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Effect of Acute Exercise on Muscle and Liver Glucose Metabolism in T1DM Rodents

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Abstract

Individuals with Type I Diabetes Mellitus (T1DM) are at an increased risk for developing secondary complications which increases their risk of premature death. While the risk of secondary complications is reduced with intensive insulin treatment (IIT) and aerobic exercise (AE), both interventional strategies increase the risk of hypoglycemia. This study examined the effects of a 60-min bout of forced treadmill AE on hepatic and muscular blood glucose (BG) metabolism. Nineteen Sprague-Dawley rats were divided into two groups: Sedentary Control Rats (SC; n=5), and Sedentary T1DM rats (DSC); T1DM rats were subcategorized into Diabetic Pre-Exercise (DPRE; n=14) and Diabetic Post-Exercise (DPOST; n=7). The study was conducted over twelve weeks and upon completion the DPOST group underwent a 60-min bout of AE. Immediately following the experimental protocol both liver and muscle tissue were analyzed to measure BG, hepatic and muscular glycogen, and several liver and muscular regulatory enzymes (G6Pase, PEPCK, AKT). The DPOST rats had significantly lower hepatic glycogen when compared to the SC group but there was no difference when compared to the DPRE rats. G6Pase protein content was greater in the DPOST when compared to the SC group. Phosphorylated AKT (P-AKT) was greater in DPOST rats when compared with both SC and DPRE groups. However, no significant differences were found in PEPCK protein content in response to diabetes and exercise. These findings suggest that during AE in T1DM rats both gluconeogenesis and both hepatic and muscle glycogen is underutilized leading to a reliance on circulating BG to meet the increased metabolic demands during exercise.

Keywords

Type 1 diabetes mellitus, exercise, skeletal muscle, glycogen, gluconeogenesis, hypoglycemia

Summary for Lay Audience

Type 1 diabetes mellitus (T1DM) is a result of the destruction of pancreatic beta cells which produce insulin. Most individuals with T1DM have an impaired ability to keep their blood sugars in a healthy range and require insulin supplementation and are thus at a higher risk for developing secondary complications such as cardiovascular disease. The two best ways to decrease the risk of developing secondary complications include exercise and intensive insulin treatment; however, both of these treatment strategies increase the risk of blood sugars dropping too low (<3.0 mM; hypoglycemia). To further understand the physiological pathways that contribute to this response during exercise this study examined several glucose-related metabolic pathways in the liver and muscle. In this study there were two groups: (1) sedentary control rats (SC) and (2) T1DM rats (DSC) which were subcategorized into pre-exercise (DPRE) and post-exercise groups (DPOST). Over twelve weeks, weekly blood sugars and body masses were measured and the DPOST group underwent a 60-min bout of AE. After completion of the experimental protocol, several enzymes (G6Pase, PEPCK, AKT) in both liver and muscle glycogen content were analyzed. Significant reductions in hepatic glycogen storage were observed between T1DM groups (DPRE, DPOST) and SC but no differences across groups were found in muscle glycogen storage. Significantly greater concentrations of G6Pase were observed in DPOST when compared with SC; while significantly greater proportions of phosphorylated AKT (P-AKT) were observed in DPOST when compared with both SC and DPRE. Additionally, no significant differences were found in PEPCK protein content between the groups. These findings suggest that during AE in T1DM rats gluconeogenesis and both muscle and liver glycogen are underutilized, leading to a reliance on circulating blood sugar to meet the demands of exercise.

Co-Authorship Statement

Dr. Jamie Melling of Western University, London, Ontario Canada, was involved in designing the project, interpretation of results and thesis revisions. Theres Tijo, also of Western University, was involved with data collection by providing invaluable help with several laboratory protocols. I Extend my gratitude to you all.

Dedication

To Emily, Matt, Casandra, Jessie, Tiffany and all of my great friends for all the love and support they provided throughout my academic journey

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List of Abbreviations

Acetyl-CoA – Acetyl Co-Enzyme A

ADP – Adenosine Diphosphate

ADA – American Diabetes Association

AE – Aerobic Exercise

AKT – Protein Kinase B

ATP – Adenosine Triphosphate

CDA – Canadian Diabetes Association

CGM – Continuous Glucose Monitoring

CVD – Cardiovascular Disease

DCCT – The Diabetes Control and Complications Trial

DSC – Type 1 Diabetic Sedentary Control

DPOST – Type 1 Diabetic Post-Aerobic Exercise Group

DPRE – Type 1 Diabetic Pre-Aerobic Exercise Group

EDIC – The Epidemiology of Diabetes Interventions and Complications study

EGP – Endogenous Glucose Production

G6P – Glucose-6-phosphate

G6Pase – Glucose-6-phosphatase

GLUT4 – Glucose Transporter Protein

HbA1c – Glycated Haemoglobin

P-AKT – Phosphorylated Protein Kinase B

SC – Sedentary Control

STZ – Streptozotocin

T1DM – Type 1 Diabetes Mellitus

T2DM – Type 2 Diabetes Mellitus

VO2max – Maximal Rate of Oxygen Consumption

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Chapter 1

1.1 Overview of Type 1 Diabetes Mellitus

Diabetes mellitus is a condition that is defined as a group of metabolic diseases associated with the body's inability to produce or effectively utilize insulin. Diabetes can be subcategorized into three main types: Type 1 diabetes mellitus (T1DM), Type 2 diabetes mellitus (T2DM), and gestational diabetes mellitus. Gestational diabetes affects women during pregnancy, increasing the risk of birth and pregnancy complications for both the mother and baby. The term 'Prediabetes' has been increasingly used to define individuals who have impaired glucose tolerance and/or impaired fasting glucose but have not yet been diagnosed with diabetes and are at increased risk for developing T2DM. T2DM, formerly known as insulin-independent diabetes, is the most common type of diabetes found world-wide (90 to 95% of all diabetic cases in the United States)[1] and is associated with a developed resistance to insulin due to many factors that include obesity, aging, and physical inactivity [1]. The inability to utilize insulin can lead to chronically elevated blood glucose (BG) concentrations (hyperglycemia). T1DM is the most common type found in children and adolescence but can occur at any age. Individuals with T1DM require external insulin and continuously adjust and monitor regularly to maintain normal BG concentration, normal mass, and normal development in children[1]. There is no means to prevent the onset of T1DM, however, it is possible for these individuals to live healthy lives if they monitor their condition strictly. Some of the variables that need to be managed include diet, exercise, BG concentration, and insulin dosage levels. Individuals with T1DM are encouraged to closely monitor their diet and nutrition to ensure they stay within a healthy BG concentration (4.0-7.0 mmol/L fasting BG). Moreover, the incidence of T1DM is increasing worldwide, with high prevalence in western countries. This

disorder affects approximately 451 million people world-wide and is estimated to reach 693 million by 2045.[2–4]

In 1922 Dr. Frederick Banting and Charles Best successfully isolated insulin that could be used to treat individuals with T1DM and shortly after their discovery insulin was distributed by a major pharmaceutical company becoming the primary drug to treat T1DM.[66] Today, insulin is self-administered through one of two ways: (1) subcutaneous injection or (2) implanted insulin pumps. Insufficient insulin leads to subsequent hyperglycemia which can result in the development of both microvascular and macrovascular complications.[5–8] Microvascular complications involve the eyes, kidneys, and peripheral nervous system, and macrovascular diseases include neuropathy, retinopathy, nephropathy and increased risk of developing cardiovascular disease [6,7].

