The Effects of Exercise Training versus Intensive Insulin Treatment on Skeletal Muscle Fibre Content in Type 1 Diabetes Mellitus Rodents

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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

In patients with Type I diabetes mellitus (T1DM) undergoing intensive insulin therapy, the development of insulin resistance (IR) is linked to the improper storage of intramyocellular lipid (IMCL) species. While both IR and IMCL are improved with combined aerobic and resistance training, it is unclear how these adaptations relate to individual fibre-type transitions and metabolic function. This study aimed to compare the effects of combined exercise training versus conventional and intensive insulin therapy on skeletal muscle fibres in T1DM rodents. Seventeen Sprague-Dawley rats were divided into groups: Control-Sedentary (CS; n=4), conventionally-treated T1DM sedentary (DCT; n=4), intensively-treated T1DM sedentary (DIT; n=5) and combined aerobic/resistance exercise-trained T1DM (DCE; n=4). After twelve weeks, muscle fibre type, IMCL, and muscle glycogen content were analyzed. Significant increases in IMCL storage solely in type I fibres implicate improvements in oxidative capacity rather than a shift towards more oxidative fibres as the primary mechanism for these improvements.

Keywords

Type 1 diabetes mellitus, exercise, skeletal muscle, intramyocellular lipids, muscle fibre/fiber type, muscle glycogen, insulin treatment
Summary for Lay Audience

Type 1 diabetes mellitus (T1DM) is caused by the body’s immune system targeting and destroying the Beta cells in the pancreas that are responsible for producing insulin. Therefore, most T1DM patients rely on insulin injections to maintain their blood sugar levels as close to normal as possible. The traditional (or conventional) insulin therapy consists of patients injecting insulin once or twice per day, with minimal monitoring of their blood sugar levels. The use of intensive insulin therapy has lessened the development of cardiovascular and other diabetes-related complications, though it is associated with the development of insulin resistance (IR). IR is a condition where injected insulin has a diminishing effect over time, requiring progressively more insulin to achieve the same reduction in blood sugar. In the current study, IR development in T1DM is linked to the accumulation of improperly stored fat within the muscle, which is readily improved with combined endurance and resistance exercise training. In this thesis we set out further to test whether the improvements in fat storage were due to a preferential shift in the number of fibres or whether the individual fibres improved the way they stored the fat. Four groups were studied: 1) healthy but sedentary rats, 2) well-controlled intensively insulin-treated T1DM rats, 3) poorly-controlled conventionally insulin-treated T1DM rats, and 4) poorly-controlled conventionally insulin-treated T1DM rats that underwent a combined exercise training program. Histochemical staining was used to determine the type of muscle fibre, and the quantity of both fat and glycogen stored in these fibres using the mixed-fibre plantaris muscle. The primary finding of the current study was that both intensive insulin therapy and combined exercise training led to a significant increase in type I fibres. However, combined exercise led to a significantly greater increase in type I fibre fat content in comparison to that of the intensive.
insulin-treated T1DM group. These findings would indicate that preferential shifts to type 1 fibres are evident following intensive insulin therapy and combined exercise training; however, exercise training leads to an increase in preferential fat storage to these oxidative fibres.
Dr. Jamie Melling of Western University, London, Ontario, Canada, was involved in project design, interpretation of the results, and thesis revisions. Dr. Michelle Dotzert, also of Western University, was involved with exercising and caring for the animals involved. Gratitude is expressed to both.
Dedication

To Mom, Dad, Thomas, and Diana. Thank you all so much for your unwavering love and support.
I would like to extend my most sincere gratitude to a number of people, without whom this project would not have been possible. First, I would like to extend my thanks to my supervisor, Dr. Jamie Melling, for his expert advice, encouragement, patience, and support throughout this challenging project. Your passion for science and great sense of humour has inspired me throughout, and it has been a privilege to have you as my advisor and mentor for the past two years. I would also like to thank Dr. Greg Marsh and Dr. Matthew Krause for their generosity in sharing their vast knowledge and expertise.

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To my family: I could never have reached this point in my education without your constant encouragement, love, and support. Thank you for always being there for me.
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List of Abbreviations

Acetyl-CoA – acetyl co-enzyme A

ADP – Adenosine Diphosphate

ADA – American Diabetes Association

Akt – Protein Kinase B

ATP – Adenosine Triphosphate

CDA – Canadian Diabetes Association

CGM – Continuous Glucose Monitoring

CS – Control non-diabetic sedentary group

CVD – Cardiovascular Disease

DAG - Diacylglycerol

DCCT – The Diabetes Control and Complications Trial

DCT – Type 1 Diabetic Conventional Insulin-Treated group

DIT – Type 1 Diabetic Intensive Insulin-Treated group

DCE – Type 1 Diabetic Conventional Insulin-treatment with Combined Exercise group

EDIC – The Epidemiology of Diabetes Interventions and Complications study

FADH+/FADH₂ – Flavin adenine dinucleotide
FAT/CD-36 – Fatty Acid Translocase/Cluster of Differentiation 36

G6Pase – Glucose-6-phosphatase

GLUT4 – Glucose Transporter Protein 4

HADH – 3-hydroxyacyl-coA dehydrogenase

HbA1c – Glycated Haemoglobin

HK - Hexokinase

IMCL – Intramyocellular Lipid(s)

IR – Insulin Resistance

IRS-1 – Insulin Receptor Substrate 1

LDL-c – Low Density Lipoprotein c

mRNA – Messenger Ribonucleic Acid

NAD⁺/NADH - Nicotinamide adenine dinucleotide

PFK – Phosphofructokinase-1

PHOS – Phosphatase

PKCθ - Protein Kinase C θ

PKCζ - Protein Kinase C ζ

STZ - Streptozotocin
T1DM – Type 1 Diabetes Mellitus

T2DM – Type 2 Diabetes Mellitus

TAG – Triacylglycerol

TCA – The Tricarboxylic Acid cycle (Krebs cycle)

VO₂max – Maximal Rate of Oxygen Consumption
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Chapter 1

1.1 Overview and Treatment of Type 1 Diabetes Mellitus

In September of 1925, Dr. Frederick G. Banting gave a lecture to a crowded room about the landmark discovery of insulin and its miraculous effects on patients with type I diabetes mellitus (T1DM), for which he and Dr. John Macleod had been awarded the Nobel Prize in Physiology and Medicine three years prior. Even though a huge step had been taken towards improving the lives of millions of people worldwide, the message of his Nobel lecture was not the triumphant celebration one might expect. Rather, Dr. Banting counselled that expectations must be managed, and that this discovery was by no means the end of the road for diabetes research. He concluded his lecture with the words “Insulin is not a cure for diabetes; it is a treatment. It enables the diabetic to burn sufficient carbohydrates, so that proteins and fats may be added to the diet in sufficient quantities to provide energy for the economic burdens of life.”

On the surface, it may not seem that a lot has changed since then in terms of our approach to T1DM treatment. Insulin is still very much a cornerstone of modern T1DM treatment strategies, and many patients still struggle with balancing optimal insulin dosage with calorie intake and exercise. However, ninety-four years later, we now have a much better understanding of the pathophysiology of T1DM and the treatment mechanisms behind the actions of the hormone, insulin.

Diabetes mellitus is an umbrella term primarily used to describe two entirely independent conditions that both lead to impaired glycemic control, though they can be
traced back to entirely different causes. In both diseases, the blunted action of insulin causes chronically raised blood glucose levels (hyperglycemia), which inhibits the body’s ability to maintain energetic homeostasis. In type 2 diabetes mellitus (T2DM), genetic and lifestyle factors lead to reduced sensitivity to insulin in the tissues, requiring progressively larger insulin dosages to elicit the same reduction in blood glucose. This condition is termed insulin resistance (IR), and as it increases, the pancreas is unable to produce enough insulin to accommodate rising blood sugar levels, resulting in chronic hyperglycemia and T2DM. It is generally agreed that T2DM is a metabolic disorder that is heavily influenced by genetic and lifestyle factors, although T2DM can often be quite manageable with a combination of medication and lifestyle changes. In contrast, T1DM is a condition based on a combination of genetic and environmental factors that contribute to a destructive autoimmune response within the pancreatic islets. This results in either a partial or total inability to produce insulin, leading to unregulated blood glucose levels that fluctuate well outside the normal, healthy range. T1DM patients must supplement themselves with exogenous insulin, or they risk severe sickness and, if left untreated, death.

In a normal, healthy body, circulating blood glucose is maintained at a relatively stable equilibrium of 3-5 mmol/L, balancing glucose influx from the digestive tract and liver, and the demand-based efflux into the various tissues of the body. The diagnosis of T1DM is made when the patient presents either two fasting blood glucose measurements of over 7 mmol/L, any single blood glucose reading over 11 mmol/L, or an HbA1c of ≥6.5%. HbA1c is a form of haemoglobin that has been covalently bonded to glucose, and with chronic hyperglycemia these glycated haemoglobin species occur at higher than
normal concentrations in the blood. Since red blood cells only have a four-month lifespan, HbA1c is used as an assessment of a three-month average of glycemic control, and has become a staple of both T1DM and T2DM diagnoses. Poor overall glycemic control is strongly associated with cardiovascular disease (CVD) and is the pivotal point in T1DM disease management, as with every 1% increase in mean HbA1c, CVD risk increases by 31%.

