Implications of Actovegin on Aerobic Performance and Mitochondrial Respiration in Trained and Untrained Mice

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ABSTRACT

Implications of Actovegin on Aerobic Performance and Mitochondrial Respiration in Trained and Untrained Mice

Brandon Kosik

Actovegin is a deproteinated calf blood extract that has been used clinically for over 60 years to treat a wide range of conditions. The drug is thought to exert its therapeutic benefits by enhancing oxidative phosphorylation and cell metabolism. It is postulated that Actovegin has insulin-like properties and can increase glucose uptake in the cell. Due to its proposed mechanism of action, Actovegin has also been speculated as an aerobic performance enhancing drug and few studies have been sought out to confirm this speculation. In addition, the effects of this drug on mitochondrial respiration have only been investigated in skeletal muscle and liver tissue through an acute administration protocol.

This thesis explores the effects of Actovegin from a functional and mechanistic perspective. The objectives were to determine if non-acute Actovegin injections every 2nd day for 14 days can increase aerobic exercise capacity and mitochondrial respiration in skeletal muscle and cardiac tissue in trained and sedentary mice versus equivalent groups receiving a placebo. Effects on blood glucose and body mass were also investigated. Using an aerobic exercise capacity test and high-resolution respirometry, the data revealed that Actovegin injections over a two-week period: (1) increased aerobic exercise capacity (2) increased skeletal muscle mitochondrial respiration, (3) did not have a significant effect on cardiac mitochondrial respiration. These results suggest that non-acute Actovegin injections may enhance aerobic exercise performance and reduce the heart's need to increase contractile capacity in a high-intensity exercise setting.

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CONTRIBUTION OF AUTHORS

The first manuscript (Chapter 2) entitled "Increased exercise capacity and skeletal muscle mitochondrial respiration in mice after 14 days of intraperitoneal Actovegin administration" has five authors. Rasmus Kinimond Hassø and Søren Lindtofte shared the first authorship and contributed to data collection, study intervention, statistical analysis and writing original manuscript. I contributed to data collection, study intervention, revision and editing of manuscript. Andreas Bergdahl was responsible for conceptualization, methodology, resources, validation, ethics, reviewing and editing manuscript, supervision. Steen Larsen was responsible for conceptualization, methodology, project administration, resources, supervision, ethics validation, reviewing and editing manuscript.

The second manuscript (Chapter 3) entitled "The effects of Actovegin on cardiac mitochondrial respiration in trained and sedentary mice" has five authors. I was the first author was responsible for data collection, study intervention, statistical analysis, writing of original manuscript. Rasmus Kinimond Hassø and Søren Lindtofte contributed to data collection, study intervention, revision of manuscript. Steen Larsen was responsible for conceptualization, methodology, resources, validation, ethics, reviewing and editing manuscript. Andreas Bergdahl was responsible for conceptualization, methodology, project administration, resources, supervision, ethics, validation, reviewing and editing manuscript, supervision.

All authors reviewed the final manuscripts and approved the contents.

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Abbreviations

ACR: Acceptor control ratio

ADP: Adenosine diphosphate

ATP: Adenosine triphosphate

BIOPS: Biopsy preservation solution

BSA: Bovine serum albumin

CO: Cardiac output

CS: Citrate synthase

EPO: Erythropoietin

ETC: Electron transport chain

$FADH_2$: Flavin Adenine Dinucleotide (2H+)

FCCP: Carbonyl cyanide-4-trifluoromethoxy phenylhydrazone

Hb_{mass}: Total body hemoglobin mass

HIIT: High-intensity interval training

HRR: High resolution respirometry

IOC: International Olympic Committee

IPO: Inositol-phospho-oligosaccharides

MIR05: Mitochondrial respiration medium

MRC: Mitochondrial respiratory capacity

NADH: Nicotinamide Adenine Dinucleotide (H+)

OXPHOS: Oxidative phosphorylation

PED: Performance enhancing drug

RCR: Respiratory control ratio

WADA: World Anti-Doping Agency

GENERAL INTRODUCTION

There has been much controversy on whether Actovegin can be considered a performance enhancing agent or not. While Actovegin is currently allowed for use in sport, the drug's ergogenic capacity remains understudied, as there has only been one limited study that tested Actovegin's acute effects on exercise performance. Therefore, the impact of chronic/non-acute Actovegin treatment on aerobic exercise performance remains unknown. Actovegin's speculated mechanism of action rests on its ability to act like insulin, in that it can increase cellular glucose uptake and enhance mitochondrial respiration. Therefore, it is merited to investigate Actovegin's effect on aerobic exercise capacity, which relies heavily on ATP production by the mitochondria.

> Manuscript 1: To determine whether 2 weeks of Actovegin injections can improve maximal aerobic exercise capacity and skeletal muscle mitochondrial respiration in trained and sedentary mice.

From a mechanistic aspect, Actovegin has been shown to acutely increase mitochondrial respiration in skeletal muscle, adipocytes, and liver. However, the chronic effects of the drug on cardiac, mitochondrial respiration currently remains unknown. Whether the drug can exert its insulin-like activity on cardiac cells to aid in increasing the rate of myocardial oxygen consumption and oxidative phosphorylation has yet to be investigated.

> Manuscript 2: To determine the chronic effects of Actovegin treatment on cardiac mitochondrial respiration in trained and untrained mice.

Hypotheses

It is hypothesized that chronic Actovegin treatment will improve maximal aerobic exercise capacity in both trained and untrained mice, with the greatest improvements observed when combining Actovegin and high intensity interval exercise. It is also hypothesized that Actovegin will enhance skeletal muscle and cardiac mitochondrial respiration in both trained and untrained mice.

CHAPTER 1: LITERATURE REVIEW

Mitochondria and Energy Production

Every organ system relies on mitochondria to produce adenosine triphosphate (ATP). Mitochondria play a fundamental role in ensuring efficient metabolic function, and ultimately, keeping our bodies alive. To meet our energy needs, the human body must produce ATP at a rate of 9×10^{20} molecules/sec, or approximately 65 kg of ATP in a single day (Zimmerman et al., 2011). Mitochondria are generally responsible for 90% of the ATP production in our bodies under aerobic conditions (Pizzorno, 2014). With such immense dependency on this organelle to produce energy, it is imperative that continuous research be conducted to prevent and treat mitochondrial deficiencies, and to find ways to improve mitochondrial respiration.

Mitochondria produce ATP primarily through the oxidation of glucose molecules. This metabolic process begins in the cell cytoplasm where glucose will first undergo glycolysis as it is metabolized to pyruvate. Pyruvate will then enter the mitochondria and pass through the citric acid cycle, generating electron carriers NADH and $FADH_2$ (Bertram et al., 2006). These carriers will then transfer their electrons to mitochondrial complexes I and II of the electron transport chain (ETC) (Martinez-Reyes & Chandel, 2020). In a process known as oxidative phosphorylation (OXPHOS), mitochondria generate energy once electrons pass through the remaining protein complexes (III and IV) embedded in the inner mitochondrial membrane. During this process, protons are pumped from the mitochondrial matrix into the intermembrane space, generating an electrochemical gradient across the inner membrane. The flow of protons back into the matrix via the ATPase complex (complex V) leads to the production of ATP, during which, electrons are passed to oxygen as the final electron acceptor (Sivitz & Yorek, 2010).

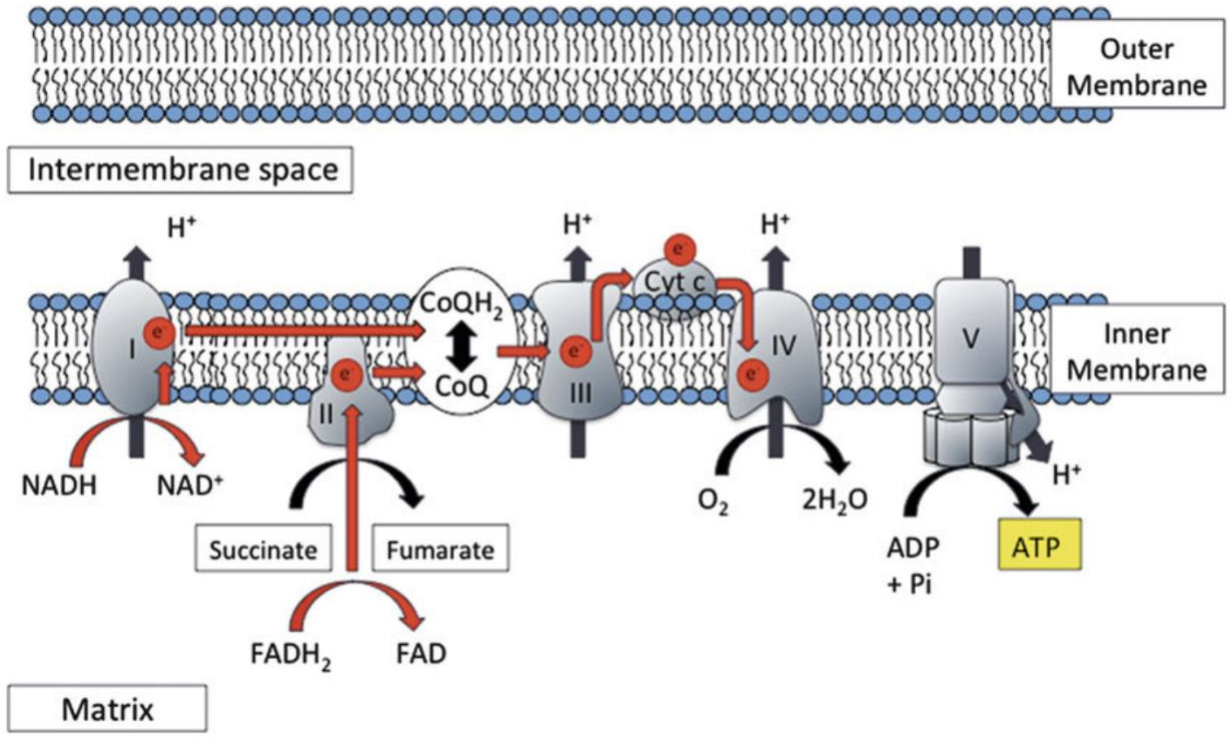

Figure 1: Electron transport chain demonstrating the 5 protein complexes and the sequence of electron flow (de Villiers et al., 2018).

Skeletal Muscle Mitochondria and Aerobic Exercise Capacity

During aerobic exercise, skeletal muscle energy demands increase significantly (Baker et al., 2010). Therefore, to maintain the efficacy of working muscles, there is a heavy dependence on mitochondrial respiration, specifically OXPHOS, to produce ATP at a sufficient rate. As the speed or exercise intensity increases, there is a proportional increase in whole body oxygen consumption (VO_2) driven mainly by increased oxygen demand and consumption in the contracting skeletal muscles (Joyner & Dominelli, 2020). This higher need for oxygen stems from the ETC requiring more oxygen molecules to ultimately drive higher ATP synthesis.

It is evident that OXPHOS capacity per skeletal muscle mass is related to training and aerobic exercise capacity (Gnaiger, 2009). Skeletal muscle mitochondrial respiration (or oxidative capacity) can be classified as one of the three main physiological variables most predictive of overall aerobic exercise performance, alongside total body hemoglobin mass (Hb_{mass}) and

VO_{2max} (Jacobs et al., 2011). Jacobs et al., (2011) found that skeletal muscle OXPHOS and electron transport system (ETS) capacity correlates strongly with overall exercise capacity in trained athletes. In another study by Jacobs & Lundby (2013), improvements in mitochondrial respiratory function were found to be positively correlated with whole body aerobic capacity, independent of mitochondrial content. This demonstrates that a more efficient ETC can lead to more ATP production in the muscles, and therefore, may translate to optimal performance. In addition, oxygen extraction significantly correlates with oxygen consumption over various workloads, and oxidative capacity in the skeletal muscle assists in facilitating the extraction of oxygen during exercise (Calbet et al., 2005). This further strengthens the notion that OXPHOS is an important factor in aerobic exercise performance. While it is true that skeletal muscle mitochondria are important determinants for overall aerobic performance, the human body requires an intricate collaboration of multiple organ systems to maintain a high level of aerobic capacity; each organ playing its role. When it comes to exercise, the heart in particular, should not be overlooked.

The Heart and Exercise

The heart's primary purpose is to pump and distribute oxygen and other nutrients to all the organ systems in our body. Providing a continuous supply of oxygen to our cells is not only essential for survival but is a major determining factor for aerobic exercise performance. During aerobic exercise, skeletal muscle oxygen consumption increases linearly with increasing work rate (Burton et al., 2004). In fact, oxygen flux during exercise can reach 100 times higher than resting rates in contracting skeletal muscles (Radak et al., 2013). Since the heart is the main source responsible for circulating oxygen, it is imperative that it maintains an efficient contractile capacity.

Cardiac output (CO) is the amount of blood the heart pumps per minute and is generally defined by the equation:

CO = Heart rate x Stroke volume (Hall, 2016).

Heart contractility plays a key role in regulating CO as well. A higher contractility reduces end-systolic volume, resulting in a greater stroke volume and thus, a greater CO (Froelicher & Myers, 2007). A greater CO during aerobic exercise means oxygen is being transported to the working skeletal muscles at a greater rate, ultimately translating to increased aerobic performance. Cardiac mitochondria play a fundamental role in the pumping efficiency of the heart. The high myocardial energy demand during aerobic exercise necessitates more ATP production to maintain contractility, and greater ATP production is directly related to greater contractile function (Lopaschuk et al., 2021). Therefore, to optimize aerobic capacity, a higher rate of OXPHOS is required to produce enough ATP.

Myocardial Substrates and Energy Production

The metabolic substrates used for cardiac ATP production at rest include mainly fatty acids (40-70%), glucose (20-30%), lactate (5-20%) and ketones (5-15%) (Gibb & Hill, 2018). During high-intensity exercise, elevated workload on the heart increases carbohydrate and fatty acid consumption to produce energy (Goodwin & Taegtmeyer, 2000). However, the primary use of fatty acid oxidation for ATP synthesis is an oxygen inefficient process when compared to glucose oxidation. Fatty acids yield less ATP per oxygen consumed than glucose (Lopaschuk *et al.,* 2010). In addition, an overreliance on fatty acids for myocardial energy can lead to a dangerous accumulation of lipids in the heart, leading to lipotoxicity and risk of cardiovascular disease (Lopaschuk, 2017). Therefore, increasing the use of glucose oxidation for energy can improve cardiac efficiency and function (Stanley *et al.,* 2005). One way of increasing glucose oxidation in the heart is to enhance glucose uptake in the cell, either through exercise or consumption of performance enhancing agents. More glucose uptake in the myocardial cell may translate to more ATP production via mitochondrial respiration, leading to more efficient pumping capacity, enhancing aerobic performance.

