon, thus there are great effects on endogenous glucagon when insulin production is impaired (40), (41). In non-diabetic individuals, the decrease in insulin secretion during exercise results in the release of its inhibitory effect on glucagon to help maintain BG. However, in individuals with T1DM, when insulin is unable to respond appropriately to metabolic demands, glucagon is not able to rise (42), (43). Thus, this beta cell failure also results in an impaired glucagon response (40), (42), (43), increasing the susceptibility of hypoglycemia development during exercise.

During aerobic exercise, circulating glucose is routed into working skeletal muscle through insulin dependent and independent (contraction mediated glucose uptake) pathways (46). In non-diabetic individuals who participate in moderate intensity aerobic exercise, plasma glucose is

maintained at or near pre-exercise concentration by a match between hepatic glucose output and peripheral glucose uptake (47). The liver generates glucose through two processes, both which rely on insulin and glucagon secretion: a) glycogenolysis, the mobilization of hepatic glycogen stores and b) gluconeogenesis, the synthesis of new glucose from smaller precursor molecules (48). The decrease in insulin secretion during exercise is believed to be essential for the activation of hepatic glycogenolysis, and the increase in glucagon secretion enhances both glycogenolysis and gluconeogenesis (49). In moderate aerobic exercise, changes in arterial plasma glucagon and insulin are quite modest. The critical determinants of controlling hepatic glucose supply during moderate aerobic exercise are glucagon and insulin concentrations in hepatic portal vein (50), (51) (52) (53).

Individuals with T1DM who require exogenous insulin therapy, face unique challenges during exercise. As insulin delivery in these patients stem predominantly from exogenous sources (medication), these patients do not experience the typical exercise induced decline in portal vein insulin . This in turn suppresses hepatic glucose production and subsequent release (49). Under these conditions, blood glucose declines, and hypoglycemia can develop during exercise, despite adequate muscle and hepatic glycogen reservoirs. This issue can be exacerbated if insulin is y elevated inappropriately through overcorrection during exercise. Higher portal vein insulin stimulates greater glucose uptake into the tissue leading to greater decreases in blood glucose.



## **1.7 Purpose and Hypothesis**

The purpose of this study was to examine how a novel insulin administration route, the OP, would affect hepatic glycogen storage and whether OP insulin administration could mitigate the typical drop in blood glucose experienced following moderate aerobic exercise. It was hypothesized that diabetic rats receiving insulin via the OP would have greater hepatic glycogen compared to the rats receiving insulin via a SQ route. Additionally, it was hypothesized that T1DM rats receiving insulin via the OP would have greater blood glucose post-exercise than the SQ group due to greater initial liver glycogen.

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## Chapter 2

### 2.1 Background

Type 1 diabetes mellitus (T1DM) is a chronic autoimmune disease where the beta cells in the Islets of Langerhans found in the pancreas are damaged or destroyed resulting in insulin deficiency (1). Insulin is a pancreatic peptide hormone that regulates blood glucose by moving glucose from the bloodstream into cells to provide glucose for ongoing cellular metabolism and/or to store for use later. Thus, T1DM is characterized by abnormally increased circulating blood glucose, termed hyperglycemia. As of 2022, approximately 300,000 Canadians live with T1DM. The number of new cases has grown 4.4% annually, much greater than Canada's population growth of 1% per year, making it one of Canada's most prevalent disorders (2). Cardiovascular diseases including coronary artery disease, myocardial infarction, and atherosclerosis are also associated with T1DM (3) (4). The current treatment methods for type 1 diabetes include the use of intensive insulin therapy (IIT) which is characterized by continuous blood glucose monitoring and rapid exogenous insulin adjustment in order to regulate blood glucose within a euglycemia range of 3.9-10.0 mmol/L (5) .

SQ insulin injections are among the most popular methods to deliver exogenous insulin into the body (6). These most commonly occur via either insulin pens or syringe injection whereby insulin is delivered into the SQ fatty tissue to provide consistent and gradual absorption of insulin into the bloodstream (7), (8). Insulin pumps, another SQ insulin administration method, involve the use of a small programmable device that delivers a continuous and controlled supply of insulin into the SQ tissue (9). Beyond SQ insulin administration, oral and transdermal methods also exist. Oral insulin delivery is an emerging method that is attractive due to its less

invasive nature. This method is administered through a pill or capsule, absorbed in the gastrointestinal tract before entry into the bloodstream (10). Finally, transdermal insulin delivery refers to the administration of insulin through the skin allowing the hormone to be absorbed usually through the use of a patch or topical formulation directly into the bloodstream (11). The choice regarding which method to use often comes down to personal preferences, lifestyle, and the specific requirements of the individual.

Although a vast literature exists outlining the effects of subcutaneous, oral and transdermal insulin delivery methods on glycemic control (6), (7),(8), (9), (10), (11), a common issue arises among these methods. Even advanced insulin delivery systems fail to accurately resemble the physiologic production and secretion of insulin. In short, exogenous insulin delivery does not trigger specific metabolic pathways in the liver that would typically be triggered with endogenous insulin production (13). The inability to trigger the proper activation of these pathways post prandially results in a decrease in hepatic glycogen storage which in turn has important implications for blood glucose regulation, especially in the context of exercise.

In non-diabetic individuals, when b-cells of the pancreas secrete insulin, glucagon secretion is inhibited (12). This is important in creating a specific insulin: glucagon ratio in the blood. The vascular network flows from the pancreas to the liver via the hepatic portal vein. Blood entering the liver is thus enriched with pancreatic hormones and there is a specific 3:1 ratio of insulin: glucagon that is unique compared to the rest of the body (13). This insulin: glucagon ratio is lost when insulin is given exogenously via SQ or transdermal causing impaired hepatic glucose production (13). Specifically, exogenous insulin administration diminishes the liver's ability to store glycogen as the insulin is released into the bloodstream, without passing through the liver



first (14), (15). The liver acts as a glucose reservoir and plays a key role in regulating glucose metabolism (13). Muscles serve a similar function of storing glucose and its regulation, and the interactions between the liver and muscles is important to optimize blood glucose regulation (18). During times of high metabolic demand (i.e. exercise), hepatic glycogen is broken into glucose at increased rates which is used subsequently to maintain blood glucose (16) (17), (18) (19), (20). Concomitantly, BG falls during exercise due to increased muscle intake. When the stores of glucose are depleted in both the muscle and liver, circulating glucose in the blood can be drastically reduced and the reliance on gluconeogenic systems become paramount. The insufficient storage of hepatic glycogen and insulin-mediated inhibition of gluconeogenesis is believed to elevate the risk of hypoglycemia in people with T1DM (21).

To combat the risk of hypoglycemia and increase hepatic glycogen stores, the omental pouch (OP) has been proposed as a novel site for insulin administration. Stice et al. described the OP as being highly vascularized with portal venous drainage (22). Insulin administration via the OP, might more closely mimic that of a non-diabetic individual. Insulin would be able to enter the hepatic portal system, travel through the hepatic portal vein and flow through the liver, creating a strong insulin gradient in the liver that would help with hepatic glycogen storage (13).

The current study's main objective was to determine whether the risk of hypoglycemia could be mitigated during a moderate bout of aerobic exercise in individuals with type 1 diabetes by administering insulin in a novel, intra-abdominal location. It was hypothesized that by administering insulin via the OP, insulin would be delivered in a way that more closely mimics that of a non-diabetic patient. As a result, hepatic glycogen would be greater and thus individuals would be better able to regulate blood glucose during exercise.

## **2.2 Materials and Methods**

### 2.2.1 Animals

32 male Sprague Dawley rats were obtained from Charles River Laboratories (St. Constant, Que., Canada) at eight weeks of age. All rats were caged in pairs and housed in a room with a 12- hour light-dark cycle, at a temperature of 20.5 degrees C, and relative humidity of 40%. Rats were provided access to standard rat chow and water ad libitum over the course of the study.

### 2.2.2 Experimental Groups

Rats were randomly assigned into one of two groups: SQ insulin administration (SQ) (n=12) and omental pouch insulin administration (OP) (n=20).

### 2.2.3 Experimental Procedures

#### *2.2.3.1 T1DM Induction Insulin Pellet Implantation*

All rats underwent an acclimatization process for one week upon arrival. During this period, the rats were not handled and given time to adjust to their climate, diet and sleep cycle. During week 2, the rats underwent seven consecutive days of intraperitoneal low-dose injections of streptozotocin (STZ; Sigma-Aldrich) to induce T1DM (Appendix A). Over the seven days, 20 mg/kg of STZ dissolved in citrate buffer (0.1M, pH 4.5) were injected within fifteen minutes of solution preparation. Diabetes was confirmed following the seven days of STZ injections by two non-fasting blood glucose measurements of ~11

mmol/L or greater (source: Association AD. 2. Classification and diagnosis of diabetes: standards of medical care in diabetes—2018. *Diabetes Care* 41: S13–S27, 2018.).

### *2.2.3.2 Insulin Pellet Implantation*

During the third week of the study, after diabetes was confirmed, insulin pellet implantation occurred for both the SQ and OP groups. Regarding the SQ group, one insulin pellet (2 IU insulin/day) was surgically implanted in each animal (Appendix B1). Blood glucose levels were monitored weekly. During week 10, additional insulin was surgically added to specific SQ animals whose blood glucose levels were above 15 mmol/L. Blood glucose was intended to be maintained between 4-9 mmol/L for each SQ rat throughout the study. In the third week of the study, 10 rats in the OP group received one insulin pellet (2 IU insulin/day) via surgical implantation (Appendix B2). At the end of week seven, the 10 OP rats who had the SQ insulin pellet, underwent a procedure where the SQ insulin pellet was removed, and half an insulin pellet was placed within the rats abdominal cavity (1 IU insulin/day). This procedure followed the protocol laid out in Appendix B2, with the only alteration being half a pellet was inserted instead of a full pellet within the abdominal wall. Although a reduced insulin quantity was provided, three rats went hypoglycemic and did not survive the first 24 hours. Blood glucose levels were monitored weekly.

### *2.2.3.3 Exercise Familiarization and Training Bout*

This study utilized a one-hour aerobic exercise bout. Seven and five days prior to the exercise bout, the rats were exposed to a brief 10-minute familiarization experience at

15m/min on the treadmill. The exercise familiarization was done in week 11 of the study. The final aerobic exercise bout involved rats running on the treadmill for one hour at 17m/min. This occurred during week 12 of the study. Rats were immediately sacrificed (within five minutes) after the exercise bout.

#### *2.2.3.4 Blood and Tissue Collection*

Rats were sacrificed during the last week (week 12) of the experiment. If the rat underwent the one-hour aerobic exercise stint, they were sacrificed immediately following exercise (within five minutes). Sacrifice was performed via anaesthetization with isoflurane, followed by cardiac exsanguination. Two blood samples of 500  $\mu$ l were collected from each rat during the exsanguination procedure. One sample (500  $\mu$ l) was also collected during week 8 of the experiment to analyze insulin levels between groups. Blood samples were centrifuged for 30 minutes at 3,000 rpm, and serum was then transferred to 1.5 ml Eppendorf tubes. Post exsanguination, both the left and right liver, vastus lateralis (red and white), gastrocnemius (red and white), plantaris and soleus muscles were all removed and immediately frozen in isopentane cooled to  $-70^{\circ}\text{C}$  by liquid nitrogen. Tissues and serum were then stored at  $-80^{\circ}\text{C}$  for future analysis.

## 2.3 Experimental Measures

### 2.3.1 Body Weight and Blood Glucose

The body weight of each rat was measured and recorded once per week over the course of the study. Weekly non-fasting blood glucose was also measured from a small blood droplet (~50  $\mu$ L) obtained from the saphenous vein. The Freestyle Lite Blood Glucose Monitoring System (Abbot Diabetes Care, INC.) was used to analyze blood glucose values which were recorded in millimoles per liter (mmol/L).