Despite these many advances in our understanding of T1DM research and treatment, global prevalence of the disease has continued to increase, particularly in youth populations, as T1DM has become one of the most frequently occurring chronic diseases in children. It is currently estimated that there are 33 million people worldwide with T1DM, each relying on lifelong insulin supplementation, and each living with increased risk of blindness, amputation, CVD, and a host of other complications.

In 1983, the Diabetes Control and Complications Trial (DCCT) set out to determine whether improved control over blood glucose could delay or even prevent the vascular complications of diabetes. Specifically, the long-term micro- and macrovascular outcomes of conventional insulin treatment were compared to outcomes in patients who utilized a more rigorous intensive insulin treatment, over a ten-year period. The traditionally used conventional insulin treatment consisted of a bolus of insulin taken once or twice per day, coupled with daily urine or blood glucose testing. In contrast, intensive insulin treatment patients monitored their blood glucose levels throughout the day, adjusting their insulin intake with the goal of maintaining blood glucose levels as close to the normal range as possible. When the DCCT group published their findings in
1993, it was clear that intensified insulin treatment would be the recommendation going forward. Within 6.5 years of the start of the trial, T1DM patients on the intensified study arm showed a risk reduction of 76% in retinopathy, 34% in kidney disease, and 69% in neuropathy, as well as improved blood pressure and lipid control relative to those undergoing conventional insulin treatment. Consequently, all study patients were then transitioned to an intensive insulin treatment regimen. It has since been confirmed that safely maintaining tight glycemic control as close as possible to a healthy range is paramount to improving lifestyle and disease outcomes in T1DM patients. As a direct result of the DCCT, intensive insulin therapy with the goal of maintaining near-normal glycemia has been adopted as the standard of care for T1DM patients.

Patient participants (93%) from the DCCT were recruited into the follow-up Epidemiology of Diabetes Interventions and Complications (EDIC) study. The EDIC aimed to rectify some of the limitations of the DCCT while continuing to study the long-term effects of early intensive insulin treatment. In the 20 year follow-up to the initial run of the DCCT, the combined DCCT/EDIC found that the incidence of CVD was 42% lower in patients who had followed the intensive treatment protocol, though that number decreased to 30% over the following decade. As CVD is the most frequent cause of mortality and disability in T1DM patients, the continued long-term benefits of intensive insulin therapy highlight the importance of introducing this treatment as early as possible to reduce the incidence of CVD throughout the lifespan of T1DM patients.

Recent advances in modern blood glucose monitoring provide an exciting future for T1DM care. Continuous glucose monitoring (CGM) systems are able to measure and record blood glucose levels uninterrupted throughout the day and night, providing
information on daily glucose fluctuations and how those values are influenced over time and by everyday activities. They are also an integral part of “artificial pancreas” devices, an emerging technology that combines CGM with an insulin pump, forming a closed-loop blood glucose control system, making it far easier for patients to live normal lives with well-controlled T1DM.

1.2 Type 1 Diabetes Mellitus: Disease Outcomes and Complications

Before the introduction of insulin therapy, an adult with T1DM had a predicted life expectancy of approximately two years after diagnosis, while diabetic children were often not expected to live longer than one year. However, since the introduction of insulin therapy and the associated improvement in glycemic control, the acute mortality of T1DM has largely been eradicated in parts of the world where insulin is available to patients. Instead, the majority of patients with well-controlled T1DM only experience associated comorbidities after many years of living with the disease. In these patients, chronic hyperglycemia brought about by insufficient insulin (hypoinsulinemia) has been linked to the development of diabetic retinopathy, myopathy, nephropathy, neuropathy, and cardiovascular disease. Though these can each contribute to disability and mortality for patients with T1DM, cardiovascular disease alone represents the most frequent cause of premature death and disability in T1DM patients worldwide, occurring two and three times as often in men and women with T1DM (respectively) in comparison to the general population.
Although hyperglycemia has received a lot of attention and is associated with many of the complications of T1DM, hypoglycemia has also been linked to long-term complications of the disease. The primary risk to patients with T1DM is through the development of severe hypoglycemia during exercise participation, due to a combination of the insulin-like action of contracting skeletal muscle, insufficient adjustment of pre-exercise insulin dose, and reduced counter-regulatory hormonal control. Additionally, those patients with T1DM who have experienced repeated severe hypoglycemic events were found to have a higher prevalence of CVD and were associated with increased mortality from all causes.

1.3 Skeletal Muscle and Type 1 Diabetes Mellitus

Skeletal muscle is the most adaptable tissue in the body, due in no small part to its composition of highly specialized fibres, each with different characteristics meant to thrive under certain circumstances, in specific metabolic environments. As a whole, skeletal muscle plays a vital role in glucose homeostasis as the primary glucose sink in the body, acting as the final destination for approximately 80% of the glucose circulating in the bloodstream. Metabolism within the skeletal muscle is driven by energy supply and demand, where the term “energy” here refers to the generation and breakdown of adenosine triphosphate (ATP). The muscle derives ATP primarily from a balance of glucose and lipids, adapting the relative fuel mix to meet the demands of the activity (although in certain situations,
such as starvation, protein can also be metabolized to provide energy). Typically, lower intensity activity requires a higher percentage of lipid-derived ATP, whereas higher intensity energetic demands require a higher percentage of carbohydrate-derived ATP. The most obvious distinction between the energy source metabolism requirements is the presence or absence of oxygen, defining aerobic and anaerobic processes.

The primary anaerobic vehicle within the skeletal muscle is through the rapid dephosphorylation of phosphocreatine paired with the conversion of adenosine diphosphate (ADP) to ATP. This reaction supplies the muscle with enough ATP for short-term contraction, fueling exercise bouts of approximately 12 seconds in duration. While this is occurring, glycogen is being broken down into glucose and then glucose-6-phosphate so that it can enter another anaerobic process, glycolysis. Glycolysis partially catabolizes glucose, providing a quickly available ATP source in the early stages of exercise and in the absence of oxygen. These two metabolic processes occur in the cytosol of the muscle fibre, close to the contractile fibres and thus are especially quick to respond to contraction.

Aerobic respiration, however, takes place in and along the inner membrane of the mitochondria. The tricarboxylic acid (TCA) cycle takes place in the mitochondrial matrix, the innermost chamber of the mitochondrion, where acetyl-CoA is enzymatically broken down and coupled to the protonation of NAD$^+$ and FADH$. These two proton carrier molecules are created both by glycolysis and the TCA cycle and are used in the electron transport chain to create a proton gradient across the inner mitochondrial membrane, which powers ATP synthase and is the most efficient metabolic contributor of ATP of all the metabolic processes in the muscle.
Each of the fibre types in skeletal muscle are specialized to support one of these energy processes above. Type I slow-twitch oxidative fibres have increased mitochondrial number and volume, while expressing greater activity of enzymes known to play crucial roles in aerobic metabolic pathways. These fibres also incorporate a greater number of capillaries per muscle fibre, improving muscle oxygen supply and aerobic efficiency. In contrast, type IIb fast-twitch glycolytic fibres rely primarily on anaerobic metabolism as their energy source. While type IIb fibres exhibit lower mitochondrial number in comparison to type I, cross-sectional area and contractile fibre size are significantly higher, allowing more forceful and quicker contractions. Type IIa fast-twitch oxidative fibres occupy a middle ground between the two extremes, combining fast-twitch contractile fibres with a moderate level of oxidative capacity.

In T1DM, skeletal muscle plays an especially important role as the major downstream contributor to systemic glycemic control. Since it acts as the primary target for insulin therapy and is responsible for the majority of insulin-stimulated glucose utilization in the body, skeletal muscle dysfunction can lead to significant short- and long-term complications for patients with T1DM, whereas maintaining skeletal muscle health can help postpone and even alleviate some of the impact of the disease. This is complicated by the fact that T1DM is known to have a differential effect on the various types of skeletal muscle fibres, as each type has distinct morphological, contractile, and metabolic properties $^{35-37}$.

It has been known for some time that chronic T1DM is associated with a decline in metabolic markers within the three major skeletal muscle fibre types. Type I fibres tend to express selectively reduced markers for oxidative metabolism such as citrate synthase
activity, which has been linked to changes in mitochondrial morphology. This includes fewer mitochondrial cristae, which are the site of the electron transport chain and therefore the location of many vital oxidative enzymes. Type IIa fibres demonstrate a decline in both oxidative and glycolytic markers, exhibiting a combination of the metabolic and morphological changes observed in the type I and IIb fibres. The T1DM related reduction in mitochondrial oxidative enzymes that occurs in the type I and IIa fibres is not seen in the type IIb fibres. Rather, type IIb fibres tend to express reduced glycolytic marker enzyme activity, including HK, PFK, and PHOS enzyme expression. As type IIb fibres do not rely on mitochondrial respiration as heavily as do type I and IIa fibres, the partial loss of mitochondrial oxidative enzymes does not affect them to the same extent, resulting in type IIb fibres’ reduced susceptibility to the serious limitations in metabolic capacity brought on by T1DM. However, this is not to say that type IIb fibres are relatively unaffected by T1DM, or that the changes brought about exclusively affect metabolic function. The presence of T1DM has been shown to adversely affect skeletal muscle mass across all fibre types, as well as preferentially reducing type IIb muscle fibre cross-sectional area.

