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Effects of Electrical Pulse Stimulation on in vitro Measurement of Mitochondrial Content and Lipid in Human Myotubes

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Review of Literature

Insulin Resistance and its Effect on Type 2 Diabetes

The main role of insulin *in vivo* is the regulation of fuel equilibrium by means of storage of glucose and free fatty acids. Insulin targets three main tissues in the body: skeletal muscle, liver and adipose tissue. Skeletal muscle is the main tissue responsible for peripheral insulin resistance and insulin stimulated glucose disposal (DeFronzo et al., 1981). The resistance to the usual action of insulin is the main contributor to the pathogenesis of common disorders: metabolic syndrome X, hypertension, and most importantly type 1 (insulin dependent) and type 2 (non insulin dependent) diabetes mellitus. The main defect in type 2 diabetes is uncertain, but it appears that a combination of environmental and genetic factors are the main contributors to the development of this progressive metabolic disorder, frequently not clinically apparent until mid-life. The main characteristic of patients with type 2 diabetes is fasting hyperglycemia, which could be the cause or consequence of a defect in insulin secretion from the pancreatic beta cells, or by peripheral insulin resistance of the adipose or skeletal muscle tissues (Zierath, Krook, & Wallberg-Henriksson, 2000).

Insulin resistance has been strongly related with obesity, more specifically with central obesity. However, the mechanisms associating central obesity and insulin resistance are not properly understood; one of the possibilities is that non esterified fatty acids, which are a product of lipolysis of the muscle's triacylglycerol stores, suppress glucose uptake either by inhibition of glucose transport (Roden et

al., 1996), or by substrate competition (Randle, Garland, Hales, & Newsholme, 1963). Forouhi et al. (1999), measured the intramyocelular lipids (IMCL) content in soleus muscle using proton magnetic resonance microscopy, percent body fat, insulin sensitivity, and visceral fat in European and Asian populations. The study concluded that the association between insulin sensitivity and IMCL in European populations was consistent with the theory that IMCL act as mediators of insulin sensitivity. However, the relationship among the Asian population suggested that IMCL content was not the sole mechanism underlining insulin sensitivity (Forouhi et al., 1999).

A reduction in the mitochondrial capacity of skeletal muscle is evident in patients with type 2 diabetes and for individuals with an increased risk to develop the disease (Moro et al., 2011). However, the extent at which mitochondrial dysfunction in patients with type 2 diabetes is reversible through physical activity and weight loss interventions is not completely clear. Toledo et al. (2007) combined daily moderate physical activity and moderate weight loss in a population with type 2 diabetes. The extent at which mitochondrial content in type 2 diabetes increased by combining both methods, as well as the relationship with improvement of hyperglycemia and insulin resistance were studied. Results of the study reported an improvement in insulin sensitivity, significant increase in mitochondrial density, mitochondrial oxidative enzymes and cardiolipin (a component of the inner mitochondrial membrane essential for the function of enzymes involved in mitochondrial energy metabolism) content. Furthermore, the total energy

expenditure during exercise directly correlated with the improvements in insulin sensitivity (Toledo et al., 2007).

Tissue insulin resistance is most likely to be the link between diabetes and obesity. When there is an increase in fat free mass, skeletal muscle cell capillaries are more widely spaced and the muscle cells experience hypertrophy. It was proposed that biophysical changes in skeletal muscle intervene in part with the effects of obesity to produce abnormal kinetics of the insulin action and reductions in insulin sensitivity of obese individuals. Additionally it was also suggested that a combination of genetic defects and changes in the biophysical properties of human skeletal muscle induced by obesity are the main cause for insulin resistance, such defects could also lead to development of non-insulin dependent diabetes mellitus (Lillioja & Bogardus, 1988).

In steady state plasma concentrations of glucose and insulin mimicking postprandial conditions, Shulman (2000) reported that muscle glycogen synthesis was almost 50% lower in subjects with type 2 diabetes, compared to non-diabetic individuals. Furthermore, during Hyperinsulinemic, hyperglycemic conditions, synthesis of glycogen in the muscles is the major pathway for glucose metabolism for diabetic and non-diabetic individuals. Finally, any defect in muscle glycogen synthesis plays a key role in the insulin resistance of patients with type 2 diabetes (Shulman, 2000). It has also been proposed that a decrease in the delivery of insulin or substrate to the muscle might be a possible cause for insulin resistance in patients with type 2 diabetes (Yang, Hope, Ader, & Bergman, 1989).

Boden, et al. (2001) reported that acute changes in plasma free fatty acids were coupled with changes in IMCL and the development of insulin resistance. It was further concluded that in accordance with previous reports, the accumulation of IMCL is one of the steps for the development of free fatty acid induced insulin resistance.

Age is also considered to be a leading cause for insulin resistance, which consequently is central to the idea that there is a higher prevalence of type 2 diabetes in older adults. Evidence supporting the idea that insulin resistance is directly related to aging is contradicting; several reports demonstrate the insulin resistance might not be associated with aging, but rather the lifestyle related to aging like obesity and reduced physical activity (Dubé et al., 2008). Amati et al. (2009) compared the insulin sensitivity of young and older adults with different exercise regimes (athlete, normal weight, obese). The results of the experiment suggested that there was no difference in insulin sensitivity between younger and older athletes, older normal weight and younger normal weight, or older and younger obese. Regardless of age, athletes were more insulin sensitive than normal weight subjects, and normal weight subjects were more insulin sensitive than obese subjects. The study concluded that insulin sensitivity might not necessarily be a consequence of aging, but rather a consequence of reduced physical activity and obesity (Amati et al., 2009)

In steady state plasma concentrations of glucose and insulin mimicking postprandial conditions, it was found that muscle glycogen synthesis was almost 50% lower in subjects with type 2 diabetes compared to normal individuals,

demonstrating that in hyperinsulinemic, hyperglycemic conditions, synthesis of glycogen in the muscle is the major pathway for glucose metabolism in diabetic and normal individuals, and that any defect in the muscle glycogen synthesis plays a key role in the insulin resistance of type 2 diabetes patients (Shulman, 2000). It has also been proposed that a decrease in the delivery of insulin or substrate to the muscle might be a possible cause for insulin resistance in patients with type 2 diabetes (Yang et al., 1989).

Alteration in protein kinase C (PKC) signaling and diacyglycerols might be responsible for lipid induced insulin resistance in human skeletal muscle. To prove or refute this hypothesis, normal volunteers were studied during euglycemic-hyperinsulinemic clamp where the levels of free fatty acids in the plasma were increased by a heparin/lipid infusion. Similar to previous experiments, the rates of insulin stimulated glucose disappearance after two hours were normal, but reduced by 43% six hours after the lipid infusion; similarly, no changes were observed in the levels of diacylcglycerols or in PCK mass in the initial two hours. However, after about six hours, both PCK activity and mass of diacylglycerols were increased; the study also reported no change in ceramide content throughout the experiment. An elevated level of free fatty acids during a euglycemic-hyperinsulinemic clamp was linked to elevated PKC activity and diacylglycerol level. Furthermore, the pathway followed by the PCK isoforms that might cause acute effects in free fatty acid induced insulin resistance in human skeletal muscle is still to be studied (Itani, Ruderman, Schmieder, & Boden, 2002).

Regardless of age, insulin resistance is the main contributor to the development of type 2 diabetes in a sedentary population. IMCL have been strongly linked to increases in insulin resistance. However, athletes have been shown to be insulin sensitive compared to other populations, even tough they have high concentrations of IMCL. The exact mechanisms through which the IMCL act on insulin action is not clear. One of the aims of the study is to investigate how the insulin signaling pathway in different populations is affected by the IMCL content, specifically the expression of the carrier protein GLUT4.