Ergogenic Aids/PEDs in Sport

The Goldman dilemma stems from a controversial study that proposed a Faustian bargain to elite athletes. Over a 15-year period, when athletes were asked whether they would take an illegal performance enhancing drug (PED) that would guarantee them an Olympic gold medal, but would kill them after five years, 52% reported they would take the drug (Goldman et al., 1984).

However, a more recent study by Connor et al. (2013) proposed the same bargain to elite level track and field athletes and found that only 1% would accept the proposition. Interestingly, 6% of athletes would take the drug if it were legal but deadly, and 12% would take the drug if it were illegal but harmless (Connor et al., 2013). These contemporary results display the new attitudes athletes have toward taking PEDs. These attitude changes are likely due to a better understanding of the risks associated with doping and a more solidified moral stance on doping (Connor et al., 2013).

While the motivation for doping today is not as extreme as it was during the time of the Goldman dilemma, elite athletes are still significantly motivated to use PEDs as their desire to win remains a top priority. It is estimated that 14-39 % of elite adult athletes have intentionally used PEDs (de Hon et al., 2015). In addition, there is also a high prevalence of dietary supplement use among elite athletes. Kanpik et al. (2016) found that 69% of elite male athletes and 71 % of elite female athletes took some form of dietary supplement with the goal of enhancing their performance. This significant aspiration to win in sport drives elite athletes to continuously seek ways to maintain an advantage over their competitors. It is therefore important to provide new information on the safety and efficacy of PEDs to ensure fair implementation of regulations surrounding these substances. One understudied substance that has had considerable speculation regarding its effectiveness as a performance enhancing agent is Actovegin.

What is Actovegin?

Actovegin is a deproteinated calf blood extract produced by Takeda Pharmaceutical Ltd, Japan. It is a clear, yellow liquid containing over 200 physiological components including electrolytes and essential trace elements. Amino acids, nucleosides, carbohydrate, and fat metabolites comprise 30% of the organic components in Actovegin (Lee et al., 2011). As a deproteinated solution that is ultrafiltered to less than 5000 Daltons, Actovegin does not contain any growth factors or hormone-like substances (Takeda, 2023).

For over 60 years, Actovegin has been used clinically to treat conditions such as acute stroke (Boiarinov et al., 1998; Derev'yannykh et al., 2008), postpartum haemorrhage (Appiah, 2002) and diabetic polyneuropathy (Ziegler et al., 2009). It has also been used as an intra-arterial

infusion for the treatment of long bone fractures (Khomutov et a., 1999) and radiation damage (Beetz et al., 1996). Actovegin can be administered as a topical agent, by tablet, infusion, or injection (Lee et al., 2011).

Actovegin is also relatively safe to use. Toxicity tests in mice have shown that, after intravenous application, Actovegin has an acute toxicity of more than 50 times its maximum therapeutic dose (Buchmayer et al., 2011). In addition, a three-month subchronic test confirmed that Actovegin is non-toxic, and no chronic pathological organic changes have been observed either macro or microscopically (Buchmayer et al., 2011).

Actovegin in the Cell

Actovegin's benefits stem from its ability to exert insulin-like activity, increasing glucose uptake and stimulating cellular metabolism (Buchmayer et al., 2011). Several studies have explored the effects of Actovegin on glucose metabolism. Parad et al. (1968) found that Actovegin has a similar effect to insulin on glucose uptake in rat lipocytes. Since Actovegin is composed of a mixture of components, it is not yet known which active ingredients are responsible for its therapeutic effects. However, it is believed that inositol-phospho-oligosaccharides (IPO) may play a considerable role in Actovegin's insulin-like activity. IPO isolated from Actovegin improves glucose uptake in adipocytes in an insulin-independent manner (Jacob et al., 1996). It has been reported that IPO activate glucose transporters, contributing up to 50% of insulin's maximum effect (Lee et al., 2011).

Actovegin has also been shown to enhance oxygen consumption and mitochondrial respiration in certain tissues. In an early study by Reichel et al. (1965), acute, ex vivo cellular oxygen uptake of rat skeletal muscle increased by 40% in the presence of Actovegin. In addition, through assessment of mitochondrial respiration, Actovegin significantly increased acute OXPHOS capacity in rat liver cells (Kuninaka et al., 1991). Actovegin has also been shown to acutely increase in-vitro OXPHOS capacity in a concentration-dependent manner in human skeletal muscle (Sondergard et al., 2016). This heightened cellular metabolism and OXPHOS may be the explanation to Actovegin's controversial ergogenic identity.

Legal Status and Ergogenic Potential

The controversy surrounding Actovegin's ergogenic potential began in the year 2000, during the Tour de France. French judicial authorities opened an investigation as they suspected that the United-States Postal Service Cycling Team was using banned substances, one of which was Actovegin (Lee et al., 2011). As a result, the International Olympic Committee (IOC) decided to ban Actovegin in December 2000 under the classification of blood-doping agents. However, two months later, the IOC lifted the ban because there was no concrete evidence suggesting that Actovegin enhances aerobic performance (Tsitsimpikou et al., 2009). According to the World Anti-Doping Agency (WADA, 2024) prohibited list, Actovegin currently stands as a non-prohibited drug in any sport. However, the WADA states that intravenous injection of any non-prohibited substance must not exceed a volume of 50 mL, and any consecutive injections must be at least 6 hours apart (WADA, 2011). Therefore, while Actovegin is permitted in sport, there are exceptions that must be considered.

Actovegin's legal status in sport is dependent on existing evidence surrounding its ergogenic ability, and there are very few studies that have explored Actovegin's effects on exercise performance. Lee et al. (2011) conducted an exhaustive arm crank ergometry test where male participants received intravenous injections of Acotvegin directly before the ergometry test. This study by Lee et al. (2011) aimed to determine the acute effects of Actovegin injections on aerobic exercise performance. They concluded that Actovegin had no ergogenic effect on peak power, blood glucose or lactate concentration, exercise efficiency, or rate of VO_2 gain. Ziegler et al. (2009) measured Actovegin's effects on strength in type 2 diabetic patients and concluded no strength changes. However, strength was measured as a secondary outcome as this study primarily explored the effects of the drug for the treatment of diabetic polyneuropathy. Therefore, Ziegler et al. (2009) cannot be considered a study on pure performance.

Actovegin is currently prohibited for human use in all parts of the world except the following countries: Austria, Poland, Ukraine, Germany, Switzerland, Russia, and the United Kingdom (Anghelescu et al., 2023).

CHAPTER 2: MANUSCRIPT 1

Increased exercise capacity and skeletal muscle mitochondrial respiration in mice after 14 days of intraperitoneal Actovegin administration

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#Shared first Authorship

Abstract

Background: The potential ergogenic effects of Actovegin have previously been investigated in humans, with no effects being found on exercise performance. Evidence exists that Actovegin has enhancing effects on mitochondrial respiration in skeletal muscle.
Objective: The aim of this study was to investigate the effects of Actovegin administration on exercise performance and mitochondrial respiratory capacity in skeletal muscle fibers.
Methods: 40 healthy male mice (38 ± 1 weeks) were randomized into 4 groups; control (C), Actovegin (A), control-trained (CT) and Actovegin-trained (AT). All mice were given intraperitoneal injections every other day for 14 days of either 0.1 ml Actovegin (10 mg/ml) or 0.1 ml saline. The training consisted of a HIIT protocol (5x1 minute at 80-90 % of maximal running speed with 2 minutes of active recovery in between bouts) performed every other day by the trained groups. All mice completed a maximal exercise capacity test before and after the intervention. High-resolution respirometry was used to measure skeletal muscle mitochondrial respiratory capacity. Mitochondrial content was assessed using citrate synthase activity.
Results: The CT and AT groups improved their exercise capacity significantly after the 14-day intervention. The increase in performance was significantly higher in the AT group compared to the CT group. Complex I + II linked mitochondrial respiratory capacity was increased in the A, CT and AT groups compared to the C group.
Conclusion: This is the first study showing enhancing effects of Actovegin on aerobic exercise performance and skeletal muscle mitochondrial respiratory capacity with in vivo administration.

Introduction

Actovegin is an ultrafiltrate derived through hemodialysis of calf blood containing more than 200 physiological compounds. The filtration process results in a molecular weight of less than 5000 Daltons, meaning Actovegin is free of proteins and hormones (Buchmayer et al., 2011). However, Actovegin contains a wide array of vitamins, minerals, and amino acids as well as lactate, succinate, and inositol-phospho-oligosaccharides (Machicao et al., 2012; Reichl et al., 2017). Since the 1960's, Actovegin has been used for numerous therapeutic treatments, including muscle injury repair (Lee et al., 2011), wound healing, burns and skin disorders (Buchmayer et al., 2011), radiation-induced damages (Beetz et al., 1996) and acute ischemic stroke (Derev'yannykh et al., 2008). Actovegin has also been shown to have insulin-like effects and is known to increase oxygen uptake and utilization during hypoxia (Machicao et al., 2012). These insulin-like, and anti-hypoxic properties have led to speculations that it may induce ergogenic effects. These speculations came to light in 2000, after the judicial authorities of France initiated an investigation regarding the use of Actovegin by the cycling team, US Postal Service, following their Tour de France victory in 2000. The investigation led to the IOC banning the use of Actovegin in December 2000 on the basis that Actovegin was believed to improve oxygen transport similarly to erythropoietin (EPO) (Tsitsimpikou et al., 2009). However, the ban was revoked just two months later due to lack of scientific evidence. Consequently, Actovegin is still permitted for athletes today, as long as intravenous administration does not exceed 100 ml every 12 hours, as is the general guidelines from the World Anti-Doping Agency regarding all intravenous administrations (World Anti-Doping Agency, 2023). Actovegin is still used in therapeutic care in hospitals and among professional athletes and sports clubs around the world today.

Few studies have examined Actovegin from an ergogenic perspective. One study investigated the effect of 40 ml of intravenously injected Actovegin in 8 male participants 2 hours prior to exhaustive arm crank exercise compared to saline injections and baseline trials (Lee et al., 2012). No differences were found for watt-peak, VO_2-peak, or respiratory compensation point between the three trials. Interestingly, 4 of the 8 participants reported that they found the exhaustive arm crank test easier after Actovegin injection compared to placebo, which the author explained with Actovegin having a potential effect on attenuating peripheral muscle fatigue. Overall, the authors

concluded that Actovegin has no ergogenic effects and that it is unlikely to enhance performance.

Actovegin's effects on muscle cell proliferation have also been tested in cultured C2C12 mouse myoblasts (Reichl et al., 2017). The Actovegin treated cells expressed increased Ki67 proliferation, increased mean area of myotubes, and increased Mf20 expression compared to controls. In the same study, the contents of Actovegin were chemically analyzed, and the authors found that Actovegin contains less testosterone and cortisol than normal adult plasma. Based on these results, the authors concluded that Actovegin cannot be classified as an anabolic solution.

One study has examined the acute effects of Actovegin on mitochondrial respiratory capacity (MRC) in permeabilized human skeletal muscle fibers ex vivo using high-resolution respirometry (HRR) (Søndergård et al., 2016). The authors observed an increase in mitochondrial respiration in a concentration-dependent manner when measured as both complex I + II stimulated respiration and the total capacity of the electron transfer system. The measured increases in mitochondrial respiration were attributed to Actovegin enhancing the effect on the intrinsic function of the mitochondria. However, whether these ex vivo results translate to an actual in vivo effect with meaningful improvements in aerobic exercise performance has yet to be investigated.

The energy required to maintain continuous aerobic exercise comes mainly from the ATP molecules generated through mitochondrial oxidative phosphorylation (OXPHOS). Although central factors such as cardiac output and total hemoglobin mass are the limiting parameters regarding maximal aerobic exercise capacity (Bassett Jr & Howley, 2000), peripheral factors such as mitochondrial function cannot be overlooked. Increased MRC contributes to increased fat oxidation, resulting in less depletion of glycogen, as well as to decreased accumulation of local fatigue-inducing factors such as ADP, P_i, H^+ and lactate (Holloszy & Coyle, 1984). These aspects all contribute to increased exercise capacity, and one study even suggests skeletal muscle MRC may be the best predictor for endurance exercise performance (Jacobs et al., 2011). Exercise training is a strong stimulus for mitochondrial plasticity, and as little as six sessions of high-intensity interval training (HIIT) has been shown to improve exercise capacity due to

improved skeletal muscle MRC (Jacobs et al., 2013). Like exercise training, drugs such as EPO increase skeletal muscle MRC in humans (Plenge et al., 2012) and myocardial MRC in rodents (Carraway et al., 2010).

The main objective of this study was to investigate the effects of a 14-day Actovegin administration on aerobic exercise performance in trained and sedentary mice. In relation to this, skeletal muscle MRC and content was assessed. Body mass and blood glucose concentration were also recorded. We hypothesized that chronic Actovegin treatment will improve maximal aerobic exercise capacity in trained and untrained mice, with the greatest improvements seen when combining Actovegin and HIIT. We also hypothesize that Actovegin will increase skeletal muscle mitochondrial respiration in both trained and sedentary groups.

Methods

All experimental procedures were carried out at the Department of Health, Kinesiology & Applied Physiology at Concordia University, Montreal, CA. The Animal Ethics Committee of Concordia University approved the study (ID: 30000259). All procedures were conducted in accordance with guidelines of the Canadian Council on Animal Care.

Animals and Drug Treatment

The mice (C57BL/6, N = 40) were 38 ± 1 weeks old males with a weight of 38 ± 1 g upon inclusion. The mice were kept in individual cages, with unlimited chow diet and water supply, in a temperature-controlled room with a 12-hour light/dark cycle. Every mouse completed a treadmill inclusion test for familiarization purposes. The mice were randomized into 4 groups: control (C), Actovegin (A), control-trained (CT) and Actovegin-trained (AT). All mice were injected every other day for 14 days with either 0.1 ml Actovegin (10mg/ml) or 0.1 ml saline solution by intraperitoneal injection. Body weight and blood glucose concentration were also registered every other day during the 14-day intervention.