While this preferential inhibition of oxidative metabolism in T1DM muscle fibres may lead to a metabolic bias favouring muscle fibres that rely on glycolytic over oxidative metabolism, it has been shown that T1DM combined with conventional insulin treatment opposes these changes and is able to maintain relatively normal function of many metabolic enzymes in type I, IIa, and IIb fibres. Furthermore, T1DM muscle exposed to regular aerobic exercise has been shown to normalize many of these metabolic enzyme activities across all fibre types independent of but similar to
conventional insulin therapy\textsuperscript{37}, suggesting that exercise training may confer benefits specialized to the T1DM-related metabolic dysfunction found in individual fibre types. However, the majority of these studies that included the effects of insulin treatment on specific muscle fibre types have only examined the effects of conventional insulin therapy\textsuperscript{35–37,43}, and thus may have limited applicability to the current standard of treatment relying primarily on intensive insulin therapy. Further study is still required as there is a noticeable shortage of research examining the way intensive insulin therapy interacts with the individual skeletal muscle fibre types in T1DM.

1.4 Intramyocellular Lipids (IMCL), the Development of Insulin Resistance, and Type 1 Diabetes Mellitus

Although the DCCT/EDIC trials have provided extensive and convincing evidence supporting intensive insulin therapy as an improvement over conventional insulin treatment, it remains unclear whether this approach is the ideal treatment strategy to reduce the incidence of cardiovascular disease in T1DM. In some cases, intensive insulin treatment has led to the development of insulin resistance (IR), a potentially catastrophic development for insulin-dependent T1DM patients, and a condition more commonly associated with T2DM\textsuperscript{44}. Though IR can occur in any of the tissues of the body, the major contributor to metabolic dysfunction in patients with T1DM is skeletal muscle IR, due to the major role that skeletal muscle plays in glycemic control. In this scenario, skeletal muscle IR leads to significantly impaired tissue glucose utilization, followed by a
compensatory effort to control blood glucose with heightened levels of circulating insulin (termed hyperinsulinemia), and eventually chronic hyperglycemia \(^{44-46}\).

The combination of T1DM and at least some level of IR occurs in approximately 20% of patients with T1DM \(^ {47,48}\). These patients have been referred to as having “double diabetes”, an unofficial diagnosis that refers to the fact that these patients simultaneously demonstrate key characteristics of both T1DM and T2DM \(^ {49}\). As one might expect, this combination of being both dependent on yet resistant to exogenous insulin is highly dangerous, and these patients have significantly higher incidence of CVD and other associated disease complications than patients with either T1DM or T2DM alone \(^ {44,49-51}\). Although the majority of IR-related research has been focused on T2DM and the development of metabolic syndrome, the presence of IR in T1DM has recently been recognized as being pathologically different from these other IR-related conditions, and intensive research efforts are required to elucidate the mechanisms behind this dangerous combination \(^ {51,52}\). Interestingly, IR and metabolic syndrome have both been recently identified as better predictors of the incidence of lifetime cardiovascular disease than HbA1c, the gold standard of T1DM diagnosis criteria \(^ {50}\). In fact, while HbA1c was shown to lack any predictive power for coronary artery disease, IR has been linked with a number of factors that predict CVD, and has demonstrated a strong association with the incidence of coronary artery disease in adults with T1DM \(^ {53,54}\).

The etiology of IR in T1DM is still widely unknown, though support for the lipotoxicity theory of IR has been steadily gaining support \(^ {45,48,55,56}\). This theory postulates that IR is caused by the improper storage and subsequent accumulation of certain IMCL metabolites that lead to interference in the insulin receptor signalling
pathway, reducing the insulin-related reduction in blood glucose and ultimately contributing to chronic hyperglycemia \(^{45,48}\). Evidence has suggested that this accumulation of IMCL is caused by insufficient insulin levels (hypoinsulinemia) leading to an increase in lipid flux within the skeletal muscle. The mitochondria are unable to accommodate this increased lipid metabolism, which leads to a metabolic “overload”, in which free fatty acids are converted to diacylglycerol (DAG) or metabolized into ceramides instead of the well-tolerated triacylglycerol (TAG), which has been shown not to interfere with insulin signalling \(^{48,57,58}\). This metabolic dysfunction feeds back upon itself, as the body’s growing accumulation of DAG, ceramides, and a number of IMCL species has been shown to interfere with insulin signalling, leading to the promotion of IR in skeletal muscle (Figure 1) \(^{19,46,48}\).

Previously, our laboratory has demonstrated that the accumulation of certain IMCL metabolites (including DAG, ceramides, arachidonic acid, linoleic acid, and palmitic acid) were shown to inhibit various elements of the insulin signalling pathway \(^{48}\). The most heavily implicated of these is DAG, which is a transitional lipid species in the conversion of free fatty acids to TAG, and has been linked with IR through its interference with the PKC-\(\theta\) component of the insulin signalling pathway (Figure 1) \(^{59,60}\). Ceramides are a precursor to several predominant cellular sphingolipids, and have been implicated as a contributing factor to IR in metabolic syndrome and T2DM by acting on PKC\(\zeta\) (protein kinase C zeta) \(^{61}\). Arachidonic acid is a polyunsaturated fatty acid precursor to a number of cytokines responsible for promoting inflammation, and many of these cytokines have been directly linked with the development of IR \(^{62}\). Linoleic acid is another polyunsaturated fatty acid, and has been associated with IR through the reduction
of muscle GLUT4 protein expression. Finally, palmitic acid, among other saturated fatty acids, contributes to IR through the inhibition of IRS-1 and PKB/Akt phosphorylation, a key step in the insulin signalling response. Our lab has previously shown that not only are all these IMCL species higher in T1DM rats when compared to control animals, but that 10 weeks of aerobic exercise training significantly reduces skeletal muscle DAG, arachidonic acid, linoleic acid, and palmitic acid content concomitant with significant improvements in insulin sensitivity in T1DM rodents.
Figure 1: The lipotoxicity theory of insulin resistance. Free fatty acids enter the cell via FAT/CD36 and are shuttled into two main pathways: free fatty acids can enter the mitochondrial matrix and undergo beta oxidation; or transition to lipid droplets and where they are stored as TAG. Improper metabolic and structural adaptations that are unable to accommodate increased lipid flux lead to enhanced fat storage, as well as the incomplete transition of fatty acids to TAG, leading to increased DAG. DAG has been shown to activate PKC-θ, which in turn inhibits IRS-1. Inactivation of IRS-1 has been shown to disrupt the insulin signalling pathway, leading to increased insulin resistance.
1.5 Hypoglycemia and Exercise in Patients with Type 1 Diabetes Mellitus

A regular course of physical activity has been positively associated with many benefits for almost all populations, T1DM patients included. Unfortunately, participation in exercise training in this particular population is limited by the reasonable fear of post-exercise hypoglycemia (low blood sugar). In fact, any adjustment to insulin dose in T1DM patients is, more often than not, based on blood glucose monitoring and the desire to avoid post-exercise hypoglycemia. There is some evidence that increasing carbohydrate intake and reducing insulin dosage prior to exercise may be a suitable approach to preventing exercise-induced hypoglycemia.

Post-exercise hypoglycemia is postulated to occur due to increased glucose uptake in exercising skeletal muscle caused by the contraction-mediated translocation of GLUT4 independent of insulin, combined with the additional impact of residual exogenous insulin circulating in the blood from normal, ongoing T1DM insulin supplementation. This combination can cause a rapid and significant drop in blood glucose, and in extreme cases can lead to diabetic coma and death. Another contributing factor may be that certain patients with T1DM may have a history of poor glycemic control, and therefore may not have built up the hepatic glycogen stores to the extent where the liver is able to counteract hypoglycemia. There is also evidence that many T1DM patients have a blunted glucagon response coupled with diminished clearance of injected insulin, which would contribute to excessively low blood glucose as well.
Despite the risk of hypoglycemia, regular physical activity is positively associated with a wide range of health benefits for patients with T1DM\textsuperscript{69}, including reduced serum cholesterol, higher cardiorespiratory fitness, improved vascular health, enhanced body composition measures, and a higher quality of life\textsuperscript{19,70}. Exercise training also decreases total cholesterol levels in T1DM patients, which is an outcome associated with reduced risk of CVD\textsuperscript{19}. Approximately 15% of children with T1DM have elevated LDL-c levels, which is concerning because not only total cholesterol, but specifically LDL-c is a well-established risk factor for CVD itself\textsuperscript{71}. Patients with T1DM who regularly engage in exercise training are also reported to have significant benefits including improved aerobic fitness (VO\textsubscript{2}max), reduced HbA1c, reduced daily insulin dosage, reduced total cholesterol\textsuperscript{24}, and cardiovascular health benefits independent of changes to body composition. It has also been shown that aerobic fitness is inversely related to CVD risk and mortality from any cause in T1DM patients\textsuperscript{29}, lending support to the claim that regular exercise training provides holistic benefits to the T1DM patient, beyond metabolic markers. The benefits of regular exercise training far outweigh the risks to patients living with T1DM, as long as exercise-related risks are suitably planned for, mitigated, and monitored, in particular the risk of exercise-related hypoglycemia\textsuperscript{72}.