Relevance of Intramyocellular Lipid (IMCL) in Muscle Metabolism

Since 1950, human skeletal muscle lipids have been considered as a substrate for energy metabolism. Neptune, Sudduth, & Foreman (1959) performed one of the earliest studies isolating and incubating rat diaphragm *ex vivo* without added substrate (fatty acid or glucose). The researchers noticed that the respiratory quotient (RQ) of the rat diaphragm after being incubated without substrate was relatively low (a range of 0.72 to 0.76). This finding indicated a greater reliance on lipids as a substrate and the utilization of intracellular lipids a fuel source. The results of this study were consistent with those of Bloor (1927), who previously proposed that endogenous fatty acids may be the main source of substrate to maintain resting cellular respiration *in vitro.*

Intramyocellular lipids (IMCL) refer to the fat stored in the muscle tissue in the form of lipid droplets. IMCL is an important form of energy for the muscles. When performing physical activity, large amounts of circulating free fatty acids are

redirected to the muscle cells to provide the required energy. During rest, the fatty acids are stored in the muscle cells in the form of triacylglycerol (TAG) for later use (Schrauwen-Hinderling et al., 2003). Excessive accumulation of IMCL results in insulin resistance, contributing to obesity, type 2 diabetes mellitus and metabolic syndrome(Ford, Giles, & Dietz, 2002) – a disorder of energy storage and utilization characterized by central obesity, elevated fasting plasma glucose, low concentration of high density lipoprotein cholesterol, elevated blood pressure, and high serum triglycerides.

IMCL is the main energy source not only at rest, but also while performing physical activity. High concentration of IMCL is associated with the development of obesity and insulin resistance and type 2 diabetes. IMCL storage is affected by the physical activity of the individuals, both type 2 diabetics and athletes have high concentrations of IMCL. Therefore the present study aims to investigate how IMCL content in different populations is affected by high intensity, short duration exercise and low intensity long duration exercise.

Intramyocellular Lipid and Insulin Resistance

IMCL is accepted as a significant source of energy, both under resting conditions and during activities with high-energy demand such as exercise. However, IMCLs are strongly associated with the development of insulin resistance in human skeletal muscle of sedentary populations (Kiens, 2006). Various studies suggest that IMCL accumulation is associated with the development of skeletal muscle insulin resistance, therefore contributing to the development of type 2

diabetes (Boden, Lebed, Schatz, Homko & Lemieux, 2001; Bachmann, 2001; Forouhi et al., 1999). Conversely, endurance trained athletes are highly insulin sensitive despite the elevated levels of intramyocellular lipid content (Bajpeyi et al., 2014). To better explain the metabolic paradox, van Loon et al. (2004), compared the IMCL storage among athletes and type 2 diabetics and using immunofluorescence microscopy identified that both have high IMCL. The results of the study suggested that there was significantly greater lipid content in athletes compared to overweight and type 2 diabetes patients; more than 40% of the muscle lipid content was accredited to the higher proportion of type I fibers in athletes, which exhibited greater lipid content compared to type 2 fibers. The differences in IMCL were accounted by the density of the lipid droplets and not by the lipid droplet size. Therefore, even though structural characteristics and distribution of the IMCL seemed similar between groups, IMCL content was significantly greater in endurance athletes.

IMCL are an important source of energy for the skeletal muscle cells. Nonetheless, it was suggested that an unusually elevated level of triglycerides in skeletal muscle is associated with low insulin sensitivity, predisposing to type 2 diabetes. (Phillips et al., 1996) examined the relationship between skeletal muscle triglyceride content and insulin sensitivity. The study related the lipid content and insulin sensitivity measured from muscle biopsies of 27 sedentary females. Insulin sensitivity was measured analyzing the activation of glycogen synthase, an enzyme regulated by insulin and a mediator for the conversion of glucose to glycogen. The results of the study showed that the IMCL content of the skeletal muscle was

negatively associated with the glycogen synthase activity. Additionally, IMCL content was correlated with insulin resistance features – such an elevated waist-to-hip ratio and fasting plasma non-esterified fatty acids. Phillips et al. (1996) concluded that elevated IMCL are associated with a lower insulin-stimulated glycogen synthase activity, and that intracellular lipid might play a major role in the insulin resistance of type 2 diabetes subjects as well as in normal subjects.

There is an association between type 2 diabetes and defects in lipid metabolism, ectopic lipid deposition in peripheral tissues including skeletal muscle, and elevated levels of free fatty acids. Ectopic lipid is generally accumulated in the form of triacylglycerol. There is an inverse relationship between IMCL content and the insulin sensitivity of sedentary individuals. It has been hypothesized that mitochondrial dysfunction is a possible reason for the reduced capacity of the skeletal muscle to oxidize fat (Amara et al., 2007). Mitochondrial dysfunction could potentially contribute to the accumulation of IMCL, insulin resistance and lipotoxicity (a metabolic syndrome resulting in the over accumulation of lipids in tissues other than adipose tissue). However, Moro et al. (2009) studied how muscle lipases influence IMCL content. The study reported that independent of muscle fiber type, the main predictor for IMCL was total body fatness. Furthermore, the IMCL concentration was higher in females than in males, and in obese than in normal weight subjects. Moreover, muscle ceramide and diacylglyceride concentrations were higher for the obese and type 2 diabetes patients and were independent of fasting free fatty acids and body fat. Moro et al. (2009) concluded that diacylglycerol

content was the main determinant of insulin resistance and that its levels are correlated with lipolytic enzymes and not with adiposity markers.

Goodpaster, He, Watkins, & Kelley (2001) hypothesized that an over accumulation of IMCL is linked to insulin resistance and possibly mediated by the muscle's oxidative capacity. The study reported that skeletal muscle of aerobically trained endurance athletes had a marked oxidative capacity, as well as were highly insulin sensitive compared to lean subjects. Furthermore, Goodpaster et al. (2001) concluded that lipid oxidative capacity is an important mediator between elevated concentrations of IMCL and insulin resistance.

Reduced lipid oxidation in skeletal muscle was hypothesized to contribute to increased IMCL content and the derivative lipotoxic intramyocellular lipids including diacylglycerols (DAG) and ceramides. DAGs and ceramides might be directly linked to insulin resistance. Furthermore, several studies demonstrated that elevated levels of lipid metabolites such as long chain fatty acyl-CoA (LCFA-CoA), DAGs and ceramides in skeletal muscle are associated with insulin resistance, obesity and type 2 diabetes (Bajaj et al, 2004; Dohm et al., 1988; Hulver et al., 2003). Studies using animal models as well as human subjects have demonstrated that LCFA-CoA, DAGs and ceramides disrupt the insulin signaling cascade at several level via protein kinase C θ (PKCθ) (Schmitz-Peiffer et al., 1997) and protein kinase B (Akt/PKB) (Adams et al., 2004). Such disruptions lead to a decrease in glucose transporter type 4 (GLUT4) protein and a decrease in GLUT4 translocation resulting in reduced glucose uptake (Han, Hansen, Host, & Holloszy, 1997).

Recently, it has become more apparent that IMCL are not the direct cause for insulin resistance, but rather lipid metabolites such as diacylglycerols and sphingolipids including ceramides are more directly linked to insulin resistance. Coen et al. (2010) focused on the previous hypothesis that diacylglycerols and sphignolipids contents are higher in the muscle of insulin resistant participants, and also that the association between insulin resistance and intramyocellular triglycerides is muscle fiber type specific. The research included a high performance liquid chromatography-tandem mass spectrometry to measure the diacylglycerol and sphingolipid contents. Gene expressions were assessed by quantitative polymerase chain reaction (qPCR). Fiber type specific intramyocellular triglyceride content was histologically assessed. The results of the study suggested that insulin resistance in human skeletal muscle is related to higher content of intramyocellular triacylglycerols in type I fibers, to higher concentration of ceramides, and to alterations in the gene expression associated with the metabolism of lipids.

The development of insulin sensitivity has been strongly associated with high IMCL content. However, some studies suggest that high IMCL content may not be the only actor leading to the development of insulin resistance. Recent studies suggest that lipid metabolities, such as diacylglycerols and ceramides play a key role in the development of insulin resistance. One of the proposed mechanisms by which IMCL content contributes to the development of insulin resistance, is by disrupting the insulin-signaling pathway. This study aims to investigate how the IMCL content is related to the expression of GLUT4, an insulin regulated carrier protein responsible for the storage of glucose in the muscle.