### 2.3.3 Western Blot

Liver, red gastrocnemius, and white gastrocnemius tissue (20 mg) were submerged 1:20 (w/v) in lysis buffer (15 mM Tris pH=7.0, 600 mM NaCl and 0.1 mM EDTA). The submerged tissue was homogenized with three, one - three second pulses using a basic mechanical homogenizer (IKA Laboratories). Tissue homogenate was transferred into Eppendorf tubes (1.5 ml) and agitated on a shaker for 2 hours on ice. Samples were then centrifuged at 12,000 rpm for 20 minutes at 4°C. Sample supernatant was extracted and transferred to a different Eppendorf tube (1.5 ml) and stored at -80°C. Total sample protein concentration and loading volumes were determined using a Bradford protein assay (source: 46. Kielkopf CL, Bauer W, Urbatsch IL. Bradford assay for determining protein concentration. Cold Spring Harb Protoc 2020: pdb-prot102269, 2020.) (Appendix E). In Eppendorf tubes, protein samples mixed with an equal volume of 2x Laemmli SDS-PAGE (4% SDS, 20% Glycerol, 10%  $\beta$ -mercaptoethanol, 0.015% bromophenol blue, 0.125M Tris, pH 6.8) were subsequently boiled at 90°C in a water bath for five minutes.

20 µg of the sample protein was then loaded into 12% polyacrylamide gels and ran at 75-150 V for 2 hours. Transfer of gel protein to nitrocellulose membranes (Bio-Rad) was conducted at 70V for 90 minutes. Following completion of the transfer, membranes were stored at 4°C in TBS-T (Tris Buffer Saline, 0.1% Tween-20), overnight. Membranes were washed in fresh TBS-T for five minutes. Following washing, membranes were blocked with a 5% w/v solution of TBS-T and skim milk powder or bovine serum albumin (BSA) for 1-2 hours. Blocked membranes were then washed for five minutes in TBS-T. Washed membranes were incubated for 2 hours at room temperature with primary antibodies detecting: Akt (Cell Signaling; 4691), phosphorylated (ser473) Akt (Cell Signaling; 4060), Anti-PCK1/PEPC (Abcam; 70358), and Anti-G-6-Pase antibody (ab93857). Anti-PCK1/PEPC, Anti-G-6-Pase, Akt and phosphorylated (ser473) Akt antibodies were used on liver samples, while only Akt and phosphorylated (ser473) Akt antibodies were used for gastrocnemius tissue. Following incubation, the primary antibody was removed, and membranes were subsequently washed in TBS-T for 10-minutes and repeated for three washes. Membranes were then incubated in a 5% w/v solution of TBS-T and skim milk powder or BSA, and secondary antibody (#170-6515 Goat anti-rabbit IgG HRP conjugate, #170-6516 Goat anti-mouse IgG HRP Conjugate; BioRad, 57 Hercules, CA, USA) at a 1:20000 dilution for 2 hours at room temperature. After incubation, membranes were washed for 10 minutes in TBS-T and repeated for three washes. Membranes were then treated with Bio-Rad chemiluminescence substrate, and the images were subsequently captured using the Bio-Rad Chemidoc MP System. Western blot image quantification was conducted using Image J (Appendix C).

### 2.3.2 Liver and Muscle Glycogen Quantification

Liver, red gastrocnemius, and white gastrocnemius tissue (~20 mg) were homogenized in 30% KOH saturated with Na<sub>2</sub>SO<sub>4</sub> and boiled for 30 minutes (Appendix D). Glycogen in each sample was precipitated with 95% ethanol and samples were left to rest on ice for an additional 30 minutes. Samples were then centrifuged for 20-30 minutes at 3000 rpm.

After centrifugation, the supernatant was discarded, and glycogen pellets were immediately resuspended in 3 ml of ddH<sub>2</sub>O. Glycogen pellets were placed on ice until a homogenous solution resulted, and then split into three one ml glass tubes for triplicate analysis. One ml of 5% phenol and five ml of sulfuric acid (96-98%) were subsequently added to each tube and allowed to stand for five minutes at room temperature and then incubated at 25-30°C for 10 minutes. The colour reaction of samples was analyzed using a spectrophotometer at a wavelength of 490 nm.

### 2.3.3 Serum Insulin Quantification

To quantify serum insulin in rat samples, ALPCO's ELISA Kit (Catalog 80-INSRT-E01) was used. The procedure involves a 96-well ELISA microplate pre-coated with a specific antibody targeting rat insulin. Samples of serum that were collected week 8 and week 12 of the experiment were used. Both samples and insulin standards were prepared according to the protocol (Appendix F) and added to the coated wells on the microplate. An incubation period followed to facilitate insulin binding to the antibody. After washing to remove unbound substances, a detection antibody specific to rat insulin was introduced to each well, allowing the formation of a capture antibody-insulin-detection antibody

complex. Subsequent washing removes excess detection antibodies. An enzyme-conjugated secondary antibody was added to bind to the detection antibody, and a substrate solution introduced, initiating an enzymatic reaction proportional to the bound insulin. This reaction induced a colour change in the wells. The enzymatic reaction was stopped with a stop solution, stabilizing the colour. The absorbance of each well was then measured spectrophotometrically at a specific wavelength (450 nm). The obtained absorbance values were utilized to quantify the concentration of rat insulin in the samples, compared to a standard curve generated by known insulin concentrations.

#### 2.3.4 Glycogen Phosphorylase Activity

To determine glycogen phosphorylase activity in red and white gastrocnemius muscle, Abcam's Glycogen Phosphorylase Assay Kit (Colorimetric) was used. Numerous reagents were prepared according to instructions found within the kit (Appendix G). Samples were prepared by cutting roughly 50 mg and homogenizing them with the provided assay buffer. After homogenizing, samples were centrifuged, and the supernatant was transferred to a new Eppendorf tube. Standards were prepared by diluting the 100mM G1P solution provided. The kit measures the rate at which glycogen phosphorylase catalyzes the conversion of an artificial substrate, producing glucose-1-phosphate. To initiate this reaction, a reaction mix consisting of Assay Buffer, Glycogen Substrate, Enzyme Mix, Development Enzyme Mix and Developer solution (all provided with kit) was made and put in each well. A background reaction mix was also prepared using the same solutions (minus the glycogen substrate) to determine the baseline level of absorbance caused by nonspecific interactions. This value was subtracted from the reaction mix value in the



calculation step to accurately measure specific glycogen phosphorylase activity. Once the reaction mixes and the background control solutions were made, and the samples were loaded into the coated microplate, the kit was ready to be measured by a microplate reader. The reaction mixes and background control solutions were added to the wells of the microplate and then the optical density was measured at 450 nm in kinetic mode (every 13 seconds for 50 consecutive intervals). Specific glycogen phosphorylase activity was calculated based on the change in optical density at two different time points during the linear portion of the reaction curve.

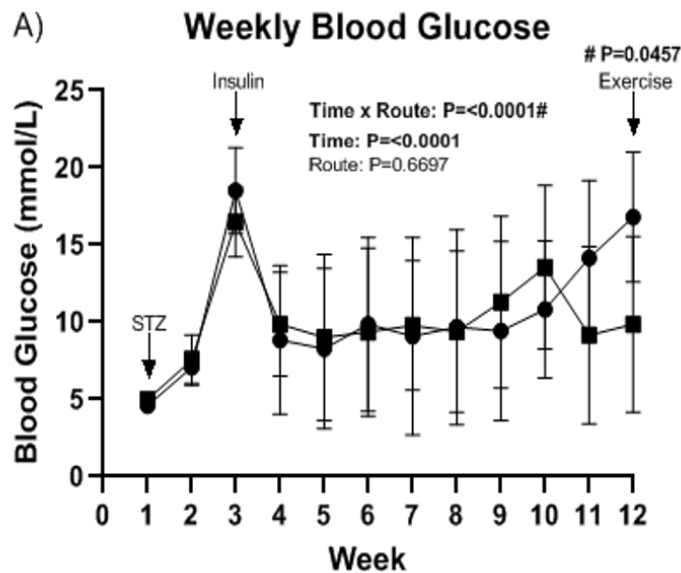
#### **2.4 Data Analysis**

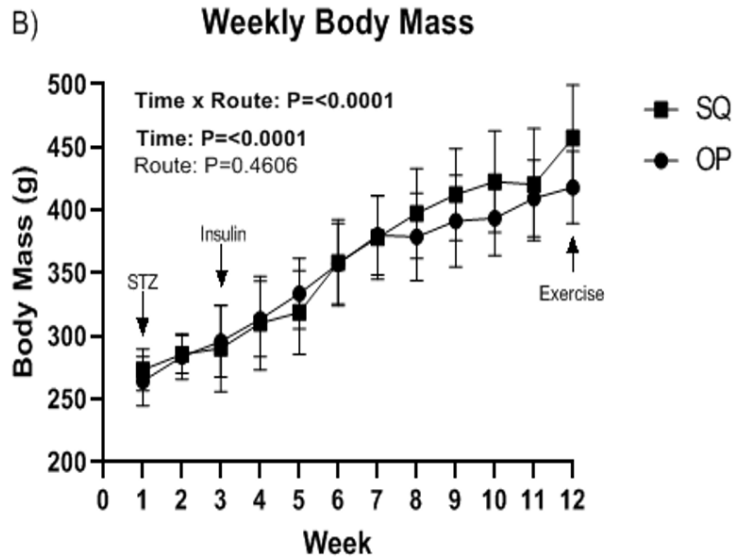
GraphPad Prism 8 (GraphPad Software, Inc.) was used to complete statistical data analysis. Weekly blood glucose and body mass measures were analyzed using a 2-way repeated measures analysis of variance (ANOVA) with time and insulin administration route as factors. Serum insulin, hepatic glycogen, red and white gastrocnemius glycogen, PAKT:AKT ratio, G6PASE and PEPC levels were analyzed using a two-way ANOVA with insulin administration route and exercise participation as factors. Tukey's multiple comparisons test was used for posthoc analysis when significant differences were observed. Levene's test for equality of variance was also performed on serum insulin concentrations to determine whether SQ and OP insulin concentrations had homogeneity of variance. Significance for all analysis was accepted at an alpha value of 0.05.

## 2.5 Results

### 2.5.1 Animal Characteristics - Body Weight and Blood Glucose

Weekly body mass (Fig. 2.1a) and non-fasting blood glucose (Fig. 2.1b) measures were recorded and analyzed to examine the influence of diabetes over time between the two groups. In terms of blood glucose, there was a significant interaction between time and insulin administration route ( $p < 0.0001$ ). This interaction is highlighted in week 12 of the study where the OP group showed significantly higher levels of blood glucose compared to the SQ group ( $p = 0.0457$ ). The main effect of time was statistically significant ( $p < 0.0001$ ). This means, independent of insulin administration route, changes in blood glucose level were statistically different over the course of the study. In terms of body mass, there was an interaction between time and insulin administration route ( $p < 0.0001$ ). The main effect of time ( $p < 0.0001$ ) was also statistically significant.



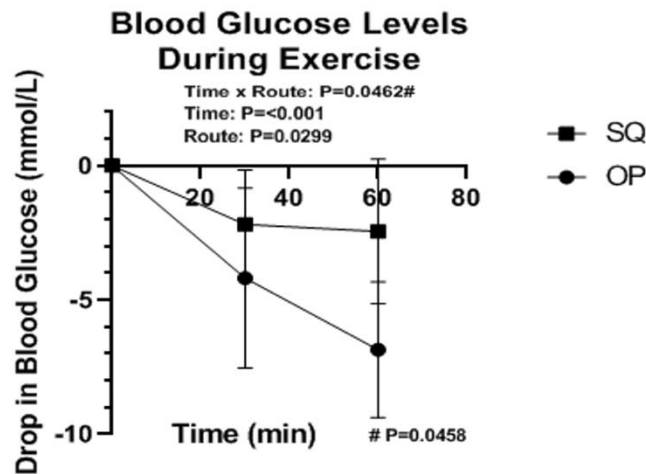


**Figure 2.1.** Weekly blood glucose (a), and body mass (b) data. ‘STZ’ indicates the initiation of streptozotocin injections to induce T1DM in both the SQ and OP animals. ‘Insulin’ indicates the start of insulin treatment for both SQ and OP animals. ‘Exercise’ indicates the week at which half the animals underwent the one-hour aerobic exercise bout. a). Mean weekly non-fasting blood glucose measures (mmol/L). Data is presented as mean  $\pm$  SD. ‘#’ indicates a significant difference between SQ and OP animals. b). Mean weekly body mass measures (g). Data is presented as mean  $\pm$  SD. No differences were observed between groups within any time point.