Aerobic exercise has received a lot of attention in T1DM research, as it is has been shown to improve oxidative capacity, muscle capillarization, and mitochondrial density, each of which alone would have profound metabolic benefits for patients with T1DM\textsuperscript{73}. It has been demonstrated that aerobic exercise is effective at reducing the changes that occur preferentially within the type I and IIa oxidative muscle fibres, opposing the diabetes-induced inhibition of mitochondrial oxidative enzymes and
contributing to a shift towards improved oxidative capacity throughout the muscle 37. There is evidence that these exercise-related adaptations are not only due to changes in metabolic activity within the various muscle fibres but are also due to the ability of certain fibres to change between primarily oxidative IIa and glycolytic IIb fibre types, as evidenced by changes in the expression of myosin heavy chain isoforms specific to each fibre type. While shifts between slow and fast-twitch fibre types have been recorded in studies of laboratory rats, there is limited evidence that human skeletal muscle is able to switch between type I and II, whereas fibre type shifting between type IIa and type IIb is far more common 74.

Recent evidence would indicate that a combined anaerobic and aerobic exercise training regimen could help to reduce the risk of post-exercise hypoglycemia, making exercise more accessible to those patients with T1DM who might otherwise entirely avoid exercise participation, and allowing them access to the benefits of regular exercise training 21,65,75. In addition, combined exercise training has been shown to cause a number of changes within the skeletal muscle that confer significant benefits to the insulin resistant T1DM patient. In healthy subjects, combined exercise training improves upon both aerobic and resistance training with significantly improved mitochondrial oxidative capacity in the skeletal muscle, as indicated by increased citrate synthase activity 76,77.

Our lab has extended this line of research to examine whether combined exercise training could improve upon skeletal muscle oxidative capacity and lipid metabolism better than aerobic exercise training alone. We found that not only did combined training improve skeletal muscle citrate synthase activity (and therefore oxidative capacity) better than aerobic training only, but combined exercise also caused a greater increase in lipid
storage capacity within the muscle than aerobic exercise training did via reduced CD36 and increased Lipin-1 \(^{56}\). This indicates that the muscle’s various adaptations to combined exercise allows for increased lipid storage, so the muscle can more effectively accommodate the metabolic overload that initially led to the accumulation of insulin-desensitizing IMCL species. Between the improved lipid storage and the increased oxidative capacity, combined exercise-trained skeletal muscle is able to funnel IMCL towards oxidation and proper IMCL storage as TAG, rather than allowing IMCL to contribute to cellular signalling disruption through the accumulation of DAG. Improving the TAG:DAG storage ratio within the muscle provides a mechanism for both the reduction of IR with combined exercise, and the Athlete’s Paradox observation of increased overall IMCL storage within the trained muscle.

Combined exercise has also been shown to more effectively maintain muscle mass across all fibre types than resistance or aerobic exercise alone, including improved cross sectional area and maintenance of hypertrophy in type IIb glycolytic fibres \(^{39}\). These improvements in insulin sensitivity, muscle fibre profile, oxidative capacity, and IMCL storage in combination with the vital reduction in post-exercise hypoglycemia contribute to the fact that many physicians, organizations, medical associations, and ministries currently recommend a regimen of combined aerobic and resistance exercise as the ideal exercise training modality for patients with T1DM \(^{6,78–80}\).
1.6 Rationale

The leading cause of mortality in T1DM patients is CVD, a condition which has been closely linked with the development of IR in patients undergoing intensive insulin therapy. Studying the pathophysiology of IR in intensively insulin-treated T1DM patients is therefore critical to understanding the development of CVD and improving the long-term outcomes for this population. The development of IR is postulated to stem from the improper storage of IMCL within the skeletal muscle, leading to the accumulation of lipid metabolites that interfere with the insulin receptor signalling pathway, blunting the action of insulin and reducing glycemic control. Exercise has been shown to reduce the buildup of these harmful IMCL species, which has occurred concomitantly with improvements in insulin sensitivity. However, many T1DM patients avoid exercise due to the risk of post-exercise hypoglycemia. A combined aerobic and resistance training regimen has been shown to not only reduce that risk, but also confers significantly improved benefits to the skeletal muscle than either aerobic or resistance training alone, including improved oxidative capacity and IMCL storage in the muscle as a whole. However, it is still unknown how combined exercise training affects individual fibre type transitions and metabolic function.

1.7 Purpose and Hypothesis

The purpose of this study was to examine the effects of a combined aerobic and resistance training regimen versus intensive insulin therapy on skeletal muscle fibre
profile in Streptozotocin (STZ)-induced T1DM rodents. Specifically, we investigated whether combined exercise led to a transition towards a more oxidative fibre profile, concomitant with improved IMCL content and stored muscle glycogen. Firstly, we hypothesized that a regimen of intensive insulin treatment would cause a shift in muscle fibre type towards a higher percentage of type IIb fibres with high IMCL content. Secondly, we hypothesized that a combined aerobic and anaerobic exercise training regimen would lead to a reduction in IMCL stores within type IIb fibres, and an overall shift in fibre composition towards an increased type IIa fast-twitch and type I slow-twitch oxidative fibre profile.
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Chapter 2

2.1 Introduction

Type I diabetes mellitus (T1DM) is an autoimmune-related disorder characterized by the destruction of insulin-producing beta cells in the pancreatic islets of Langerhans. This results in chronically low levels of circulating insulin and the subsequent loss of glycemic control. The inability to maintain blood glycemic equilibrium can result in elevated blood glucose levels, cumulating in a condition known as hyperglycemia. Over time, uncontrolled hyperglycemia can lead to a host of health complications. These include the development of cardiovascular disease (CVD), neuropathy, retinopathy, myopathy, and nephropathy, which represent the greatest contributors to morbidity and mortality for patients with T1DM. Despite the many advances made in T1DM research since the discovery of insulin, global prevalence of the disease has continued to rise, particularly in youth populations. As of 2018, T1DM was one of the most frequently diagnosed chronic diseases in children and youth, and it is expected to continue to increase in prevalence.

With the publication of the Diabetes Control and Complications Trial (DCCT) in 1993, a large amount of evidence supported the long-term benefits of intensive insulin therapy over conventional insulin therapy. This observation ultimately led to the early completion of the DCCT trial and recommendation that all future patients with T1DM utilize an intensive insulin treatment. Since then, further research efforts have confirmed that focusing on tight blood glucose management through intensive insulin therapy significantly improves lifestyle, longevity, and disease outcomes in patients with T1DM.
Specifically, intensive insulin therapy leads to significant reductions in the long-term
development of CVD (42%), nephropathy (34%), retinopathy (76%), and neuropathy
(69%) in patients with T1DM.

In approximately 20% of patients with T1DM, long-term reliance on intensive
insulin therapy has been associated with the development of insulin resistance (IR), a
phenomenon more commonly associated with type 2 diabetes mellitus (T2DM). Termed “double diabetes”, these patients are at an increased risk for CVD and other
associated morbidities in comparison to patients with either T1DM or T2DM alone.
Though the majority of IR-related research has been focused on patients with metabolic
syndrome or T2DM, recently the presence of IR in T1DM has been recognized to be
pathologically different from other IR-related conditions. The pathogenesis of IR in
T1DM in this already high-risk population remains unknown. Thus, research examining
the mechanisms of IR development in patients with T1DM and exploration of
interventional tools to mitigate its onset have recently become an emerging area of study.

One of the more promising theories to be postulated surrounding the development
of “double diabetes” in T1DM is called the muscle-lipotoxicity theory of IR. Initially proposed as a cause for IR development in T2DM, the theory posits that the
improper storage of intramyocellular lipid (IMCL) metabolites in the skeletal muscle
leads to the disruption of insulin receptor signalling function. As a consequence,
inadequate insulin-mediated reduction in blood sugar occurs leading to sustained
increases in blood glucose and chronic hyperglycemia. At the onset of the condition, it
is believed that insufficient levels of insulin lead to an increase in lipid flux which the
mitochondria are unable to accommodate. This “metabolic overload” ultimately results in
the conversion of free fatty acids into diacylglycerol (DAG), or the metabolization into ceramides. The resulting accumulation of IMCL initiates the development of IR, as both DAG and ceramides have been shown to interfere with the insulin signalling pathway and have been positively correlated with IR severity.

Though the lipotoxicity theory currently carries the most weight, there are other theories surrounding the development of IR in T1DM. It has been proposed that IR develops as a direct result of the autoimmune related mechanisms surrounding T1DM, while other theories focus on glucotoxicity and the accumulation of advanced glycation end products. Moreover, IR develops through increased glucotoxicity in the muscle cell, leading to the activation of protein kinase C through the activity of c-Jun N-terminal kinase, which then contributes to inhibition of the insulin signalling pathway.