In 1993, the Diabetes Control and Complications Trial (DCCT) was published, and there were two aims of this trial. The first aim was to test the 'glucose hypothesis', and the second aim was to compare conventional insulin treatment (CIT) with intensive insulin treatment (IIT).[6,7] The 'glucose hypothesis' focused on determining if achieving euglycemia would reduce the risk of long-term complications associated with diabetes. The CIT group maintained glycated hemoglobin (HbA1c) values at 13% or lower using insulin injections. Whereas the IIT group maintained more strict control over their HbA1c values at about 6.5% via multiple daily injections of insulin. The findings of the DCCT confirmed that the glucose hypothesis was correct, and the IIT intervention led to near-euglycemia and reduced the percentage of microvascular and cardiovascular complications associated with T1DM. These findings led to the recommendation of IIT for patients with T1DM as standard care. The findings were so strongly in support of IIT that the CIT strategy was halted, and all CIT groups were switched to IIT treatment.[6,7] Building on these studies, the Epidemiology of Diabetes Interventions and Complications (EDIC) trial was conducted from 1994

to 2017, in which they reorganized and followed the individuals from the DCCT[6,7]. This trial examined measures associated with atherosclerosis and despite having all individuals being converted from CIT to IIT, the newly converted patients showed worse outcome measures compared to the intensive only treatment[6,7]. These findings suggest that prior metabolic control can impact complications in individuals with T1DM even after changes occur and this is known as metabolic memory.[6,7]

It is still unknown why autoimmunity in T1DM is specific to the insulin producing B cells and what the specific mechanism for autoimmunity is. Regarding environmental factors that could potentially contribute to the onset of T1DM, there are several proposed theories. These include the accelerator and overload hypotheses; the hygiene hypothesis; the fertile field hypothesis; old friends' hypothesis; and the threshold hypothesis. The Accelerator and Overload hypothesis suggests that environmental stressors increase insulin demand, which overloads islet cells and accelerates B cell damage.[1] The Hygiene hypothesis suggests that the rising incidence of autoimmune disease in general is due to a reduced or altered stimulation of the immune system by environmental factors (ex. children not being exposed to as many germs at an early age).[1] The Fertile Field hypothesis suggests that microbial infection induces a temporary state in which other antigens can more easily react, yielding autoreactive T cells.[1] Old Friends hypothesis suggests that dietary exposure may be a direct regulator of the immune system and of self-tolerance by altering gut microbia and permeability.[1] Other environmental factors that have been linked to T1DM development include infectious agents, and nutritional influences such as lack of breastfeeding, low vitamin D, and high consumption of cow's milk.[9]

1.2 Fuel Utilization in Exercise: Healthy vs. T1DM

Glycogen is a complex molecule in which glucose molecules are stored in the body in a way that makes it readily available. This is important as glucose is the primary fuel for our bodies. There are two main sites in the body in which glycogen is stored: liver and skeletal muscle. The concentration of glycogen is much greater in the liver than in muscle tissue, however more glycogen is stored in muscle tissue due to its larger overall mass.

It is well established that an acute bout of exercise is associated with a large increase in glucose production (GP) to meet the metabolic needs of the working muscle. Increases in GP can be accounted for by increasing net hepatic glycogenolysis and/or increases in gluconeogenesis. Hepatic glycogenolysis occurs when glycogen is broken down into free glucose molecules which can then be released into the blood stream. Gluconeogenesis occurs when substrates other than glucose such as lactate, free fatty acids and amino acids are converted into new glucose molecules which can then be transported in the blood and used as a fuel source by other tissues. Studies have shown that exercise induces both increases in gluconeogenesis and hepatic glycogenolysis to account for increased metabolic needs.[11,12] Individuals with T1DM have an observed increase in resting rates of GP leading to reduced concentrations of hepatic glycogen in both the fasting state and after mixed-meal intake.[13]

In non-diabetic patients, exercise increases GP proportionally to the intensity of exercise and this is accounted for by a matched increase in the rate of net hepatic glycogenolysis. In contrast, individuals with T1DM experience an increase in the rate of GP that can be attributed to excessive rates of gluconeogenesis both at rest and during exercise.[13] In 2004, Petersen et al. found that individuals with T1DM had hepatic glycogen concentrations that were 33% lower than in

healthy controls.[13] Despite this reduced hepatic glycogen concentration, rates of resting hepatic glycogenolysis were similar to those of healthy controls. However, since rates of resting GP were increased in T1DM individuals, net hepatic glycogenolysis accounted for only about 20% of GP and thus the increased rate of gluconeogenesis is the major factor responsible for the increased rates of GP.[13] Some of the earliest studies to examine GP and gluconeogenesis were conducted by Felig and Wahren in 1970.[14] This work demonstrated that hepatic gluconeogenesis contributed to about 20-25% of GP at rest and during exercise this decreased to about 6-11%.[14,15] In 2004, Petersen et al found that the high rate of GP at rest and during exercise in patients with T1DM was a result of a 2-2.7-fold increase in gluconeogenesis which is consistent with previous findings.[13]

The hormonal regulation of gluconeogenesis and glycogenolysis during exercise have been studied thoroughly in both human and animal models[16–18]. It is generally accepted that the increases in GP during exercise is mainly due to an increase in plasma glucagon and a decrease in plasma insulin.[13] Previous research has suggested that a decrease in insulin is necessary to limit excessive BG uptake and an increase in glucagon is required for the promotion of hepatic GP to prevent hypoglycemia.[13] Furthermore, Wahren et al. (2007), found that in individuals with T1DM glucose infusion failed to inhibit GP which is contrary to what occurs in non-diabetic individuals.[11] Exercise-induced increases in GP can be attributed to stimulation of net hepatic glycogenolysis which is accomplished by coordinated hormonal responses including a decrease in the insulin/glucagon ratio and an increase in plasma epinephrine and norepinephrine concentrations [13]. Epinephrine and Glucagon are two key hormones that signal the need for glycogen breakdown. In response to muscular activity there is an increased release of epinephrine which in turn stimulates glycogen breakdown in muscle and to a lesser extent in the liver. The liver responds more to the release of glucagon which occurs when BG concentrations are lowering (<5 mmol/L).

However, it is important to turn off glycogen breakdown quickly to prevent the depletion of glycogen stores once the energy needs are met.

The liver plays a major role in maintaining euglycemia and phosphorylated glucose produced by glycogen breakdown. Glucose-6-phosphate (G6P) is not readily transported out of cells into the blood stream and requires a liver specific enzyme known as glucose 6-phosphatase (G6Pase) which cleaves the phosphoryl group from G6P creating a free glucose molecule (and an inorganic phosphate). G6Pase also releases free glucose at the completion of gluconeogenesis and can be viewed as a rate-limiting enzyme for this process.[19] Phosphoenolpyruvate carboxykinase (PEPCK) is also an important rate-limiting enzyme for BG homeostasis. PEPCK is the enzyme that converts oxaloacetate into phosphoenolpyruvate (PEP) and carbon dioxide (CO_2). It has been observed in rodents that overexpression of PEPCK can lead to chronically elevated BG concentrations and the development of T2DM.[20,59,60] PEPCK activity is stimulated by both cortisol and glucagon and in turn increases gluconeogenesis. In contrast, PEPCK is inhibited by insulin in circumstances which require gluconeogenesis to be reduced (i.e. feeding). In patients with T1DM, the PEPCK activation and deactivation is impaired due to altered glucagon release and requirement of exogenous insulin, which in turn increases the risk of hypoglycemia.[20,21]

Another important protein that helps regulate the insulin pathway is Protein Kinase B (AKT). AKT has three isoforms which include AKT1, AKT2, and AKT3, which are all responsible for different physiological functions. AKT2 facilitates insulin-induced translocation of the glucose transporter 4 (GLUT4) to the plasma membrane. This mechanism contributes to the uptake of glucose from the blood stream in muscle, adipocytes, liver and other tissues. It has been suggested that AKT has two principal roles in glucose metabolism. First, after being stimulated by insulin, AKT associates with GLUT4 transports, and this causes increased plasma glucose uptake through

translocation of GLUT4 from intracellular stores to the plasma membrane. Moreover, in response to insulin stimulation AKT increases glycogen synthesis via inactivation of glycogen synthase kinase-3 (GSK-3), an enzyme that inhibits glycogen synthase. AKT plays an important role in BG homeostasis and overexpression of AKT can lead to an increase in glycogen synthesis and removal of BG into surrounding tissue[22]. Thus, it is plausible that in poorly controlled T1DM, there is an elevation in glycogen synthesis and glucose uptake and a subsequent decrease in glycogenolysis and glucose release into the blood stream.[23,24]