### 2.5.2 Blood Glucose Levels During Exercise

Blood glucose levels of SQ and OP animals were measured at three time points over the course of the one-hour exercise bout, pre-exercise (0 minutes), half-way (30 minutes), and post-exercise (60 minutes) (Fig. 2.2). These values were recorded and analyzed to determine whether insulin administration route affected the utilization of blood glucose levels during exercise. A statistically significant interaction between time and insulin administration route was also seen ( $p=0.0462$ ). This interaction is highlighted by the statistically different blood glucose levels at

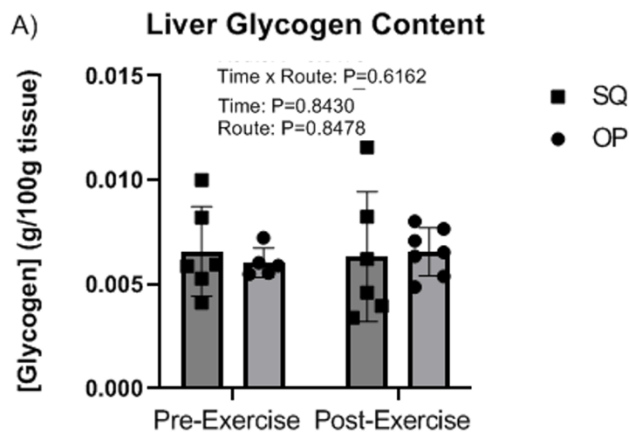
the 60-minute mark of exercise ( $p=0.0458$ ). The SQ group showed a statistically significant smaller drop in blood glucose levels post-exercise when compared to the OP group. The main effects of time and insulin administration route were also statistically significant ( $p<0.001$ ) and ( $p=0.0299$ ), respectively.

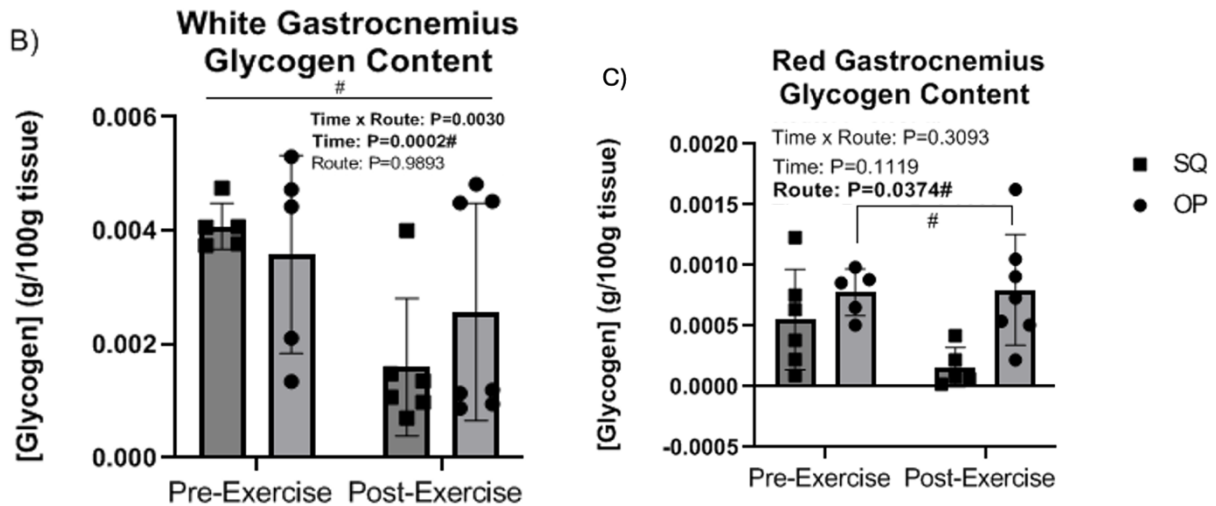


**Figure 2.2.** Drop in blood glucose levels (mmol/L) at three time points over the course of the 1-hour aerobic exercise bout; pre-exercise (0 minutes), half-way (30 minutes), and post-exercise (60 minutes). Data is presented as mean  $\pm$  SD. ‘#’ indicates a statistical difference between the SQ and OP animals.

### 2.5.3 Liver and Muscle Glycogen Content

Liver glycogen content (Fig 2.3a) did not differ in terms of insulin administration route ( $p>0.005$ ) or with respect to time (pre- and post-exercise levels) ( $p>0.05$ ). Glycogen content in the white gastrocnemius (Fig. 2.3b) showed significant differences pre- and post-exercise ( $p=0.0002$ ) and had a significant interaction between time and administration route ( $p=0.003$ ). There was no statistical difference between insulin administration routes, independent of other variables ( $p>0.05$ ). When analyzing the drop in glycogen content in the white gastrocnemius tissue (Fig 2.3c), the SQ group demonstrated a significantly greater drop in glycogen content compared to the OP group ( $p=0.0031$ ). In terms of glycogen content in the red gastrocnemius (Fig 2.3d), there was no statistical difference with the main effect of time ( $p>0.05$ ) nor was there an interaction between time and insulin administration route ( $p>0.05$ ). However, there was a statistically significant difference between insulin administration routes, independent of other variables ( $p=0.037$ ). There was a drop in glycogen content in the white gastrocnemius tissue (Fig. 2.3e), there was no significant difference between the SQ and OP groups ( $p>0.05$ ).

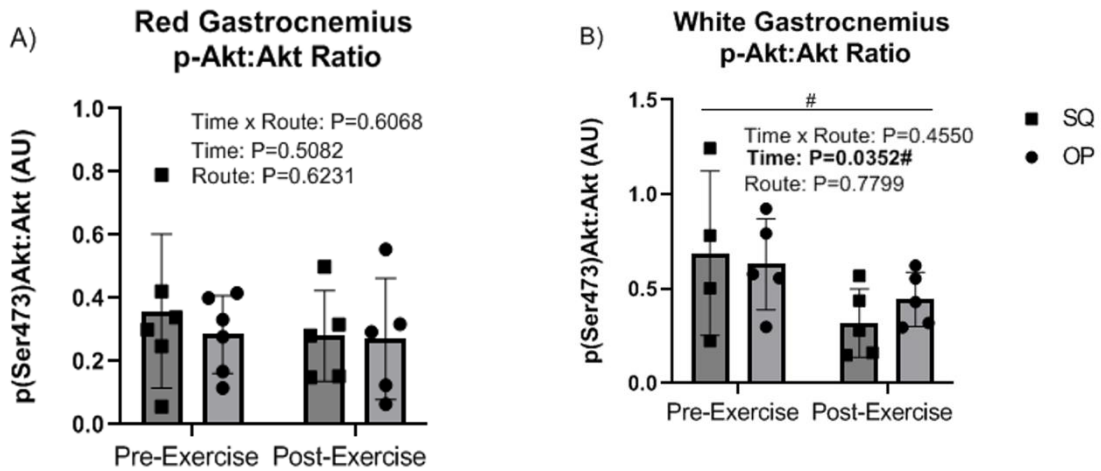




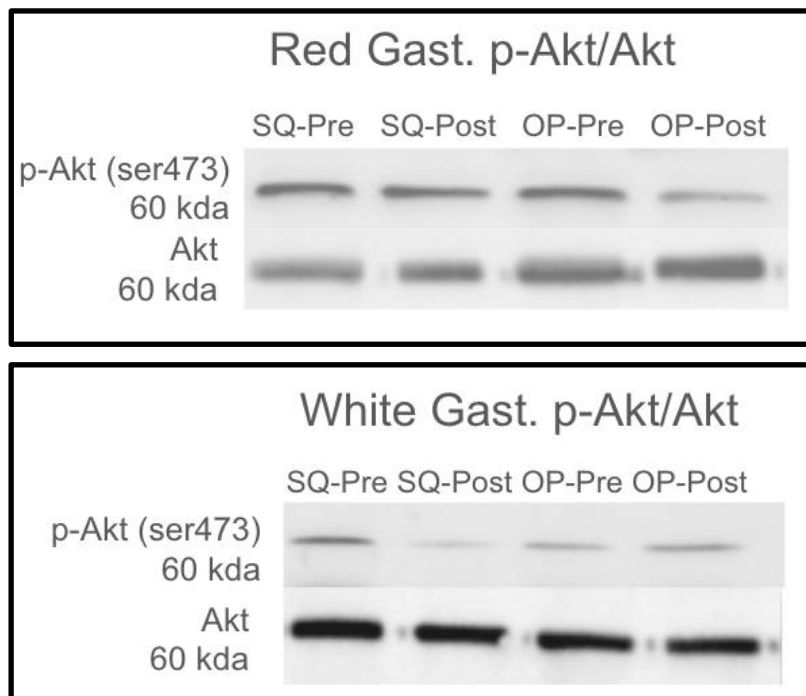
**Figure 2.3.** Liver glycogen content (g/100g tissue) (a), white gastrocnemius glycogen content (g/100g tissue) (b), red gastrocnemius glycogen content (g/100g tissue) (c). a. Mean liver glycogen content. Data is presented as mean  $\pm$  SD. No differences were observed between groups. b. Mean white gastrocnemius glycogen content. Data is presented as mean  $\pm$  SD. ‘#’ Denotes a significant difference between pre- and post-exercise levels. c. Mean red gastrocnemius glycogen content. Data is presented as mean  $\pm$  SD. ‘#’ denotes a significant difference between SQ and OP insulin administration routes.

#### 2.5.4 Muscle Protein Content

In the red gastrocnemius, the ratio of p(ser473) Akt to total Akt (Fig. 2.4a) was not statistically different for either main effect, time or insulin administration route ( $p > 0.05$  for both). In the white gastrocnemius, there were no statistical differences found in insulin administration route or in terms of an interaction between the main effects ( $p > 0.05$  for both). The ratio of p(ser473) Akt to total Akt (Fig. 2.4b) showed significant differences for the main effect of time ( $p = 0.0352$ ). This highlighted that independent of insulin administration route, the level of p-Akt(ser473) to total Akt post-exercise was significantly lower than pre-exercise levels.



C)



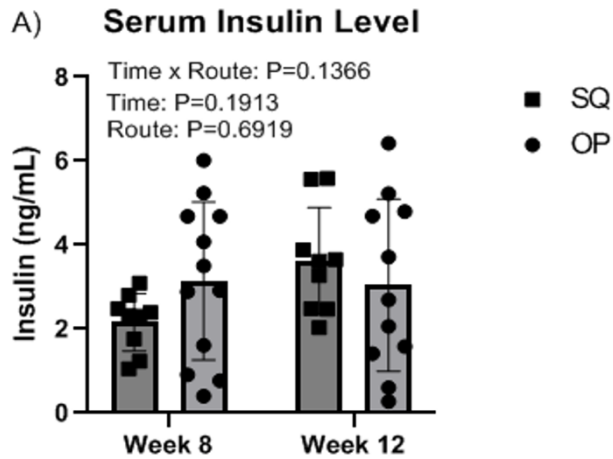
**Figure 2.4.** Red gastrocnemius p(ser473) Akt: Akt ratio (a), white gastrocnemius p(ser473) Akt: Akt ratio (b), and representative western blot images (c). a. Mean red gastrocnemius p(ser473) Akt: Akt ratio. Data is presented as mean  $\pm$  SD. ‘#’ Denotes a significant difference between pre-

and post-exercise levels. b. Mean white gastrocnemius p(ser473) Akt: Akt ratio. Data is presented as mean  $\pm$  SD. No differences were observed between groups.

#### 2.5.6 Serum Insulin Levels

Serum samples were taken at two time points during the study. The first sample was taken at the beginning of week 8 and the second sample was taken at the end of week 12. There were no significant differences for either main effect, time or insulin administration route ( $p > 0.05$  for both; Fig 2.6a). Levene's test of variance was also conducted on the data to determine whether the sample variances were statistically different. Two Levene's tests were conducted. The first test compared the variances of the SQ and OP serum insulin levels at week 8 (Fig. 2.6b) and the second compared the variances at week 12 (Fig. 2.6c). The Levene's test at week eight revealed the variances were statistically different ( $p = 0.007$ ). At week 12, the Levene's test also showed significant differences in the variances between groups ( $p = 0.044$ ).