Skeletal Muscle Oxidative Capacity and Insulin Sensitivity

The major site for fatty acid oxidation is the mitochondria. Fatty acid oxidation has been hypothesized to play a key role in the prevention of lipotoxicity (Bajpeyi et al., 2011). Simoneau, Colberg, Thaete, & Kelley (1995) demonstrated that an increased glycolytic capacity relative to the skeletal muscle oxidative capacity is associated with insulin resistance in obese females. Additionally it was also demonstrated that a reduction in the volume of the mitochondria had a strong relationship with an increase in insulin resistance (Kelley, He, Menshikova, & Ritov, 2002).

Obesity is one of the main factors that influence insulin action, resulting in an increase insulin resistance. The components of the cardiometabolic syndrome including dyslipidemia, hypertension, and type 2 diabetes, provide a considerable risk for cardiovascular disease. Insulin does not only target fat, liver, and skeletal muscle, but also cardiovascular tissue. The energy for the cell is dependent on the mitochondrial metabolism of lipids and glucose. Thus, when the oxidation of nutrients is not sufficient, the ratio of oxygen consumption to adenosine triphosphate (ATP) production is low, which in turn leads to the production of superoxide anions. The formation of reactive oxygen species (ROS) may have consequences that increase the degree of mutagenesis and stimulate the pro-inflammatory processes. Contributors of mitochondrial dysfunction include the formation of ROS, aging, reduced mitochondrial biogenesis and genetic factors. Insulin resistance caused by mitochondrial dysfunction may contribute to metabolic irregularities and promote cardiovascular disease (Kim, Wei, & Sowers, 2008).

Endurance trained athletes have been found to have high storages of IMCL, but at the same time they are highly insulin sensitive, suggesting that IMCL are not the only regulator for insulin action. Recently, data suggests that an increased rate of skeletal muscle lipid synthesis helps protect against lipid induced insulin resistance. Bergman et al. (2010) investigated the effects of intramuscular triglycerides at rest in endurance-trained cyclists. It was hypothesized that the rate of intramuscular triglyceride synthesis was increased when compared to sedentary men. Using an intravenous glucose tolerance test, insulin action was measured. The results of the study suggest that endurance trained athletes possess a higher synthesis of skeletal muscle triglycerides and also a decreased DAGs content in the skeletal muscle. The results of the experiment suggest that increased IMCL synthesis rate is a chronic adaptation of exercise, and not part of the recovery period after exercise Bergman et al. (2010).

Insulin resistance is linked to high IMCL content and low mitochondrial content. An increase in mitochondrial content and function as chronic adaptation to aerobic exercise, results in an increased synthesis of IMCL to be used as an energy source. Furthermore mitochondrial dysfunction has been strongly associated with the development of cardiovascular disease. Mitochondrial adaptations to exercise have been studied in the past. However there is little information regarding adaptations in type 2 diabetics and different exercise modalities. This study aims to address how mitochondrial content, including type 2 diabetics, is affected by different exercise modalities.

Use of Cell Culture Model for Studies on Skeletal Muscle Metabolism

From Hippocrates' early observations associating longevity and wellbeing with muscular fitness, to the effects of aerobic training on the remodeling of the mitochondrial reticulum (Kirkwood, Packer, & Brooks, 1987), and the effects of increased physical activity in the improvement of the cardio-metabolic risk factors of severely obese individuals (Goodpaster et al., 2010), skeletal muscle adaptations to exercise training have been thoroughly studied. Throughout time, exercise has been known to alter muscle function and metabolism. However, the molecular mechanisms leading to the muscular adaptations are not completely understood.

Use of skeletal muscle samples, obtained from a muscle biopsy, is perhaps the best available model to study *in vivo* muscle metabolism and is ideal for clinical intervention studies. However, obtaining a muscle biopsy is invasive, provides limited amounts of tissue, and provides a model where confounding environmental factors are difficult to control (e.g. effects of diet and physical activity, consistency of muscular contractions to follow an exercise prescription, etc.). *In vitro* cell culture models, on the other hand, represent a novel and powerful model to study metabolism with the possibility of precisely manipulating a specific stimulus/condition and by eliminating confounding environmental factors, such as diet and physical activity. Cultured myotubes maintain the genetic background of the donor, including the biochemical, morphologic and metabolic properties of adult skeletal muscle (Bajpeyi et al., 2014; Gaster, Kristensen, Beck-Nielsen, & Schroder, 2001; Ukropcova et al., 2005).

Cultured human primary myotubes retain the metabolic characteristics of the donor. Bourlier et al. (2013) designed a study to investigate the preservation of metabolic adaptations to endurance exercise training in cultured myotubes. Middle-aged subjects completed an 8-week supervised aerobic exercise training program and muscle biopsies of the *vastus lateralis* muscle were collected before and after the aerobic exercise intervention. Cultured skeletal muscle adaptations were similar to the adaptations *in vivo*. Adaptations included improved glucose oxidation, improved glycogen synthesis, and inhibition of palmitate oxidation by glucose. In addition to *in vivo* improvement in aerobic fitness measured by VO_2max and glucose metabolism measured by western blotting, the primary myotubes cultured from the muscle biopsy showed similar adaptations *in vitro*. It was concluded that the effects of aerobic exercise training *in vivo* are preserved in primary human cultured myotubes (Bourlier et al., 2013).

Human skeletal muscle is composed of a mixture of different types of fibers that exhibit a range of metabolic capacities and contractile speeds from the oxidative slow-twitch type I fibers to the glycolytic fast-twitch type II fibers. Moreover, the rate of ATP synthesis is dependent on the oxidative capacity of the fibers. Finally the greater oxidative capacity of the type I fibers allow for greater sustained contractions compared to the type II fibers. The metabolic and contractile properties of the fibers are critical for sustaining skeletal muscle performance (Blei, Conley, Odderson, Esselman, & Kushmerick, 1993).

As proposed by Amara et al. (2008), one of the most important aerobic training adaptations of skeletal muscle is the increased mitochondrial content.

Mitochondrial function as well as mitochondrial content is reflected by the mitochondrial capacity to utilize oxygen to produce ATP. Muscular metabolic adaptation following a short training period of 5 to 7 days, have been reported to increase muscle oxidative capacity. Long-term exercise adaptations (12 week intervention) include: lower lactate production, decreased depletion of the glycogen storages, and decreased hydrolysis of phosphocreatine (Holloszy & Coyle, 1984). Furthermore, Green, Jones, Ball-Burnett, Farrance, & Ranney (1995) reported that after 8 weeks of aerobic exercise training, there was a decreased reliance on glycolysis and high-energy phosphate metabolism for ATP production. After the initial 4 weeks of training, the oxidative capacity of the muscle increased by 31%, and plateaued for the final weeks of training, suggesting an upper limit to the mitochondrial adaptations to exercise.

A study by Ukropcova et al. (2005) aimed to test the capacity of the myotubes to oxidize fat *in vitro* to assess if the myotubes exhibited the same characteristics of the donor. Muscle biopsies of the *vastus lateralis* were cultured and differentiated into myotubes. The results of the study concluded that the ability of glucose to suppress fat oxidation is preserved in cultured myotubes. Furthermore, when cells were exposed to high levels of palmitate, fat oxidation was increased; supporting the hypothesis that variability in metabolic phenotypes is preserved in human skeletal muscle.

Human myotubes have been shown to retain the genetic characteristics of the donor, providing an alternative, non-invasive model to study different metabolic pathways. Cultured myotubes from sedentary lean, type 2 diabetics and athletes can

be used to investigate the effects of an *in vitro* model of exercise. The present study will use exercise mimetic, electrical pulse stimulation (EPS) in a cell culture model to investigate the exercise adaptations of the different metabolic groups (athletes, lean and type 2 diabetics) on mitochondrial and lipid content and expression of GLUT4.

Use of Electrical Pulse Stimulation as a Model for Exercise *in vitro*

Electrical pulse stimulation (EPS) is a method that can be used to mimic the motor neuron activations of skeletal muscle fibers in differentiated skeletal muscle myotubes. Contraction induced by chronic, low frequency stimulation in human skeletal muscles, by a slow motor nerve, or by electrical stimulation, normally induce the manifestation of proteins associated with the slow-twitch muscle phenotype, but at the same time representing different forms of equivalent fast fast-twitch proteins (Thelen, Simonides, & van Hardeveld, 1997).