1.3 Exercise and Hypoglycemia in Individuals with T1DM

Exercise and physical activity (PA) are important modifiable risk factors associated with many cardiovascular and metabolic disorders. Some of the benefits associated with PA in individuals with T1DM include improved body composition, increased cardiorespiratory fitness, increased endothelial function, improved blood lipid profile, decreased total daily insulin requirements, decreased stress, and depression; and increased quality of life (QOL).[25] One of the biggest barriers to exercise for individuals with T1DM is the risk and fear of hypoglycemia. Hypoglycemia is a drop in blood sugar to concentrations below 4.0 mmol/L and when this occurs as a response to exercise it is termed exercise-induced hypoglycemia (EIHG). EIHG can be asymptomatic or symptomatic in which the symptoms can vary from minor issues with athletic performance to more severe acute symptoms such as nausea, loss of conscious or even death. The most common form of EIHG is nocturnal hypoglycemia (NHG), in which the individual enters a hypoglycemic state during the sleeping hours and can be most detrimental since the individual is not awake to make the proper adjustments, which include reducing insulin dosage and immediate carbohydrate consumption. There are many factors that a patient with T1DM needs to consider

when exercising to maintain proper BG concentrations such as exercise parameters, environmental factors, regimen changes, bodily concerns, and hypoglycemia-associated autonomic failure (HAAF).[26,44]

HAAF is the phenomenon that occurs in patients with T1DM in which recent bouts of hypoglycemia causes both defective glucose counterregulation and hypoglycemia unawareness which in combination causes a cycle of recurrent hypoglycemia.[43] Glucose counterregulation is defective due to the attenuated response of epinephrine because of elevated insulin concentrations. [43] Furthermore, hypoglycemia unawareness is largely caused by attenuated sympathetic neural response to declining BG concentrations.[43] The reason for the attenuated sympathetic neural response is poorly understood but several hypotheses for why this occurs is outlined in a review by Cryer et al. 2013. [43]

Exercise parameters that can affect BG concentrations include the exercise type, order of exercise, frequency, intensity, duration/timing, and training status (how often and how long an individual has been performing specific exercise training). [26] Many different types of exercise have been examined in patients with T1DM to better understand their effects on BG during and post-exercise recovery. Some of the most researched modes of exercise include aerobic exercise (AE), resistance exercise (RE), and bouts of high intensity interval training (HIIT). AE, also known as 'cardio', is steady state exercise that targets the cardiovascular system by maintaining an elevated heart rate over long periods of time. AE has proven to be extremely beneficial for preventing many comorbidities with T1DM such as cardiovascular and metabolic disorders; however, it has also been associated with increased risk of EIHG.[26] While AE benefits include a decreased risk for cardiovascular disease, coronary artery disease, and many macrovascular and microvascular complications, AE has been associated with increased risk of EIHG.

In 2015, Bohn et al. observed that less than one in five adults with T1DM met the physical activity (PA) recommendations[27]. The PA recommendations for this population includes 150-mins of moderate AT or 75-mins of vigorous AE, two days of RE per week and no more than 2 consecutive days of physical inactivity.[25] Previous research has shown that PA is beneficial for glycemic control in T2DM, in which a combination of AE and RE is best.[25] For T1DM individuals it is important to manage insulin dosage up to 90-mins before the start of a bout of exercise, however, this is difficult as different types of exercise result in different energy expenditures resulting in improper glycemic control.[25] Energy expenditure has been shown to be lower with RE than AE; however, AE has been shown to have much more rapid benefits to cardiovascular health which has been shown in as little as six days. [63,64] Whereas the benefits of RE on cardiovascular health takes longer as shown by a study by Doustar et al. (2012) [61] which reported no significant exercise benefits in response to a 4-week RE program. Rather, improvements in cardiovascular health were evident after 12-weeks of RE training.[62] Limited stores of muscle and liver glycogen are used as energy substrates during both AE and RE, with the source and rate of glycogen depletion dependent upon the type and intensity of training[25]. Many studies have shown that glucose uptake by exercising muscle may be enhanced for many hours after exercise extending into the overnight hours. [25] Thus, increased energy consumption after both exercise modalities may result in depleted glycogen stores and increased risk of EIHG.[25]

In a study by Yardley et al. in 2013, the effects of AE on BG concentrations during, immediately after and 24 hours post-exercise in individuals with T1DM were studied. The authors found that during AE BG declined rapidly, resulting in significant changes from baseline within the first ten mins of exercise[28]. The AE group exhibited significant increase in BG at 50-60-mins recovery when compared to the start of recovery.[28] Furthermore, McDonald et al. (2016) observed

that hepatic glycogen content in T1DM rats was deficient despite elevations in glycogenic proteins in the liver and this affect was apparent even after 10-weeks of AE training.[29] Furthermore, even after 10-weeks of training they found that in response to high-intensity exercise there was no change in the magnitude of exercise-induced BG reduction acutely. [29] Moreover, in a study by Petersen et al. (2004) they reported that the increase in GP during an acute bout of exercise was almost fully contributed to through hepatic glycogenolysis in non-T1DM individuals and in contrast individuals with T1DM favoured gluconeogenesis.[56] It has been reported that gluconeogenesis cannot solely provide enough BG to maintain steady concentrations during exercise.[30]. Thus, deficient hepatic glycogen stores in T1DM rats may explain why they demonstrate impaired BG control in response to exercise.[29]

1.4 Rationale

One of the leading causes of death in patients with T1DM is cardiovascular disease, which can be significantly decreased with regular AE. The biggest barrier to AE in this population is the fear of hypoglycemia and to increase the participation in AE a better understanding of the relationship between AE and hypoglycemia is needed. Previous studies have found hepatic glycogen is reduced in T1DM, while muscle glycogen levels are elevated. Moreover, despite associated enzymes with hepatic glucose remaining present and elevated following training.[29]. An aim of this study was to look at glucose production and usage in the liver and muscle, respectively, following a single bout of AE in T1DM rats.

1.5 Purpose and Hypothesis

The purpose of this study was to examine hepatic and muscle glycogen utilization, as well as gluconeogenesis in diabetic rats following an acute 60-min bout of vigorous intensity aerobic exercise (70-80% VO2Max). It was hypothesized that exercised T1DM animal would exhibit a reliance on BG and increased gluconeogenesis enzyme activity with no change in hepatic and muscle glycogen concentrations at the end of 60-min of acute AE.

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Chapter 2: Effect of Acute Exercise on Muscle and Liver Glucose Metabolism in T1DM Rodents

2.1 Introduction

Type 1 Diabetes Mellitus is an auto-immune disorder that involves the destruction of pancreatic beta cells resulting in insufficient endogenous insulin production and thus the requirement of external insulin supplementation. The incidence of diabetes has been rising dramatically over the past 50 years and it is estimated that 451 million adults are living with the disease worldwide and it is predicted that this number will reach 693 million by 2045.[2,3,31] Globally, it is estimated that diabetes-related health costs were at 966 billion USD in 2021 and that by 2045 this number will reach 1054 billion USD.[32] A major therapeutic goal for this population involves stabilizing blood glucose (BG) concentrations within a healthy range (euglycemia; 4-7mM) in order to attenuate the development of secondary complications. The risk of developing these secondary complications is associated with chronic hyperglycemia which can be significantly reduced with proper BG management using intensive insulin treatment (IIT)[7,32–34]. High values of glycated hemoglobin (HbA1c), a marker of chronic hyperglycemia, is associated with a significant increase in cardiovascular events and mortality in patients with T1DM regardless of underlying variables such as blood pressure, body mass, blood lipids and cigarette smoking.[35] Despite the benefits of proper glucose management, less than a third of adults with T1DM achieve their target HbA1C values.[26]

There are many complications commonly associated with T1DM including nephropathy, neuropathy, retinopathy, atherosclerosis, ischemic heart disease (IHD) and cardiovascular disease

(CVD).[34] Currently, the most effective ways to reduce the risk of developing complications of T1DM include IIT and regular exercise.[36] Studies show that physical activity can reduce the frequency and severity of complications in individuals with T1DM.[28] Additionally, physical activity can lead to improved body composition[37] increased cardiorespiratory fitness[38] decreased total daily insulin requirements[39] and increased quality of life.[40,25] Moreover, work from our research group has shown that aerobic exercise (AE) training can lead to beneficial hypertrophy of the heart, along with increases in stroke volume index suggesting improved systolic and diastolic functioning; characteristic of enhanced both micro- and macrovascular health.[16] This suggests that AE is a viable way to reduce the risk of cardiovascular complications and also reduce the severity of the outcomes if a cardiovascular event does occur.