B)

**Assumption Checks**

Test of Equality of Variances (Levene's)

	F	df <sub>1</sub>	df <sub>2</sub>	p
WK 8 SQ vs. OP	9.275	1	19	0.007

C)

**Assumption Checks**

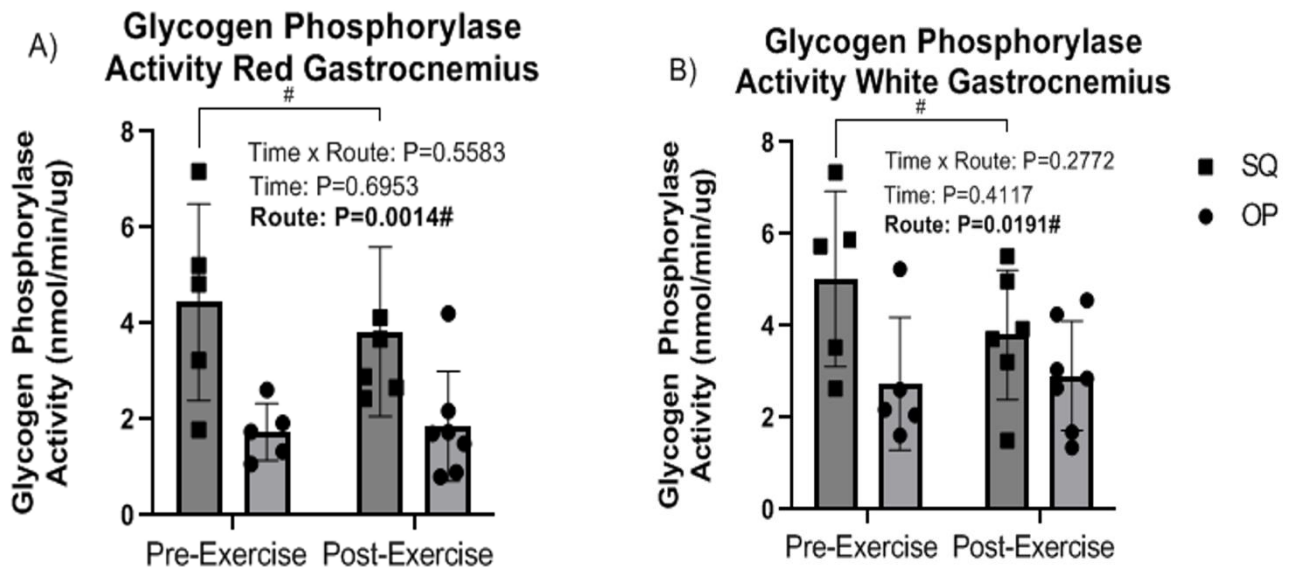
Test of Equality of Variances (Levene's)

	F	df <sub>1</sub>	df <sub>2</sub>	p
WK 12 SQ vs. OP	3.484	1	18	0.044

**Figure 2.5.** Serum insulin levels (a), Levene's test of variance on week 8 serum insulin levels (b), and Levene's test of variance on week 12 serum insulin levels (c). a. Mean serum insulin levels in both SQ and OP groups at week 8 and week 12 of the study. Data is presented as mean ± SD. No differences were observed between groups. b. Levene's test of variance week 8. P-value lower than 0.05 indicates significant difference between SQ and OP serum insulin level variances at week 8. c. Levene's test of variance week 12. P-value lower than 0.05 indicates significant difference between SQ and OP serum insulin level variances at week 12.

### 2.5.6 Glycogen Phosphorylase Activity

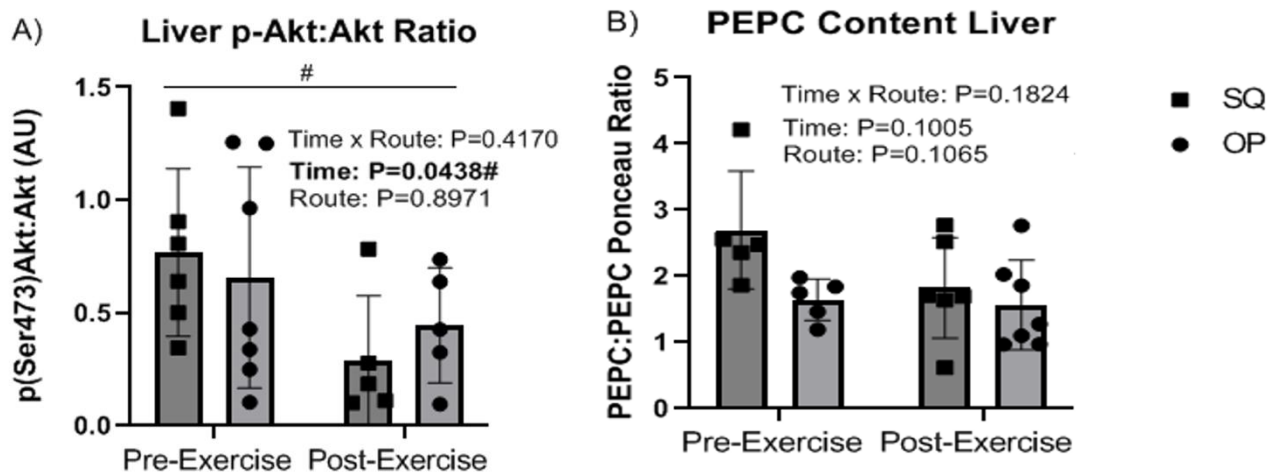
Glycogen phosphorylase activity was measured in both the white and the red gastrocnemius muscle. In the red gastrocnemius muscle (Fig. 2.7a), no interaction between time and insulin administration route was found ( $p>0.05$ ). However, there was a significant difference in the method of insulin administration ( $p=0.0014$ ). This highlighted that independent of the time period (pre/post-exercise), the SQ group had significantly higher levels of glycogen phosphorylase activity in the red gastrocnemius muscle than the OP group. A similar pattern was witnessed in the white gastrocnemius muscle (Fig. 2.7b). There was no interaction between time and insulin administration route ( $p>0.05$ ). However, there was a significant difference in terms of the method of insulin administration ( $p=0.0191$ ). This highlighted that independent of the time (pre/post-exercise), the SQ group had significantly higher levels of Glycogen Phosphorylase activity in the white gastrocnemius than the OP group.

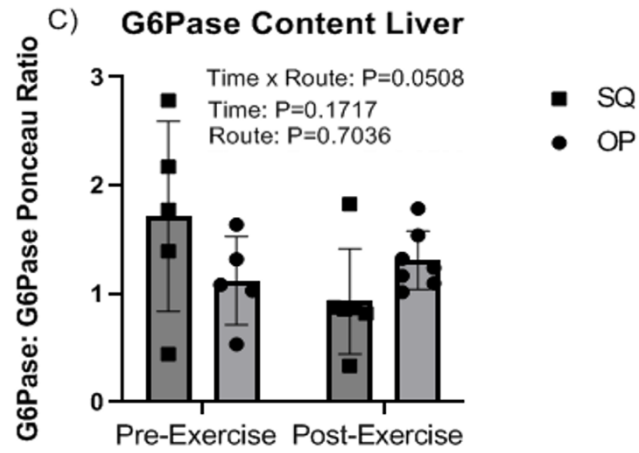


**Figure 2.6.** Glycogen Phosphorylase activity in the red gastrocnemius (a), and Glycogen Phosphorylase activity in the white gastrocnemius (b). a. Mean Glycogen Phosphorylase activity in the red gastrocnemius. Data is presented as mean  $\pm$  SD. ‘#’ Denotes a significant difference between SQ and OP insulin administration routes. b. Mean Glycogen Phosphorylase activity in the white gastrocnemius. Data is presented as mean  $\pm$  SD. ‘#’ Denotes a significant difference between SQ and OP insulin administration routes.

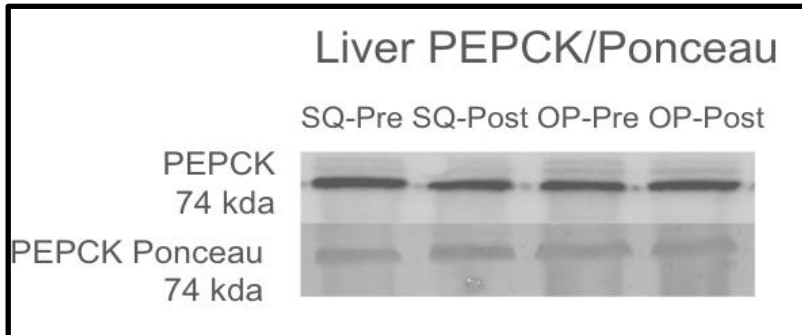
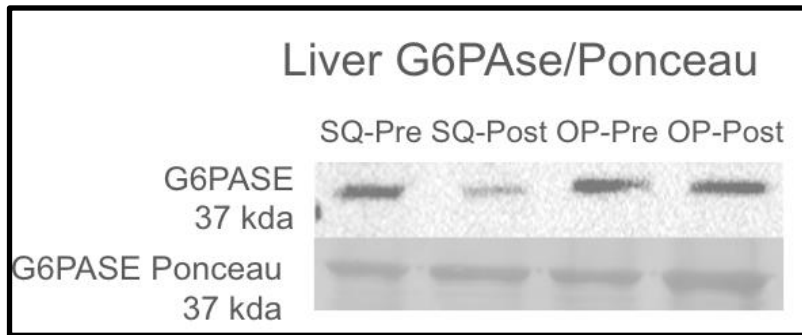
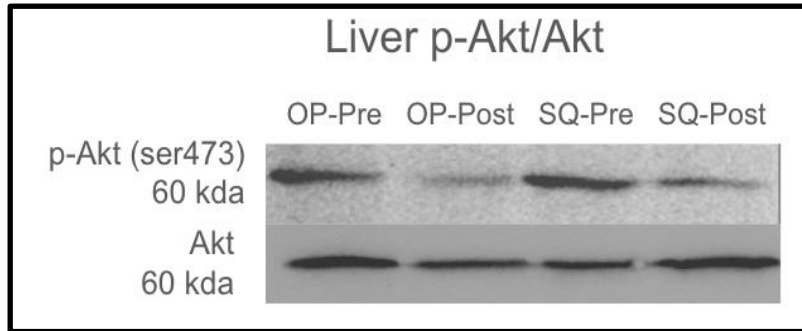
### 2.5.7 Liver Protein Content

In the liver, the ratio of p-Akt(ser473) to total Akt (Fig. 2.5a) showed no statistical differences found in insulin administration route or in terms of an interaction between the main effects ( $p > 0.05$  for both). However, significant differences for the main effect of time ( $p = 0.0352$ ) were present. This highlighted that independent of insulin administration route, the level of p-Akt(ser473) to total Akt post-exercise was significantly lower than pre-exercise levels. There were no significant differences in liver PEPC content (Fig. 2.5b) for either main effect, time or insulin administration route ( $p > 0.05$  for both). For liver G6Pase levels (Fig. 2.5c) there were also no significant differences for either main effect, time or insulin administration route ( $p > 0.05$  for both). For PEPC and G6Pase, protein content was normalized to ponceau staining of membranes.





D)



**Figure 2.7.** Liver p(ser473) Akt: Akt ratio (a), liver PEPC: PEPC Ponceau ratio (b), liver G6Pase:G6Pase Ponceau ratio (c) a. Mean liver p(ser473) Akt: Akt ratio (d), representative western blot images. Data is presented as mean  $\pm$  SD. ‘#’ Denotes a significant difference between pre- and post-exercise levels. b. Mean liver PEPC: PEPC Ponceau ratio. Data is presented as mean  $\pm$  SD. No differences were observed between groups. c. Mean liver G6Pase:G6Pase Ponceau ratio. Data is presented as mean  $\pm$  SD. No differences were observed between groups.

## 2.6 Discussion

Aerobic exercise has numerous acute and long-term benefits for individuals with type 1 diabetes. Such benefits include but are not limited to increases in cardiorespiratory fitness, decreased insulin resistance, and improved blood lipid concentration and endothelial function (23), (24), (25), (26). While these benefits exist, individuals with type 1 diabetes also face unique challenges during exercise which present a serious risk for hypoglycemia (27), (28), (29), (30). This risk for hypoglycemia stems from the fact that individuals with T1DM experience poor glucagon regulation as well. During exercise, sufficient blood glucagon release is necessary to prevent BG from dropping to hypoglycemic states. However, for individuals with T1DM, glucagon does not rise due to an elevated circulation of exogenous insulin, potentiating the exercise-mediated reduction in BG, leading to an increased risk of hypoglycemia onset (32), (34), (35), (21).