Aerobic Exercise Capacity Test and Exercise Intervention

On experimental day 0, the mice performed a baseline maximal aerobic exercise capacity test until exhaustion (refer to Table 1). The test was performed with a 10-degree incline on a multi-

lane treadmill specifically designed for mice (Bouganim & Bergdahl, 2017). The exercise capacity test consisted of a 3-minute warmup at a speed of 13.3 m/min, with a speed increase of 3.3 m/min every minute until exhaustion. After completion, maximal running speed (m/min) was noted for each mouse. The two trained groups performed a HIIT protocol specifically designed for mice (Caru et al., 2019) every other day. The HIIT protocol consisted of a 5-minute warmup at a speed of 13.3 m/min. Afterwards, 5 intervals of one minute duration were performed, with an intensity of 80 – 90 % of their predetermined maximal running speed (precise intensities were calculated to 84.01 % for the CT group and 85.74 % for the AT group). In between intervals, 2 minutes of active recovery were performed at 13.3 m/min (60 - 65 % of maximal running speed). The HIIT protocol ended with a 5-minute cooldown at 13.3 m/min. To evaluate changes in aerobic exercise performance, the day after the two-week intervention period, a final aerobic exercise capacity test was conducted on all four groups using the same protocol as the baseline test.

Table 1: Maximal aerobic exercise capacity test adapted from Caru et al., 2019

Stage	Speed (m/min)	Incline (degree slope)	Duration (min)
1/Warm-up	13.3	10	3
2	16.7	10	1
3	20	10	1
4	23.3	10	1
5	26.7	10	1
6	30	10	1
7	33.3	10	1

Table 2: HIIT protocol adapted from Caru et al., 2019

Stage	Speed (% of maximal speed)	Duration (min)
Warm-up	~ 60	5
Bout 1	80-90	1
Recovery	< 50	2
Bout 2	80-90	1
Recovery	< 50	2
Bout 3	80-90	1
Recovery	< 50	2
Bout 4	80-90	1
Recovery	< 50	2
Bout 5	80-90	1

Body Mass and Blood Glucose

Using the One Touch Verio Glucometer, blood glucose concentration was measured every second day from the mouse tail vain before any HIIT or body mass measurements. Body mass was measured immediately after blood glucose using an electronic balance scale.

Tissue Preparation and Preservation

All mice were euthanized using CO_2 asphyxiation 24 hours after completion of the post intervention exercise capacity test. Immediately after euthanasia, the left and right vastus lateralis muscles were extracted and placed in physiological saline solution (NaCl 135.5 mM, KCl 5.9 mM, MgCl 1.2 mM, glucose 11.6 mM, HEPES 11.6 mM, pH 7.35). One of the vastus lateralis muscles was preserved at -80 °C for subsequent enzyme analysis, while the opposite vastus lateralis was kept on ice and prepared for high resolution respirometry (HRR) analysis. The muscle fibers were dissected into smaller bundles using two needles and subsequently placed into 3 ml of BIOPS (CaK_2EGTA 2.77 mM, K_2EGTA 7.23 mM, Na_2ATP 5.77 mM, $MgCl_2 \cdot 6H_2O$ 6.56 mM, Taurine 20 mM, Na_2Phosphocreatine 15 mM, Imidazole 20 mM, Dithiothreitol 0.5 mM, MES 50 mM, pH 7.1) and 30 µl saponin (5mg/ml - Sigma S7900) for 30 minutes to permeabilize the muscle fibers. The muscle tissue then underwent two 10-minute washes in 5 ml of MiR05 (mitochondrial respiration medium) (EGTA 0.5 mM, $MgCl_2 \cdot 6H_2O$ 3.0 mM, K-

lactobionate 60 mM, Taurine 20 mM, KH_2PO_4 10 mM, HEPES 20 mM, Sucrose 110 mM, BSA 1 g/L, pH 7.1). The described procedures are commonly used as the standard protocol for tissue preparation and permeabilization in mitochondrial respiration studies (Veksler et al., 1987; Letellier et al., 1992).

Mitochondrial Measurements

MRC was assessed by high resolution respirometry (HRR) using the Oxygraph-2k (Oroboros Instruments, Innsbruck, Austria). HRR is widely regarded as the gold-standard method for measuring ex-vivo mitochondrial oxygen consumption of tissues (Scheiber et al., 2019). 2 mg of skeletal muscle tissue was used in each chamber of the Oxygraph. The titration protocol included malate (1 mM) and glutamate (10 mM) for complex l linked LEAK assessment. Cytochrome C (10 µM) was added to test the integrity of the mitochondrial membrane. An ADP-titration followed (0.025 – 0.05 – 0.1 – 0.25 – 0.5 mM) with the addition of MgCl (3 mM) for assessment of complex l-linked respiration. Succinate (10 mM) was added to assess complex l + ll linked respiration. All experiments were carried out at 37°C and at an oxygen range of 200 - 450 nmol/L to ensure oxygen was not a limiting factor. Mitochondrial content was assessed by using citrate synthase (CS) activity as biomarker (Larsen et al., 2012). Mitochondria specific flux, also known as mitochondrial intrinsic respiratory capacity, was calculated as mass specific MRC divided by mitochondrial content. Data was analyzed using Oroboros DatLab version 6.1.0.7.

Enzyme Analysis

CS activity was measured using spectrophotometry. Skeletal muscle tissue was homogenized in 600 µl 0.3 M K_2HPO_4, 0.05 % bovine serum albumin (BSA) (pH 7.7) for 2 min on a Tissuelyzer (Qiagen, Venlo, Limburg, Netherlands). 6 µl of 10 % triton was added and the samples were left on ice for 15 min. The homogenate was diluted 50 times in a solution containing 0.33 mM acetyl-CoA, 0.6 mM oxaloacetate, 0.157 mM 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), 39 mM Tris-HCl (pH 8.0). The change in DTNB to TNB at 37 °C was measured spectrophotometrically at 415 nm on an automatic analyzer, Cobas 6000, C 501 (Roche Diagnostics, Mannheim, Germany). CS activity is expressed as micromoles substrate per minute per gram dry weight of tissue.

Statistical Analysis

Data was analyzed using ANOVA and linear mixed effects models, with Tukey's multiple comparisons post-hoc test. The obtained p-values were adjusted according to the total number of tests to minimize the risk of type I errors. Statistical and graphical data processing were carried out in RStudio version 4.3.0 and GraphPad Prism version 9.4.1. All statistical analyses were performed with an alpha level of 0.05. The sample size for each group was N = 10, unless stated otherwise. Data is presented as means ± SD. On the following figures, one asterisk (*) indicates $p < 0.05$, two asterisks (**) indicate $p < 0.01$, three asterisks (***) indicate $p < 0.001$ and four asterisks (****) indicate $p < 0.0001$.

Results

Aerobic Exercise Capacity Test

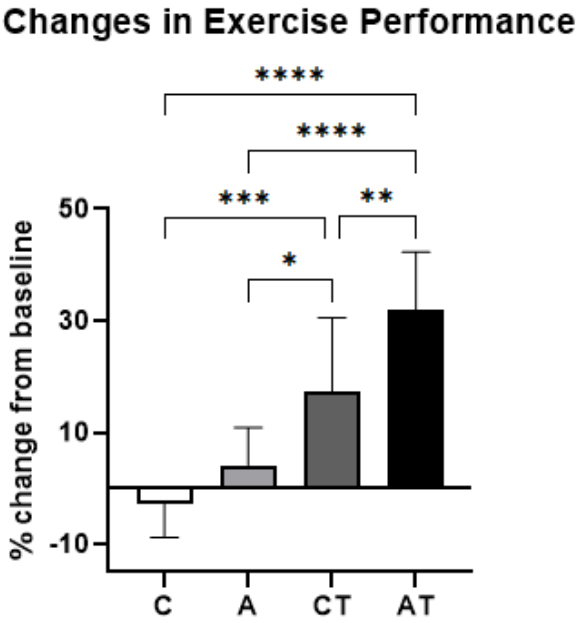

Figure 1: Changes in aerobic exercise performance demonstrated as a percent change in maximal running speed from baseline to post intervention. The groups C, A, CT, and AT represent Control (n = 10), Actovegin (n = 10), Control-Trained (n =10) and Actovegin-Trained (n = 10) groups, respectively.

Analysis of the exercise capacity test revealed a significant main effect of both administration ($p < 0.001$) and training ($p < 0.0001$) on running speed, indicating that exercise performance was increased across groups. No significant differences between groups were observed in pre-

intervention running speed. Pairwise comparisons revealed significant pre to post improvements in exercise capacity for both the CT and AT groups following the intervention (CT: 17 % increase, $p < 0.0001$; AT: 32 % increase, $p < 0.0001$ for AT). The CT group displayed a significantly higher increase in running speed than the C and A groups ($p < 0.001$ and $p < 0.05$, respectively), while the AT group demonstrated a higher increase in running speed compared to all other groups ($p < 0.01$) (*Fig. 1*).

Mitochondrial Respiration

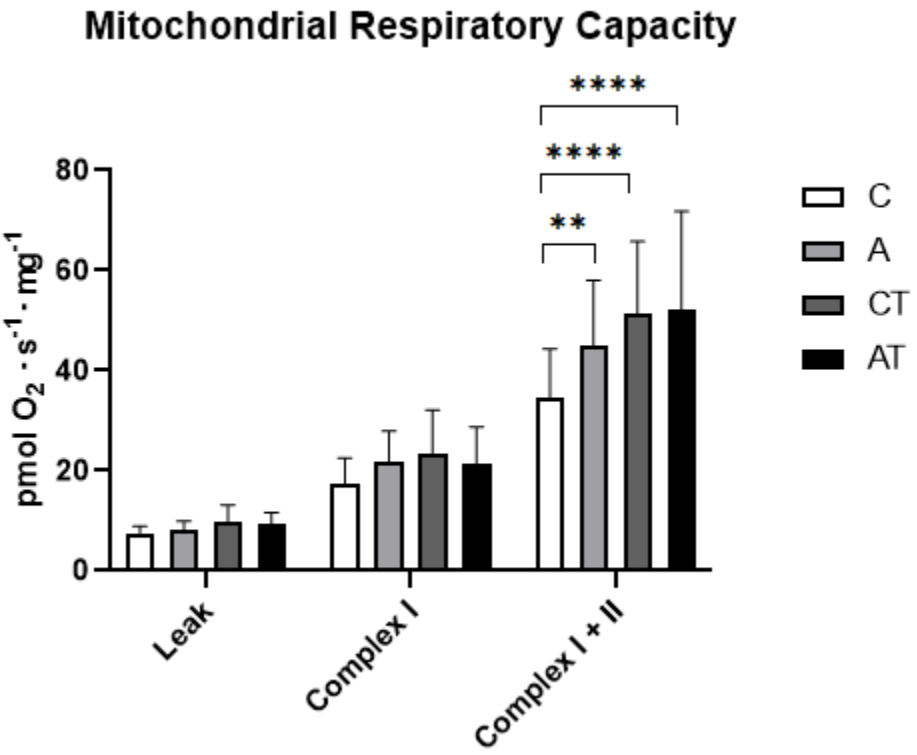

Figure 2: Mitochondrial respiration in permeabilized skeletal muscle fibers. LEAK respiration measured with malate and glutamate. Complex I-linked respiration measured with malate, glutamate and ADP. Complex I + II linked respiration measured with malate, glutamate, ADP and succinate. The groups C, A, CT, and AT represent Control (n = 10), Actovegin (n = 10), Control-Trained (n =10) and Actovegin-Trained (n = 10) groups, respectively.

The A group displayed a 30% increase ($p < 0.01$) in complex I + II-linked MRC compared to the C group. In contrast, the CT group displayed a 49% higher ($p < 0.0001$) MRC while the AT group displayed a 51% greater ($p < 0.0001$) MRC compared to the C group. No significant differences were observed between groups for LEAK or complex I-linked respiration.

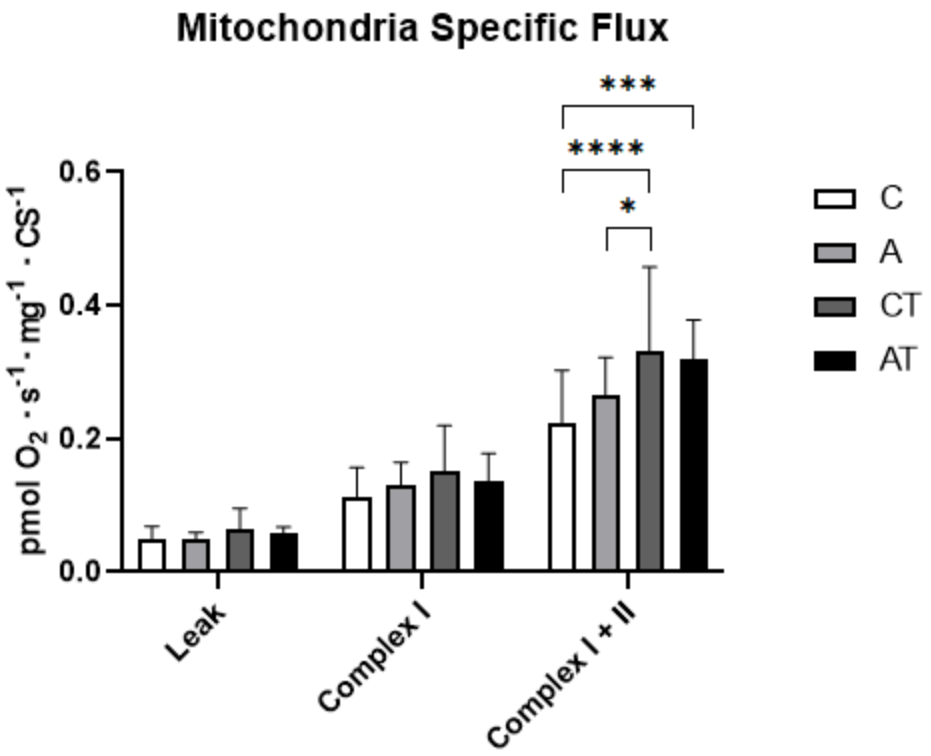

Figure 3: Mitochondrial respiratory capacity normalized to citrate synthase activity. The groups C, A, CT, and AT represent Control (n = 10), Actovegin (n = 8), Control-Trained (n =10) and Actovegin-Trained (n = 10) groups, respectively.

The Actovegin group consisted of n = 8 due to 2/10 tissue samples resulting in unsuccessful CS measurements. There were no significant differences observed between groups regarding CS activity. When normalizing MRC to CS activity, the CT group displayed a significantly higher mitochondria specific flux than both the C and A group ($p < 0.0001$ and $p < 0.05$, respectively). In addition, the AT group demonstrated a significantly higher mitochondria specific flux compared to the C group ($p < 0.001$) (Figure 3).

Body Mass and Blood Glucose

All groups experienced a loss in body mass over the 14-day intervention, however, there were no significant differences between groups for changes in body mass (C: -2.03 ± 1.34g vs. A: -1.68 ± 1.89g vs: CT: -2.83 ± 1.83g vs. AT: -3.10 ± 1.20g, $P > 0.05$). While each group also experienced a drop in blood glucose concentration by Day 14, compared to baseline, there were

no significant differences between groups (C: -1.80 ± 1.29 mmol/L vs. A: -1.79 ± 1.41 mmol/L vs. CT: -1.46 ± 1.59 mmol/L vs. AT: -1.53 ± 2.31 mmol/L, $P > 0.05$).