While exercise is an effective means to manage T1DM, it has been reported that less than one in five adults with T1DM meet the physical activity guidelines.[9] Indeed, many insulin-dependent patients with T1DM avoid physical activity because of fears surrounding exercise-induced hypoglycemia (EIHG).[41] EIHG is very common in these individuals because muscle contraction stimulates additional glucose uptake on top of which is already promoted by circulating insulin.[42] This can be problematic if insulin and carbohydrate (CHO) intake are not properly aligned prior to exercise.[26] Further, the glucose counter regulatory response (GCR) to BG reductions is impaired in patients with T1DM. The GCR stimulates the release of several key hormones that work to elevated and stabilize BG concentrations.[43] Some of these hormones include glucagon, adrenaline (epinephrine), cortisol and growth hormone (GH) which are released in a sequential order at specific BG thresholds.[11] Many studies have shown that shortly after diagnosis of T1DM the glucagon secretion is reduced in response to hypoglycemia onset.[44,45]

Furthermore, the release of epinephrine which mirrors glucagon in its ability to stimulate hepatic glucose production is diminished in response to prior bouts of hypoglycemia and exercise.[45]

In addition to an impaired GCR, the risk of hypoglycemia in T1DM may be partly attributed to lower rates of endogenous glucose production (EGP), increased glucose uptake or a combination of both.[46] EGP is the release of glucose resulting from glycogenolysis (glycogen breakdown) and gluconeogenesis (new glucose production) that occurs naturally in the body without external CHO supplementation.[25,47,48] The liver plays a major role in EGP, as it is responsible for increasing EGP to stabilize BG via gluconeogenesis in a fasted state or in response to hypoglycemia onset.[46] During exercise in non-T1DM individuals, the alpha cells in the pancreas are stimulated to release glucagon while beta cells reduce insulin secretion when BG concentrations are too low (<4.0 mM).

In a study by Basu et al. (2014) it was reported that eight of twelve diabetic patients in their study experienced a bout of asymptomatic hypoglycemia within 15-mins after onset of exercise despite being fed a 600-kcal mixed meal 2 hours prior to exercise.[53,54] These results would support other findings that have shown both EGP and hepatic glycogen storage are impaired in T1DM individuals which in turn potentiates the risk for hypoglycemia in this patient population.[48,53] Further, it has been observed that individuals with T1DM exhibit impaired hepatic glycogen synthesis and reduced glycogenolysis in response to hypoglycemia.[12] Despite this deficiency, McDonald et al. (2016) found that several key regulatory hormones involved in hepatic glycogen/glucose synthesis are elevated in T1DM-induced rats which include phosphoenolpyruvate carboxykinase (PEPCK), glycogen synthase (GS), and glucokinase (GK).[29] This might suggest that gluconeogenesis is the primary means to generate and replace BG during and following exercise in these patients. In contrast to liver, it has been shown that muscle

glycogen is unchanged – or even higher following a bout of exercise in comparison to non-T1DM [54]. It is not clear why muscle glycogen is underutilized; however, it is plausible that circulation of insulin may impede glycogen breakdown through inhibition of muscle glycogen phosphorylase activation.[55] Few studies have investigated the effect of exercise on muscle and liver glycogen utilization and glucose production in patients with T1DM.

The purpose of this study was to examine hepatic and muscle glycogen utilization, as well as hepatic glycogenolysis/gluconeogenesis in T1DM rats following an acute 60-min bout of vigorous intensity AE (70-80% VO_2Max). It was hypothesized that exercised T1DM rats would exhibit a reliance on BG and increased gluconeogenesis enzyme activity with little change in hepatic and muscle glycogen concentrations.

2.2 Materials and Methods

2.2.1 Ethics Approval

All of the protocols in this study were approved by the University Council of Animal Care of Western University in accordance with the standards set by the Canadian Council of Animal Care.

2.2.2 Animals

Nineteen male Sprague-Dawley rats at an age of eight weeks were obtained from Charles River Laboratories (Quebec, Ontario). The subjects were caged in pairs and held in a 12-hour dark/light cycle with a constant room temperature of 20 ± 1^{o}C and humidity was about 50% throughout the duration of the study. All rats were fed standard rat chow and water ad libitum for the duration of the experiment. A power analysis was completed to determine a minimum of n = 5 for each group: a priori to detect a significant difference at $p < 0.05$ and power of $\beta = 0.8$.

2.2.3 Experimental Groups

Individual male rats were randomly assigned into one of two treatment groups. Control Sedentary (SC; n=5) and T1DM animals (DSC; n=14). The DSC group was then further divided into two subgroups at the end of 12 weeks: T1DM Pre-Exercise (DPRE; n=6) and T1DM Post-Exercise (DPOST; n=8). One rat was removed from the DPOST group due to complications that occurred during exercise (DPOST; n=7).

2.2.4 Experimental Procedures

T1DM Induction and Insulin Pellet Implantation

Upon arrival the nineteen rats were acclimatized to their new homes for five days. After acclimatization the two T1DM treatment groups were given an intraperitoneal (IP) injection of 20mg/kg of streptozotocin (STZ; Sigma-Aldrich) daily over five days to destroy pancreatic beta cells and induce T1DM. The STZ injections were prepared and given within 5-mins after being dissolved in a 0.1 ml citrate buffer (pH 4.5). T1DM was confirmed after non-fasted BG measurements of ≥ 18 mmol/L were observed for two consecutive days. After confirming T1DM induction, each individual rat had an insulin pellet surgically implanted into the subcutaneous region of their abdomen (1 pellet; 2U insulin/day; Linplant, Linshin). Insulin treatment was controlled through addition or removal of insulin pellets in order for each group to maintain BG concentrations between 7-10 mmol/L. BG was measured twice weekly over the 12-week duration of the study.

Exercise Training Protocols

Animals in the both the DPRE and DPOST group were familiarized with exercise equipment over a five-day period in which they underwent exposure to the treadmill belt used for the AE intervention. To do so, animals ran for 15-mins on a motorized treadmill at progressively higher speeds (up to 30m/min at 0^{o} incline) 3 days prior to experimental bout of AE. The acute

bout of vigorous-intensity AE protocol involved running for 1 hour at 27 m/min on a 6% incline gradient to elicit an exercise intensity of 70-80% their maximum rate of oxygen consumption (VO_{2max}).[65] The animals were motivated through extrinsic means via short term bursts (~20 bursts/animal) of compressed air triggered when they stopped running or if they were not keeping up with the pace.

2.2.5 Experimental Measures

Body Masses and Blood Glucose

Animal body masses were measured and recorded weekly throughout the entirety of the study. Non-fasted BG readings were taken twice weekly at 9am by collecting a droplet of blood (~50 µl) via the saphenous vein. BG was also taken for the DPOST group immediately prior to exercise, at 30-mins of exercise and immediately after 60-mins of exercise. BG values were analyzed via Freestyle Lite BG Monitoring System and are reported in millimoles per litre (mmol/L).

Tissue Collection

The SC and DPRE groups were sacrificed between 9:00 and 10:00am at week 12. The DPOST group was sacrificed immediately following the 60-min acute bout of aerobic treadmill exercise between 10:00 and 11:00am via anaesthetization with isoflurane, followed by cardiac exsanguination. Lower limbs were dissected and the plantaris, soleus, and red portion of the gastrocnemius were rapidly frozen via liquid nitrogen and stored at – 70 ^{o}C until glycogen and protein analysis would be completed. Times of removal and freezing post-death were recorded and timed to approximately 5-mins from sacrifice to tissue freezing to minimize tissue dehydration.