The majority of research regarding muscular adaptations to T1DM has included markers of metabolic function gathered from whole skeletal muscle. While this work has shown that T1DM leads to significant decreases in muscle oxidative capacity, only a few studies have examined differential changes in metabolic function between the three major muscle fibre types. In T1DM skeletal muscle, type I slow-twitch oxidative fibres displayed significantly reduced citrate synthase activity, a marker for oxidative phosphorylation, which is often associated with changes in mitochondrial morphology. Type IIa fast-twitch oxidative fibres exhibit a combination of reduced enzymatic activity for both oxidative and glycolytic processes in patients with T1DM. Type IIb fast-twitch glycolytic muscle fibres of patients with T1DM express reduced enzymatic activity for markers of glycolytic flux, including hexokinase (HK), phosphofructokinase-1 (PFK), and phosphatase (PHOS). Since type IIb fibres do not rely on oxidative
phosphorylation to the same extent as types I and IIa, the T1DM-related inhibition of oxidative capacity has a lesser effect on type IIb fibres. However, it has been shown that T1DM leads to a preferential reduction in type IIb fibre cross-sectional area (CSA), resulting in a decrease in the overall skeletal muscle mass.\textsuperscript{23,27–29}

Regular physical activity has been positively associated with a host of benefits for patients with T1DM, including increases in overall muscle mass and muscle fibre CSA, and the normalization of altered metabolic enzyme function.\textsuperscript{24,30} These changes are evident across all fibre types, suggesting that exercise may confer both whole-muscle benefits as well as fibre type-specific changes. Though exercise training in the T1DM patient population has these muscular benefits, actual participation in this healthy behaviour is limited by the fear of post-exercise hypoglycemia (low blood sugar).\textsuperscript{31} Hypoglycemia onset following exercise occurs due to the reliance on circulating exogenous insulin and the increased glucose uptake in skeletal muscle long after the cessation of exercise. In extreme cases, recurrent or overly prolonged exercise can lead to acute cognitive dysfunction, diabetic coma and even death. In an effort to avoid this, many patients (and especially those utilizing a more conventional insulin treatment regimen) attempt to counter the anticipated exercise-related drop in blood sugar by reducing their insulin dosage and elevating their blood glucose through carbohydrate intake prior to the onset of exercise. However, the safety of intentionally elevating blood glucose levels is questionable, and exercising patients with T1DM who follow this trend tend to have increased HbA1C values in comparison to sedentary T1DM patients.\textsuperscript{32}

Recent evidence has shown that with a combined aerobic and resistance exercise training regimen, this post-exercise hypoglycemia risk can be reduced.\textsuperscript{9,33} Additionally,
when compared to aerobic or resistance training alone, combined exercise training confers many improved benefits to the patient with T1DM. Our laboratory has recently shown that the combination of aerobic and resistance exercise training improves skeletal muscle oxidative capacity and maintains muscle mass across all fibre types better than aerobic or resistance exercise alone \(^{18,27}\). Interestingly, combined exercise training also led to a greater reduction in skeletal muscle DAG than aerobic or resistance exercise alone, which was associated with improvements in IR and suggests that combined training may be the ideal exercise modality for patients with T1DM \(^{12}\). Further research efforts are needed to investigate the specific transitional and metabolic changes to muscle fibre types resulting from combined exercise in T1DM skeletal muscle. Moreover, these ongoing studies need to explore these exercise-related changes in skeletal muscle morphology in comparison to the modern standard of care, intensive insulin therapy.

The purpose of this study was to examine the effects of a combined exercise training regimen versus intensive insulin therapy on the skeletal muscle fibre profile of T1DM rodents. Specifically, we investigated whether combined exercise led to a transition towards more oxidative type I fibres, concomitant with improved intramyocellular lipid content and stored muscle glycogen content. Firstly, we hypothesized that a regimen of intensive insulin treatment would cause a shift in muscle fibre type towards a higher percentage of type IIb fibres with high intramuscular fat stores. Secondly, we hypothesized that a combined aerobic and anaerobic exercise training regimen would oppose this change, leading to a reduction in IMCL stores in type IIb fibres, and a shifting of fibre type towards an oxidative type I and IIa fibre profile.
2.2 Materials and Methods

2.2.1 Ethics Approval

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

2.2.2 Animals

Seventeen male Sprague-Dawley rats were obtained from Charles River Laboratories (St. Constant, Quebec, Canada) at eight weeks of age. The rats were caged in pairs and held on a consistent 12-hour dark/light cycle, with room temperature held at 20 ± 1°C and relative humidity at 50% for the duration of the study. All rats were given access to standard rat chow and water ad libitum throughout the study.

2.2.3 Experimental groups

Each rat was randomly assigned into one of four treatment groups. Control sedentary (CS; n=4), diabetic sedentary with conventional insulin therapy (DCT; n=4), diabetic sedentary with intensive insulin therapy (DIT; n=5), and diabetic with combined exercise training and conventional insulin therapy (DCE; n=4).

2.2.4 Experimental Procedures

2.2.4.1 T1DM Induction and Insulin Pellet Implantation
Following five days of acclimatization, the rats in the three diabetes groups were given an intraperitoneal low dose injection of 20mg/kg streptozotocin (STZ; Sigma-Aldrich) every day for five days, in order to induce T1DM. All STZ injections were given within five minutes of preparation, dissolved in a 0.1 M citrate buffer (pH 4.5). T1DM was confirmed using non-fasted blood glucose measurements of ≥18 mmol/L on two consecutive days. Following T1DM confirmation, abdominal subcutaneous insulin pellets were surgically implanted into the three diabetic groups of rats receiving insulin therapy (1 pellet; 2U insulin/day; Linplant, Linshin). The intensity of insulin therapy was controlled through addition or removal of insulin pellets as needed, so that DCT rats were able to maintain a blood glucose range of 9-15 mmol/L, DIT rats were maintained at blood glucose range of 7-9 mmol/L, and DCE were maintained at BG range of 9-15 mmol/L. Blood glucose ranges were maintained throughout the duration of the study.

2.2.4.2 Exercise Training Protocols

Rats in the DCE experimental group spent a week becoming familiar with the exercise equipment and then proceeded to undergo a combined regimen of aerobic and resistance exercise training over the next 12 weeks. Combined resistance and aerobic exercise training involved alternating for five days per week for 12 weeks, where the first week would have resistance (R) training performed three days of the week, alternating with two days of high-intensity aerobic (A) exercise (R-A-R-A-R), followed by a week of the opposite (with three days of aerobic exercise separated by two days of resistance exercise; A-R-A-R-A).
Familiarization with the aerobic exercise protocol was established through running for 15 minutes at progressively higher speeds on a motorized treadmill (up to 30m/min at a 0° incline) at five and three days before the start of training. The high intensity aerobic training program involved the DCE rats running for one hour at 27m/min on a 6% incline gradient, eliciting an exercise intensity of between 70-80% of VO$_2$max $^{36}$. The rats were encouraged to maintain pace by short bursts of compressed air triggered when a rat slowed and broke a photoelectric beam at the back of the treadmill.

Resistance exercise training was accomplished with a weighted ladder climb. At the top of the ladder was an open, dark box where the rats could shelter. Weights were placed into small fabric bags attached to the base of their tails and the rats were then placed on the bottom of the ladder so they could climb. This process was repeated with increasing weights as the rats adapted to each weight load, in order to maintain appropriate stimulus to the muscle so as to reproduce a resistance training protocol. Familiarization involved 10 ladder climbs with progressive weight increases up to 35% of body mass at 5 and 3 days before the onset of training. Following each climb, the rats were permitted to rest in the box for ~2 minutes. Pre-training maximal carrying capacity was determined by initial loading of 75% body mass, with each subsequent climb adding 30g of weight until animals were unable to successfully complete the climb to the preferred dark box destination at the top of the ladder. During training, the DCE rats were loaded with 50%, 75%, 90%, and 100% of their predetermined maximal carrying capacity for single climbs, and then repeated 100% load climbs until exhaustion or unwillingness to climb despite pressurized air bursts to encourage climbing. Maximal
carrying capacity was re-evaluated every four exercise sessions to ensure the rats were undergoing a progressive resistance exercise regime.

2.2.5 Experimental Measures

2.2.5.1 Body Weights, Blood Glucose

Body weights were measured and recorded weekly throughout the course of the study. Blood glucose measurements were also made weekly throughout the study and were taken by collecting a small droplet of blood (~50µL) from the saphenous vein. Blood glucose values were analyzed via a Freestyle Lite Blood Glucose Monitoring System (Abbot Diabetes Care, INC.) and reported in millimoles per litre (mmol/L).

2.2.5.2 Tissue Collection

Rats were sacrificed 72 hours following their last training session via anaesthetization with isoflurane, followed by cardiac exsanguination. The lower limbs were dissected and the plantaris muscles were removed, mounted in Cryomatrix™ embedding medium (Lot No. 225229, Thermo Fisher Scientific) and rapidly frozen in isopentane cooled to -70°C by liquid nitrogen. Plantaris was chosen due to its 50:50 mix of red and white muscle tissue, with the intention of highlighting any changes to both oxidative and glycolytic fibre types. Serial cryosections were cut in varying thicknesses (depending on the requirement for the histochemical analysis) at -20°C with a Leica CM350 Cryostat (Leica Biosystems) and adhered to VWR Superfrost® Plus Microslides (Cat. No. 48311-703, VWR International). All sections were stored at -30°C until analysis.
2.2.6 Histochemical Analysis

2.2.6.1 Hematoxylin and Eosin

Slides were incubated in Harris Haematoxylin for 20 minutes, then rinsed in an alkaline tap water substitute and exposed to 1% HCl in 70% ethanol for 10 seconds. Following another rinse in the alkaline tap water, slides were incubated for 3 minutes in a 5% eosin hematology solution. Slides were rinsed in tap water to remove excess stain, dehydrated in ascending alcohol, cleared with xylenes, and mounted with toluene-based mounting media.