(Silveira, Pilegaard, Kusuhara, Curi, & Hellsten, 2006), reported genetic and metabolic adaptations induced by EPS *in vitro* using rat skeletal muscle cells. Continuous electrical stimulation led to a consistent increase in peroxisome proliferator-activated receptor-gamma coactivator alpha (PGC-1 α), a transcriptional co-activator that acts as a central inducer of mitochondrial biogenesis in cells cultured from rat muscle. EPS also increased mitochondrial uncoupling protein 3 (UCP3) mRNA, which facilitates the transport of anions of the inner mitochondrial membrane to the outer mitochondrial membrane. Finally EPS

also facilitates the transfer return of the protons from the outer mitochondrial membrane to the inner mitochondrial membrane.

Using an *in vitro* model, Nicolic et al. (2012) recently studied the molecular mechanisms underlying the physiological adaptations induced by physical training. A high frequency, acute electrical pulse stimulation model resulted in an increased production of lactate, as well as an increase in deoxyglucose uptake. Furthermore, Chronic, low-frequency electrical pulse stimulation resulted in an increased oxidative capacity of the cultured human myotubes. This adaptation was observed by the increase in glucose uptake, glucose oxidation and the complete oxidation of fatty acids. The findings of the study concluded that electrical pulse stimulation of human skeletal muscle cells could be used to investigate the effects of exercise *in vitro* (Nicolic et al, 2012).

Sparks et al. (2011) reported the use of a pharmacological cocktail of palmitate, forskolin, and ionomycin (PFI) to mimic exercise *in vitro*. These drugs mimic exercise by increasing free fatty acids in the interstitial space as seen in exercise, increasing cyclic-AMP levels as seen with adrenergic stimulation that occurs with exercise, and cause the release of calcium ions from the sarcomere to induce contraction *in vitro*, respectively. Use of PFI resulted in increased glucose uptake, increased fat oxidation, increased mitochondrial content, as well as increased IMCL.

Electrical pulse stimulation in human myotubes is a novel way to mimic exercise *in vitro.* EPS can be used as a non-invasive method to investigate the effects of exercise on human myotubes. Recent studies have reported similar results *in*

vitro using EPS to *in vivo* measurements of mitochondrial content, glucose uptake and lipid oxidation. This study will use this novel *in vitro* model of exercise to study the effects of different exercise modalities on human myotubes.

Problem Statement

A study by Bajpeyi et al. (2014) investigated the relationship between lipid content measured *in vitro* in myotubes and *in vivo* measurements of insulin sensitivity, intramyocellular lipids (IMCL), oxidative capacity, and physical activity level. The results of the experiment showed a higher lipid content in myotubes cultured from physically active subjects, but not in myotubes cultured from donors with type 2 diabetes. Whereas, diacylglycerols and ceramide content were significantly higher in myotubes cultured from donors with type 2 diabetes. Moreover, diacylglycerols and ceramides in myotubes were inversely associated with insulin sensitivity – demonstrating that it is not intramyocelullar lipid content, but rather DAGs and ceramides that are more directly associated with insulin resistance.

Recent study by Nikolic´et al. (2012) investigated the effects of EPS induced muscle contraction on energy metabolism in skeletal muscle cells cultured from extremely obese individuals with and without type 2 diabetes. However, it is not clear whether this EPS induced adaptation is dependent on EPS duration (time) and time of cell harvest (acute (immediate) vs. chronic (24 hour after the EPS cessation) adaptation). Therefore, the purpose of this study is to compare and contrast the metabolic adaptations to acute and chronic EPS stimulation.

In this study we investigated the effects of different Electrical Pulse Stimulation conditions (acute and chronic) on mitochondrial and lipid content and insulin signaling using an *in vitro* cell culture model. We used cultured myotubes pooled from: athletes, sedentary lean and type 2 diabetic donors. Acute and chronic *in vitro* models of exercise training were induced using the protocol from Nikolic´et al. (2012). Human myotubes were pooled to account for individual differences regarding activity levels and diet in each cohort and was also cost effective.

Mitochondrial content and insulin signaling molecules (insulin substrate receptor 2, GLUT4) were assessed by western immunobloting, and lipid content was measured by immunohistochemistry.

Specific Aims

Specific Aim 1: Examine the effects of acute vs. chronic exercise training in an *in vitro* cell culture model on mitochondrial and lipid content using Electrical Pulse Stimulation. To accomplish this aim, we used human myotubes from sedentary non-obese subjects. **To examine the effects of different exercise modalities, we measured lipid by immunohistochemistry, mitochondrial content by western immunoblotting of the five complexes of Oxidative Phosphorylation, and insulin signaling by measuring the Akt phosphorylation and GLUT4 content by western immunoblotting.**

Hypothesis: Both acute and chronic EPS will increase mitochondrial content, lipid content, Akt phosphorylation, and GLUT4 content. Chronic EPS will result in greater increases in the abovementioned parameters compared to acute EPS.

Specific Aim 2: Compare and contrast the exercise-induced adaptations of mitochondrial and lipid content after EPS in myotubes collected from lean, athletes, and type 2 diabetics. To accomplish this aim, we used human myotubes from sedentary non-obese subjects, athletes, and type 2 diabetics. **Lipid content was measured by immunohistochemistry, mitochondrial content by western immunoblotting of the five complexes of Oxidative Phosphorylation, and insulin signaling by measuring GLUT4 content using western immunoblotting.**

Hypothesis: Mitochondrial content, lipid content, Akt phosphorylation, and GLUT4 content will be greater in myotubes from athletes compared to myotubes cultured from lean or donors with type 2 diabetes. We hypothesized that the

greatest improvements in mitochondrial content, lipid content, and insulin signaling would be observed in cells cultured from athletes compared to cells cultured from lean or type 2 diabetic subjects.

Methods

Study population

Pooled skeletal muscle cells from sedentary lean, athletes, and type 2 diabetes donors were used. Samples were obtained at Pennington Biomedical Research Center. Primary muscle cultures were established from muscle biopsies obtained from the *vastus lateralis* muscle. Pooled samples from five lean, healthy Caucasian male donors (23 ± 1.9 yrs and BMI 24.2 ± 0.6 kg/m^2), four type 2 diabetes mellitus donors (43 ± 4 yrs and BMI 40.2 ±2.2 kg/m^2) and four endurance-trained athletes (23 ± 1 yrs and BMI 24.4 ± 0.9 kg/m^2) were used for the study.

Biopsy and culturing of myotubes

Following an overnight fast and local anesthesia (lidocaine/bupivacaine), skeletal muscle samples were collected from the *vastus lateralis* using the Bergstrom technique with suction (Tarnopolsky, Pearce, Smith, & Lach, 2011) (Propper Manufacturing Co., Long Island City, NY). Skeletal muscle progenitor cells were immuno-sorted using the 5.1H11 antibody provided by the Hybridoma Bank (University of Iowa) and the magnetic activated cell sorting (MACS) column system (Miltenyi Biotec, Auburn, CA). Myoblasts from each donor were then grown simultaneously to approximately 90% confluence and then pooled together by group (sedentary lean, athlete, or type 2 diabetes).

Cultures were seeded with the same number of cells in each well and grown in 6-well plates. Cells were counted using a hemocytometer, each well contained about 96.000 cells. Cell were grown using Dulbecco's Modified Eagle Medium

(DMEM. Life Technologies, Carlsbad, CA) supplemented with 10% Fetal Bovine Serum (FBS. Life Technologies, Carlsbad, CA) and incubated in a humidified chamber with 95% oxygen and 5% carbon dioxide at 37°C. When myoblasts reached ~90% confluence in each well, cells were differentiated into multinucleated myotubes by incubating in Alpha Modified Eagle Medium (α MEM. Life Technologies, Carlsbad, CA) supplemented with 2% FBS (Life Technologies, Carlsbad, CA).

Electrical Pulse Stimulation (EPS)

Fully differentiated cells were stimulated using a C-PACE culture pacer and a C-Dish carbon electrode (Ion Optix Milton, MA) by two different methods.