Glycogen Quantification

Glycogen content in liver and skeletal muscle tissue was determined colourmetrically, as described previously by Lo and Russell (1970). For skeletal muscle tissue samples, we examined the red portion of the gastrocnemius muscle. In brief, tissue samples were submerged in 30% potassium hydroxide solution that was saturated with sodium sulfate (Na2SO4). Samples were further immersed in boiling water for 30-mins in order to digest the tissue and followed by a 30-min precipitation in 95% ethanol on ice. Samples were then centrifuged at 3000 r/min (1500 g) for 30-mins and then resulting supernatant was discarded. Glycogen precipitates were then dissolved in ddH_2O and the phenol-sulfuric acid colour reaction was used. In sequential order, 5% phenol, followed by 96%-98% sulfuric acid (H2SO4) was rapidly added to the glycogen solution. After 10-mins the solution was agitated and set in a 25-30 ^oC water bath for 20-mins. Finally, each sample was analyzed in triplicates on a spectrophotometer at 490-nm wavelength.

Key Regulatory Liver Enzymes

Protein content for phosphoenol pyruvate carboxykinase (PEPCK), glucose-6 phosphatase (G6Pase) and phosphorylated/non-phosphorylated protein kinase B (P-AKT) were determined via a Western Blot protocol. Liver and red gastrocnemius tissue were homogenized in buffer (100 mM NaCl, 50 mM Tris base, 0.1 mM EDTA, and 0.1EGTA, pH~7.5) using a polytron, and total protein content was determined using the Bradford protein assay (See Appendix E). Homogenates (40–80μg of protein) were subsequently mixed with equal volumes of sample buffer (0.125 M Tris, 20% glycerol, 4% SDS, 10%β-mercaptoethanol, 0.015% bromophenol blue, pH~6.8), separated by SDS-PAGE (4% stacking, 10% separating) and then transferred to nitrocellulose membranes. Membranes were blocked by submerging them in 5% non-fat dairy milk in tris-buffered saline (TTBS) (10 mM Tris, 100 mM NaCl, and 0.1% Tween-20, pH 7.5) for 1 hour and incubated overnight at 4°C with primary antibodies diluted in TTBS with 2% nonfat dairy milk. Following

washes in TTBS, membranes were exposed to corresponding secondary antibodies (IgG-HRP conjugated, Bio-Rad) in TTBS with 2% nonfat dairy milk for 1 hour at room temperature. After successive washes in TTBS, protein bands were visualized with a luminol-based chemiluminescent substrate imaged with the Chemidoc XRS System, and analyzed with Quantity One Software. Protein concentrations were measured in Arbitrary Units (AU) using ImageJ software.

Statistical Analyses

Weekly BG concentrations and body masses were compared using a two-way repeated measures analysis of variance (ANOVA) and Sidak's multiple comparisons test. BG concentrations were also taken for the DPOST group immediately prior to exercise, at 30-mins of exercise and immediately following exercise at 60-mins and these were compared using a one-way repeated measures ANOVA. Muscle and liver glycogen content and PEPCK, G6Pase, and P-AKT protein content were all compared using a one-way ANOVA. When a significant effect was found, a Tukey's post hoc test was used. A significance level of $p < 0.05$ was used and all values were reported as means ± SE. All statistical analyses were performed using Graphpad 8 statistical software.

2.3 Results

2.3.1 Animal Characteristics

Weekly BG concentrations (mmol/L) and body mass (g) were measured twice weekly throughout the 12-week study. Complete body mass and BG measurements were collected for all 19 animals (DSC, n= 14; and SC, n=5). Weekly body mass showed a significant difference between SC and DSC in which DSC weekly body mass was lower than SC ($P<0.05$; Figure 1a).

Weekly BG measurements for DSC were significantly greater than the SC group (P<0.05; Figure 1b). These results are presented in *Figure 1a* and *Figure 1b*, respectively.

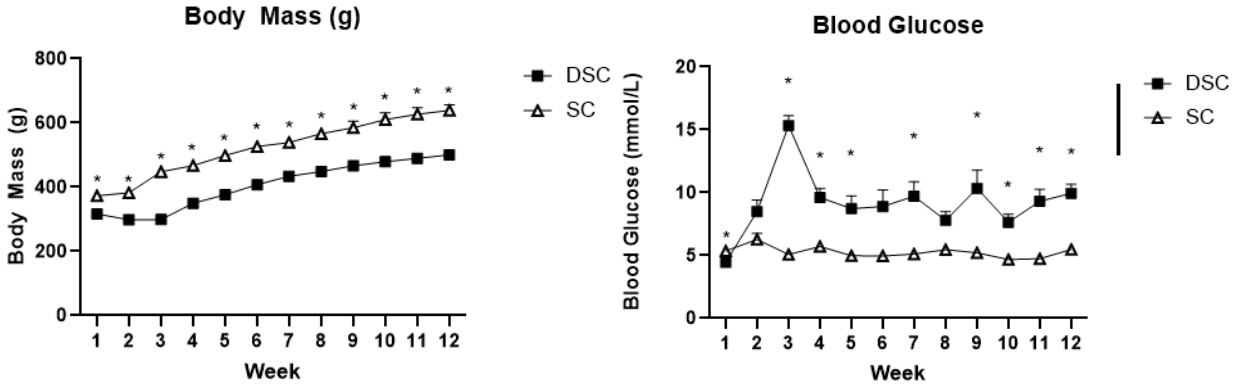

***Figure 1*:** (a) Mean non-fasted weekly body mass measurements in grams. (b) Mean non-fasted weekly BG measurements (mmol/L). All data presented as mean ± SEM. * denotes P<0.05

2.3.2 Liver and Muscle Glycogen

Liver and muscular glycogen content was measured in SC, DPRE and DPOST in all 19 animals except 1 (SC, n=5; DPRE, n=6; DPOST, n=7) that had to be removed from the exercise group due to complications. After running a one-way ANOVA on hepatic glycogen content, it was found that the SC group had significantly higher concentrations of glycogen than both the DPRE group (P = 0.0329; Figure 2a) and the DPOST group (P = 0.0241); however, there was no difference found between the DPRE and DPOST groups. Additionally, there was no significant difference in muscle glycogen content between any of the three groups. (P=0.8569; Figure 2b). These results are presented in *Figure 2 and Figure 3*, respectively.

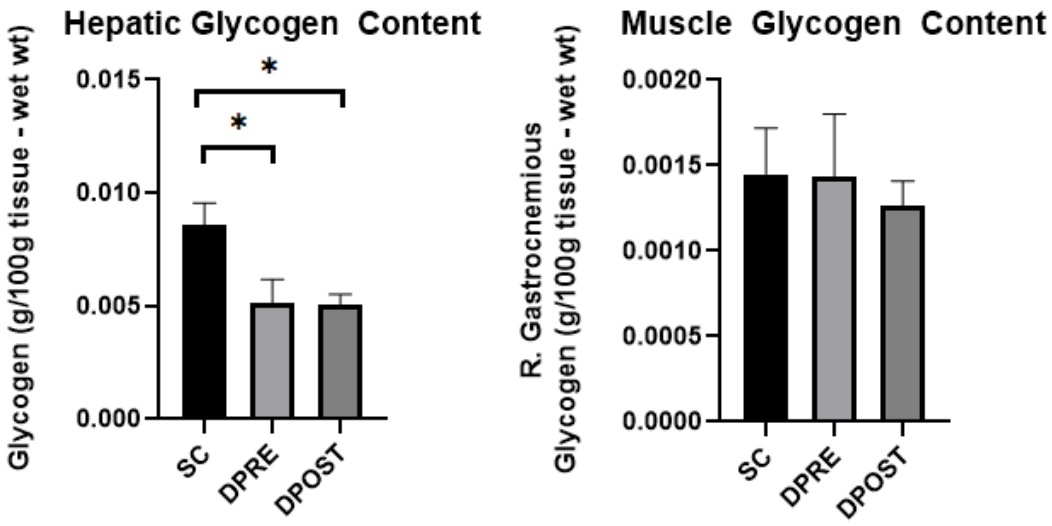

Figure 2: (a) Mean hepatic glycogen content from each treatment group. (b) Mean intramuscular glycogen content from each treatment group as determined via phenol-sulfuric acid glycogen assay. All data are presented as mean ± SEM. * denotes $P < 0.05$.