The current study demonstrated that rats who were given the OP insulin experienced a significantly greater decline in blood glucose 60 minutes post moderate aerobic exercise when compared to the SQ group (figure 2.2a). While this finding was contrary to the hypothesis, it

may be attributed to abnormally high insulin in the hepatic portal vein which in turn would inhibit glucagon release (36), (37), (38). In non-diabetic individuals, during moderate aerobic exercise, pancreatic insulin is inhibited due to increased sympathetic activity (39). This decrease in insulin is critical to promote exercise-induced hepatic glycogenolysis, the breakdown of stored hepatic glycogen. While hepatic glycogen plays many roles, one of its primary responsibilities is to be broken down into glucose and released into the bloodstream to counteract declining blood glucose during exercise (40). Omer et al. (2004), studied the kinetics of islet secretion transplanted in various locations of a diabetic rodent. This study examined how different transplant sites affected glycolytic control in diabetic rodents post-exercise. Of particular importance was the attempt to transplant islets deep within the peritoneal cavity. Rats receiving encapsulated islet transplants into the peritoneal cavity were able to return blood glucose back to normal within seven days of exercise, while rats receiving islet transplantations in the liver and SQ remained hypoglycemic and required a second transplantation one week post initial transplant (56). This study further supported the idea that delivering insulin to the OP more closely resembles how a non-diabetic individual would make insulin. Although the results of Omer et al. (2004) and the current study do not align, this could be attributed to how the studies were carried out. Omer et al. used encapsulated islets to treat the T1DM, whereas the current study utilized insulin pellets instead.

Given that the OP drains directly into the hepatic portal vein (41), the rats in the current study which received insulin via the OP group had a direct source of insulin to hepatic tissues. If the usual exercise triggered decrease in portal vein insulin concentration is overridden and portal vein insulin concentrations increase beyond the physiological range, then the exercise induced stimulation of liver glycogenolysis is instead rapidly suppressed (42). This is a plausible

mechanism for why blood glucose dropped more substantially during exercise in the OP group. Furthermore, when exercise was initiated, the OP rats were unable to appropriately downregulate the production of insulin, further contributing to abnormally high insulin in hepatic portal vein. This theory is supported by the fact that hepatic glycogen levels in the OP group were the same pre- and post-exercise (figure 2.3a). The OP group was unable to stimulate hepatic glycogenolysis, resulting in the similar quantities of hepatic glycogen pre- and post-exercise. While the SQ group did not experience significant changes in hepatic glycogen pre- and post-exercise either, they approached significance, unlike the OP group, indicating more effective hepatic glycogenolysis. Future work should examine insulin concentration in the hepatic portal vein to determine if they indeed are abnormally high. In turn, research can then focus on augmenting insulin delivery such that insulin concentrations are more sensitive to exercise.

The current study also sought to determine the effect of OP insulin delivery on the daily management of blood glucose and insulin. Two separate measures of blood insulin were taken during the study, at weeks 8 and 12 (figure 2.6a). While the mean insulin concentrations, reported in (ng/mL), were not significantly different between the OP and the SQ group at either time point, clear differences in the variance of the data existed between groups. Levene's test of equality of variances was performed at both time points, revealing that the OP group has significant differences in the variance of the blood insulin compared to the SQ group (figure 2.6b & figure 2.6c) at both week 8 and 12. To state this alternatively, the OP insulin administration group had significantly more variance in blood insulin than the SQ group.

Many factors, including drug dosage, individual differences within the test subjects, and mechanism of drug action, impact how a drug is absorbed and regulated in the bloodstream (43).

Factors such as social interaction, diet, exercise, sleep/awake schedule, body mass and blood glucose were all closely controlled between individual animals.

Greater variation in blood insulin due to the OP insulin administration route may lead to inconsistent efficacy among different patients. Future studies on OP insulin could consider explaining which aspects of omentum delivery, i.e. integration with the hepatic portal system led to greater blood insulin level variance.

The final aim of the study was to determine how different insulin administration routes affect an individual's ability to utilize glycogen stored in the muscle. A difference between the OP and SQ groups regarding hepatic glycogen was expected. It was theorized that OP administration penetrates deep within the abdominal cavity, triggering the bodies native insulin production pathway. This in turn, stimulates glycogenesis. The results of the study did not align with this hypothesis; there was no statistical difference found between hepatic glycogen in the SQ and OP groups. Although insulin delivery via the OP might mimic physiological insulin secretion better, it may not significantly affect other metabolic pathways influencing hepatic glycogen such as glycogenesis. Since hepatic glycogen were not statistically different in either group, the current study shifted its focus to analyzing glycogen stored in the muscle.

When looking at the white gastrocnemius fibers, a significant difference was seen pre- and post-exercise ( $p=0.0002$ ) in the glycogen content for both the SQ and OP group (figure 2.3b). This highlights the fact that the white gastrocnemius fibers played a role in providing glucose during exercise. A combined effect of time and administration route also yielded significant differences ( $p=0.003$ ). This combined reveals differences existed between the two routes of administration. The SQ group demonstrated a more drastic fall in white gastrocnemius glycogen content



compared to the OP group post-exercise, indicating the animals in the SQ group were better able to utilize stored glycogen content in the white gastrocnemius. This finding can be attributed to the fact the OP group had abnormally high insulin concentration and were unable to down regulate insulin release to allow for glycogenolysis during exercise (39), (42). This pattern of better glycogen utilization for the SQ group seen in the white gastrocnemius was also seen in the red gastrocnemius, however the effects were not as significant. In terms of red gastrocnemius content, once again a significant difference existed between the SQ and the OP groups ( $p=0.037$ ). The SQ group experienced a drop in glycogen content post-exercise, while the OP group gastrocnemius glycogen content remained unchanged post-exercise (figure 2.3c). This finding that the red gastrocnemius saw a smaller decrease post-exercise compared to the white gastrocnemius aligns with the preferred energy utilization in red and white tissue. Red tissue fibers tend to be smaller in diameter and contain both a high density of mitochondria and capillary networks. These fibers, oxidative by nature, are also known as red-oxidative tissue and primarily use fat as fuel. White tissue fibers, also known as anaerobic, or non-oxidative fibers, have larger diameters, a lower density of mitochondria and capillary networks and are glycolytic in nature. As a result of being glycolytic, white tissue fibers are more reliant on glucose as an energy source compared to red tissue fibers (50), (51), (52). Thus, greater differences in glycogen utilization were seen in the white gastrocnemius compared to the red.

Glycogen phosphorylase acts on stored glycogen by catalyzing the first step of glycogenolysis, a process that ultimately yields molecules of glucose (53). There were significantly greater glycogen phosphorylase activity in the SQ group for both red and white gastrocnemius tissue (figure 2.7 a & b). Greater glycogen phosphorylase activity is correlated with greater breakdown of stored glycogen (54), (55). Thus, the reason why the current study observed statistically

greater glycogen utilization in the SQ group could potentially be explained by the SQ groups' significantly greater glycogen phosphorylase activity.

The final aspect to call to attention is the potential for different hormones (i.e. catecholamines, glucagon) to play a role in the observed glucose metabolism. Although the current study did not measure these hormones; it is still important to consider the potential implications of these hormones on the findings. It has been shown that catecholamine levels in rats are elevated following surgical preparation (57). Laparoscopic surgeries (an intra-abdominal surgery) for example, have been shown to elicit an acute stress response leading to increased blood catecholamine levels (57). Increased catecholamines in turn may alter glucose metabolism causing a stimulation of liver glycogenolysis and an elevation in blood glucose levels (54), (55). Marana et al. reported that a neuroendocrine response is elevated following inhalational anesthetics including isoflurane. However, it was shown that this elevation in blood catecholamine levels are transient, and levels do not remain elevated for extended periods postoperatively (58). Although the effect of omentum pouch surgery on catecholamine release is likely minimal, it is still a consideration and a potential impact on the presented liver glucose metabolism measures.

## **2.8 Conclusion**

The results of the current study highlight multiple factors which suggest OP insulin administration is not a viable alternative to traditional SQ insulin administration methods. Rats with T1DM who were given insulin via the OP saw a greater decrease in blood glucose post moderate aerobic exercise. OP rats also showed signs of poor regulation of blood insulin as indicated by high variance in blood insulin measures. Finally, OP rats displayed a reduced ability

to utilize glycogen stored in the muscle when compared to SQ rats as explained by the decreased glycogen phosphorylase activity. Taken together, these findings support the idea that current OP insulin delivery is not a superior alternative to SQ insulin administration.

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## Appendices

### Appendix A: Multiple Low-dose Streptozotocin Induction

Purpose: To induce Type I diabetes in rats

Materials: Gloves, Lab Coat, Streptozotocin (STZ), 5X Stock Citric Acid/Citrate Buffer (Anhydrous Citric Acid, Sodium Citrate Dihydrate, MilliQ Deionized Water), 13M HCl, 3 Falcon Tubes, Sterile Filter.

Equipment: Biological Safety Cabinet, Weigh Scale, pH Meter

Procedure:

#### *Preparing 5X Citric Acid/Citrate Buffer*

1. For a pH 4.6 buffer at 765 mM (5X stock solution), in a beaker, Add:
  1. a. 13.8g Anhydrous Citric Acid (Sigma) or 15.1g Citric Acid Monohydrate
  2. b. 23.8g Sodium Citrate Dihydrate (Sigma), Mix into...
  3. c. 175mL of MilliQ water

The pH should be at 4.6, Add HCl or NaOH to adjust (do not overshoot pH)

2. Once the proper pH is obtained, add MilliQ water until you are close to the 200 ml mark (pH will move slightly). If satisfied with the pH, adjust volume in a 250 ml graduated cylinder and filter in a 0.2 $\mu$ m filter.
3. Store at room temperature. This is the 5X stock solution.

*Making up Streptozotocin (STZ) for Injection \*\*NOTE Animals should be pre-weighed prior to making up STZ to ensure accurate amounts of STZ to be prepared.*

1. Using the pre-made buffer, put 1 mL of buffer in a 50 mL Falcon Tube and add 4 mL of distilled water filtered through a 0.2µm syringe filter. Check the pH. This gives you a working concentration of 153 mM.
2. The desired pH is between 4.5-4.7. Under the fume hood, add 1 drop at a time of concentrated HCl to the buffer, checking pH in between until desired pH is reached.
3. Once pH is reached, add 1 mL distilled water (sterile filtered through a 0.2µm syringe filter as before). If pH is below 4.5, restart.
4. Weigh out an appropriate amount of STZ for the number of animals (see calculations below) that will be injected in a 15-minute time frame.
  - a) Ex. Rats will be injected at 20 mg/kg, so for 10 animals at an ideal weight of 200g (avg. weight of rats to be injected), you will require a minimum of 40mg.  $20 \text{ mg/kg} \times 0.2\text{kg} = 4 \text{ mg per animal}$
  - b) The amount of STZ weighed out should be more than the minimum as some solution will be lost in filtering.  $(4\text{mg (per animal)} \times 12 \text{ rats} = 48 \text{ mg total (0.048g)})$
5. Dissolve the STZ into a buffer (keeping in mind a comfortable injection volume). Shake to dissolve powder (approx. 1 min). Sterile filter using a 0.2µm syringe filter. Ex.  $48\text{mg STZ} \div 3 \text{ mL buffer} = 16 \text{ mg/mL solution}$   $4 \text{ mg} \div 16 \text{ mg/mL solution} = 0.25\text{mL}$
6. STZ is time dependent and must be used within 15 minutes.

*Injecting and Follow-Up of the Animals:*

1. Promptly inject each rat with the solution (intraperitoneal) at a dosage rate of 20 mg/mL (in this example, 0.25mL). Do not use any STZ solution more than 15 minutes after it has been dissolved in the sodium citrate buffer.

2. Dispose of any container having come into contact with the STZ (in either powder or dissolved form) into a biohazardous waste receptacle. Dispose of needles into a sharps container.
3. Return injected rats to their cage. Record the date of STZ injection and add a biohazard label to the cage (leave biohazard label on cage for at least 3 days following the last injection).
4. Repeat this procedure the following day.
5. Check blood glucose daily. Diabetes is achieved with two non-fasting blood glucose readings of  $>18$  mmol Diabetes should be achieved after 5-8 injections (i.p. 20 mg/kg).