- Acute, high-frequency EPS (pulse trains of bipolar pulses 99 Hz for 200 ms given every 5th second, 30V, for 60 min),
- Chronic, low-frequency EPS (single, bipolar pulses of 2 ms, with 30 V and 1Hz continuously for 24 or 48 hours).

Cell media for control and experimental conditions was replaced every 12 hr during chronic stimulation.

Control and experimental cells were maintained inside the incubator during stimulation. Cells for each condition were treated equally, contained the same amount of differentiation media (2 mL/well) and harvested at the same time, EPS being the only difference between control and experimental.

Harvesting of cultured myotubes

After acute or chronic EPS, myotubes were collected immediately following the EPS stimulation period ("Early Harvest") and at 24 hours post-EPS stimulation ("Late Harvest"). Control and experimental myotubes were incubated in differentiation media during the 24 hours preceding the late harvest conditions. Myotubes were collected for western blotting and immunohistochemistry.

Western blotting

Differentiation media was discarded and myotubes were washed with Dulbecco's Phosphate Base Saline (DPBS; Life Technologies, Carlsbad, CA). Then Ripa buffer (Sigma-Aldrich, St. Louis, MO) supplemented with 2% phosphatase inhibitor cocktail 2, 2% protease inhibitor cocktail and 2% phosphatase inhibitor cocktail 3 (Sigma-Aldrich, St. Louis, MO) were added. Cells were scrapped and collected in 1.6 mL microcentrifuge tubes and stored at -80°C until analyzed.

Solubilized proteins (20 μg) were run on a 12% mini protean TGX gel (Bio Rad, Hercules, CA), transferred onto a polyvinylidene (PVDF) membrane (Bio Rad, Hercules, CA), and incubated in primary antibodies (1/500 dilution) OXPHOS cocktail (AbCam, Cambridge, MA) and (1/500 dilution) GLUT4 (Cell Signaling, Beverly, MA), followed by secondary antibodies (1/5000 dilution) IRDye 800 CW (LiCor, Lincon, NE). Subsequently, immunoactive proteins were analyzed and quantified using the Odyssey CLx Scanner and Image Studio Ver. 2.1 (LiCor, Lincon,

NE.). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (AbCam, Cambridge, MA) was used as a protein loading control.

Immunohistochemistry

Fully differentiated myotubes were fixated in 2 ml of 10% formalin per well at room temperature. Fixed myotubes were permeabilized using 500 µl of 0.1% saponin (Sigma, St. Louis MO) and incubated in primary antibody (1/200 dilution) MitoProfile (Abcam, Cambridge, MA), followed by secondary antibody (1/200 dilution) Alexa Fluor 594 (Life Technologies, Carlsbad, CA). Following primary and secondary incubation, myotubes were incubated in (1/500 dilution) Bodipy 488 (Life Technologies, Carlsbad, CA) to co-stain for lipids and (1/10 000 dilution) DAPI (Life Technologies, Carlsbad, CA) was used to normalize by nucleus content.

Lipid, nucleus and mitochondrial content were quantified by measuring florescence signal using Fluoroskan Ascent Fluorescence and Luminescence Microplate Reader (Thermo Fisher, Waltham, MA).

Image Processing

Zeiss confocal microscope was used in the UTEP analytical cytology core facility to capture images of myotubes stained for lipid, nucleus, and mitochondria (using fluorescent immunohistochemistry).

Statistical Analysis

Data were analyzed using PRISM GraphPad Software, version 6.0 (GraphPad Software, La Jolla, CA). Paired student t-test was used to compare baseline vs. post intervention parameters, when applicable. A p-value <0.05 was considered statistically significant.

Results

Effects of electrical pulse stimulation (EPS) on lipid and mitochondrial contents measured by immunohistochemistry.

Lipid Content

Myotubes from the sedentary lean group resulted in a significant higher lipid content (bodipy normalized to nuclei content) after 24 hours of EPS stimulation (early harvest) compared to unstimulated control ($p<0.05$; Figure 1B). There was no significant difference in any of the other conditions or collection times as shown in figures 1A and 1C).

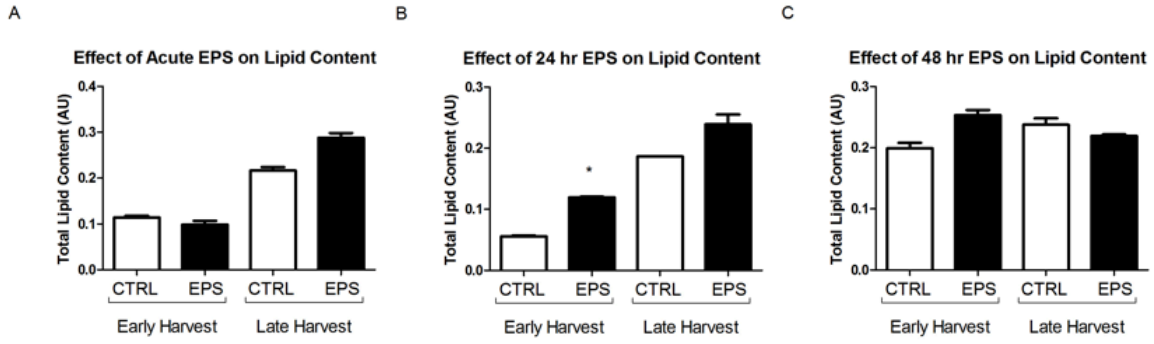

Figure 1. Total lipid content (Bodipy/nuclei) in myotubes from sedentary lean individuals was significantly higher after 24 hr early harvest EPS. *p<0.05 vs. control . N=3.

As seen in figure 2, acute EPS had a different effect depending on collection time. Lipid content in myotubes cultured from athletes was significantly higher ($p<0.05$) after acute and 24 hours early harvest EPS (figures 2A and 2B), and significantly lower ($p<0.05$) after 48 hr early harvest EPS (figure 2C). Late harvest EPS produced no significant difference in lipid contents in the athlete group as shown in figures 2A, 2B and 2C.

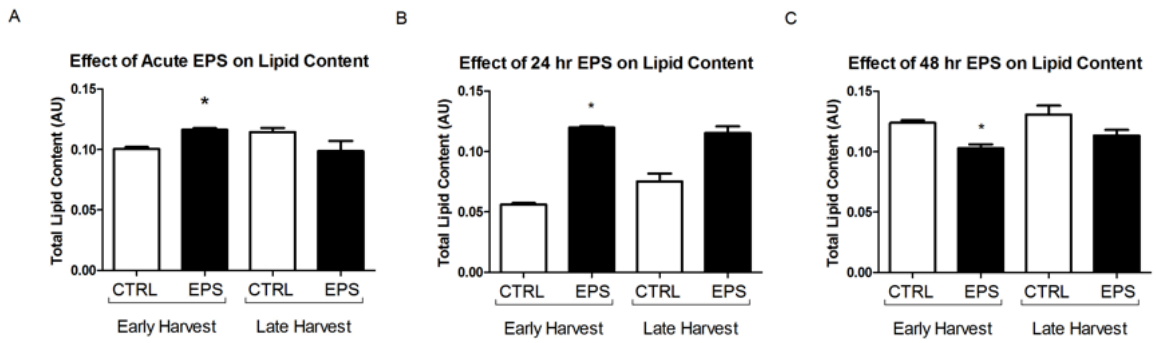

Figure 2. Lipid content (Bodipy/nuclei) in myotubes from athletes were significantly higher after acute and 24 hr early harvest EPS. Lipid content was significantly lower after 48 hr early harvest EPS. *p<0.05 vs. control, N= 3.

Type 2 diabetes EPS group was not significantly different after acute, 24 hr and 48 hr early harvest EPS, shown in figures 3A, 3B and 3C. Lipid content after 24 hr late harvest EPS was significantly lower ($p<0.05$) as shown in figure 3B. There was no significant difference ($p<0.05$) after acute and 48 hr late harvest EPS shown in figures 3B and 3C.