Discussion

The main novel finding in this study is that Actovegin administration significantly enhanced aerobic exercise performance, with an even greater enhancement seen when combined with HIIT. This was demonstrated by the AT group showing a significantly larger increase in aerobic exercise capacity compared to all other groups. Secondly, the observed improvements in exercise capacity were accompanied by an increase in MRC for the CT and AT groups compared to the C group. Furthermore, an increase was observed in MRC for the A group, indicating an isolated effect of Actovegin on MRC without exercise training. To our knowledge, this is the first study demonstrating performance enhancing properties of Actovegin, as well as an increase in skeletal muscle MRC with in vivo administration in healthy mice.

As the observed increases in exercise performance were accompanied by increases in mitochondrial respiratory capacity, it is relevant to determine to which degree these two factors are correlated. The analysis demonstrated no correlation between the two factors ($R^2 = 0.054$). This suggests that the observed increases in performance cannot be attributed to the observed differences in MRC, despite concurrent increases in both parameters. While other studies have found stronger correlations between MRC and exercise performance (Jacobs et al., 2013; Jacobs et al., 2011), it is possible that due to the exercise test being of shorter duration and higher intensity in the present study, it is limited by mitochondrial capacity to a lesser degree than exercise tests chosen in other studies. In the present study both trained groups followed the exact same HIIT protocol, and no differences were observed in the exercise training intensities between the CT and AT groups (84.01% and 85.74% of maximal running speed, respectively). Also, no differences were observed between groups in pre intervention exercise capacity. As such, the observed differences in post intervention exercise performance between the CT and AT groups is most likely a result of the Actovegin administration. During the intervention, all groups presented a significant reduction in body weight. Weight loss was uniform across groups and is a common response for mice being transferred from grouped to individual housing, as they were in the present study upon inclusion (Bartolomucci et al., 2003). A linear regression between

observed weight loss and the observed changes in exercise performance showed no correlation ($R^2 = 0.042$), indicating that the loss of body weight observed in the present study cannot explain the increases seen in exercise performance.

A significant increase was observed in complex I + II-linked MRC for the A, CT and AT groups compared to the C group, showing that MRC can be improved in mice with just 14 days of Actovegin administration, HIIT, or a combination of both. Increases in MRC following HIIT similar to our findings have been reported in humans before (Jacobs et al., 2013). One former study has also reported increases in human muscle fiber MRC with Actovegin, although Actovegin was administered acutely ex vivo and in large concentrations (Søndergård et al., 2016). Another study reported increased mitochondrial respiration in Actovegin-treated type 1 diabetic mice compared to saline-treated controls, indicating Actovegin may be an effective agent for attenuating mitochondrial dysfunction associated with type 1 diabetes (Kosik et al., 2023).

In the present study there were no differences in CS activity between groups, indicating that neither Actovegin administration nor 14 days of HIIT is sufficient to induce changes in mitochondrial content in mice. Similar results have previously been shown in human studies, reporting no changes in CS activity following periods of HIIT (Gorostiaga et al., 1991; Granata et al., 2016). However, the findings regarding CS activity and HIIT are generally contrasting, as other studies do report increases in CS activity following HIIT (Burgomaster et al., 2008; Gillen et al., 2016; MacInnis et al., 2017). As such, the relation between HIIT and mitochondrial content is yet to be fully understood. As CS activity was uniform across all four groups in the present study, the increases seen in MRC in the A, CT and AT groups can be attributed to an increase in mitochondrial intrinsic function, supported by our findings on mitochondria specific flux. The changes observed in MRC and mitochondria specific respiratory capacity may be the result of adaptations and regulation of proteins that could be altered independently of citrate synthase activity. Such intrinsic adaptations could include increases in mitochondrial cristae density, which has been shown to be increased in trained populations compared to untrained populations Nielsen et al., 2017). Improved cristae density may allow for a higher volume-specific inner mitochondrial surface area and thus potentially a higher capacity for OXPHOS. Other authors

have hypothesized that increases observed in MRC and mitochondrial intrinsic function may be explained by the formation of mitochondrial supercomplexes, in which existing mitochondrial complexes can be reorganized in larger supercomplexes, allowing for improved mitochondrial respiration (Bianchi et al., 2004). The formation of supercomplexes increases with exercise training in both humans (Greggio et al., 2017) and rodents (Han & Yan, 2022), which may explain the results observed in the present study as well.

Due to the many physiological components in Actovegin it is difficult to identify the precise mechanisms of action. The content of succinate in Actovegin has been thought to play a possible role in relation to the effects of Actovegin on mitochondria, as succinate is a potent stimulator of mitochondrial respiration (Gnaiger, 2020). In a study by Søndergård et al. (2016), an increase in MRC was observed with acute Actovegin incubation. The authors controlled for the effects of succinate in Actovegin by running concurrent measurements with succinate concentrations corresponding to the contents in Actovegin. While they did see an increase in MRC with succinate addition, the increase was of a lower magnitude than with Actovegin. As such, they concluded that the content of succinate may explain some of the effects of Actovegin on mitochondria, but not all (Søndergård et al., 2016). It is important to note that the experiments done by Søndergård et al. were conducted with acute Actovegin incubation and direct addition of Actovegin to the Oxygraph chambers. In the present study, Actovegin was administered in vivo, and no additional Actovegin was added to the tissue post-mortem. As such, the levels of Actovegin-derived succinate were much lower in the present study than in the study done by Søndergård. Other authors have stated that membrane stabilizing effects of Actovegin could explain the increases in MRC observed in experiments using saponin to permeabilize cell membranes (Brock et al., 2018; Lee et al., 2016). It is true that too much exposure to saponin may result in harmful cell membrane damages (Bo ̈tger & Melzig, 2013). However, the permeabilization of cell membranes is a prerequisite for the substrates added during titration protocols to be able to affect the mitochondria within cells (Pesta & Gnaiger, 2012). The duration of saponin exposure has been shown to affect mitochondrial respiration, with 30 minutes of saponin exposure resulting in maximal respiratory rate compared to 0, 15 or 45 minutes of exposure (Tonkonogi et al., 1998). In the present study, cell membrane integrity was controlled using cytochrome C during the titration protocols. No samples responded abnormally to

cytochrome C, indicating no harmful effects of the saponin exposure. As such, the potential membrane stabilizing effects of Actovegin is highly unlikely to have influenced mitochondrial measurements in the present study. Besides its effects on mitochondria, it is also possible that Actovegin could affect other physiological parameters related to exercise capacity, including blood volume, hemoglobin mass, capillarization or functional properties of the skeletal or cardiac muscle. However, no such measurements were carried out in the present study.

Conclusion

For the first time, this study showed ergogenic effects of Actovegin. Aerobic exercise capacity was increased in the CT group as a result of 14 days of HIIT, but was further improved in the AT group, demonstrating an additive effect of Actovegin administration with training. Complex I + II-linked MRC was increased in the A, CT, and AT groups, showing an isolated effect of both HIIT and Actovegin administration on mitochondrial respiration. No additive effect was seen on MRC. Measurements of CS activity resulted in no differences between groups for mitochondrial content, suggesting that increases in MRC were attributed to increased intrinsic respiratory capacity. Despite concurrent increases in exercise capacity and MRC, the two factors did not correlate in the present study. The performance enhancing effect of Actovegin demonstrated in the present study has yet to be explained.

This study contributes to a better understanding of how Actovegin affects aerobic exercise capacity and mitochondrial function in skeletal muscle. In opposition to previous studies rejecting that Actovegin has any ergogenic effects on performance (Brock et al., 2018; Lee et al., 2011), this study sheds new light on Actovegin as a potential performance enhancing substance. As this is the first study reporting such effects, future studies should aim to elaborate these findings.

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CHAPTER 3: MANUSCRIPT 2

The effects of Actovegin on cardiac mitochondrial respiration in trained and sedentary mice

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Background: Actovegin is a calf blood extract shown to acutely enhance mitochondrial respiration in skeletal muscle. In addition, there is much speculation surrounding Actovegin's status as a performance enhancing agent. However, few studies have investigated the effect of Actovegin on the heart. It is unknown whether non-acute Actovegin injections influence cardiac mitochondrial respiration.

Objective: Determine the effect of Actovegin on aerobic exercise capacity and cardiac mitochondrial respiration in trained and untrained mice. Outcomes on blood glucose and body mass were also investigated.

Methods: Male C57Bl/6 mice were randomized to 4 groups: control (C), Actovegin (A), control-trained (CT) and Actovegin-trained (AT). Every second day for 14 days, the A and AT groups were injected intraperitoneally with 0.1ml (10mg/ml) Actovegin, while the C and CT groups were injected with 0.1 ml saline solution. On treatment days, the AT and CT groups underwent a high-intensity interval training (HIIT) protocol designed for mice, using a rodent treadmill. Aerobic capacity was measured using a graded running test at baseline (Day 1) and Day 14. Mitochondrial respiration of the heart was measured using high resolution respirometry.

Results: After two weeks, high resolution respirometry revealed there were no significant differences between groups for any state of cardiac mitochondrial respiration ($P > 0.05$).

Conclusion: Actovegin administration enhances aerobic exercise capacity when combined with HIIT in mice. However, there is no direct effect of Actovegin on cardiac mitochondrial respiration. Future studies should investigate Actovegin's chronic effects on diseased heart models to better understand its influence on cardiac tissue.

Introduction

Actovegin is a hemodialysate of calf blood which has been used for over 60 years for the treatment of acute stroke (Boiarinov et al., 1998; Derev'yannykh et al., 2008), postpartum haemorrhage (Appiah, 2002), diabetic polyneuropathy (Ziegler et al., 2009) radiation damage (Beetz et al., 1996) and muscle tears (Pfister & Koller, 1990). Actovegin is a clear, yellow liquid that contains over 200 physiological components and is ultrafiltered to 6000 Da, making it free of any proteins, growth factors and hormones (Lee et al., 2011). Although having demonstrated a wide range of therapeutic effects, the main active ingredients in Actovegin remain unidentified. Actovegin is thought to exert its benefits through insulin-like activity, meaning that it increases glucose uptake into cells, promoting overall oxidative metabolism (Buchmayer et al., 2011). Several studies have explored the drug's acute effect on tissue oxygen consumption and mitochondrial respiration in vitro; two parameters associated with oxidative cell metabolism. Reichel et al., (1965) found that cellular oxygen uptake of rat skeletal muscle increased by 40% in the presence of Actovegin. In addition, Actovegin acutely increases concentration dependent OXPHOS capacity in human skeletal muscle (Sondergard et al., 2016).

The enhanced OXPHOS capacity and tissue oxygen consumption associated with Actovegin has led to the belief that the drug may possess ergogenic properties. However, there have been few studies on the matter and there is limited evidence supporting Actovegin's effectiveness as a performance enhancing drug (PED). Lee et al., (2011) found that acute injections of Actovegin had no effect on peak power, exercise efficiency, or rate of VO_2 gain in male participants, concluding that Actovegin does not enhance aerobic exercise performance.

When it comes to aerobic exercise performance, the heart is an essential organ. Left ventricular contractile capacity and force are key parameters to consider when associating the heart with aerobic exercise (Berman et al., 2022). The cardiac mitochondria are of specific interest as they play an essential role in the pumping efficiency of the heart. During aerobic exercise, a high myocardial energy demand requires more ATP production to maintain contractility, which is directly associated with greater contractile capacity (Lopaschuk et al., 2021). Contractility plays an important role in regulating cardiac output (CO) as well. A higher contractility reduces end

systolic volume, resulting in a greater stroke volume and thus, a greater CO (Froelicher & Myers, 2007). In turn, a greater CO during aerobic exercise will circulate oxygen to the working skeletal muscles at a greater rate, ultimately increasing aerobic performance.

While the heart uses mainly fatty acids to produce energy, fatty acids yield less ATP per oxygen consumed than glucose, making this a relatively oxygen inefficient process (Lopaschuk et al., 2010). Therefore, increasing the reliance on glucose oxidation for ATP synthesis may improve cardiac efficiency and function (Stanley et al., 2005). This becomes especially relevant when considering cardiac ATP synthesis and contractile function during aerobic exercise. Since Actovegin has been shown to increase glucose uptake in the cell, perhaps the drug may influence cardiac OXPHOS, translating to increased contractile capacity, and ultimately, increased CO.

While the effects of Actovegin have been investigated in skeletal muscle and liver, there have been no studies exploring the effect of Actovegin on heart mitochondria. The objectives of this study were to (1) determine the chronic effects of Actovegin treatment on cardiac mitochondrial respiration in trained and untrained mice and (2) determine whether 2 weeks of Actovegin injections every second day can improve maximal aerobic exercise capacity in trained and untrained mice. We hypothesize that Actovegin will improve aerobic exercise capacity in trained and untrained mice. We also hypothesize that chronic injections of Actovegin will enhance cardiac mitochondrial respiration in trained and untrained groups.

Methods

Animal Care

Male C57Bl/6 mice (aged 6-8 months) from the Concordia University breeding colony were selected as the sample population for this study. The mice were individually housed in standardized cages in a room with a 12-hour light/dark cycle set at a constant temperature of 22°C. All mice were provided with fresh tap water and standard rodent chow. All procedures and measures were approved by the Concordia University Animal Research Ethics Committee (protocol #30000259) and conducted in accordance with the guidelines of the Canadian Council of Animal Care.

Treadmill Screening and Group Randomization

One day prior to the beginning of the experimental intervention, mice were screened for their ability and willingness to run on the treadmill. This was done by placing 5 mice at a time from the same litter on a rodent treadmill (Bouganim & Bergdahl, 2017) and running them at a low speed of 10 m/min for 5 minutes. The mice from the same litter who demonstrate an ability to keep up with the pace were included in the study and were evenly divided into four groups: control, Actovegin, control-trained, and Actovegin-trained. Dividing the qualifying mice from the same litter among the four groups served to control for certain litters being naturally stronger runners. A mouse was deemed unable or unwilling to run if it (1) continuously attempted to escape the treadmill while in motion or (2) remained in contact with the bristles at the back of the treadmill for more than 10 seconds or was unable to run consistently at the front of the treadmill. Treadmill screening continued until 10 mice had been allocated to each group for a total of 40 mice.