2.2.6.2 Metachromatic myosin ATPase for muscle fibre type analysis

Slides were incubated in an acidic pre-incubation solution (0.49% Potassium Acetate, 0.26% calcium chloride dihydrate, pH 4.38 adjusted with glacial acetic acid) for 7 minutes at room temperature, then washed 3 times for 2 minutes each in 0.1 Tris Buffer. Slides were then incubated for 30 minutes at room temperature in an ATP incubation solution, then rinsed in a 1% calcium chloride dihydrate solution. Slides were stained in 0.1% toluidine blue for 90 seconds, cleared in running ddH$_2$O, dehydrated in ascending alcohols, and then cleared with xylenes. Slides were mounted in toluene-based mounting media. Type I muscle fibres stained dark blue, type IIa fibres stained light blue, and type IIb fibres stained white (see Figure 4b for an example).

2.2.6.3 Glycogen Periodic Acid Schiff for glycogen staining

Slides were placed directly in Carnoy’s fixative (ethanol, chloroform + glacial acetic acid) at -30°C for 5-10 minutes, then removed from the freezer and allowed to
continue to incubate while returning to room temperature. Once at room temperature, the Carnoy’s fixative was discarded and the slides were rinsed in 3 changes of ddH₂O, before being placed in 0.5% periodic acid solution for 5 minutes at room temperature. Slides were then left to incubate in 37.5°C Schiff’s reagent for 30 minutes, and then cleared with running 37°C tap water for 10 minutes. Slides were then dehydrated in ascending alcohols, cleared with xylenes, and mounted with toluene-based mounting media. Fibres containing high glycogen stores stained a bright purple, while those with low glycogen stores stained white (see Figure 4c for an example).

2.2.6.4 Oil Red O for quantification of neutral lipids

Muscle sections were surrounded by an immunoedge pen barrier and covered with 0.5% Oil Red O solution in propylene glycol. Slides were left to incubate for 5 hours in a humidity chamber at which point they were washed in running ddH₂O and mounted with aqueous mounting media (10% PBS, 90% glycerol). Fibres with high IMCL stores stained dark reddish/brown, while those with low IMCL stores were a light-pinkish/yellow colour in comparison (see Figure 4d for an example).

2.2.7 Microscopy Image Collection and Muscle Fibre Analysis

All microscopic imaging was performed on a Zeiss AxioVert S100 microscope equipped with a Canon EOS Rebel T2i camera adapted for use with an NDPL-1(2x) attachment. Muscle sections were captured at 10x magnification in a grid pattern so that each region of each slide was photographed, allowing the same region to be compared between each of the histochemical slides.
The grid photographs were printed and quantified, ensuring the same fibres were examined across each stain. Fibre numbers were tallied so that a minimum of 200 fibres were included per animal, with each of the fibres containing data from four different stains (H/E, fibre type, Fat content, glycogen content). High/low IMCL/glycogen storage content and muscle fibre identity were evaluated based on staining colour specific to each procedure, as outlined in Methods section 2.2.6, Histochemical Analysis. Muscle fibres were only included if they were clearly defined across all four stains, with a clear distinction between those fibres containing high IMCL/glycogen stores versus those with low stores, and clear distinction between fibre type, so as to avoid incomplete or imprecise analysis.

2.2.8 Data Analysis

Muscle fibre type, fibre glycogen content, and fibre IMCL content were compared within and between groups using a one-way analysis of variance (ANOVA) with GraphPad Prism 6 (GraphPad Software, Inc.). When significant differences were observed, pairwise post-hoc analysis was performed using the Tukey test. For results demonstrating a significant Brown-Forsythe test for equality of group variances, post-hoc analysis was performed using the Welch’s ANOVA for unequal variances. Significance was determined for an alpha value of 0.05.
2.3 Results

2.3.1 Animal Characteristics

Animal physical characteristics included weekly blood glucose (mmol/L) and weekly body mass (g) measures. Complete body weight and blood glucose data were collected for 17 animals (CS n=4; DCT n=4; DIT n=5; DCE n=4). These results are presented in Figure 2 and Figure 3 respectively. Weekly glucose measures were significantly different between all groups (P ≤ 0.0001) except for DCT vs. DCE, for which no significant difference was found (Figure 2). These measurements were taken on non-fasted animals at 9:00 am, and through adjusting the number of insulin pellets implanted in the animals, attempts were made to maintain DCT animals within blood glucose levels between 9-15mmol/L, and DIT animals within 7-9mmol/L, respectively. Weekly body mass analysis identified a significant difference between the DIT and DCE groups (P=0.0059), although no other significant differences between the diabetic groups (DCT, DIT, and DCE) were identified. The body mass for all three diabetic groups was significantly lower than the mass of the CS animals (P<0.0001) (Figure 3).

2.3.2 Muscle Fibre Analysis Overview

A total of 14,520 skeletal muscle fibres were quantified across the four groups, resulting in full profile information about muscle fibre type, IMCL content, glycogen storage content, and tissue quality/context for 3,630 fibres, ranging from the fewest completely quantifiable fibres in the DCT group (803), to the greatest number of
completely quantifiable fibres in the DIT group (1,043). Histochemical analysis examples are demonstrated in *Figure 4*.

2.3.3 Diabetes, Exercise, and Insulin Treatment Protocol induced changes to Type I fibres

With respect to the animals in the DIT group, there was a significantly greater percentage of type I fibres containing high IMCL in both the CS and DCT groups (P<0.0001), as well as the DCE group (P=0.0003) (*Figure 5a*). There were also a significantly smaller number of these high IMCL type I fibres in the DCE group when compared to CS (P=0.0047) (*Figure 5a*). No other significant differences were observed between groups with respect to the percentage of type I fibres containing high IMCL stores. No significant differences were observed between all groups with regards to the percentage of type I fibres containing high glycogen stores (*Figure 5b*).

When compared to CS animals, there was a significantly higher percentage of type I fibres found in both the DIT group (P=0.0017) and the DCE group (P=0.0445) (*Figure 5c*). The animals in the DCT group also had significantly smaller percentage of type I fibres than the animals in the DIT group (P=0.0104) (*Figure 5c*). No other significant differences were observed between groups with respect to the overall percentage of type I fibres analyzed within the muscle sections.

2.3.4 Diabetes, Exercise, and Insulin Treatment Protocol induced changes to Type IIa fibres

There were no significant differences observed between all groups with regards to the percentage of type IIa fibres containing high IMCL stores (*Figure 6a*). It was
observed that the animals in the DIT group had a significantly higher percentage of type IIa fibres containing high glycogen stores than the CS animals (P<0.0001) (Figure 6b). No other significant differences were observed between groups with respect to the percentage of type IIa fibres containing high glycogen stores. There were no significant differences observed with regards to the overall percentage of type IIa fibres analyzed between all groups (Figure 6a).

2.3.5 Diabetes, Exercise, and Insulin Treatment Protocol induced changes to Type IIb fibres

There were no significant differences observed between all groups with regards to the percentage of type IIb fibres containing high IMCL stores (Figure 7a), type IIb fibres containing high glycogen stores (Figure 7b), or the overall percentage of type IIb fibres analyzed (Figure 7c).

2.3.6 Diabetes, Exercise, and Insulin Treatment Protocol induced changes to Overall Muscle Fibre profile

With respect to the animals in the DCE group, there was a significantly lower percentage of high IMCL-containing muscle fibres in both the CS and DIT groups (P=0.0002), as well as the DCT group (P=0.0094) (Figure 8a). No other significant differences were observed between groups with respect to the overall percentage of fibres containing high IMCL stores. There were no significant differences observed between all groups with regards to the percentages of muscle fibres containing high glycogen content (Figure 8b).
Figure 2: Mean non-fasted weekly blood glucose measures (mmol/L). All data presented as mean ± SEM. * denotes $P \leq 0.0001$.