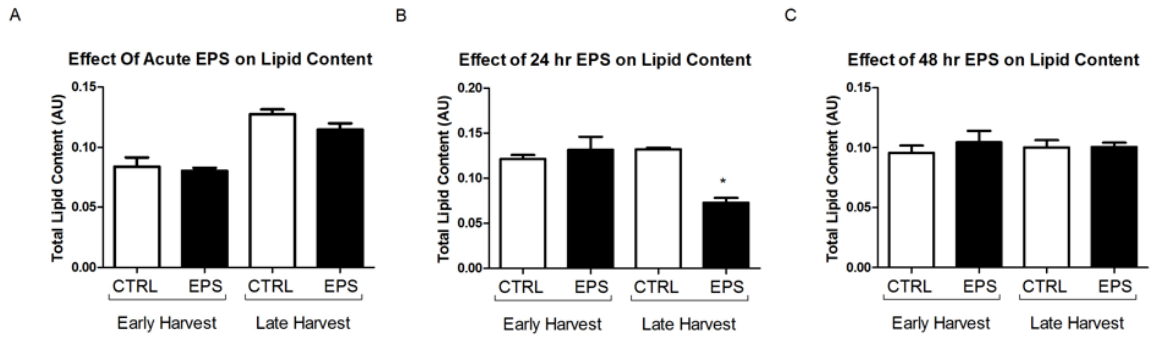

Figure 3. Lipid content (Bodipy/nuclei) in T2D myotubes was significantly lower after 24 hr of early harvest EPS. All other conditions showed no significant differences. *p<0.05 vs. control, N= 3.

Mitochondrial content

Acute, 24 and 48 hr early harvest EPS in the sedentary lean group produced significantly higher ($p<0.05$) mitochondrial content (normalized to nuclei content) shown in figures 4A, 4B and 4C. Acute late harvest produced a significantly lower ($p<0.05$) mitochondrial content shown in figure 4A. 24 and 48 hr late harvest EPS did not result in a significant difference in mitochondrial content as shown in figures 4B and 4C.

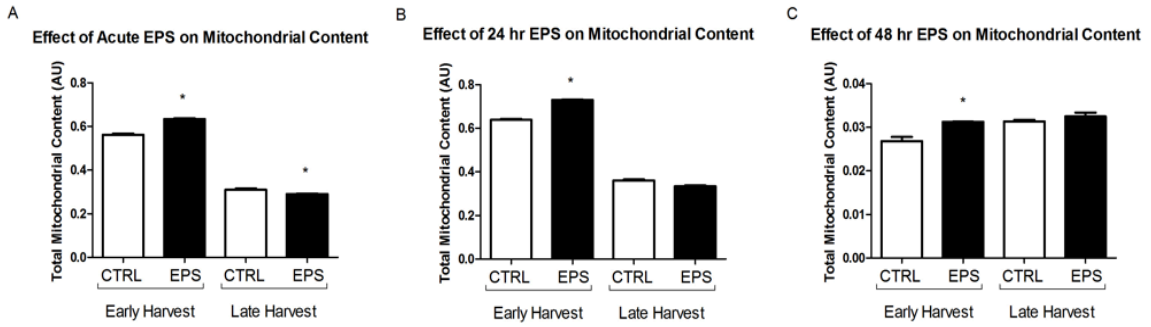

Figure 4. Total Mitochondrial content (MitoProfile/nuclei) in myotubes from sedentary lean individuals were significantly higher after acute, 24 hr and 48 hr of early harvest EPS and significantly lower after acute late harvest EPS. *p<0.05 vs. control, N= 3

Cells from the athlete group showed significantly higher mitochondrial content following 24 and 48 hr early harvest EPS ($p<0.05$) represented in figures 5B and 5C. Acute early harvest EPS did not produce a significant difference in mitochondrial content as shown in figure 5A. Acute late harvest EPS produced significantly higher ($p<0.05$) mitochondrial content compared to control, represented in figure 5A. 24 hr late harvest EPS produced significantly lower ($p<0.05$) mitochondrial content as shown in figure 5B. 48 hr late harvest EPS did not show any significant differences as shown in figure 5C.

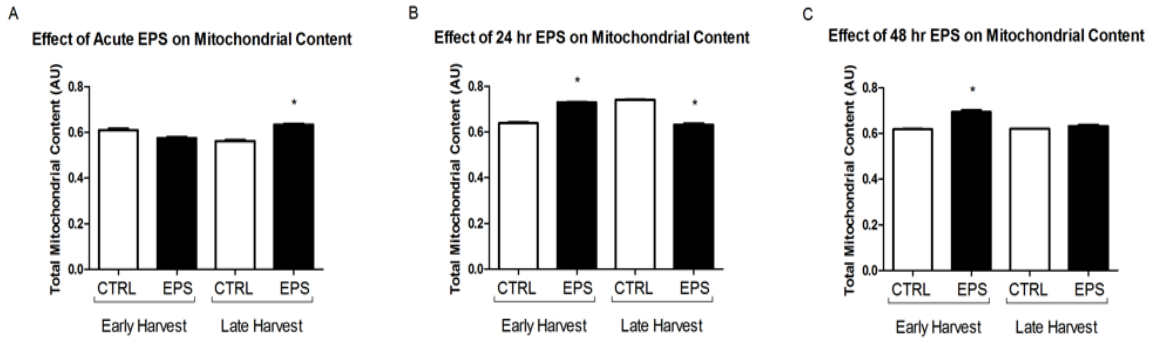

Figure 5. Mitochondrial content (MitoProfile/nuclei) in athlete's myotubes was significantly higher after 24 hr and 48 hr of early harvest EPS and after acute late harvest stimulation. Mitochondrial content was significantly lower after 24 hr late harvest EPS. *p<0.05 vs. control, N= 3

The T2D myotubes showed a significantly lower ($p<0.05$) mitochondrial content after acute and 24 hr early harvest EPS represented in figures 6A and 6B. 48 hr early harvest EPS did not show significant difference as shown in figure 6C. 24 hr late harvest EPS produced significantly higher ($p<0.05$) mitochondrial content as shown in figure 6B. Acute and 48 hr late harvest EPS produced significantly lower ($p<0.05$) mitochondrial content as shown in figures 6A and 6B.

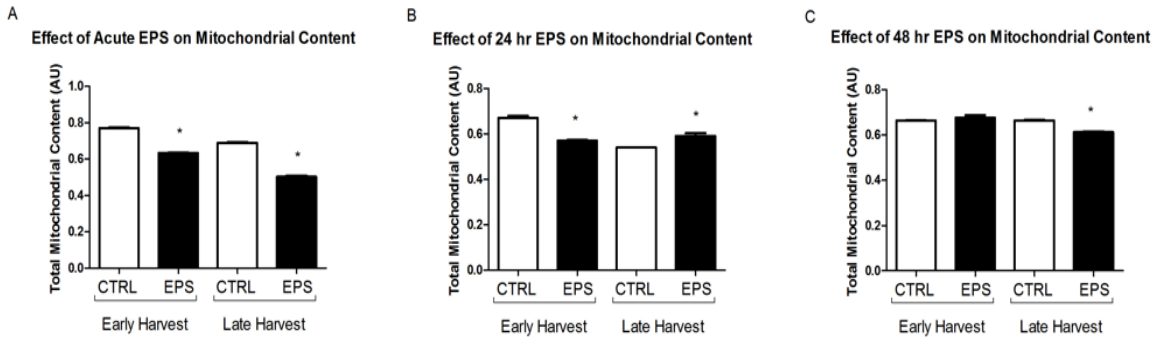

Figure 6. Mitochondrial content (MitoProfile/nuclei) in T2D myotubes was significantly higher after 24 hr late harvest EPS. Mitochondrial content was significantly lower after acute early and late harvest EPS, 24 hr early harvest EPS and 48 hr late harvest EPS. *p<0.05 vs. control, N= 3

Effect of EPS on mitochondrial content measured by western immunoblotting

Mitochondrial content was further assessed by the measurement of the OXPHOS complex and quantified using the expressions of all five complexes in myotubes from lean subjects. Results of early harvest EPS are shown in figure 7 and results of late harvest EPS are shown in figure 8.