**References:**

Low dose STZ induction protocol. Animal Models of Diabetic Complications  
Consortium AMDCC Protocols. 2003

## **Appendix B1: Insulin Pellet Implantation Protocol Subcutaneous**

Materials: LinShin LinPlant Insulin Pellet, Rat anesthetic - Isoflurane, Ampicillin, Sterile water, 1 ml syringe with 25 g needle, 10% povidone-iodine solution, gauze (or swab), Tissue forceps, Scalpel handle and blades (or scissors), Silk suture, Needle drivers.

Equipment: Isoflurane Anaesthetic Machine, Hair clippers, Heat lamp.

Special Safety: Must don lab coat and gloves before handling rodents. Any bite or scratch that breaks the skin must be thoroughly scrubbed with soap and water (report to Occupational Health and Safety).

### Procedure:

1. Anesthetize the animal using the isoflurane machine by placing it in the induction chamber. Set isoflurane to 4-5% with an O<sub>2</sub> flow rate of 1 L/min. Open the stopcock valve so gas reaches the chamber. Keep in the chamber until the animal is unconscious.
2. Remove the animal and place it, stomach-side up, with its nose in the nose cone on the surgery table, reduce the isoflurane to 3% to maintain the plane of anesthesia.
3. Shave the area where the pellet is to be implanted.
4. Using gauze (or a swab), apply 10% povidone-iodine solution to the skin, followed by 70% ethanol, to disinfect the site of insertion.
5. Hold the skin with forceps and make a SQ incision (2-3cm) on the inferior left portion of the abdomen.
6. Using forceps, briefly immerse the pellet in 10% povidone-iodine solution, rinse with saline and insert into the SQ region.
7. Pinch the skin closed after the last pellet is inserted.

8. Close the incision by suturing.
9. Place the animal under a heat lamp and monitor until it recovers from anesthesia.
10. Record on the cage card that insulin pellets have been implanted.

## **Appendix B2: Insulin Pellet Implantation Protocol Omental Pouch**

Materials, Equipment, Special Safety: Same as Appendix B1

1. Anesthetize the animal using the isoflurane machine by placing it in the induction chamber. Set isoflurane to 4-5% with an O<sub>2</sub> flow rate of 1 L/min. Open the stopcock valve so gas reaches the chamber. Keep in the chamber until the animal is unconscious.
2. Remove the animal and place it, stomach-side up, with its nose in the nose cone on the surgery table, reduce the isoflurane to 3% to maintain the plane of anesthesia.
3. Shave the area where the pellet is to be implanted.
4. Using gauze (or a swab), apply 10% povidone-iodine solution to the skin, followed by 70% ethanol, to disinfect the site of insertion.
5. Hold the skin with forceps and make a SQ incision (2-3cm) on the inferior left portion of the abdomen. Once the SQ incision is made, find the abdominal wall using forceps and create another incision (2-3cm) in the abdominal wall.
6. Using forceps, briefly immerse the pellet in 10% povidone-iodine solution, rinse with saline and insert the pellet through both the SQ and abdominal wall layer to least 2 cm.
7. Use forceps to pinch the abdominal wall together after the pellet is inserted.
8. Close the abdominal incision first by suturing it independently from the SQ incision.
9. Once the abdominal incision is closed, suture the SQ opening.
10. Place the animal under a heat lamp and monitor until it recovers from anesthesia.
- 11.** Record on the cage card that insulin pellets have been implanted.

## **Appendix C: Western Blotting Protocol**

Western blotting is a method for the quantification of protein expression and phosphorylation of AKT, G6Pase, and PEPC.

**Tissues tested:** Liver and Red and White Gastrocnemius

### **Sample preparation:**

1. Place homogenized samples on ice.
2. Load the amount of protein in each well previously determined by a protein loading curve.
3. The volume of homogenate needed is determined from a Bradford protein quantification assay (See Bradford Assay protocol, Appendix E).
4. Label a new set of Eppendorf tubes with the appropriate sample names.
5. Dilute the volume of sample in sample buffer (1:1 ratio) and vortex.
6. Boil sample and buffer mixture for up to 5 minutes at 90°C.
7. Prior loading samples into gel allow samples to return to room temperature. It may be necessary to vortex or quickly centrifuge samples before loading.

### **Preparation of gels:**

1. Clean short glass plates with 70% ethanol before use and then prepare gel cassette.
2. Prepare separating gel according to chart relative to the number of gels and percent acrylamide to be used (12% separating gel recipe was used for all blots in this study).
3. After 10 minutes of mixing, pour separating gel using Pasteur pipette while trying to eliminate bubbles.
4. Immediately overlay the gel with water saturate isobutanol to ensure a continuous charge from separating to stacking gel.

5. Wait approximately 45-60 minutes for gel to polymerize and rinse off overlay solution with ddH<sub>2</sub>O and dry clean with filter paper when stacking gel (4%) is ready to pour.
6. Prepare and pour stacking gel (4%) according to the chart below:
7. Place the correct sized comb between the glass plates, ensuring no air bubbles are trapped in the wells and continue to pour stacking gel mixture on the ends of the comb.
8. Prepare 1L of 1x running buffer per 2 gels and store in the refrigerator.
9. Once the stacking gel has polymerized (30 minutes), gently remove the comb and fill wells with 1x running buffer.
10. Load the correct amount of sample and ladder using a micropipette with a loading tip.
11. Once loading is complete place gels in the running unit.
12. Fill the running unit with a cold 1x running buffer (chamber inside the cassette and the outside).
13. Run gels at 70V until through the stacking portion of the gel (~30 minutes) and then 125-130V until sample dye has reached the front of the glass.

14. During the running period, prepare the transfer buffer and keep it in the refrigerator.

#### SEPARATING GEL

<b>10 % Gel</b>				
	<b>2 gels</b>	<b>4 gels</b>	<b>6 gels</b>	<b>8 gels</b>
<b>ddH<sub>2</sub>O</b>	7.996 mL	15.992 mL	23.988 mL	31.984 mL
<b>Acrylamide solution<sup>1</sup></b>	6.667 mL	13.333 mL	20 mL	26.667 mL
<b>Separating gel buffer<sup>2</sup></b>	5 mL	10 mL	15 mL	20 mL
<b>SDS solution<sup>3</sup></b>	200 µL	400 µL	600 µL	800 µL
<b>10% APS solution</b>	125 µL	250 µL	375 µL	500 µL
<b>TEMED</b>	12.5 µL	25 µL	37.5 µL	50 µL
<b>Total Volume</b>	20 mL	40 mL	60 mL	80 mL

#### STACKING GEL

<b>4 % Gel</b>				
	<b>2 gels</b>	<b>4 gels</b>	<b>6 gels</b>	<b>8 gels</b>
<b>ddH<sub>2</sub>O</b>	5.992 mL	11.983 mL	17.975 mL	23.967 mL
<b>Acrylamide solution<sup>1</sup></b>	1.333 mL	2.667 mL	4 mL	5.333 mL
<b>Stacking gel buffer<sup>5</sup></b>	2.5 mL	5 mL	7.5 mL	10 mL
<b>SDS solution<sup>3</sup></b>	100 µL	200 µL	300 µL	400 µL
<b>10% APS solution</b>	62.5 µL	125 µL	187.5 µL	250 µL
<b>TEMED</b>	12.5 µL	25 µL	37.5 µL	50 µL
<b>Total Volume</b>	10 mL	20 mL	30 mL	40 mL



## SEPARATING GEL

<b>10 % Gel</b>				
	<b>2 gels</b>	<b>4 gels</b>	<b>6 gels</b>	<b>8 gels</b>
<b>ddH<sub>2</sub>O</b>	7.996 mL	15.992 mL	23.988 mL	31.984 mL
<b>Acrylamide solution<sup>1</sup></b>	6.667 mL	13.333 mL	20 mL	26.667 mL
<b>Separating gel buffer<sup>2</sup></b>	5 mL	10 mL	15 mL	20 mL
<b>SDS solution<sup>3</sup></b>	200 µL	400 µL	600 µL	800 µL
<b>10% APS solution</b>	125 µL	250 µL	375 µL	500 µL
<b>TEMED</b>	12.5 µL	25 µL	37.5 µL	50 µL
<b>Total Volume</b>	20 mL	40 mL	60 mL	80 mL

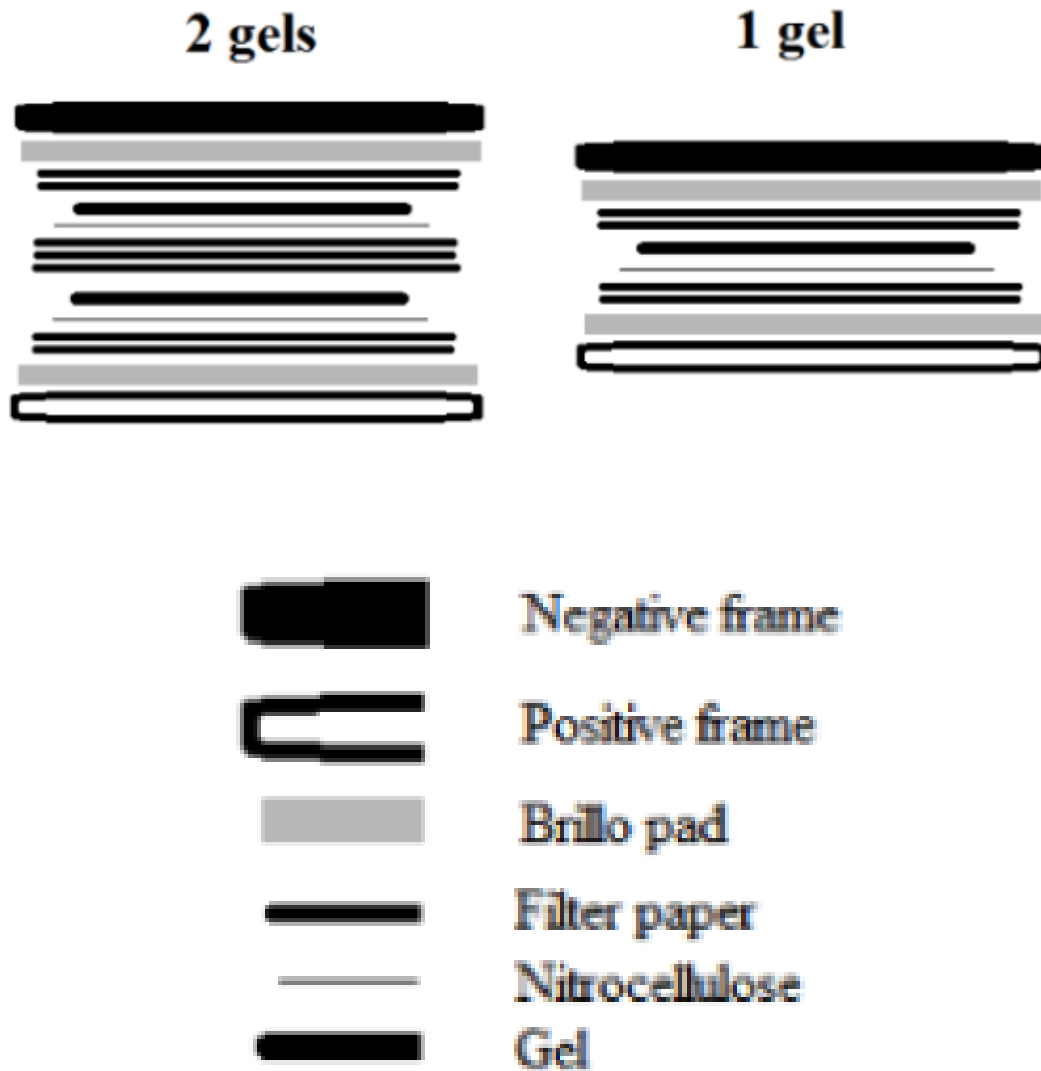
## STACKING GEL

<b>4 % Gel</b>				
	<b>2 gels</b>	<b>4 gels</b>	<b>6 gels</b>	<b>8 gels</b>
<b>ddH<sub>2</sub>O</b>	5.992 mL	11.983 mL	17.975 mL	23.967 mL
<b>Acrylamide solution<sup>1</sup></b>	1.333 mL	2.667 mL	4 mL	5.333 mL
<b>Stacking gel buffer<sup>5</sup></b>	2.5 mL	5 mL	7.5 mL	10 mL
<b>SDS solution<sup>3</sup></b>	100 µL	200 µL	300 µL	400 µL
<b>10% APS solution</b>	62.5 µL	125 µL	187.5 µL	250 µL
<b>TEMED</b>	12.5 µL	25 µL	37.5 µL	50 µL
<b>Total Volume</b>	10 mL	20 mL	30 mL	40 mL

### Transfer of Gels to Nitrocellulose:

1. Cut filter paper and nitrocellulose to appropriate size (short plate size).
2. Soak filter paper, nitrocellulose, and Brillo pads in the cold transfer buffer for 20 minutes.
3. Once the running period is complete, assemble the transfer apparatus (“sandwich”) as shown below, making sure to remove all air bubbles between gel and nitrocellulose paper (keep the sandwich completely submerged in the transfer buffer at all

times).