Aerobic Exercise Capacity Test

The day after group randomization and screening, this randomized control trial began with an assessment of baseline aerobic exercise capacity of all four groups by conducting a maximal aerobic exercise capacity test designed for mice. The mice performed the exercise capacity test on a rodent treadmill using a protocol adapted from Caru et al., 2019. The test began with a warm-up stage at a speed of 13 m/min and an incline of 10 degrees for 3 minutes. The speed was then increased by approximately 3.3 m/min every 1 minute until the animal reached exhaustion, at which point, their maximum speed was recorded (refer to Chapter 1, Table 1). Exhaustion was defined as the point where the mice remained in contact with the bristles at the back of the treadmill for 10 consecutive seconds.

Drug Administration

Following the baseline aerobic exercise test, every second day for a period of two weeks, the Actovegin and Actovegin-trained groups were injected with 0.1ml (10mg/ml) intraperitoneal injections of Actovegin, while the control and control-trained groups received 0.1 ml intraperitoneal injections of physiological salt solution (sham). Injections were always administered immediately after a training session.

High-Intensity Interval Training Protocol

During the treatment period, only the Actovegin-trained and control-trained groups underwent a high intensity-interval training (HIIT) regimen designed for mice and adapted from Caru et al., 2019. HIIT was used as the choice of exercise because of its ability to induce physiological adaptations similar to moderate intensity continuous training, with less time commitment (Gray et al., 2016). Using the rodent treadmill, the mice began with a warm-up stage, running at a speed of 13 m/min for 5 minutes. The mice then ran five 1-minute bouts at 80-90% of their pre-determined maximum baseline speed. In between each bout, there was a 2-minute recovery period ran at 50% of their maximum baseline speed (refer to Table 1). The mice were trained every second day, prior to injections. To evaluate changes in aerobic performance, the day after the two-week treatment period, a final aerobic exercise capacity test was conducted on all four groups using the same protocol as the baseline test.

Table 1: HIIT protocol adapted from Caru et al., 2019

Stage	Speed (% of maximal speed)	Duration (min)
Warm-up	~ 60	5
Bout 1	80-90	1
Recovery	< 50	2
Bout 2	80-90	1
Recovery	< 50	2
Bout 3	80-90	1
Recovery	< 50	2
Bout 4	80-90	1
Recovery	< 50	2
Bout 5	80-90	1

Blood Glucose and Body Mass Measurements

Body mass was measured every second day during the two-week intervention period using an electronic balance scale. Non-fasted blood glucose concentrations were measured from the mouse tail vein in the morning at baseline (Day 1), Day 7, and Day 14, using the One Touch Verio Flex glucometer (Kennard et al., 2021). All blood glucose measurements were taken immediately after body mass assessments and at least 2 hours prior to any injection, training, or aerobic exercise test, to allow ample time for tail bleeding to stop.

Tissue Extraction and Permeabilization

The day following the final aerobic exercise capacity test, the mice were euthanized by CO_2 and cervical dislocation, and the heart was immediately extracted. Next, fiber bundles of the heart apex were isolated using forceps and the tissue was permeabilized in a buffer solution containing 50 μg/ml of saponin and 2 ml of Biopsy Preservation Solution (BIOPS) on ice consisting of (in mM): CaK_2EGTA 2.77, K_2EGTA 7.23, Na_2ATP 5.77, $MgCl_2 \cdot 6H_2O$ 6.56, Taurine 20, Na_2Phosphocreatine 15, Imidazole 20, Dithiothreitol 0.5, MES 50, pH 7.1 (Fontana-Ayoub et al., 2016). To ensure that the tissue was clear of any residual saponin, using a platform rotator, two consecutive 10-minute washes of the tissue were performed in a mitochondrial respiration medium (MiRO5) consisting of (in mM): EGTA 0.5, $MgCl_2 \cdot 6H_2O$ 3.0, K-lactobionate 60, Taurine 20, KH_2PO_4 10, HEPES 20, Sucrose 110, BSA 1 g/L, pH 7.1 (Kuznetsov et al., 2008). The described procedures are commonly used as the standard protocol for tissue preparation and permeabilization in mitochondrial respiration studies (Veksler et al., 1987; Letellier et al., 1992).

Mitochondrial Respiratory Measurements

Following tissue permeabilization and washing, mitochondrial oxygen consumption of the heart was measured using a high-resolution respirometer (Oxygraph-2k, Oroboros Instruments, Innsbruck, Austria). High resolution respirometry is widely regarded as the gold-standard method for measuring ex vivo mitochondrial oxygen consumption of tissues (Scheiber et al., 2019). Approximately 1.2 to 2.0 mg of cardiac tissue was placed in the respirometer chambers containing 2.0 ml of MirO5 at 37°C. All respiratory measurements were carried out in a hyper-oxygenated environment to avoid oxygen diffusion limitations.

Mitochondrial Respiratory Protocol

To assess mitochondrial respiration of the heart in the absence of adenosine diphosphate (ADP) (state 2/LEAK respiration), pyruvate (6 mM) and malate (1 mM) were added to the respirometer chambers. Subsequently, respiration across complex V of the electron transport chain (state 3 respiration) was assessed by adding a saturating concentration of ADP (5 mM). Cytochrome C (10 μM) was then added to test the integrity of the outer mitochondrial membrane. Succinate (10 mM) was then added to measure maximal OXPHOS across complex I and II (maximal coupled state 3 respiration). To measure any respiration uncoupled to ATP production (state 4 respiration), oligomycin (2.5 μg/ml) was added to block complex V. Next, titrations of uncoupling agent carbonyl cyanide-4-trifluoromethoxy phenylhydrazone (FCCP) (0.25 μM) were performed to assess maximum non-coupled respiration capacity fueled by complex II. Finally, to measure any background oxygen consumption in the absence of mitochondrial respiration, antimycin A (2.5 μM) was added to inhibit complex III.

Acceptor Control Ratio and Respiratory Control Ratio

The respiratory control ratio (RCR), or ratio of mitochondrial respiration supporting OXPHOS needed to offset proton leak, is typically used to define mitochondrial efficiency (Salin et al., 2018). The RCR was calculated by dividing the O_2 flux of maximal state 3 respiration by that of state 4 (Succinate/Oligomycin). The Acceptor control ratio (ACR) represents the coupling between oxidation and phosphorylation and was obtained by dividing the O_2 flux of state 3 respiration by that of state 2 (ADP by Pyr + Mal).

Citrate Synthase Activity

Citrate synthase activity was measured using spectrophotometry and is a standard procedure for determining the quantity of mitochondria present, and therefore, the intrinsic mitochondrial respiration heart. The following procedures were previously described by Larsen et al. (2015). Approximately 2 mg of tissue was homogenized in 600 μL of 0.3M K_2HPO_4, 0.05% bovine serum albumin (BSA), pH 7.7 for 2 min on a Tissuelyzer (Qiagen, Venlo, Limburg, the Netherlands). 6 μL of 10% triton was then added to the tissue and the samples were left on ice for 15 minutes before being stored at −80 °C for future analysis. The homogenate was then diluted 50 times in a solution containing 0.33 mM acetyl-CoA, 0.6 mM oxaloacetate, 0.157 mM

DTNB, 39 mM Tris-HCl (pH 8.0). The change in 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) to TNB at 37 °C was measured spectrophotometrically at 415 nm (Srere, 1969) on an automatic analyzer, Cobas 6000, C 501 (Roche Diagnostics, Mannheim, Germany).

Statistical Analysis

All data is presented as mean ± standard error of mean (SEM) and statistical significance is defined as $P < 0.05$. Mitochondrial respiration was analyzed as total O_2 flux per milligram of tissue mass for each substrate added to the Oxygraph. Normality of all data was tested using the Shapiro-Wilk test, with $P > 0.05$ indicating a normal distribution. A One-Way ANOVA was used to compare the mean differences between groups for changes in mitochondrial respiration and citrate synthase activity, ACR, and RCR. A Tukey Post-Hoc test was used to determine the significance of any differences found between groups. In addition, a Grubbs outlier test was used to remove any outliers presented in the citrate synthase data. All statistical analyses were performed using IBM SPSS Statistics for Macintosh (Version 29.0).

Results

Cardiac Mitochondrial Respiration

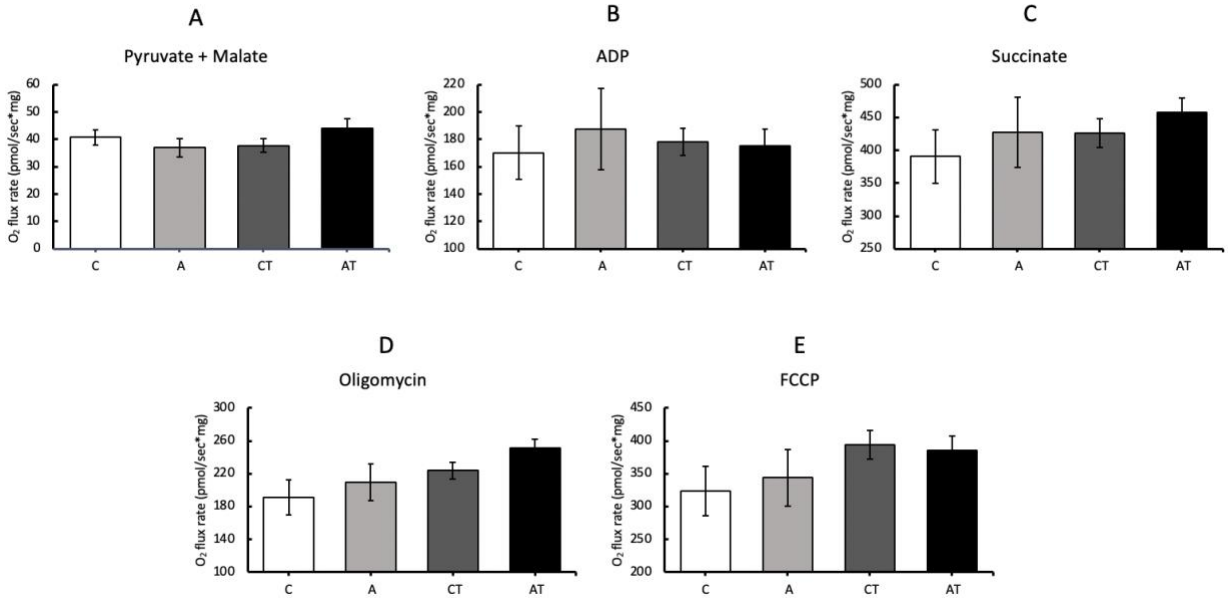

Figure 1: Cardiac mitochondrial respiration represented as O_2 flux per milligram of tissue following subsequent addition of substrates pyruvate + malate **(A)**, ADP **(B)**, succinate **(C)**, oligomycin **(D)**, FCCP **(E)**. The groups C, A, CT, and AT represent Control, Actovegin, Control-

Trained and Actovegin-Trained groups, respectively. A One-Way ANOVA was performed for each substrate to compare the difference in O_2 flux between the Control (n = 10), Acotvegin (n = 10), Control-Trained (n = 10), and Actovegin-Trained (n = 10) group. Values are expressed as means ± SEM *P < 0.05.

State 2 respiration was measured by the simultaneous addition of substrates pyruvate and malate to determine the oxygen consumption of the cardiac tissue in the absence of ADP or OXPHOS, also known as LEAK mitochondrial respiration. High resolution respirometry revealed no significant differences in oxygen consumption rate between any of the 4 groups (C: 40.8 ± 2.8 pmol/sec*mg vs. A: 40.0 ± 3.4 pmol/sec*mg vs. CT: 37.8.7 ± 2.6 pmol/sec*mg vs. AT: 44.0 ± 3.6 pmol/sec*mg, P = 0.768) (refer to Figure 1A).

State 3 respiration, measured after the addition of ADP, determines the complex I-stimulated tissue oxygen consumption with OXPHOS activated. High resolution respirometry revealed there were no significant differences in oxygen consumption rate between groups (C: 170.3 ± 19.3 pmol/sec*mg vs. A: 187.5 ± 29.8 pmol/sec*mg vs. CT: 178.3 ± 9.8 pmol/sec*mg vs. AT: 175.6 ± 11.7 pmol/sec*mg, P = 0.931) (refer to Figure 1B). State 3 maximal respiration was also measured after the addition of succinate. This determines the maximal mitochondrial respiration of the tissue at maximum OXPHOS, also known as complex I+II-stimulated tissue oxygen consumption. The addition of succinate revealed there were no significant differences in oxygen consumption rate between groups (C: 391.0 ± 40.7 pmol/sec*mg vs. A: 427.7 ± 53.3 pmol/sec*mg vs. CT: 426.1 ± 28.8 pmol/sec*mg vs. AT: 457.5 ± 21.9 pmol/sec*mg, P = 0.684) (refer to Figure 1C).

The addition of oligomycin revealed there was no significant difference between groups for mitochondrial respiration uncoupled to ATP production (state 4 respiration) (C: 191.2 ± 21.2 pmol/sec*mg vs. A: 209.9 ± 22.3 pmol/sec*mg vs. CT: 223.9 ± 10.4 pmol/sec*mg vs. AT: 250.9 ± 10.8 pmol/sec*mg, P = 0.142) (refer to Figure 1D). The addition of FCCP revealed that there were no significant differences between groups for maximal non-coupled respiration capacity (C: 323.4.7 ± 37.3 pmol/sec*mg vs. A: 343.9 ± 43.1 pmol/sec*mg vs. CT: 394.6 ± 21.8 pmol/sec*mg vs. AT: 385.8 ± 21.6 pmol/sec*mg, P = 0.385) (refer to Figure 1E). Finally, the addition of antimycin A revealed that there were no significant differences in background oxygen

consumption, independent of mitochondrial respiration, between groups (C: 14.3 ± 3.2 pmol/sec*mg vs. A: 16.9 ± 2.0 pmol/sec*mg vs. CT: 19.0 ± 3.3 pmol/sec*mg vs. AT: 19.7 ± 3.6 pmol/sec*mg, P = 0.604).