2.3.3 Blood Glucose, Aerobic Exercise and Duration of Exercise

BG measurements were taken immediately prior to AE, at 30 min of AE and at 60 min of AE. A significant decrease in BG from Pre-Exercise (0 MIN) to 30 min of AE (P=0.0028), and from Pre-Exercise to 60-mins of AE (P=0.0002; Figure 3). There was no significant difference in BG between 30-mins and 60-mins of AE (P=0.4941; Figure 3). These results are presented in Figure 3.

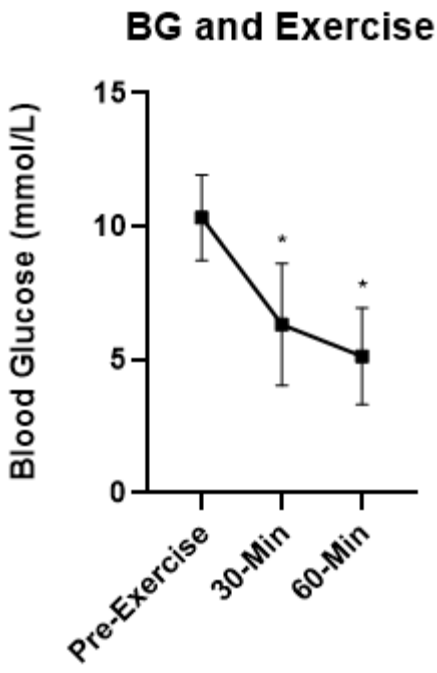

Figure 3: Mean BG measurements for the DPOST group were taken immediately prior to AE, at 30-mins and at 60-mins of AE. All data are presented as mean ± SEM. * denotes significant difference from Pre-exercise time-point $P<0.05$.

2.3.4 Key Liver Regulatory Enzymes Pre and Post Aerobic Exercise

There were no significant differences between the SC and DPRE when comparing concentrations of G6Pase (P=0.0722, Figure 4a). However, when comparing SC to DPOST, liver G6Pase was significantly elevated in the DPOST group (P=0.0134, Figure 4a). When comparing protein concentrations of PEPCK, no groups were significantly different from each other (P=0.9021, Figure 4b). The ratio of P-AKT to total AKT protein content was used to determine AKT activity. DPOST had significantly greater proportion of P-AKT when compared to both the SC (P=0.0046, Figure 4c) and the DPRE group (P=0.0037, Figure 4c) while the SC and DPRE groups were not significantly different (P=0.9434, Figure 4c). The results of these findings are presented in Figure 4a, Figure 4b and Figure 4c, respectively.

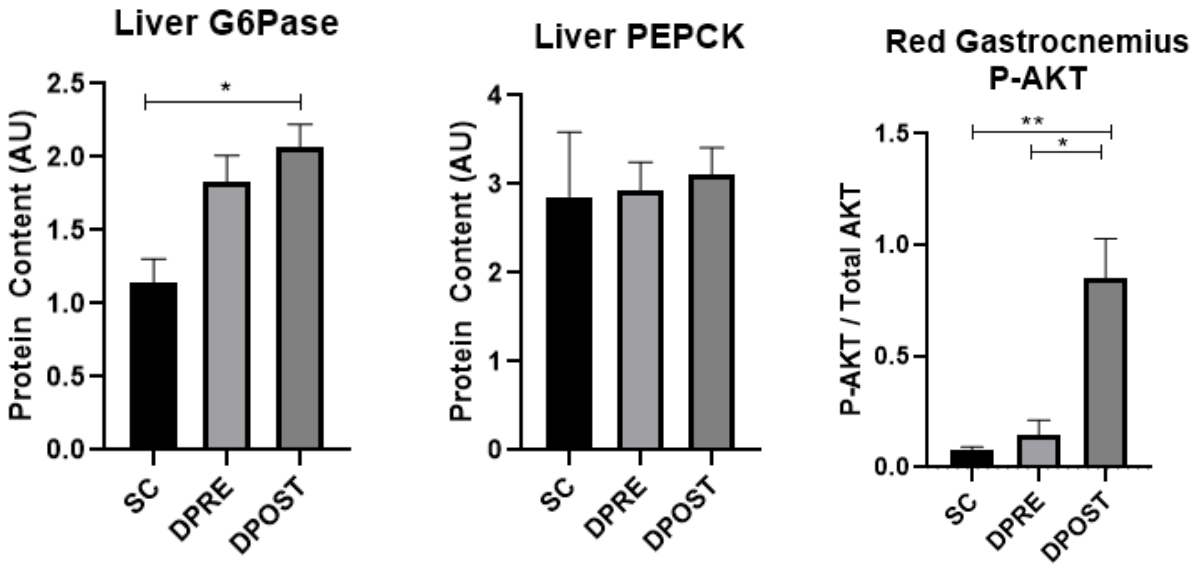

Figure 4: (a) Mean liver glucose-6-phosphatase protein content (AU) as determined via Western Blot analysis between all treatment groups. ***(b)*** Mean liver Phosphoenolpyruvate Carboxykinase (PEPCK) protein content (AU) as determined via Western Blot analysis between all treatment groups. ***(c)*** Mean ratio of phosphorylated protein kinase B (P-AKT) protein content (AU) as a ratio of total AKT of the red gastrocnemius muscle as determined via Western Blot analysis between all treatment groups. All data are presented as mean ± SEM. * denotes $P<0.05$.

2.4 Discussion

We originally hypothesized that an acute bout of moderate to vigorous AE (70-80% VO_2Max) would result in no significant change (utilization) in hepatic and muscle glycogen concentrations in T1DM rats. The results from this study supported our hypothesis as there were no change in glycogen concentration when comparing DPRE and DPOST groups for both hepatic and muscle glycogen. Moreover, we hypothesized that since we expected no change in glycogen concentrations in these tissues there would be a reliance on BG and thus a decrease in BG concentrations over the duration of the exercise intervention. The results from this study supported our hypothesis as there was a significant decrease in BG concentrations from pre-exercise concentrations to both the 30 min and 60 min BG concentrations. Finally, we hypothesized that there would be an increase in gluconeogenesis in response to AE in T1DM rats. While the results showed that there was no significant differences between SC, DPRE and DPOST groups for PEPCK, there was a significant increase in G6Pase between SC and DPOST suggesting that an increase in gluconeogenesis was evident by the completion of AE in T1DM rats.

In a non-T1DM population, increases in BG concentrations during exercise are primarily accounted for via glycogenolysis, whereas T1DM patients primarily use gluconeogenesis to replenish BG concentrations.[56] However, as glycogen stores become depleted in these patients' gluconeogenesis is insufficient to match the increased peripheral glucose uptake that occurs during moderate and high intensity exercise. [57] Since there was no observed decreases in muscle and liver glycogen, this suggests that there is a lack of sufficient glycogenolysis at both the exercised muscle and the liver. Moreover, since there was no increase in PEPCK (G6Pase only) with AE in T1DM rats it suggests that a potential increase in gluconeogenesis evident towards the later stage of exercise. However, without a concomitant increase in PEPCK the gluconeogenic substrate

including lactate and amino acids are unlikely contributing to generation and release of glucose from the liver. These findings suggest that both gluconeogenesis and glycogenolysis, at both liver and local muscle tissue, are underutilized and consequently there is a significantly reliance on BG to sustain the energy requirements of exercise in T1DM rats.

Glycogenolysis is the breakdown of glycogen into free glucose molecules. Hepatic glycogenolysis is a major source of replenishing BG concentrations during fasting or exercise periods, whereas glycogenolysis that occurs at the local skeletal muscle tissue is used in response to exercise as an energy source. [67] Previous research has found that hepatic glycogen is deficient in both male and female T1DM rodents (McDonald et al. in 2016. Larocque et al. in 2022).[29, 54] This deficiency in hepatic glycogen increases the risk of EIHG but the mechanisms surrounding this phenomenon are poorly understood. McDonald et al. (2016) found that there was deficient hepatic glycogen despite increased concentrations of glycogen synthesis-mediated enzymes.[29] Despite the apparent capability to generate hepatic glycogen it was reported that overactive sympathetic nerves at rest and or exercise may deplete glycogen stores.