4. Place “sandwich” into the transfer holding tank making sure the black, negative side is facing the black transfer unit. Fill the tank with the cold transfer buffer and add an ice pack into the unit to keep transfer period cold throughout.
5. Connect to the power supply and run at 70V for 1.5 hours.

**Blocking:**

1. Prepare 1L 1x 2. TBS per 2 blots.
2. After transfer, gently remove gel and place in small container with solution 5% blocking (optional, rinse gel once with 1x TTBS for 5 minutes before blocking) Incubate up to 2 hours on shake at room temperature.
3. After blocking prepare primary antibody (minimum 20 ml of solution)
4. Wash blots 1x in TTBS for 5 minutes.
5. Incubate blots in primary antibody solution overnight at 4 °C. or for two hours at room temperature.
6. Once finished, the primary antibody solution can be stored in the refrigerator for use within a week or stored in the freezer for long term storage.
7. Wash blots 3x in TTBS for 10 minutes each.
8. Prepare secondary antibody (confirm HRP, not AP) solution (1:5000-25000)
9. Incubate blots for 1 hour on shaker at room temperature.
10. Wash blots 3x in TTBS for 10 minutes each. Keep in 1x TBS for long storage.

## Western Blotting

### Solutions:

Name	Components, concentrations, pH	Example amounts	Storage
1. Acrylamide solution	Acrylamide – 30% (w/v) Bis-acrylamide – 0.8% (w/v)	Acrylamide – 150 g Bis-acrylamide – 4 g Total V - 500 mL	4°C
2. Separating gel buffer	Tris – 1.5 M SDS – 0.4 % (w/v) pH – 8.8	Tris – 90.9 g SDS – 2 g Total V- 500 mL	4°C
3. SDS solution	SDS – 10% (w/v)	SDS – 10 g Total V - 100 mL	Room temperature
4. 2X Laemmli SDS-PAGE sample buffer	Tris - 0.125 M Glycerol – 20% (v/v) SDS – 4% $\beta$ –mercaptoethanol – 10% (v/v) Bromophenol blue – 0.015% (w/v) pH – 6.8	Tris – 7.57 g Glycerol – 100 mL SDS – 20 g $\beta$ –mercaptoethanol – 50 mL Bromophenol blue – 0.075 g Total V – 500 mL	Room temperature, fumehood
5. Stacking gel buffer	Tris – 0.5 M SDS – 0.4% (w/v) pH – 6.8	Tris – 30.3 g SDS – 2 g Total V – 500 mL	4°C
10X running buffer	Tris – 0.25 M Glycine – 1.92 M SDS – 1% (w/v)	Tris – 60.6 g Glycine – 288 g SDS – 20 g Total V – 2 L	4°C

6.	1X running buffer	10X running buffer – 10% (v/v)	10 X running buffer – 100 mL Total V – 1 L	4°C
7.	1X transfer buffer	10X running buffer – 10% (v/v) Methanol – 20% (v/v)	10X running buffer – 100 mL Methanol – 200 mL (add last) Total V – 1 L	4°C or colder
	10X TBS	Tris – 0.1 M NaCl – 1 M pH – 7.5	Tris – 24.2 g NaCl – 116.9 g Total V – 2 L	Room temperature
8.	1X TTBS	10X TBS – 10% (v/v) Tween-20 – 0.1% (v/v) pH – 7.5	10X TBS – 200 mL Tween-20 – 2 mL Total V – 2 L	Room temperature
9.	Blocking solution	a. Non-fat, dry milk protein – 5% (w/v) b. Bovine serum albumin – 5% (w/v) 1X TTBS – 95%	a. Non-fat, dry milk protein – 1.25 g b. Bovine serum albumin – 1.25 g 1X TTBS – top up to 25 mL	Room temperature
10.	Primary antibody solution 1	Non-fat, dry milk protein – 2% (w/v) 1X TTBS – 98% Antibody against protein of interest	Non-fat, dry milk protein – 0.4 g 1X TTBS – top up to 20 mL	4°C (-20°C long term)
11.	Primary antibody solution 2	Bovine serum albumin – 5% (w/v) 1X TTBS – 95% Antibody against protein of interest	Bovine serum albumin – 1 g 1X TTBS – top up to 20 mL	4°C (-20°C long term)
12.	Secondary antibody solution	Non-fat, dry milk protein – 2% (w/v) 1X TTBS – 98% Antibody against primary antibody	Non-fat, dry milk protein – 0.4 g 1X TTBS – top up to 20 mL	Room temperature
13.	1X TBS	10X TBS – 10% (v/v) pH – 7.5	10X TBS – 10 mL Total V – 100 mL	Room temperature

## References:

Murray, Michael, "The Effect of Aerobic Exercise Training on Hepatic Glycogen Metabolism in Type 1 Diabetic Rats" (2015). Electronic Thesis and Dissertation Repository. 2726. Appendix E: Phenol <https://ir.lib.uwo.ca/etd/2726>

## Appendix D: Glycogen Assay

Materials: Tissue samples, Fume hood, 3 x 100mm glass test tubes, Potassium hydroxide (KOH) pellets, Sodium sulfate (Na<sub>2</sub>SO<sub>4</sub>), 95% ethanol, Glycogen powder, Phenol crystals, 96-98 % Sulfuric Acid (H<sub>2</sub>SO<sub>4</sub>), 490 nm spectrophotometer.

### Standard Curve:

Prepare a standard curve with a stock glycogen solution (1mg/mL).

Standard	[Glycogen] (mg/ml)	Volume (µl)	Water (µl)
1	0.000	0	1000
2	0.005	5	995
3	0.010	10	990
4	0.050	50	950
5	0.100	100	900
6	0.200	200	800
7	0.300	300	700

### Colour Reaction:

Add 1 ml of 5 % phenol, rapidly add 5 ml of 96-98 % H<sub>2</sub>SO<sub>4</sub>.

Let samples stand for 10 min.

Vortex and place in a water bath (25 to 30C) for 10 to 20 minutes.

Read tubes at 490 nm.

Procedure:

1. Cut and weigh approximately 20 mg of tissue samples and place in glass test tube.
2. Saturate 30% KOH stock solution with sodium sulfate ( $\text{Na}_2\text{SO}_4$ )
3. Add 0.5 ml of 30 % KOH saturated with  $\text{Na}_2\text{SO}_4$ . Make sure that tissue is completely submerged.
4. Put caps on tubes and immerse in boiling water bath until homogenous solution is obtained (30 minutes).
5. Place tubes on ice.
6. Precipitate glycogen with 1mL of 95 % ethanol for 30 minutes (on ice).
7. Spin tubes at 840 x g (3 000 rpm on Sorval) for 20 to 30 minutes.
8. Remove supernatants and immediately dissolve precipitates in 3mL ddH<sub>2</sub>O. Do not allow precipitated to dry.
9. Pipette 1 ml glycogen solution into 3 separate glass test tubes (3 x 100mm). - 3 separate tubes of glycogen solution are necessary for analysis in triplicates. 9. Add 1 ml of 5 % phenol.
10. Rapidly add 5 ml of 96-98 % H<sub>2</sub>SO<sub>4</sub>.
11. Let samples stand for 10 min.
12. Vortex and place in a water bath (25 to 30C) for 10 to 20 minutes.
13. Read tubes at 490 nm.
14. Calculate glycogen content by:

$$\text{g of glycogen} / 100\text{g tissue} = \frac{A_{490}}{k} \times \frac{V}{v} \times \frac{10^{-4}}{W}$$

where, V = total volume of glycogen solution; v = volume of aliquot used in colour reaction; A490 = absorbance at 490 nm; W = weight of tissue samples in grams; k = slope of standard curve; units = 1 per microgram glycogen

Solutions:

Glycogen Stock Solution (1 mg/ml)	
100 ml	ddH2O
100 mg	Glycogen Powder

30% Potassium Hydroxide Stock Solution	
300g	Potassium Hydroxide Pellets (KOH)
1L	ddH2O
Reaction is exothermic; therefore, it might be necessary to put on ice.	

Spectrometer Procedure:

1. Turn on ~20 minutes before needed (TRANS)
2. Set desired  $\lambda$  & insert appropriate filter.
3. Insert a blank tube (dH2O)
4. With spec. on "TRANS", set to 0.000 using "zero" button & while pressing "zero set."
5. Release "zero set" but adjust value to 100.00 with spec set to "TRANS" using "100% T/OA" adjustments.



6. Set spec from “TRANS” to “ABS” using LHS button.
7. Check that “ABS” reading is 0.00; if not adjust with “100%T/OA.”
8. Check that a true a zero reading has been reached by reading the abs values of 2 other blank tubes.
9. Read 3 reagent tubes (blank)

**Reference:**

Lo, S.L., Russell, J.C., and Taylor, A.W. (1970) *J Appl. Physiol*, 28(2), 234-6.

## Appendix E: Bradford Protein Assay

Analytical procedure used to calculate the concentration of protein in liver and muscle samples.

### Procedure:

1. Dilute 1 part dye reagent with 4 parts ddH<sub>2</sub>O (Bio Rad 500-0006). One microplate requires 25 mL reagent.
2. Filter the diluted solution through Whatman 1 filter paper (store solution at room temperature up to two weeks).
3. Add the indicated amounts of BSA (1 mg/mL) or unknown protein sample, water, and reagent respectively into a 96-well microplate.

Protein (ug)	Water (uL)	BSA (uL)	Unknown sample (uL)	Reagent (uL)
0	10	0	-	200
1	9	1	-	200
2	8	2	-	200
3	7	3	-	200
4	6	4	-	200
5	5	5	-	200
6	4	6	-	200
unknown	9.5	-	0.5	200

4. Shake and incubate at room temperature for a couple of minutes.
5. 5. Read absorbance at 595 nm.

### References

- Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, 72(1–2), 248–254. [https://doi.org/10.1016/0003-2697\(76\)90527-3](https://doi.org/10.1016/0003-2697(76)90527-3)
- Murray, Michael, "The Effect of Aerobic Exercise Training on Hepatic Glycogen Metabolism in Type 1 Diabetic Rats" (2015). *Electronic Thesis and Dissertation Repository*. 2726. <https://ir.lib.uwo.ca/etd/2726>

## Appendix F: Rat Insulin ELISA Kit

Sample Handling: Serum and plasma samples are appropriate for use in this assay. No dilution or treatment of the sample is required. However, if a sample has a greater concentration of insulin than the highest standard, the sample should be diluted in Zero Standard and the analysis should be repeated.

It is recommended to 1) thoroughly vortex each sample before use and 2) perform pipetting actions without pausing.

Samples can be stored at 2-8°C for 24 hours prior to analysis in this assay. For longer periods, storage at < -20°C is recommended. Avoid repeated freezing/thawing of the sample.

Reagent Preparation: All reagents must be equilibrated to room temperature prior to preparation and subsequent use in the assay:

**Conjugate Stock** is to be diluted with 10 parts Conjugate Buffer. For example, to prepare enough Working Strength Conjugate for one complete microplate, dilute 0.9 mL of Conjugate Stock (11X) with 9 mL of Conjugate Buffer.

**Working Strength Conjugate** is stable for 4 weeks at 2-8°C. Wash Buffer Concentrate is to be diluted with 20 parts distilled water. For example, to prepare Working Strength Wash Buffer, dilute 20 mL of Wash Buffer Concentrate (21X) with 400 mL of deionized water. Working Strength Wash Buffer is stable for 4 weeks at room temperature (18-25°C).