Respiratory Control Ratio and Acceptor Control Ratio

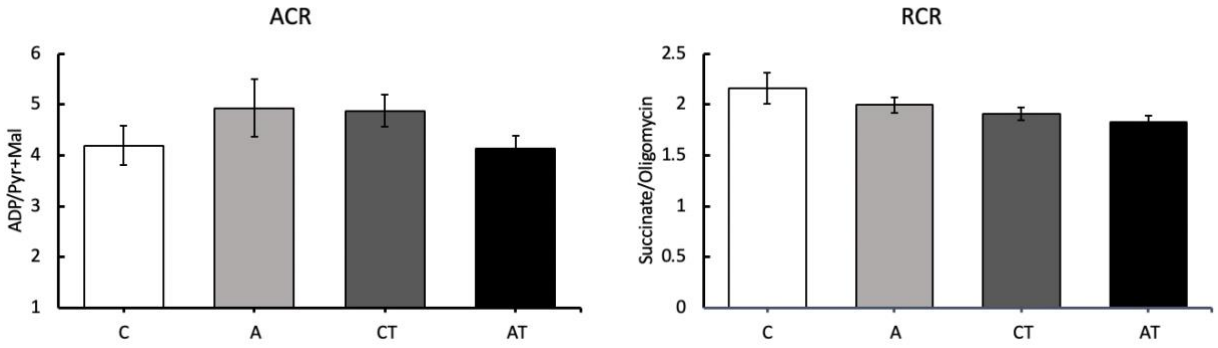

Figure 2: Respiratory control ratio (RCR) and acceptor control ratio (ACR). The groups C, A, CT, and AT represent Control, Actovegin, Control-Trained and Actovegin-Trained groups, respectively. A One-Way ANOVA was performed for each substrate to compare the difference in RCR and ACR between the Control (n = 10), Acotvegin (n = 10), Control-Trained (n = 10), and Actovegin-Trained (n = 10) group. Values are expressed as means ± SEM *P < 0.05.

After taking the ratio of maximal state 3/state 4 respiration, there were no significant differences between groups for RCR (C: 2.16 ± 0.15 vs. A: 2.00 ± 0.07 vs. CT: 1.9 ± 0.06 vs. AT: 1.83 ± 0.06, P = 0.106. After dividing the O_2 flux of ADP by Pyr + Mal (state 3/state 2 respiration), there were also no significant differences between groups for ACR (C: 4.19 ± 0.38 vs. A: 4.93 ± 0.56 vs. CT: 4.88 ± 0.32 vs. AT: 4.13 ± 0.25).

Citrate Synthase Activity

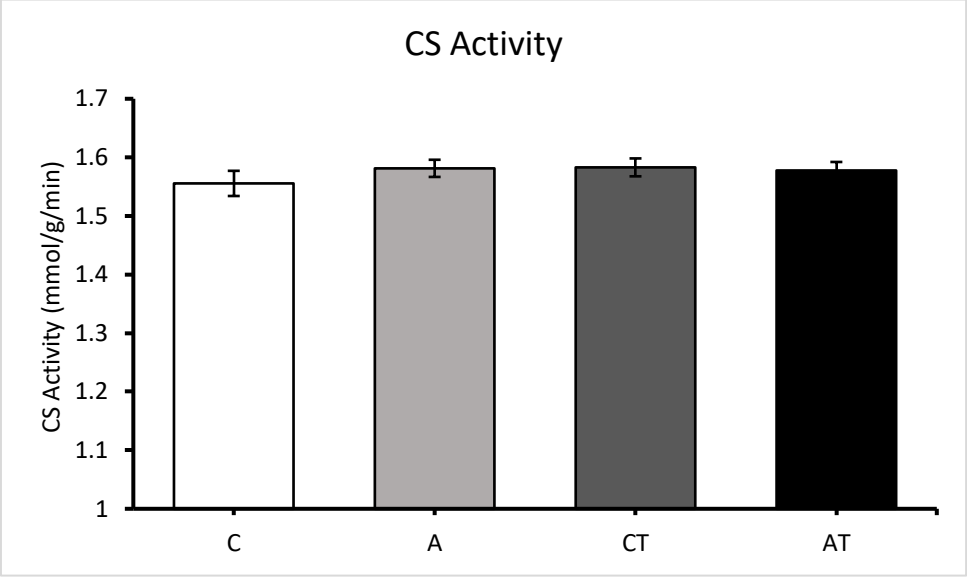

Figure 3: Citrate Synthase (CS) Activity displayed in mmol/g/min. The groups C, A, CT, and AT represent Control, Actovegin, Control-Trained and Actovegin-Trained groups, respectively. A One-Way ANOVA was to compare the difference in citrate synthase activity between the Control (n = 8), Acotvegin (n = 10), Control-Trained (n = 10), and Actovegin-Trained (n = 10) group. Values are expressed as means ± SEM *P < 0.05.

A Grubb's test revealed two CS activity outliers in the control group, which were removed from the data sample. A One-Way ANOVA revealed there were no significant differences in CS activity between any of the groups (C:1.56 ± 0.02 mmol/g/min vs. A:1.58 ± 0.01 mmol/g/min vs. CT: 1.58 ± 0.01 mmol/g/min vs. AT: 1.57 ± 0.01 mmol/g/min, P = 0.679).

Remaining Parameters

The data for maximal aerobic exercise capacity, blood glucose concentration, and body mass are reported and discussed in Chapter 2 *(Increased exercise capacity and skeletal muscle mitochondrial respiration in mice after 14 days of intraperitoneal Actovegin administration).*

Discussion

To our knowledge, this was the first study investigating the effects of Actovegin on heart mitochondrial respiration. It is also the first study to implement chronic injections of Actovegin in association with exercise.

The first main finding of this study is that there was no significant difference between any of the 4 groups for state 2, state 3 (complex I) or maximal state 3 (complex I+II) mitochondrial respiration (O_2 flux) following the two-week intervention. The similarities between groups for State 2 (LEAK respiration) indicate that Actovegin administration on its own or in combination with exercise does not alter the cardiac tissue's ability to consume oxygen in an ADP-restricted environment.

From Figure 1B, it is apparent that when complex V and OXPHOS are activated following the addition of ADP, chronic Actovegin injections have no significant effect on cardiac mitochondrial respiration, regardless of whether it is combined with HIIT. However, although insignificant, a trend is observed in Figure 1C suggesting that the combination of HIIT with Actovegin may increase maximal mitochondrial respiration. It is known that mitochondrial respiratory capacity increases in response to aerobic exercise, mainly due to a physiological adaptation that increases the quantity of mitochondria (Sondergard et al., 2016). In addition, Jacobs et al. (2013) found that 6 sessions of HIIT were sufficient to improve whole body exercise capacity and endurance performance in humans. This was mainly due to a 20% increase in skeletal muscle mitochondrial content, driving improvements in respiratory capacity and oxygen extraction. It is also important to consider that this slight trend observed in Figure 1C may be due to the effects of HIIT and not Actovegin alone, since there is not enough of a difference in O_2 flux between the AT and CT groups to pin the outcome on Actovegin. This lack of significant difference between the AT and CT group does not align with our initial hypothesis and demonstrates that, while significantly effective in skeletal muscle, non-acute Actovegin injections do not increase cardiac maximal mitochondrial respiration in trained or untrained mice.

In healthy myocardial cells, fatty acids supply 50-70% of the substrates needed to fuel OXPHOS (Kolwicz, 2018). However, during exercise, myocardial glucose uptake doubles at 55% VO_2 max and is significantly reduced at 75% VO_2 max (Kemppainen et al., 2002). Since Actovegin enhances glucose uptake in the cell, and glucose is the more oxygen efficient substrate for energy production in the heart (Lopaschuk et al., 2010), we expected an increased glucose uptake in the AT and A groups to increase the rate of OXPHOS and ultimately ATP production in the heart. However, this significant increase in OXPHOS was not observed, and the reason may be that Actovegin is not potent enough to enhance glucose oxidation in the heart during high-intensity aerobic exercise, unlike its potency in skeletal muscle (Kosik et al., 2023; Søndergård et al., 2016). Or perhaps, the 80-90% max exercise intensity was too high to efficiently metabolize glucose in combination with Actovegin. In these high intensity exercise states, lactate is the dominant source for energy production in the heart. Takala et al. (1983) found that myocardial lactate levels increased sevenfold in rats during a 20-minute swimming exercise. The increased lactate levels are thought to serve as a cardioprotective mechanism during ischemic stress and heightened levels of cardiac work (Kemppainen et al., 2002).

The second main finding of this study is that there were no significant differences between groups for state 4 mitochondrial respiration after the addition of oligomycin. However, in figure 1D, it is apparent that the AT group trends toward a higher state 4 respiration compared to the control. Since oligomycin inhibits complex V of the ETC, this trend might suggest that Actovegin in combination with HIIT increased uncoupled mitochondrial respiration when compared to a sedentary group without the treatment of Actovegin. Typically, in state 4 respiration, any tissue oxygen consumption observed is due to either a proton leak through the inner mitochondrial membrane since there is no ATP production via ATP synthase, or an increase in mitochondrial quantity. In this case, mitochondria will likely respire on other physiological substrates such as pyruvate or fatty acids (Korzeniweski, 2015). Therefore, the combination of Actovegin and exercise may elicit some response that causes cardiac mitochondria to slightly enhance uncoupled respiration. It is likely that the AT group trending toward a higher state 4 respiration is due to the group having developed a naturally higher baseline OXPHOS level. There was also no significant difference between any of the groups for maximum non-coupled respiration following the addition of FCCP (figure 1E) and background oxygen consumption.

Since aerobic exercise training naturally increases mitochondrial content, we wanted to ensure that any differences in cardiac mitochondrial respiration/oxygen consumption were due to improvements in intrinsic mitochondrial function (respiratory capacity per mitochondria) and not due to sheer number of mitochondria. Therefore, we controlled for this by measuring the intrinsic activity of the mitochondria by normalizing the O_2 flux to citrate synthase activity (Larsen et al., 2012). From figure 3, it is apparent that there is no significant difference in citrate synthase activity between any of the groups, meaning that each group had a similar quantity of mitochondria after the 14-day intervention. We can therefore consider the mitochondrial respiration data in figure 1 as intrinsic mitochondrial respiration. The similarity in CS activity observed among the groups also suggests that Actovegin injections over 14 days do not influence mitochondrial biogenesis. A study on acute ex vivo Actovegin treatment of skeletal muscle fibers also showed that alterations in mitochondrial respiratory capacity were due to the Actovegin effecting the intrinsic function of the mitochondria (Larsen et al., 2012).

In addition, the ACR and RCR were also calculated. The similarities between groups for the ACR (Figure 2) suggests that chronic Actovegin injections alone or in combination with HIIT have no direct effect on cardiac mitochondrial coupling. Furthermore**,** the similarities between groups for the RCR indicate that Actovegin alone or in combination with HIIT does not directly offset proton leak and effect mitochondrial efficiency.

While this study directly emphasizes the effects of Actovegin on cardiac mitochondrial respiration, a limitation of the study is that the implications of this drug on cardiac output and contractile capacity were not directly measured. While increased cardiac mitochondrial respiration is associated with increased contractile capacity, directly measuring the force of contraction of the left ventricle would have been ideal in assessing this parameter.

Conclusion

This study demonstrates that chronic Actovegin injections over 2 weeks do not enhance cardiac mitochondrial respiration in healthy trained or sedentary mice. Therefore, we cannot directly attribute an increase in maximal aerobic exercise capacity to cardiac mitochondrial respiration. If Actovegin's main mechanism of action is through increasing glucose uptake into the cell, then cardiac cells may not benefit from this drug with respect to mitochondrial respiration and contractility. Further investigation using different exercise intensities or diseased animal models is warranted to determine how this versatile drug can influence the heart.

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CHAPTER 4: SUMMARY, CONCLUSION AND FUTURE DIRECTIONS

The objective of this thesis was to further understand Actovegin from a functional and mechanistic perspective. We specifically wanted to determine the effects of non-acute Actovegin injections on aerobic exercise performance, skeletal muscle mitochondrial respiration, and cardiac mitochondrial respiration. The first manuscript demonstrated that Actovegin injections enhance aerobic exercise capacity, especially when paired with HIIT. It also showed that Actovegin increases skeletal muscle mitochondrial respiration, when taken alone and when combined with HIIT. The second manuscript interestingly displayed how Actovegin has no significant effect on cardiac mitochondrial respiration, providing valuable information on how this drug influences different tissue mitochondria. The data presented in these manuscripts should serve to help generate new hypotheses, and hopefully guide a future clinical trial on Actovegin and exercise performance. We hope that the novel findings of these studies will add insights surrounding Actovegin's status as a performance enhancing drug. However, to unravel this dispute, and gain the WADAs attention, there must be a focus on clinical trials. Future studies should explore the effects of chronic Actovegin injections on aerobic performance in humans using different forms of aerobic exercise tests (cycling, running, rowing). Studies should also focus on the composition of Actovegin to better understand which of the many physiological components are the main active ingredients, for therapeutic and ergogenic outcomes.

Lastly, with the knowledge obtained from these projects, if I had the opportunity to repeat it, I would make the following additions: (1) Experiment with different concentrations of Actovegin to investigate the optimal dosage for aerobic performance. (2) Determine how Actovegin effects the production of reactive oxygen species in the heart and skeletal muscle of healthy and diseased murine models. (3) Investigate Actovegin's effects on oxygen extraction and mitochondrial respiration in the brain.

APPENDIX

Effects of Actovegin on liver and cardiac mitochondrial respiration in type 1 diabetes

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Background: Insulin deficiency in type 1 diabetes leads to an impairment of glucose metabolism and mitochondrial function in cardiomyocytes and hepatocytes. Actovegin is a hemodialysate of calf blood which has been shown to enhance glucose uptake and cell metabolism in healthy human skeletal muscle. The objectives of this study were to determine the effect of Actovegin on cardiac and liver mitochondrial respiration in a type 1 diabetic mouse model. Outcomes on body mass, and water consumption were also investigated.

Methods: Type 1 diabetic, male, C57Bl/6 mice were randomized to an Actovegin and a control group. Every third day, for 14 days, the Actovegin (n = 10) and control group (n = 9) were injected intraperitoneally with 0.1 ml (10 mg/ml) Actovegin and 0.1 ml physiological salt solution, respectively. Mitochondrial respiratory capacity of the heart and liver was measured by high resolution respirometry (Oroboros Oxygraph). Body mass and water consumption were evaluated every second day using an electronic balance scale. An independent samples T-test was used to establish between-group differences for cardiac mitochondrial respiration, body mass and water consumption. A Mann-Whitney-U test was used to compare the mean difference between groups for liver mitochondrial respiration.

Results: After 14 days, compared to the control group, the Actovegin group demonstrated a significantly higher maximal liver mitochondrial respiration (Actovegin: 120 ± 7 pmol/sec*mg versus control: 100 ± 8 pmol/sec*mg, P = 0.028). There were no significant differences between groups for maximal cardiac mitochondrial respiration (Actovegin: 327 ± 30 pmol/sec*mg versus control: 256 ± 40 pmol/sec*mg, P = 0.187). There were no significant differnces between groups for body mass or water consumption (P > 0.600).

Conclusion: Actovegin injections over two weeks can significantly improve oxidative phosphorylation capacity of liver mitochondria in type 1 diabetes. Further investigation into the mechanistic action of Actovegin is warranted to establish an understanding of how liver and cardiac tissue may benefit from this drug.