Figure 3: Mean weekly body mass measures (g). All data presented as mean ± SEM. * denotes $P=0.0059$, ** denotes $P<0.0001$. 
Figure 4: Histochemical stains used in quantification, from left to right: (a) Hematoxylin and Eosin (context stain used to help identify fibres), (b) Metachromatic myosin ATPase (stain to identify muscle fibre type), (c) Glycogen Periodic Acid Schiff (stain to identify high/low glycogen content), and (d) Oil Red O (stain for high/low IMCL content). See Appendix G for quantification example.
Figure 5: (a) The percentage of total type I fibres identified as containing high neutral IMCL stores via Oil Red O staining. (b) The percentage of total type I fibres identified as containing high glycogen stores via glycogen periodic acid Schiff staining. (c) The percentage of total muscle fibres identified as type I via metachromatic myosin ATPase staining. All data are expressed as mean ± SEM. * denotes 0.05 > P > 0.005; ** denotes P = 0.0047; *** denotes P = 0.0017; **** denotes P = 0.0003; ***** denotes P < 0.0001.
Figure 6: (a) The percentage of type IIa fibres identified as containing high neutral IMCL stores via Oil Red O staining. (b) The percentage of total type IIa fibres identified as containing high glycogen stores via glycogen periodic acid Schiff staining. (c) The percentage of total muscle fibres identified as type IIa via metachromatic myosin ATPase staining. All data are expressed as mean ± SEM. **** denotes P<0.0001.
Figure 7: (a) The percentage of total type IIb fibres identified as containing high neutral IMCL stores via Oil Red O staining. (b) The percentage of total type IIb fibres identified as containing high glycogen stores via glycogen periodic acid Schiff staining. (c) The percentage of total muscle fibres identified as type IIb via metachromatic myosin ATPase staining. All data are expressed as mean ± SEM.
Figure 8: (a) The percentage of total fibres across all fibre types identified as containing high neutral IMCL stores via Oil Red O staining. (b) The percentage of total muscle fibres across all fibre types identified as containing high glycogen stores via glycogen periodic acid Schiff staining. All data are expressed as mean ± SEM. ** denotes \( P=0.0094 \); *** denotes \( P=0.0002 \).
2.4 Discussion

It is well known that both T1DM and exercise place unique energetic demands upon skeletal muscle as a whole; however, the mechanisms by which the various muscle fibre types adapt to meet the combination of these energetic demands remains to be understood. A limitation of previous research examining diabetic skeletal muscle health and function is that most studies have only examined whole muscle physiology, rather than investigating the differences between individual fibre types. Given that skeletal muscle fibres vary drastically in their metabolic profiles, their relative energy substrate usage, and their response to exercise, we set out to determine how a combined endurance and resistance exercise training regimen would compare with the standard treatment of care for patients with T1DM (intensive insulin treatment). Specifically, we wanted to examine how T1DM impacts muscle fibre composition and energy substrate storage, and how these transitions adapt following a 12-weeks exercise intervention.

First, we hypothesized that a regimen of intensive insulin treatment of T1DM rats would cause a shift in muscle fibre type towards a higher percentage of type IIb fibres with high intramuscular lipid (IMCL) stores. Second, we hypothesized that a combined aerobic and anaerobic exercise training regimen would reduce IMCL stores in type IIb fibres, shifting fibre type composition back towards a more oxidative type IIa profile. Interestingly, results from the current investigation have demonstrated that only the type I muscle fibres showed any significant changes in IMCL storage, whereas neither type IIa nor type IIb fibres displayed the predicted changes postulated in our hypothesis.
Although the percentage of overall high fat fibres was not different between CS and DCT groups, both had significantly more high-fat fibres than the DIT group (*Figure 5a*). One possible explanation for this observation stems from work done previously by our lab, where it was demonstrated that T1DM rats treated with intensive insulin therapy had reduced skeletal muscle lipid content in conjunction with improved IR when compared to conventional insulin treatment. This improvement in IR with intensive insulin therapy is believed to be primarily due to a reduction in diacylglycerol (DAG) in skeletal muscle fibres. However, the Oil Red O procedure stains all neutral lipids, and cannot distinguish between DAG, triacylglycerol (TAG), and other neutral lipids. It is plausible that in the current study, while a significant reduction in the accumulation of DAG and other disruptive neutral lipid species may have occurred, the DIT group may have maintained its stores of TAG, an energy-rich IMCL species that does not inhibit insulin signalling.

In contrast, the type I fibres in the DCE group had significantly more IMCL stores than those in the DIT group (*Figure 5a*). While this finding did not support our hypothesis, it has been documented that exercise in non-diabetic patients leads to increases in IMCL storage. This observation lends support for the Athlete’s Paradox, the phenomenon in which exercise training leads to an increase in muscle IMCL accompanied by improved metabolic indicators, such as improved insulin sensitivity. Combining this observation with this investigation’s findings on fibre-specific data and IMCL storage demonstrates that the Athlete’s Paradox likely occurs in T1DM rats primarily because of the changes in type I fibres. In support of these findings, it has recently been shown that trained healthy patients also increase their IMCL storage.
primarily in the type I fibres, which are well equipped to handle this change and thereby allow the muscle to benefit from the increased IMCL stores 40.

Our data support the theory that lipotoxicity-induced IR may well be muscle fibre type dependent. It is accepted that intramuscular triglycerides are primarily contained and used within type I muscle fibres, a conclusion which is supported by the observation that intramuscular triglyceride content is three times higher in type I oxidative fibres than in type IIb glycolytic fibres 47. This suggests that lipid toxicity (and by extension lipid metabolism-induced IR) is more closely associated with and may be more dependent on type I muscle fibres than type II fibres 45. The data from the current study has shown that the only changes in IMCL storage in T1DM occur within the type I fibres, and therefore any change in lipid-based IR would likely stem from the differences found within these fibres.

The current study also examined muscle IMCL and glycogen content without mapping either energy store to specific fibre types (Figures 8a and 8b, respectively). Although there were no differences observed regarding whole muscle glycogen stores between groups (Figure 8b), whole-muscle IMCL stores in the DCE group were significantly greater than those in any other group. Indeed, these results are in agreement with a number of previous studies that showed an association between exercise training and significant increases in IMCL storage 18,39,40.

In the current study, the only significant difference observed in muscle glycogen storage was between CS and DIT type IIa fast-twitch oxidative fibres (Figure 6b). Muscle glycogen is stored in the cytosol of the muscle fibre, so it is readily available as a
fuel source for the contractile filaments. Previous studies have found that muscle glycogen storage did not change significantly between intensive insulin treated T1DM patients and healthy controls, although these studies did not examine muscle glycogen storage in specific fibre types. With exercise training, both glycolytic and oxidative enzyme activity have been shown to increase, though whole muscle glycogen storage remained unchanged, pre-training and post-training. This earlier research shows that whole muscle glycogen storage is relatively unaffected by exercise training, though the data from the present study shows that while this phenomenon may apply to skeletal muscle as a whole, individual type IIa muscle fibres may be susceptible to change under intensive insulin treatment and T1DM.

Finally, when looking at the overall muscle fibre profile of the four study groups, we identified significant differences in the percent of total fibres that stained as type I fibres between the various groups, but no differences between the relative percentage of type IIa or type IIb fibres. Preliminary data from our lab has shown that there is no evident change in the percentage of type IIa and IIb fibres, though type II fibres may increase cross-sectional area and expression of hybrid muscle fibre myosin heavy chain isoforms in response to diabetes and exercise. This preliminary work is supported by the current study, where there was also no observed difference in relative percentage of type IIa and IIb fibres. Future research into the changing energy storage capacity of these T1DM hybrid myofibers could yield interesting results and would help to further elucidate our understanding of T1DM skeletal muscle metabolism.
2.5 Conclusion

In conclusion, this study has demonstrated evidence against the hypothesis that the changes within the skeletal muscle are due to shifting fibre type rather than subcellular improvements to IMCL metabolism. We hypothesized that a regimen of intensive insulin treatment would cause a shift in muscle fibre type towards a higher percentage of type IIb fibres with high IMCL stores. Secondly, we hypothesized that a combined aerobic and resistance exercise training regimen would lead to a reduction in IMCL stores in type IIb fibres coupled with a shift towards more type I and IIa oxidative fibres. Instead we found that there was no change within the type IIb fibres, and that the fat storage mechanisms within type I skeletal muscle fibres were preferentially reduced by the presence of T1DM and intensive insulin therapy. This finding was also evident in the conventionally-treated sedentary animals but was readily improved with a 12-week regimen of combined exercise training. These changes occurred within type I fibres only, implying that instead of being caused by a shift between type IIa and IIb fibres, much of the skeletal muscle-related dysfunction occurring in T1DM can likely be traced to malfunctioning fat metabolism specifically within the type I fibre.

The current study provides support for the recommendation that patients with T1DM, and especially those with “double diabetes”, may benefit by supplementing their insulin treatment with a regular exercise training regimen consisting of both aerobic and resistance exercise in order to improve insulin sensitivity. This investigation also provides evidence to improve future efforts to mitigate the IMCL-related dysfunction within the skeletal muscle by focusing on improving oxidative capacity specifically
within the type I muscle fibre. This observation allows for identification of the type I myofiber as a possible target for interventions specifically tailored to improve IMCL storage within the fibre, including pharmacological and exercise treatments focused on improving mitochondrial biogenesis, morphology, and density to divert fatty acids away from storage and into oxidation. Future directed efforts to study the reduction of IR in T1DM would benefit from metabolomic analysis of the type I myofiber IMCL stores in conjunction with mitochondrial LD analysis, helping to further elucidate the fibre-specific adaptations to exercise and the intensity of insulin therapy within T1DM skeletal muscle.
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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.
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. 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.

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. 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. 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.

4. 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)) 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. STZ is time dependent and must be used within 15 minutes.

5. 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. 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).