Hypoglycemic-associated autonomic failure (HAAF) occurs in patients with T1DM in which a recent hypoglycemic-event causes both defective glucose counter regulation and a reduced awareness of a subsequent hypoglycemic event (hypoglycemia unawareness).[43] The glucose counter regulatory response (GCR) involves a feedback system that senses declining BG concentrations and stimulates that release of several hormones that work to stabilize BG concentrations.[43] Some of these hormones include glucagon, adrenaline (epinephrine), cortisol and growth hormone (GH) which are released in a sequential order at specific thresholds to stabilize BG.[11]The GCR is impaired due to the attenuated response of epinephrine as a result of increased

circulating insulin concentrations and the inability to reduce insulin secretion and increase glucagon secretion. [43]

Gluconeogenesis is the conversion of molecules other than glucose into new glucose molecules. Substrates used for gluconeogenesis include lactate, free fatty acids and amino acids. Gluconeogenesis primarily occurs in the liver and is increased both at rest and during exercise in T1DM. [13] Glucose-6-phosphate (G6P) is a key regulatory enzyme for gluconeogenesis and requires a liver specific enzyme known as glucose-6-phosphatase (G6Pase) to cleave the phosphoryl group from G6P to produce a free glucose molecule. Phosphoenolpyruvate carboxykinase (PEPCK) is another rate-limiting enzyme for gluconeogenesis. PEPCK activity is stimulated by cortisol and glucagon which in turn increases gluconeogenesis. PEPCK is inhibited by insulin which results in decreased gluconeogenesis.

One of the major findings of this study was that there was a significant change in G6Pase enzyme concentrations with moderate intensity treadmill exercise in T1DM rats. Petersen et al. 2004, reported that individuals with T1DM exhibited little change in gluconeogenesis from rest to moderate exercise (35% VO_2Max) but there was a significant increase when exercise was high intensity (70% VO_2Max) during 50-min treadmill running. [13] In contrast to our work, in a study by McDonald et al. 2016, it was also observed that PEPCK concentrations were greater in both untrained and trained T1DM rats compared to their non-T1DM counterparts. It unclear why elevations in PEPCK are not consistent across experimental studies; however, the duration of diabetes, training status, and insulin treatment may explain these discrepancies. It is important to note that we have recently reported that G6Pase in elevated in female T1DM animals without an increase in PEPCK enzyme levels.[54]

Protein Kinase B (AKT) is another important protein that helps regulate the insulin pathway. AKT has three isoforms which include AKT1, AKT2, and AKT3 and they are all responsible for different physiological functions. AKT2 facilitates insulin-induced translocation of the glucose transporter 4 (GLUT4) to the plasma membrane. This promotes the uptake of glucose from the blood stream into local tissue and organs. It has been suggested that AKT has two major roles in glucose metabolism. First, when stimulated by insulin, AKT associates with GLUT4 transporter leading to increased plasma glucose uptake. Moreover, in response to insulin stimulation, AKT increases glycogen synthesis via inactivation of glycogen synthase kinase-3 (GSK-3), an enzyme that inhibits glycogen synthase. [23,24]

In skeletal muscle, when protein kinase B (AKT) is activated, it leads to the suppression of muscle glycogen breakdown. It is well documented that insulin activates AKT in skeletal muscle and since individuals with T1DM often have high exercising insulin concentrations it could lead to the preservation of muscle glycogen. Since we did not see any change in muscle glycogen storage in response to AE when looking at the DPRE and DPOST groups it could be possible that they relied on BG to fuel working muscle and maintain BG concentrations. In contrast, in non-T1DM there is a suppression of insulin release during exercise which would allow for muscle glycogen stores to be used to help fuel working muscle. Furthermore, in non-T1DM, glycogenolysis is the primary pathway for maintaining BG concentrations via EGP. However, in individuals with T1DM glycogenolysis contributes less to EGP and these individuals are forced to rely on gluconeogenesis. Despite an increase in G6Pase, the inability to utilize gluconeogenic substates lactate and amino acids, gluconeogenesis is unlikely to match the glucose disposal associated with prolonged AE, which can lead to an increased risk of hypoglycemia.[56,16] The role AKT plays in the diabetic population has not been studied extensively and more research needs to

be done on this pathway to further understand its role in reduced hepatic glycogen storage and EIHG.

2.5 Limitations

The current study had several limitations which include the exclusion of female rats and the small sample size of the groups. Female rats were not used in this study because they have been observed to have a less severe decline in BG as a response to exercise then their male counterparts and the focus of this research was to examine the effects of an acute bout of exercise on glucose metabolism to better understand EIHG.[54] Furthermore, this study was limited to a small sample size (SC: n=5; DPRE: n=6; DPOST: n=7). A power analysis was completed to determine the minimum n required which was determined as an n=5 for each group as determined by a Power Analysis: a priori to detect a significant difference at $p< 0.05$ and power of $\beta = 0.8$. Moreover, an animal was removed from the study at the end of the 12-weeks in the DPOST group due to complications from the treadmill exercise intervention which reduced the total number of subjects in this group by one; however, the data for this rat was used for the DSC but not the DPOST group. Lastly, a limitation is that this study used forced treadmill exercise rather than voluntary treadmill exercise which could have affected the hormonal responses of the animals and influenced the results.

2.6 Conclusion & Summary

The current study provides further support that hepatic glycogen storage is impaired in T1DM and that this may contribute to the associated decrease in BG during exercise. Furthermore, this is the first study examine both muscle and liver tissue immediately prior to exercise and immediately following exercise. The major findings of this study demonstrate that with AE, T1DM

animals demonstrate little change in hepatic and muscle glycogen and a partial elevation in the gluconeogenic enzymes (G6Pase only). This would suggest that patients with T1DM heavily rely on BG to supply the energy requirements of AE. Furthermore, the significantly greater concentrations of P-AKT in muscle in response to AE in T1DM rats suggest that muscle glycogen use is impaired during exercise due to elevated concentrations of insulin in T1DM and this may potentiate a reliance on BG. Taken together, these finding suggest that hepatic and skeletal muscle glycogen metabolism is impaired leading to a reliance on BG to support energy needs during AE in patients with T1DM.

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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:

    a. 13.8g Anhydrous Citric Acid (Sigma) or 15.1g Citric Acid Monohydrate

    b. 23.8g Sodium Citrate Dihydrate (Sigma), Mix into…

    c. 175mL of MilliQ water The pH should be at 4.6, Add HCl or NaOH to adjust (do not over-shoot 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 0.2µm filter.

3. Store at room temperature. This is your 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. 68

1. Using 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-m time frame. Ex. Rats will be injected at 20mg/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. 20mg/kg X 0.2kg = 4mg per animal

3. The amount of STZ weighed out should be more than the minimum as some solution will be lost in filtering. (4mg (per animal) X 12 rats = 48mg total (0.048g) 5. Dissolve the STZ into buffer (keeping in mind a comfortable injection volume). Shake to dissolve powder (approx. 1min). Sterile filter using a 0.2µm syringe filter. Ex. 48mg STZ ÷ 3 mL buffer = 16mg/mL solution 4mg ÷ 16mg/mL solution = 0.25mL 6. STZ is time dependent and must be used within 15 minutes

4. Injecting and Follow-Up of the Animals 1. Promptly inject each rat with the solution (intraperitoneal) at a dosage rate of 20mg/mL (in this example, 0.25mL). Do not use anymore 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. 69 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. 20mg/kg).

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Appendix B: Insulin Pellet Implantation

Pellet implantation (for a rat):

1. Anesthetize the animal using the isoflurane machine by placing it in the induction chamber. Set isoflurane to 4-5% with an O2 flow rate of 1L/min. Open the stopcock valve so gas reaches the chamber. Keep in chamber until the animal is unconscious.

2. Remove the animal and place its nose in the nose cone, 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 subcutaneous incision.

6. Cleanse a 12g trocar with 10% povidone-iodine solution and insert it through the puncture site to a depth of at least 2 cm.

7. Using forceps, briefly immerse the pellet in 10% povidone-iodine solution, rinse with saline and insert into the subcutaneous region.

8. Use 1 pellet for the first 350g of body weight.

9. Pinch the skin closed after the last pellet is inserted. Place a drop of 10 % povidone-iodine solution over the opening.