Introduction

Type 1 diabetes (T1D) is a chronic autoimmune metabolic disorder that leads to the destruction of pancreatic b-cells. Since b-cells are responsible for producing insulin, T1D causes an absolute insulin deficiency in the affected individual, ultimately leading to hyperglycemia. T1D is evaluated based on a fasting blood glucose (BG) concentration > 7 mmol/L or a random BG concentration > 11.1 mmol/L (DiMeglio et al., 2018). Management of this disease typically requires daily insulin injections, insulin pump therapy or automated insulin delivery system, as well as glucose monitoring with a continuous glucose monitor (Lucier & Weinstock, 2023). Being one of the most frequent chronic diseases in children, T1D can develop at any age, with up to 50% of cases occurring in adulthood and as many as half of those cases being misdiagnosed as type 2 diabetes (T2D)(Thomas et al., 2018). In addition, T1D is quite prevalent worldwide, at 9.5% or 15 per 100,000 people living with the disease (Mobasseri et al., 2020).

The lack of insulin in T1D results in cells being unable to take up glucose which can lead to a significant impairment in mitochondrial function and ATP production (Karakelides et al., 2007). The heart is of specific interest since T1D is associated with a significant increased risk of cardiovascular disease (Schofield et al., 2019). Using animal models, several studies have shown that cardiac mitochondrial respiration is reduced in T1D. Early studies have reported that T2D diabetic mice have impaired state 3 (complex I-linked) cardiac mitochondrial respiration, reducing OXPHOS capacity and leading to harmful metabolic alterations (Kuo et al., 1985). In terms of energy metabolism, the heart relies mainly on the oxidation of fatty acids (60-70%), glucose, lactate, and other substrates (30-40%) for ATP production. However, in diabetic animals, glucose oxidation is significantly inhibited and there is an even greater reliance on fatty acids for ATP production (Bugger & Abel, 2010).

The liver is another organ that is negatively impacted by T1D. Glycemia, lipid availability, and oxidative stress are key factors that contribute to hepatic mitochondrial function (Gancheta et al., 2016). Insulin resistance associated with T1D reduces glucose uptake by the hepatocytes and alters hepatic energy homeostasis (Dewidar et al., 2023). Recent studies have shown that liver OXPHOS and ATP synthesis is reduced in T1D (Wolf et al., 2019; Kupriyanova et al., 2021; Gancheva et al., 2016).

As indicated, T1D is a disease that affects energy homeostasis and mitochondrial respiration of essential organ systems. Currently, insulin is the main treatment for the long-term management of T1D (Lucier & Weinstock, 2023). However, given the severity and prevalence of the disease, it is warranted investigate other drugs that may counter the effects of T1D. One of these drugs is Actovegin, a deproteinated calf blood extract. Actovegin has been used clinically for over 60 years for the treatment of a wide variety of conditions such as stroke (Boiarinov et al., 1998; Derev'yannykh et al., 2008), post-partum hemorrhage (Appiah, 2002), diabetic polyneuropathy (Ziegler et al., 2009), and radiation damage (Beetz et al., 1996). Actovegin is composed of over 200 physiological components such as electrolytes, amino acids, nucleosides, carbohydrate, and fat metabolites (Lee et al., 2011).

Actovegin is thought to exert its action through insulin-like activity, increasing glucose uptake and stimulating cell metabolism (Buchmayer et al., 2011). In addition, Actovegin has been shown to acutely enhance OXPHOS capacity and oxygen consumption in rat skeletal muscle (Reichel et al., 1965) and liver cells (Kuninaka et al., 1991), as increase in-vitro OXPHOS in human skeletal muscle (Sondergard et al., 2016). Interestingly, chronic Actovegin injections in T1D mice were found to enhance skeletal muscle mitochondrial respiration when compared to a control group receiving a placebo (Kosik et al., 2023).

While Actovegin has shown to enhance mitochondrial respiration in skeletal muscle, its chronic effects on cardiac and liver mitochondrial respiration remain unknown. The objective of this study was to investigate the effects of non-acute Actovegin injections on cardiac and liver mitochondrial respiration in T1D mice. Body mass, food and water consumption were also assessed.

Methods

Population and Group Randomization

Male C57B1/6 mice (aged 6-8 months) from the Concordia University breeding colony were selected as the sample population for this study. The mice were first randomized and divided into 2 groups: Actovegin (n = 10) and Control (n = 9). Mice were individually housed in standardized

cages in a room with a 12-hour light/dark cycle set at a constant temperature of 22°C. All mice were provided with fresh tap water and standard rodent chow. All procedures and measures were approved by the Concordia University Animal Research Ethics Committee and the Canadian Council for Animal Care (protocol #30000259).

Experimental Intervention

T1D was induced by intraperitoneal injection of streptozotocin (150mg/kg). To confirm that a diabetic state had been reached, BG concentrations were measured 2 days post-injection from the mouse tail vein using the One Touch Verio Flex glucometer. Concentration readings of greater than 14 mmol/L were considered diabetic (DiMeglio *et al.,* 2018). Over 13 days, the experimental group received 0.1 ml intraperitoneal injections of (25% v/v) Actovegin very third day, beginning on Day 1 and ending on Day 13 (Kosik et al., 2023). In contrast, the control group received intraperitoneal injections of 0.1 ml physiological salt solution consisting of (in mM): NaCl 135.5, KCl 5.9, MgCl 1.2, glucose 11.6, HEPES 11.6, pH 7.35, every third day, over the same period. Once the mice were confirmed to be diabetic, Day 1 of the experimental intervention began.

Body Mass, Food, and Water Measurements

Body mass was measured every second day during the two-week intervention period using an electronic balance scale. In addition, food and water consumption were also monitored and recorded every second day during the intervention.

Tissue Extraction and Permeabilization

After Day 14, the mice were euthanized by CO_2 asphyxiation and cervical dislocation. The heart and liver were then immediately extracted. Next, fiber bundles of the heart apex were isolated using forceps and the right lobe of the liver was diced in to small fragments and the tissues were permeabilized in a buffer solution containing 50 μg/ml of saponin and 2 ml of Biopsy Preservation Solution (BIOPS) on ice consisting of (in mM): CaK_2EGTA 2.77, K_2EGTA 7.23, Na_2ATP 5.77, $MgCl_2 \cdot 6H_2O$ 6.56, Taurine 20, Na_2Phosphocreatine 15, Imidazole 20, Dithiothreitol 0.5, MES 50, pH 7.1 (Fontana-Ayoub et al., 2016). To ensure that the tissue was clear of any residual saponin, using a platform rotator, two consecutive 10-minute washes of the

tissue were performed in a mitochondrial respiration medium (MiRO5) consisting of (in mM): EGTA 0.5, $MgCl_2 \cdot 6H_2O$ 3.0, K-lactonionate 60, Taurine 20, KH_2PO_4 10, HEPES 20, Sucrose 110, BSA 1 g/L, pH 7.1 (Kuznetsov et al., 2008).

Mitochondrial Respiratory Measurements

Following tissue permeabilization and washing, mitochondrial oxygen consumption of the heart and liver were measured using a high-resolution respirometer (Oxygraph-2k, Oroboros Instruments, Innsbruck, Austria). High resolution respirometry is widely regarded as the gold-standard method for measuring ex vivo mitochondrial oxygen consumption of tissues (Scheiber et al., 2019). Approximately 1.2 to 2.0 mg of cardiac and liver tissue were placed in the respirometer chambers containing 2.0 ml of MirO5 at 37°C. All respiratory measurements were carried out in a hyper-oxygenated environment to avoid oxygen diffusion limitations.

Mitochondrial Respiratory Protocols

Standard Protocol

To assess mitochondrial respiration of the heart in the absence of adenosine diphosphate (ADP) (state 2/LEAK respiration), pyruvate (6 mM) and malate (1 mM) were added to the respirometer chambers. Subsequently, respiration across complex V of the electron transport chain (state 3 respiration) was assessed by adding a saturating concentration of ADP (5 mM). Cytochrome C (10 μM) was then added to test the integrity of the outer mitochondrial membrane. Succinate (10 mM) was then added to measure maximal OXPHOS across complex I and II (maximal coupled state 3 respiration). To measure any respiration uncoupled to ATP production (state 4 respiration), oligomycin (2.5 μg/ml) was added to block complex V. Next, titrations of uncoupling agent carbonyl cyanide-4-trifluoromethoxy phenylhydrazone (FCCP) (0.25 μM) were performed to assess maximum non-coupled respiration capacity fueled by complex II. Finally, to measure any background oxygen consumption in the absence of mitochondrial respiration, antimycin A (2.5 μM) was added to inhibit complex III and terminate OXPHOS.

Fatty Acid Titration Protocol of the Heart

To assess the mitochondrial respiration of the heart driven by beta-oxidation, a fatty acid titration protocol was used. Firstly, malate (1mM) was added to the respirometer, followed by ADP

(5mM) to activate complex V and OXPHOS. Next a titration of medium chain fatty acid octanoylcarnintine (Oct) was performed in the following order: Oct (0.3mM), Oct (0.75mM), Oct (1.5mM), Oct (2.25mM), Oct (3mM).

Liver Protocol

To assess the mitochondrial respiration of the liver, the following substrates were added in order: Malate (1mM) + Glutamate (10mM), ADP (5mM), Succinate (10mM), FCCP titration with steps of 0.25μM.

Statistical Analysis

All data is presented as mean ± standard error of mean (SEM) and statistical significance is defined as $P < 0.05$. Mitochondrial respiration was analyzed as total O_2 flux per milligram of tissue mass for each substrate added to the Oxygraph. Normality of all data was tested using the Shapiro-Wilk test, with $P > 0.05$ indicating a normal distribution. An independent samples T-Test was used to compare the mean differences between groups for mitochondrial respiration of the heart (standard protocol and beta oxidation protocol). A Mann-Whitney U test was used to determine differences between groups for liver mitochondrial respiration. A repeated measures ANOVA was used to assess any differences between groups for total food and water consumption/gram body mass. All statistical analyses were performed using IBM SPSS Statistics for Macintosh (Version 29.0).

Results

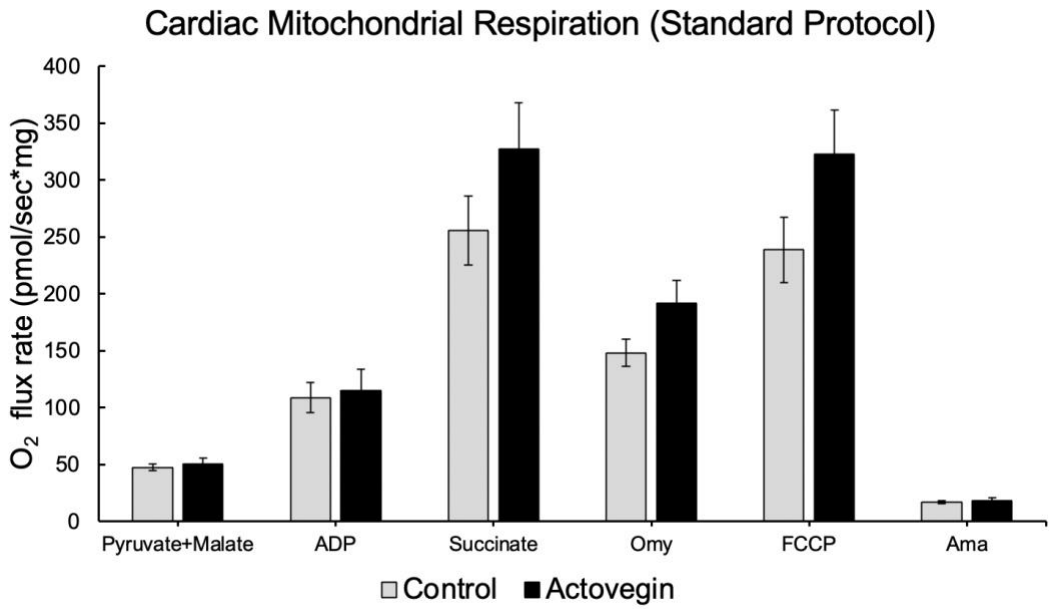

Figure 1: Cardiac mitochondrial respiration represented as O_2 flux per milligram of tissue following subsequent addition of substrates pyruvate + malate, ADP, succinate, oligomycin (Omy), FCCP, and antimycin A (Ama). An independent samples T-Test was performed for each substrate to compare the difference in O_2 flux between the Control (n = 9), Acotvegin (n = 10), group. Values are expressed as means ± SEM. *P < 0.05.

State 2 respiration was measured by the simultaneous addition of substrates pyruvate and malate to determine the oxygen consumption of the cardiac tissue in the absence of ADP, also known as LEAK mitochondrial respiration. High resolution respirometry revealed there were no significant differences in oxygen consumption rate between the two groups (Control: 47.6 ± 2.8 pmol/sec*mg vs. Actovegin: 50.4 ± 5.0 pmol/sec*mg, P = 0.636).

State 3 respiration, measured after the addition of ADP, determines the complex I-stimulated tissue oxygen consumption with OXPHOS activated. High resolution respirometry revealed there were no significant differences in oxygen consumption rate between groups (Control: 108.7 ± 13.3 pmol/sec*mg vs. Actovegin: 114.9 ± 19.0 pmol/sec*mg, P = 0.797). State 3 maximal respiration was also measured after the addition of succinate. This determines the maximal mitochondrial respiration of the tissue at maximum OXPHOS, also known as complex I+II-stimulated tissue oxygen consumption. The addition of succinate revealed there were no significant differences in oxygen consumption rate between groups (Control: 255.6 ± 30.5

pmol/sec*mg vs. Actovegin: 327.1 ± 40.9 pmol/sec*mg, P = 0.187). However, as seen in Figure 1, the Actovegin group is trending slightly higher.

The addition of oligomycin revealed that there was no significant difference in cardiac mitochondrial respiration uncoupled to ATP production (state 4 respiration) between groups (Control: 148.0 ± 11.9 pmol/sec*mg vs. Actovegin: 191.9 ± 19.6 pmol/sec*mg, P = 0.08). The addition of FCCP revealed that there were no significant differences between groups for maximal non-coupled respiration (Control: 238.6 ± 28.4 pmol/sec*mg vs. Actovegin: 322.5 ± 39.1 pmol/sec*mg, P = 0.107). Finally, the addition of antimycin A revealed that there were no significant differences in background oxygen consumption, independent of mitochondrial respiration, between groups (Control: 17.0 ± 1.5 pmol/sec*mg vs. Actovegin: 18.5 ± 2.4 pmol/sec*mg, P = 0.736).