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

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Melling CWJ, Grisé KN, Hasilo CP, Fier B, Milne KJ, Karmazyn M, Noble EG. A model of poorly controlled type 1 diabetes mellitus and its treatment with aerobic exercise training. Diabetes Metab 39: 226–235, 2013.
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: **Histochemical Procedures – Hematoxylin and Eosin**

- Alkaline Tap water substitute
  - 0.35g sodium bicarbonate
  - 2.0g magnesium sulphate
  - 100mL distilled water
- Harris Haematoxylin (prepared commercially)
- Acid Alcohol
  - 1% HCL – 5mL
  - 70% EtOH
    - 346.5 mL EtOH
    - 148.5 mL H₂O
• 1% Eosin Solution - stock

Procedure

1. Defrost frozen sections in humidity chamber for 1 hour

2. Hematoxylin for 20 min

3. Scott’s Tap water substitute – sections should end up purple blue
   a. Rinse 5x
   b. Fill for 1 min
   c. Rinse 5x

4. Acid Alcohol 10 sec – sections should be reddish colour

5. Scott’s Tap water - sections should end up purple blue
   a. Rinse 5x
   b. Fill for 1 min
   c. Rinse 5x

6. Eosin 3 min

7. Rinse with tap water 10-15 times to wash off surplus stain

8. Dehydrate
   a. 65% EtOH 2 min
   b. 80% EtOH 2 min
   c. 95% EtOH 2 min
   d. Anhydrous EtOH 2 min
   e. Xylenes 1 min

9. Mount with toluene-based mounting media
Appendix D: Histochemical Procedures - Metachromatic myosin ATPase

- Acidic pre-incubation solution – 500 mL
  - 0.49% KCH$_3$COO (w/v) – 2.45g
  - 0.26% CaCl$_2$-2H$_2$O (w/v) – 1.3g
  - Adjust pH to 4.38 with glacial acetic acid immediately prior to use

- 0.1 Tris buffer
  - 12.10g Trizma Base
  - 2.60g CaCl$_2$-2H$_2$O
  - Makes 1L – bring pH to 7.8 and double check pH EVERY time

- ATP Incubation solution – 50 mL
  - 0.4% glycine (w/v) ---------- 0.2g
  - 0.42% CaCl$_2$-2H$_2$O (w/v) --- 0.21g
  - 0.32% NaCl (w/v) ----------- 0.16g
  - 0.19% NaOH (w/v) ----------- 0.095g
  - 0.3% ATP (w/v) ------------- 0.15g
  - Adjust pH to 9.4 with 5N HCL immediately prior to use
  - Prepare this solution immediately prior to staining

- 1% Calcium Chloride Dihydrate Solution
  - 10g CaCl$_2$-2H$_2$O
  - Add to 1L ddH$_2$O

- 0.1% Toluidine Blue solution
  - 0.1% toluidine blue (w/v) for desired volume
  - 125mL of ddH$_2$O + 125mg toluidine blue
o Mix well (vortex) before use

- Dehydration solutions
  o 95% EtOH
  o 2x 100% EtOH
  o 100% Xylenes

Procedure

1. Defrost frozen sections in humidity chamber for 1 hour

2. Prepare ATP incubation solution, adjust pH of Tris buffer and Acidic pre-incubation solution

3. Incubate slides for 7 min at room temperature in acidic pre-incubation solution

4. Wash 3x for 2min each in Tris buffer

5. Incubate for 30 min at room temperature in ATP incubation solution

6. Dip 4x in 3 changes of CaCl₂·2H₂O solution

7. Incubate for 90 seconds in 0.1% toluidine blue solution

8. Rinse in running ddH₂O for 30 sec max – just make sure the excess is gone

9. Dip 5x in 95% EtOH

10. Dip 5x each in 2 changes of 100% EtOH

11. Incubate for 1 min in Xylenes

12. Mount with permount (toluene based mounting medium)
Appendix E: **Histochemical Procedures – Oil Red O**

*Procedure*

1. Defrost frozen sections in humidity chamber for 1 hour
2. Circle each section on slide with Immunoedge pen (or wax pencil)
3. Drop 0.5% Oil Red O in propylene glycol solution on the section until it’s completely covered
4. Incubate for 5 hours in closed humidity chamber
5. Wash with running ddH$_2$O
6. Mount with aqueous mounting media

Appendix F: **Histochemical Procedures – Glycogen Periodic Acid Schiff**

- **Carnoy’s Fixative**
  - 95-100% EtOH 6.4mL
  - Chloroform 1.2mL
  - Glacial acetic acid 0.4mL

- **Periodic Acid Solution – make up fresh**
  - Periodic Acid (H$_3$IO$_6$) 0.05g
  - Distilled H$_2$O 10 mL

- **Schiff’s Reagent - stock – Fischer/Sigma**

*Procedure*

1. Put Schiff’s reagent in 37.5°C water bath to warm up (need ~80mL)
2. Make Periodic Acid solution, and allow to warm up to room temp
a. 0.5g Periodic Acid
b. 100mL Distilled Water

3. Make up Carnoy’s Fixative in the fume hood, then put in the fridge (in copland jar)
   a. 6.4 mL 95-100% EtOH
   b. 1.2 mL Chloroform
   c. 0.4 mL Glacial Acetic Acid

4. Put cut sections into Carnoy’s in copland jar in fridge for 5-10 min

5. Take out and allow the copland jar to come to room temperature (covered/in fume hood, on stirrer, low)

6. Discard Carnoy’s, rinse 3x in 3 changes of ddH₂O (copland jar w/slides + beaker, ddH₂O back and forth 3 times, then change. Rpt 3x)

7. Add Periodic Acid solution for 5 min @ room temperature

8. Discard Periodic Acid solution, rinse in ddH₂O 3x

9. Add Schiff’s reagent for ~30min @ 37.5° in water bath (stirring low)

10. Discard Schiff’s carefully (it stains!!), run in 37° tap water for ~30 min (until water is not pink)

11. Dehydrate
   a. 3 min in 65% EtOH
   b. 2 min in 80% EtOH
   c. 2 min in 95% EtOH
   d. 2 min in 100% EtOH
   e. 1 min in Xylenes
12. Mount with toluene-based media

**Appendix G: Quantification Example**

![Quantification Example Images]

| Type 1 | Type 1a | Type 1b |
|--------|---------|---------|
| High Fat | Low Fat | High Fat | Low Fat | High Fat | Low Fat |
| High Fat | Low Fat | High Fat | Low Fat | High Fat | Low Fat |
| High Fat | Low Fat | High Fat | Low Fat | High Fat | Low Fat |

![Quantification Example Table]
Quantification consisted of identifying the exact same fibre across all four stains, ensuring there was a clear distinction between high/low and fibre type, and then using a tally sheet to collect data.

Appendix H: Ethics Approval

![Image of Ethics Approval Certificate]

AUP Number: 2014-009
PI Name: Noble, Earl
AUP Title: Innovation To Reduce Muscular And Cardiovascular Complications Of Diabetes
Approval Date: 06/26/2014

Official Notice of Animal Use Subcommittee (AUS) Approval: Your new Animal Use Protocol (AUP) entitled "Innovation To Reduce Muscular And Cardiovascular Complications Of Diabetes" has been APPROVED by the Animal Use Subcommittee of the University Council on Animal Care. This approval, although valid for four years, and is subject to annual Protocol Renewal.2014-009:

1. This AUP number must be indicated when ordering animals for this project.
2. Animals for other projects may not be ordered under this AUP number.
3. Purchases of animals other than through this system must be cleared through the ACVS office. Health certificates will be required.

The holder of this Animal Use Protocol is responsible to ensure that all associated safety components (biosafety, radiation safety, general laboratory safety) comply with institutional safety standards and have received all necessary approvals. Please consult directly with your institutional safety officers.

Submitted by: Copeman, Laura
on behalf of the Animal Use Subcommittee
University Council on Animal Care
Curriculum Vitae

Name: David McBey, B.Sc., R.Kin.

Education:

• Master of Science in Kinesiology, University of Western Ontario, London, Ontario, 2017-2019 (In Progress)

• Bachelor of Science Honours Subject of Specialization in Kinesiology, Queen’s University, Kingston, ON, 2008-2013

Honours and Awards:

• Ontario Graduate Scholarship, University of Western Ontario, September 2019-August 2020

• UCBeyond National Scholarship, September 2018-April 2019

• Western Graduate Research Scholarship, University of Western Ontario, January 2017-December 2018

• Sick Kids Hospital Silver Volunteer Award, The Hospital for Sick Children, May 2015

• Canadian National Millennium Scholarship, Queen’s University, September 2008-April 2012

Related Work/Volunteer Experience

• Registered Kinesiologist with the College of Kinesiologists of Ontario, 2015-Present
• Teaching Assistant, Introductory Exercise Physiology, University of Western Ontario, September 2018-December 2018

• Teaching Assistant, Physiology of Exercise Training, University of Western Ontario, January 2018-April 2018

• Teaching Assistant, Laboratory in Exercise Physiology, University of Western Ontario, September 2017-December 2017

• Teaching Assistant, Introduction to Psychomotor Behaviour, University of Western Ontario, January 2017-April 2017

• Full-time Research Assistant, The Hospital for Sick Children Rheumatology Department, Toronto, Ontario, May 2014-May 2015

• Research Assistant, The Human Vascular Control Research Laboratory, Queen’s University, Kingston Ontario, September 2013-April 2014 & September 2015-April 2016

• Research Lab Assistant, Drug Discovery Group, Ontario Institute for Cancer Research (MaRS), Toronto, Ontario, May 2013-Sept 2013

• Research Assistant, The Hospital for Sick Children Surgical Research Team, Toronto, Ontario, May 2009-August 2009