10. Close the incision by suturing.

11. Place the animal under a heat lamp and monitor until it recovers from anesthesia.

12. Record on the cage card that insulin pellets have been implanted.

Pellet removal:

1. Anesthetize the animal as described above for implantation.

2. Shave and palpate the area of implantation to locate pellets. Sterilize this area by applying 10% povidone-iodine solution followed by 70% ethanol.

3. Using a scalpel (or scissors), make an incision through the skin superficial to the location of the pellets.

4. Using forceps, remove the pellet. Some connective tissue may need to be cut away using scissors. Discard the pellet.

5. Close the incision by suturing.

6. Place the animal under a heat lamp and monitor until it recovers from anesthesia.

7. Record on the cage card that the pellets have been removed.

Appendix C: Phenol-Sulfuric Acid Assay for Glycogen Quantification

Part A: Solutions

<u>30% KOH (w/v)</u>

- 30 g of KOH pellets
- 100 mL of ddH2O
- EXOTHERMIC REACTION — combine in an Erlenmeyer flask placed in an ice bath

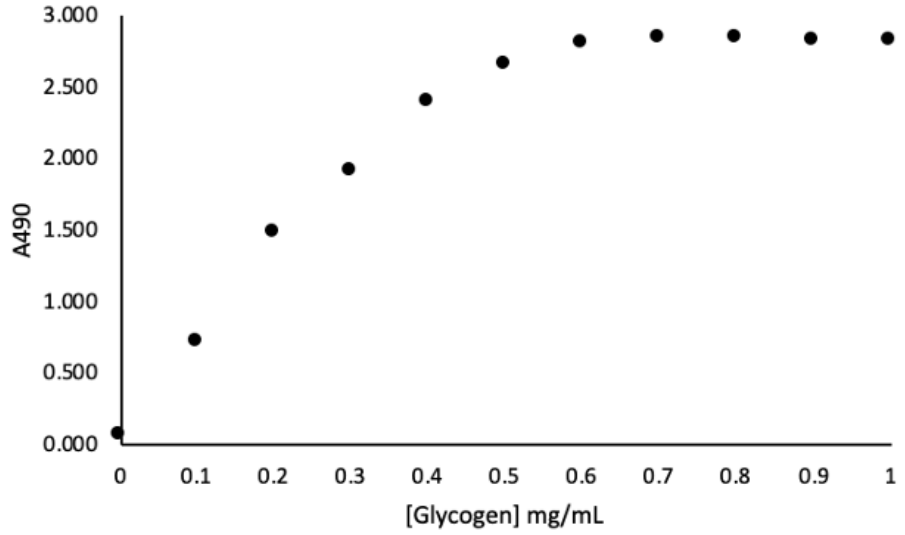
<u>95% Ethanol (v/v)</u>

- 95 mL of 100% Ethanol
- 5 mL of ddH2O

<u>Glycogen Standards:</u> 0.1, 0.2, 0.3, 0.4, 0.5 and 0.6 mg/mL

- Prepare large volumes of standards and store in 15 mL Falcon Tubes

- Standard curve flattens out after 0.6 mg/mL (see below)
    - Linear portion spans from 0 to 0.5 or 0.6 mg/mL



    - With a 5% (w/v) Phenol solution, the standard curve was linear from 0.0 – 1.0 mg/mL glycogen concentrations with an R2 = 0.99.

<u>100% Sulfuric Acid (Stock)</u>

<u>5% Phenol (v/v)</u>

- 5 mL of 100% phenol (stock)
- 95 mL of ddH2O

Part B: Tissue Homogenization and Glycogen Isolation

1. Cut and weigh approximately 20 mg of tissue, place into a 2.0 mL Eppendorf tube and keep on ice until the next step.
2. For 12 samples, saturated ~10 mL of 30% (w/v) KOH solution with Na2SO4.
3. Boil approximately 0.75 L of water and pour into the metal tray on the hot plate. Set the hot plate to MAX.

4. Add 500 µL of the Na2SO4-saturated KOH solution to each Eppendorf tube, ensuring that each sample is completely submerged.
5. Place samples in a boiling water bath for 30 minutes. Half way through, agitate the tubes until no pieces of tissue are visible.
6. Place tubes on ice and precipitate glycogen by adding 1 mL of 95% ethanol for 30 minutes.
7. Centrifuge tubes at 3000 rpm for 30 minutes. 8. Discard the supernatant and immediately dissolve the pellet in 1 mL of ddH2O.
    a. Do NOT allow the pellet to dry

Part C: Glycogen Quantification

1. Turn on the hot water bath and set it to ~70 °C.
2. In a flat-bottom polystyrene 96-well microplate, pipette 50 uL aliquots of ddH2O, glycogen standards and sample glycogen solutions in triplicate.
3. Add 150 µL of sulfuric acid to each well.
4. Quickly add 30 µL of 5% phenol to each well.
    a. Works best with a repeater pipette. Use a 5 mL CombiTip set to 150 µL for the sulfuric acid, and a 0.5 or 1.0 mL CombiTip set to 30 µL for phenol.
5. Cover plate in ParaFilm and place in a static hot water bath for 10 minutes.
6. Dry the microplate with KimWipes and measure absorbance using a microplate reader at 490 nm (1.0 seconds).
7. Calculate glycogen concentration (units: µmol of glycogen/g of tissue):

$$\frac{\mu\text{mol of glycogen}}{\text{g of tissue}} = \frac{A490}{k \times W \times MM_{Glycogen}}$$

Where: A490 = Adjusted Absorbance at 490 nm (Sample Absorbance – Blank Absorbance)

k = slope of the standard curve (units µg-1)

W = mass of tissue sample used

MMGlycogen = molar mass of glycogen (666.5777 g/mol)

Materials

- Microplate reader
- Centrifuge
- 2.0 mL microcentrifuge tubes
- Flat bottom polystyrene 96-well microplate
- Glass plate, razor blade, tweezers
- Eppendorf tube rack
- Metal tray and hot plate
- Kettle • Repeater pipette with 5.0 mL and 0.5 or 1.0 mL CombiTips (optional, but recommended)
- Static hot water bath

References

Masuko, T., Minami, A., Iwasaki, N., Majima, T., Nishimura, S.-I., & Lee, Y. C. (2005). Carbohydrate analysis by a phenol-sulfuric acid method in microplate format. Analytical Biochemistry, 339(1), 69–72. https://doi.org/10.1016/j.ab.2004.12.001

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 D: Western Blotting Protocol. Western blotting method for the quantification of protein expression and phosphorylation of AKT, G6Pase, and PEPCK.

Tissues tested: Skeletal muscle and liver

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).
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. 80
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 ddH2O 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 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), gentle remove comb and fill wells with 1x running buffer.
10. Load correct amount of sample and ladder using micropipette with loading tip.
11. Once loading is complete place gels in running unit.
12. Fill running unit with 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 transfer buffer and keep in refrigerator.

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 cold transfer buffer for 20 minutes.
3. Once 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 sandwich completely submerged in transfer buffer at all times).

4. Place “sandwich” into transfer holding tank making sure the black, negative side is facing the black transfer unit. Fill tank with cold transfer buffer and add ice pack into the unit to keep transfer period cold throughout.
5. Connect to power supple 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 20ml 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% β –mercaptoethanol – 10% (v/v) Bromophenol blue – 0.015% (w/v) pH – 6.8	Tris – 7.57 g Glycerol – 100 mL SDS – 20 g β –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: Phenolhttps://ir.lib.uwo.ca/etd/2726

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 ddH2O (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. 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

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: Ethics Approval

eSirius 3G

Western

PI :	Melling, Jamie
Protocol #	2018-063
Status :	Approved (w/o Stipulation)
Approved :	07/01/2018
Expires :	07/01/2022
Title :	The role of exercise on ameliorating the negative metabolic and cardiovascular effects of Type 1 diabetes.

Table of Contents

Protocol Introduction

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Note that species selection is part of this introductory page

Curriculum Vitae

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Publications:

Murphy K, Camenzuli J, Myers SJ, et al. Assessment of executive function in a rodent model of Type 1 diabetes. *Behavioural Brain Research.* 2023; 437 (2023) 114130. doi: doi.org/10.1016/j.bbr.2022.114130