As seen in figure 7A, complex I content was higher after 24 hr and 48 hr and lower after acute early harvest EPS. Complex II content was lower after acute early harvest EPS and higher after 24 hr early harvest EPS as seen in figure 7B. Figure 7C shows an increase in complex III content after 24 and 48 hr EPS. Complex IV content was higher after acute and 24 hr early harvest EPS and lower after 48 hr early harvest EPS as shown in figure 7D. Figure 7E shows a lower complex V content after acute early harvest EPS, and no change after 24 hr and 48 hr early harvest EPS. Representative western blot image of the five OXPHOS complexes are shown in figure 7F.

Acute late harvest EPS showed higher complex I content, 24 hr late harvest EPS showed a lower complex I content, and there was no change after 48 hr late harvest EPS as shown in figure 8A. 48 hr late harvest EPS showed a higher complex II content and there was no change after acute and 24 hr late harvest EPS as shown in figure 8B. 24 hr late harvest EPS showed lower complex III content, 48 hr late harvest EPS showed higher complex III content, and no change after acute late harvest EPS represented in figure 8C. Figure 8D shows higher complex IV content after 24 hr and 48 hr late harvest EPS, and no change after acute late harvest EPS. 24 hr and 48 hr late harvest EPS showed higher complex V content, and acute late

harvest EPS showed lower complex V content shown in figure 8E. Representative western blot image of the five OXPHOS complexes are shown in figure 8F.

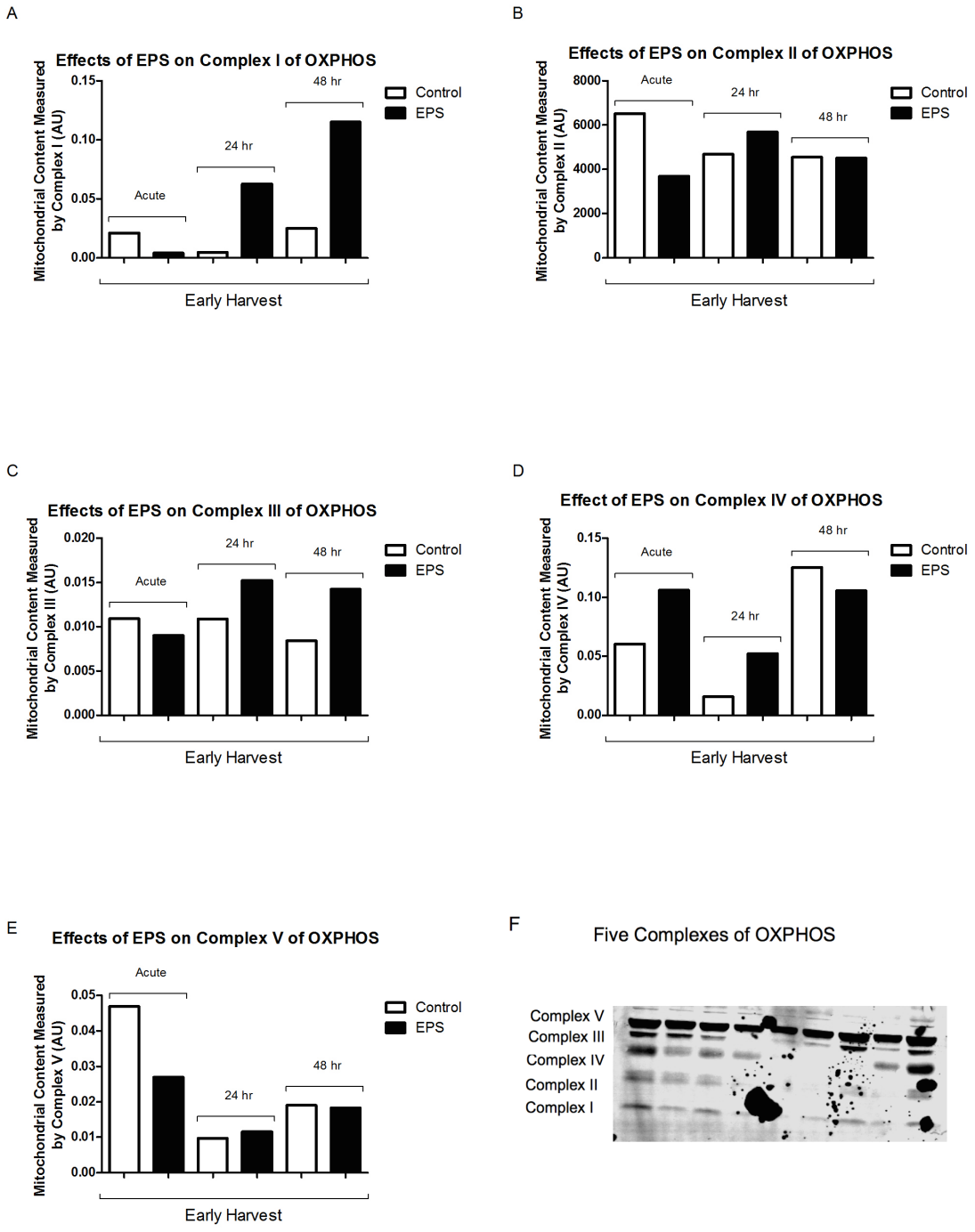

Figure 7. Mitochondrial content represented by OXPHOS protein expression was higher after 24 hr early harvest EPS. ATP production represented by complex V was lower after acute early harvest EPS and there was no difference after 24hr and 48 hr early harvest EPS. N=3

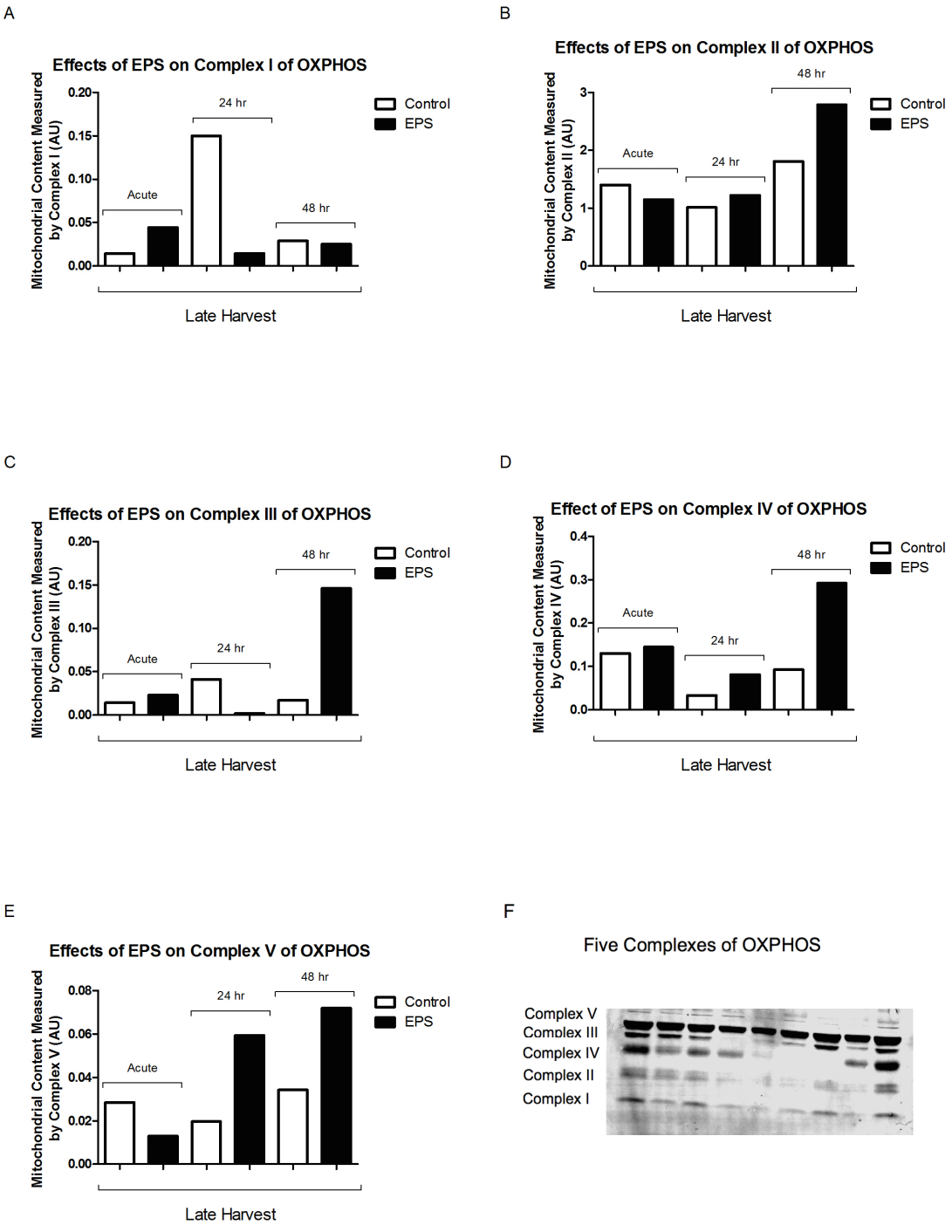

Figure 8. Mitochondrial content represented by OXPHOS protein expression was higher after 48 hr late harvest EPS. ATP production represented by complex V was higher after 24 and 48 hr late harvest EPS, and lower after acute late harvest EPS. N=3