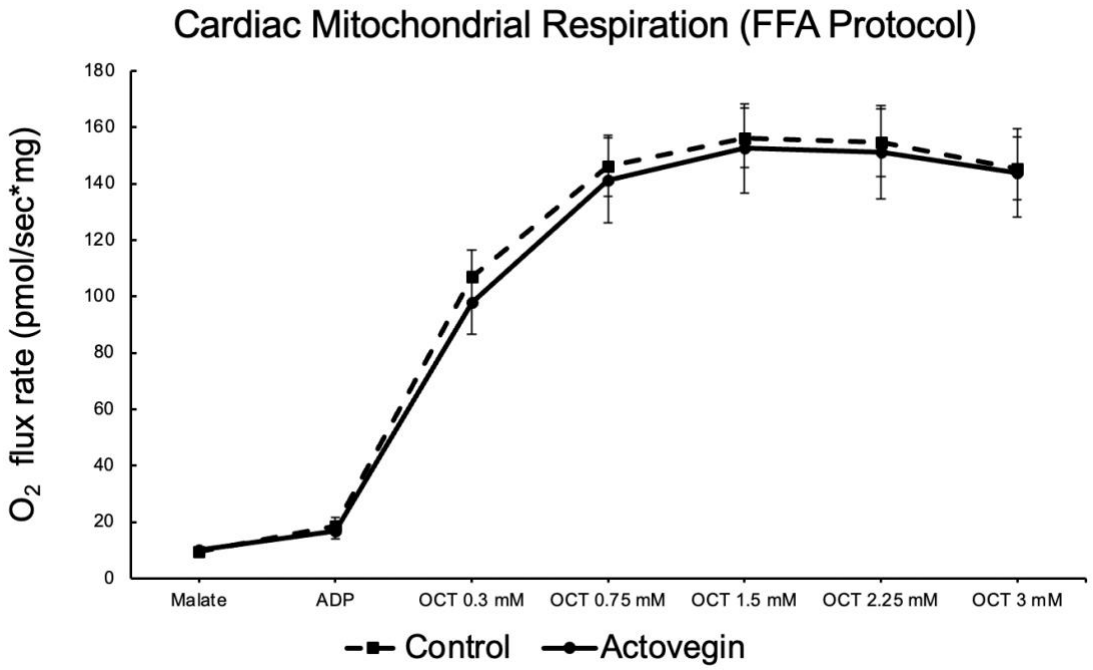

Figure 2: Beta oxidation-driven cardiac mitochondrial respiration represented as O_2 flux per milligram of tissue following subsequent addition of substrates Malate, ADP and Oct 0.3 mM, 0.75 mM, 1.5 mM, 2.25 mM, 3 mM. An independent samples T-Test was performed for each substrate to compare the difference in O_2 flux between the Control (n = 9), Acotvegin (n = 10), group. Values are expressed as means ± SEM. *P < 0.05.

The addition of malate (Control: 9.6 ± 1.6 pmol/sec*mg vs. Actovegin: 10.1 ± 1.0 pmol/sec*mg, P = 0.786) and ADP (Control: 18.5 ± 3.0 pmol/sec*mg vs. Actovegin: 16.7 ± 2.8 pmol/sec*mg, P = 0.655) to the respirometer chambers revealed no significant differences between groups for cardiac mitochondrial respiration due to OXPHOS. The sequential addition of Oct as a titration of increasing concentration was performed to stimulate beta oxidation and assess cardiac mitochondrial respiration under these conditions. While the addition of Oct increased overall oxygen tissue oxygen consumption, there were no significant differences between groups for beta oxidation-driven mitochondrial respiration for any concentration of Oct (0.3 mM: P = 0.536; 0.75 mM: P = 0.758, 1.5 mM: P = 0.851, 2.25 mM: P = 0.871, 3 mM: P = 0.941).

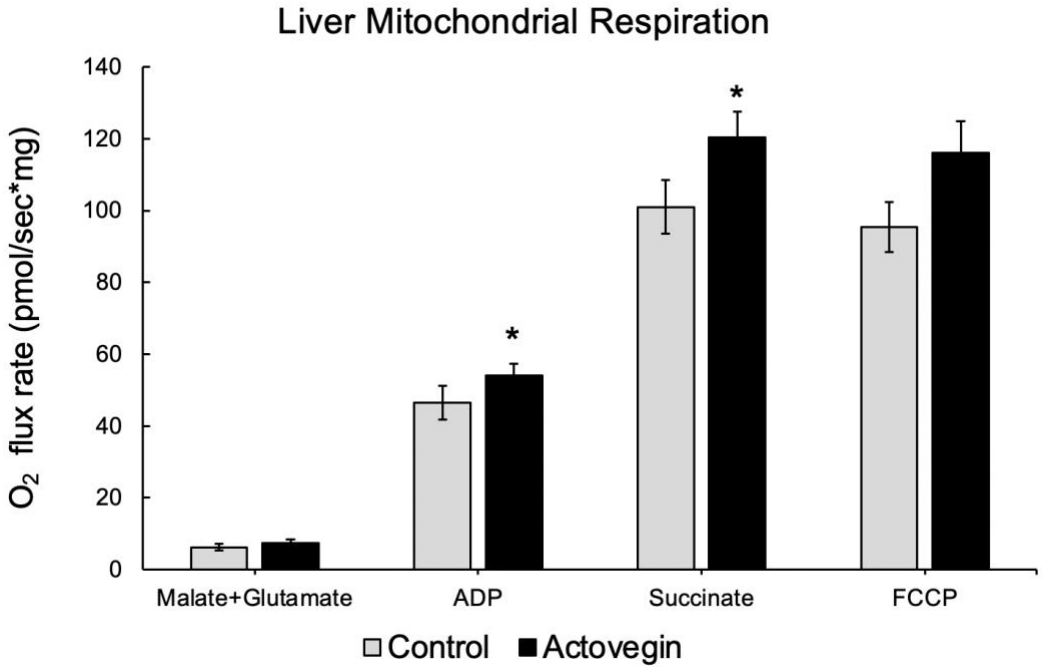

Figure 3: Liver mitochondrial respiration respresented as O_2 flux per milligram of tissue following subsequent addition of substrates malate + glutamate, ADP, succinate, and FCCP. A Mann-Whitney U test was performed for each substrate to compare the difference in O_2 flux between the Control (n = 9), Acotvegin (n = 10), group. Values are expressed as means ± SEM. *P < 0.05.

To determine state 2 respiration of the liver, substrates malate and glutamate were added to the respirometer chambers. High resolution respirometry revealed there were no significant differences in state 2 oxygen consumption rate between the two groups (Control: 6.2 ± 0.9 pmol/sec*mg vs. Actovegin: 7.4 ± 1.0 pmol/sec*mg, P = 0.211). The addition of ADP revealed

that the Actovegin group had a significantly greater state 3 liver mitochondrial respiration compared to the control group (Control: 46.5 ± 4.8 pmol/sec*mg vs. Actovegin: 54.0 ± 3.3 pmol/sec*mg, P = 0.035). After the additon of succinate, the Actovegin group also displayed a significantly greater maximal liver mitochondrial respiration compared to the control group (Control: 100.1 ± 7.5 pmol/sec*mg vs. Actovegin: 120.4 ± 7.1 pmol/sec*mg, P = 0.028). Lastly, the addition of FCCP revealed that there were no significant differences between groups for maximal non-coupled respiration of the liver (Control: 95.4 ± 7.0 pmol/sec*mg vs. Actovegin: 116.0 ± 8.9 pmol/sec*mg, P = 0.113).

Body Mass, Total Water and Food Consumption

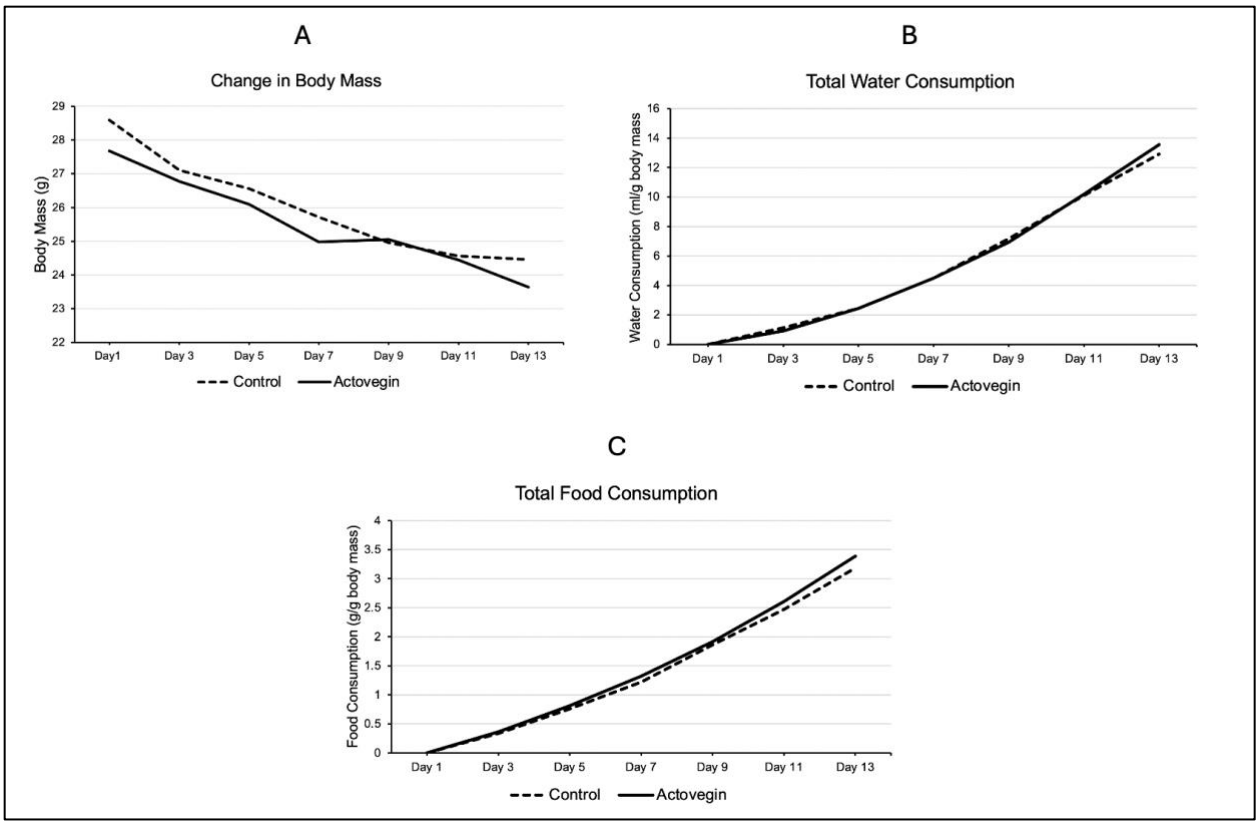

Figure 4: Body mass (A) measured every second day. Total water consumption (B) and total food consumption (C) measured every second day and presented as grams of food/body mass. A repeated measures ANOVA was used to determine any differences between Actovegin (n = 10) and control (n = 9) groups. Values are expressed as means ± SEM. *P < 0.05.

Both groups experienced a decline in body mass after the 13-day intervention, however, there were no significant differences between groups for changes in body mass ($P > 0.05$). Food and water consumption were also measured every second day throughout the intervention. There were no significant differences in food or water consumption between groups for any of the monitored days ($P > 0.05$).

Discussion

To our knowledge, this was the first study investigating the non-acute effects of Actovegin on heart and liver mitochondrial respiration in T1D mice. The first novel finding of this study is that the Actovegin group had a significantly higher complex I+II-linked (state 3) mitochondrial respiration in the liver compared to the control group. This finding suggests that chronic Actovegin treatment enhances submaximal and maximal OXPHOS capacity in T1D mice. As previously mentioned, hepatocyte OXPHOS is significantly reduced in T1D (Wolf et al., 2019; Kupriyanova et al., 2021; Gancheva et al., 2016). Therefore, through some unknown mechanism, Actovegin may be able to attenuate the negative impact of T1D on liver energy homeostasis. This finding is in line with our hypothesis and similar to a study that showed higher OXPHOS in skeletal muscle of T1D mice treated with Actovegin compared to a control group (Kosik et al., 2023).

The second main finding of this study was that there were no significant differences in cardiac mitochondrial respiration between groups. A standard protocol was used to assess glucose-driven OXPHOS, and a fatty acid titration protocol was used to assess beta-oxidation-driven OXPHOS. For the standard protocol, the Actovegin group trended toward a higher maximal cardiac mitochondrial respiration (Complex I+II-linked) and non-coupled respiration (addition of FCCP). This trend suggests that Actovegin may enhance maximal OXPHOS in T1D mice compared to an untreated group. The limited research on Actovegin's effect on the heart makes it difficult to compare the results revealed in this study to existing literature. However, one study showed that Actovegin inhibits spontaneous formation of reactive oxygen species produced by blood phagocytes of patients with heart failure (Astashkin et al., 2014). Although not directly

targeting the heart, the aforementioned study demonstrates that Actovegin might have a therapeutic effect in a condition associated with heart disease.

The fatty acid titration protocol was used because we wanted to assess cardiac mitochondrial respiration using the heart's main energy source at rest, free fatty acids, as the substrate. We originally wanted to determine if Actovegin's glucose uptake ability would reduce the heart's metabolic demand for fatty acids in a compensatory nature. As such, we expected to see a difference between groups for beta-oxidation-driven OXPHOS, however, no significant difference was observed. This finding implies that Actovegin does not increase cardiomyocyte glucose uptake enough to offset fatty acid metabolism.

Body mass was recorded over the 2-week intervention to determine if Actovegin could mitigate T1D-associated weight loss in mice. Actovegin was not successful in offsetting the weight loss in the T1D mice. This finding is contrary to that of a study by Kosik et al. (2023) where both groups of T1D mice (Actovegin and control) had a significant reduction in body mass, but the Actovegin group had a lesser reduction compared to the control. However, the intervention by Kosik et al. (2023) was slightly different, as the mice underwent aerobic endurance testing 3 times over 2 weeks.

Lastly, we wanted to determine if Actovegin can influence two of the main symptoms associated with T1D, polyphagia and polydipsia (Atkinson et al., 2014). As seen in figure 4B and 4C, there were no significant differences between the groups for total food and water consumption, indicating that Actovegin had no effect on attenuating polyphagia and polydipsia.

Conclusion

This study shows that a 14-day treatment period with intraperitoneal injections of Actovegin enhances liver OXPHOS capacity in T1D mice and does not influence cardiac mitochondrial respiration, including beta-oxidation-driven OXPHOS. In addition, the dose of Actovegin administered in this study is not potent enough to attenuate polyphagia and polydipsia in T1D. Future studies using different dosages of Actovegin are warranted to further understand the efficacy of this drug in T1D.

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