**Controls (Levels 2 and 3)** are provided in a lyophilized form. Please refer to the Certificate of Analysis provided with each kit for the appropriate volume of deionized water for reconstitution. Close the vial with the rubber stopper and cap, gently swirl the

vial, and allow it to stand for 30 minutes prior to use. The contents of the vial should be in solution with no visible particulates. The reconstituted controls are stable for 7 days stored at 2-8°C. If desired, the controls can be stored at  $\leq -20^{\circ}\text{C}$  in aliquots for up to 6 months. The controls should not be repeatedly frozen and thawed.

#### Assay Procedure:

All reagents and microplate strips should be equilibrated to room temperature (18-25°C) prior to use. Gently mix all reagents before use. A standard curve must be performed with each assay run and with each microplate if more than one is used at a time. All standards, controls, and samples should be run in duplicate.

1. The microplate should be equilibrated to room temperature prior to opening the foil pouch. Designate enough microplate strips for duplicate determinations of the standards, controls, and samples. The remaining microplate strips should be stored at 2-8°C in the tightly sealed foil pouch containing the desiccant.
2. Pipette 10  $\mu\text{L}$  of each standard, control, and sample into their respective wells. See Reagent Preparation and Certificate of Analysis for control reconstitution instructions.
3. Pipette 75  $\mu\text{L}$  of Working Strength Conjugate (see Reagent Preparation) into each well.
4. Cover microplate with a plate sealer and incubate for 2 hours at room temperature, shaking at 700-900 rpm on a microplate shaker.
5. Decant the contents of the wells and wash the microplate 6 times with 350  $\mu\text{L}$  of Working Strength Wash Buffer per well (see Reagent Preparation) using a microplate washer. Alternatively, fill the wells with Working Strength Wash Buffer using a wash

- bottle equipped with a wash nozzle. (It is not recommended to use a multichannel pipette. Wash buffer must be dispensed with adequate and equal force in order to properly wash the wells.) Between washes, invert the microplate to discard the liquid and firmly tap the inverted microplate on absorbent paper towels. After the final wash, (automated or manual), remove any residual Wash Buffer and bubbles from the wells by inverting and firmly tapping the microplate on absorbent paper towels.
6. Pipette 100  $\mu$ L of TMB Substrate into each well.
  7. Cover microplate with a plate sealer and incubate for 15 minutes at room temperature, shaking at 700-900 rpm on a microplate shaker.
  8. Pipette 100  $\mu$ L of Stop Solution into each well and gently shake the microplate to mix the contents. Remove any bubbles before proceeding with the next step.
  9. Place the microplate in a microplate reader capable of reading the absorbance at 450 nm. The microplate should be analyzed immediately after the addition of the Stop Solution, and no longer than 30 minutes after.

## **Reference**

ALPCO Rat Insulin ELISA Kit. (Catalog 80-INSRT-E01). Salem, NH: ALPCO. Retrieved from [<https://www.alpco.com/rat-insulin-elisa.html>]

## Appendix G: Abcam Glycogen Phosphorylase Assay Kit (Colorimetric)

Materials (not supplied): 96-well clear flat-bottom plate, multi-well spectrophotometer, 50% Glycerol, PBS.

### Reagent Preparation:

Briefly centrifuge small vials at low speed prior to opening.

1. **Assay Buffer XXXVII/Assay Buffer:** Ready to use as supplied. Warm bottle to room temperature before use. Store at 4°C. 2.
2. **Enzyme Mix XVII/Enzyme Mix:** Reconstitute with 220  $\mu$ L of Assay Buffer XXXVII/Assay Buffer. Pipette up and down to dissolve completely. Keep on ice while in use. Store at -20°C and use within 2 months.
3. **Development Enzyme Mix IX/Developer:** Reconstitute with 220  $\mu$ L of Assay Buffer XXXVII/Assay Buffer. Pipette up and down to dissolve completely. Keep on ice while in use. Store at -20°C and use within 2 months.
4. **Developer Solution III/Substrate Mix:** Reconstitute with 220  $\mu$ L of Assay Buffer XXXVII/Assay Buffer. Pipette up and down to dissolve completely. Keep on ice while in use. Store at -20°C and use within 2 months.
5. **G1P Standard:** Reconstitute with 100  $\mu$ L of water to generate 100 mM G1P. Store at -20°C and use within 2 months.
6. **Glycogen Substrate/Glycogen:** Reconstitute with 1.2 mL of water. Pipette up and down to dissolve. Keep cold while in use. Store at -20°C and use within 2 months. \

7. **Glycogen Phosphorylase:** Reconstitute with 50  $\mu\text{L}$  of 50% glycerol (not included). Keep cold while in use. Aliquot and store at  $-20^{\circ}\text{C}$ . Avoid repeated freeze thaw cycles.

Sample Preparation:

1. Homogenize tissue (50 mg) or cells (10<sup>6</sup> cells) with 200  $\mu\text{L}$  of ice-cold Assay Buffer XXXVII/Assay Buffer.
2. After homogenization, keep the lysates on ice for 15 min.
3. Centrifuge at 10,000 x g and  $4^{\circ}\text{C}$  for 15 min. Transfer the clear sample supernatant to a new tube.
4. For supernatants prepared from tissues, use PBS to further dilute the supernatant.
5. For each tested sample, prepare two parallel wells: Sample and Background Control

Standard Curve:

1. Mix 10  $\mu\text{L}$  of 100 mM G1P with 990  $\mu\text{L}$  of water to prepare 1 mM G1P Standard. Keep on ice while in use.
2. Add 0, 2, 4, 6, 8 and 10  $\mu\text{L}$  of the 1 mM G1P Standard into the desired wells to generate 0, 2, 4, 6, 8 and 10 nmole of G1P per well, respectively.
3. Adjust the volume to 50  $\mu\text{L}$  with Assay Buffer XXXVII/Assay Buffer.

Assay Procedure:

Thaw all reagents thoroughly and mix gently.

1. Mix enough reagents for the number of assays to be performed. For each well, prepare 50  $\mu\text{L}$  of the appropriate Reaction Mix containing:

Reaction mixes: Prepare enough reagents for the number of assays to be performed:

Component	Reaction Mix	Background Reaction Mix
Assay Buffer XXXVII/Assay Buffer	34 $\mu\text{L}$	44 $\mu\text{L}$
Glycogen Substrate/Glycogen	10 $\mu\text{L}$	-
Enzyme Mix XVII/Enzyme Mix	2 $\mu\text{L}$	2 $\mu\text{L}$
Development Enzyme Mix IX/Developer	2 $\mu\text{L}$	2 $\mu\text{L}$
Developer Solution III/Substrate Mix	2 $\mu\text{L}$	2 $\mu\text{L}$

2. **Samples:** For each tested sample prepare two parallel wells: Sample (S) and Background Control (BC) wells by adding 2  $\mu\text{L}$  of the supernatant (see Section 10) to the desired wells in a 96-well clear flat-bottom plate. Adjust the volume to 50  $\mu\text{L}$  with Assay Buffer XXXVII/Assay Buffer.
3. **Positive Control:** For Positive Control (PC) well, add 2  $\mu\text{L}$  of the glycogen phosphorylase enzyme into designated wells in the plate. Adjust the volume to 50  $\mu\text{L}$  with Assay Buffer XXXVII/Assay Buffer.
4. To start the reaction, add 50  $\mu\text{L}$  of Reaction Mix to each well containing Standards, Samples (S) or Positive Control (PC).
5. To all Sample Background Controls (BC) add 50  $\mu\text{L}$  of Background Mix to the desired Sample wells.



6. Measure the OD at 450 nm in kinetic mode at 30°C for 60 min. After the reaction completes, the OD 450 nm signal may start to decrease. Therefore, use the maximum OD 450 nm for calculation.

Calculations:

1. Subtract 0 pmol Standard from all Standard readings.
2. Plot the G1P Standard Curve.
3. Select two time points within the linear portion of the curve t1 and t2.
4. Subtract the Sample Background OD reading from Sample reading for these two time points.
5. Calculate the glycogen phosphorylase activity of the Sample: OD 450 nm = OD2 – OD1 at time points t2 and t1.
6. Apply the  $\Delta$ OD 450 nm to the G1P standard curve to get A nmol of G1P generated during the reaction time ( $\Delta t = t2 -$

$$\text{Specific activity} = \frac{A \times D}{(\Delta t \times X \times M)} \text{ (mU/mg)}$$

t1).

Where, A = G1P from Sample calculated using the Std Curve equation (nmol)  $\Delta t$  = Reaction time, D = Dilution Factor and M = Sample used (mg)

## **Reference**

Abcam Glycogen Phosphorylase Assay Kit (Colorimetric) (Catalog ab273271). Retrieved from [\[https://www.abcam.com/products/assay-kits/glycogen-phosphorylase-assay-kit-colorimetric-ab273271.html#:~:text=The%20Glycogen%20Phosphorylase%20Colorimetric%20Assay,a%20variety%20of%20sample%20types.\]](https://www.abcam.com/products/assay-kits/glycogen-phosphorylase-assay-kit-colorimetric-ab273271.html#:~:text=The%20Glycogen%20Phosphorylase%20Colorimetric%20Assay,a%20variety%20of%20sample%20types.)

## **Appendix H: Ethics Approval**

The protocols utilized in this study were approved and monitored by the Canadian Council on Animal Care of Western University (London, Ontario, Canada).

## **Curriculum Vitae – Amit Sayal**

University of Western Ontario | Master of Science – Exercise Biochemistry & Metabolism,

Kinesiology: 2022 – 2024

University of Western Ontario | Bachelor of Arts (Western Scholars), Honors Specialization

Kinesiology, Western Scholars Electives Program: 2018 – 2022

### **AWARDS, HONORS & SCHOLARSHIPS**

- University of Western Ontario Dean’s Honor List – 2018-2023
- Western University Howard G. Ferguson Award – 2022
- OUA Dale Iwanoczko Award of Merit - 2022
- USPORTS Academic All Canadian – 2018-2023
- OUA All Academic – 2018-2023
- UWO In-Course Scholarship Year II - 2019
- The Western Scholarship of Excellence – 2018

## **PUBLICATIONS**

**1. Authors:** Mitchell J. Sammut, David P. McBey, **Amit P. Sayal**, C. W. James Melling

**Title:** The Effects of Resistance Exercise Training on Insulin Resistance Development in Female Rodents with Type 1 Diabetes

**Journal:** Journal of Diabetes Research

**Citation:** Mitchell J. Sammut, David P. McBey, Amit P. Sayal, C. W. James Melling, "The Effects of Resistance Exercise Training on Skeletal Muscle Metabolism and Insulin Resistance Development in Female Rodents with Type 1 Diabetes", Journal of Diabetes Research, vol. 2024, Article ID 5549762, 14 pages, 2024. <https://doi.org/10.1155/2024/5549762>

## **PRESENTATION & CONFERENCE EXPERIENCE**

**1.** York University 14th Muscle Health Awareness Day. Toronto, ON | May 2023

**Title of Presentation:** How Different Insulin Administration Routes Effect Hepatic Glycogen Levels after an Aerobic Exercise Bout in Male Rats with Type 1 Diabetes

## **RESEARCH EXPERIENCE**

1. Academic Skill Transmission Between Mothers and Their Children Periodical or Conference Name: Western University - Scholar's Electives Thesis Presentations - May 2021
2. London Health Sciences Centre Summer Research 1st author of article "5 Things To Know About Oxygen Poisoning". Discusses dangers of administering oxygen in various conditions. Conducted literature review & wrote the article. May – June 2023

3. St. Michael's Hospital Neuroscience Lab Internship (Concussion) "Do Neural Network Connectivity Deficits Persist Chronically in Concussed Athletes?". Identified gaps in concussion literature. Screened hundreds of fMRI scans for study relevance. Studied the effects of concussions in athletes on neural networks. June – August 2023
4. The Hospital for Sick Children Research Assistant Duchenne Muscular Dystrophy (DMD). screened 10,000+ patient charts for DMD & analyzed sleep (REM, titration/BiPAP etc.) to determine if eligible for study. May 2019
5. The Hospital for Sick Children Research Assistant Neuromuscular Weakness (NMW). analyzed 200+ NMW research studies. Assessed eligibility of each article for the meta-analysis. June – July 2019

## **TEACHING & MENTORING EXPERIENCE**

1. Captain Western Men's Varsity Volleyball Team - September 2018 – April 2024
2. Student Athletic Mentorship Program Mentor - September 2020 – April 2022
3. Founder of "Mustangs in the House" Community Outreach Program – September 2021 – April 2024