GLUT4 content measured by western immunoblotting

Sedentary lean myotube expression of GLUT4 protein content was higher after 24 hour early harvest, and lower after acute and 48 hr early harvest EPS represented in figure 9. Acute and 24 hr late harvest EPS did produced a difference in GLUT4 content, however, 48 hr late harvest EPS produced higher GLUT4 as shown in figure 10. Representative western blot images are shown in figures 11.

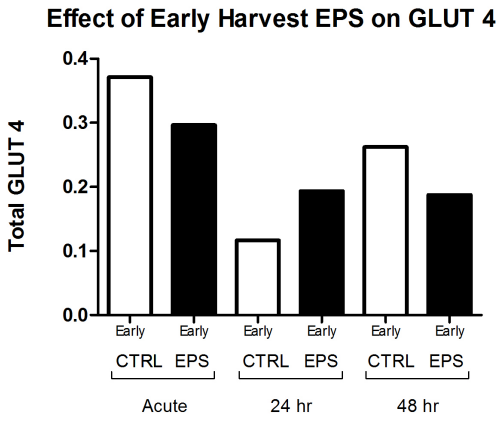

Figure 9. GLUT4 content in lean myotubes was higher after 24 hr early harvest stimulation, and lower after acute and 48 hr early harvest stimulation. N= 3

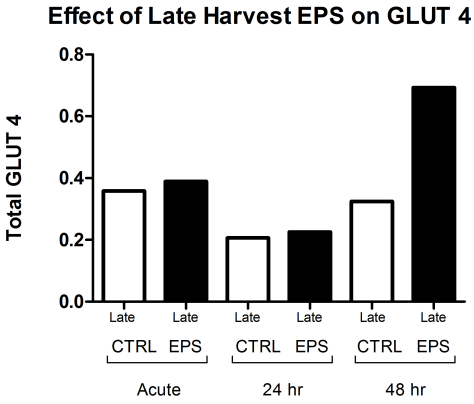

Figure 10. Glut4 content increased after 48 hr late harvest EPS stimulation, there was a small increase after acute and 24 hr late harvest stimulation. N= 3

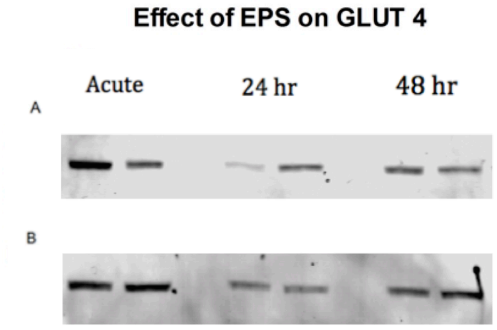

Figure 11. Representative figure of GLUT4 Content. Figure A shows early harvest EPS and figure B shows late harvest EPS

Discussion

Higher lipid content in skeletal muscle in sedentary, lean and obese individuals has been shown to be associated with insulin resistance (Galgani et al. 2008). It has also been demonstrated that myotubes retain the physiological characteristics of the donors including the oxidative capacity and insulin sensitivity (Ukropcova et al., 2005). Our results, however, suggested that IMCL content in type 2 diabetic patients was not elevated in myotubes. Furthermore, higher lipid content in myotubes is associated with better insulin sensitivity, mitochondrial function and physical activity level (Bajpeyi et al., 2014). Therefore, we investigated the effects of electrical pulse stimulation (EPS) and cell harvesting time on lipid content to better understand the exercise effects on lipid accumulation in different populations such as sedentary lean, athletes and type 2 diabetes. Lipid content in sedentary lean myotubes was higher following chronic electrical pulse stimulation (Figure 1A 1B, 1C), which is consistent with the pattern demonstrated by Bajpeyi et al. (2014) and van Loon et al. (2004). However, myotubes cultured from type 2 diabetics and athletes showed differential patterns in lipid content depending on EPS duration. Type 2 diabetic cells showed minimal differences, and even lower lipid content after acute, 24 hr and 48 hr EPS at early and late harvest (Figure 3A, 3B, 3C), while athletes cells showed higher lipid content after acute and 24 hrs EPS at early harvest followed by lower lipid content after 48 hr EPS at late harvest (Figure 2A, 2B, 2C).

Major explanation for differences in IMCL content include a greater oxidative capacity in athletes, therefore having a greater IMCL content in the muscle to be oxidized and used as a fuel source. IMCL in skeletal muscle is one of the primary fuel sources at rest, and during activities of high-energy demand (Kiens, 2006). Type 2 diabetes patients have a decreased oxidative capacity in the muscles, consequently increasing the IMCL content in the muscle because of their inability to use them as a fuel source (Toledo et al. 2007). We expected to see elevated IMCL content in myotubes after chronic stimulation regardless of cohort. However, contrary to the expected results, 48 hr of stimulation in the athletes showed a significant decrease in lipid content (Figure 2C), a possible reason for the lower lipid content may be that the lack of available substrate did not support substrate replenishment, and further studies ensuring adequate availability of lipid would be necessary to evaluate this result.

Given the complexities of muscle lipid accumulation and muscle lipid oxidation, continuing with measures of mitochondrial content *in vitro* via immunohistochemistry seemed the next logical step. Our results showed that EPS revealed differences in exercise response among these three cohorts in terms of mitochondrial content. Sedentary lean individuals followed the same expected pattern demonstrated in previous research by Bajpeyi et al. (2014), subjects in the exercise group had higher mitochondrial and lipid content when compared to control. However, myotubes from athletes and T2D showed that exercise duration has a differential effect on mitochondrial content (Figures 5A–C, 6A–C). The results from both the athlete and T2D group suggest that there is a disparity in EPS induced

changes in mitochondrial content among different metabolic groups as well as depending on EPS duration.

The disparities between changes in mitochondrial content between athletes and T2D myotubes may be linked to their mitochondrial content/activity prior to exercise stimulation. It has been shown that athletes cells already have elevated mitochondrial content (Bajpeyi et al., 2014), therefore leaving little room for improvement during chronic stimulation. The results of the T2D group support the idea that there is a difference between acute and chronic effects of exercise because of the difference in mitochondrial content after 24 hr. When myotubes were harvested immediately at the end of EPS there was a significant decrease in mitochondrial content, but when myotubes were harvested 24 hr after stimulation, there was a significant increase in mitochondrial content in donors with type 2 diabetes. A possible explanation for the results may be that T2D myotubes suffer from mitochondrial malfunction (Moro et al., 2011; Toledo et al., 2007) and immediate changes in mitochondrial content after EPS are not achieved.

Though we measured mitochondrial content in lean, athletes and type 2 diabetic subjects by immunohistochemistry, we also evaluated mitochondrial content in lean subjects via western blotting of the five complexes of OXPHOS, to confirm the results obtained by immunohistochemistry. Similar to the immunohistochemistry results, western blotting in lean myotubes displayed differences in acute verses chronic effects of exercise. The chronic effect of stimulation showed higher complex V content after 24 hr late harvest EPS of stimulation and that effect appears to be further increased after 48 hr of stimulation

(Figure 8E). Early harvest EPS showed that there is a small increase after 24 hr of stimulation and no change after 48 hr of stimulation (Figure 7E). The end results being that in sedentary lean myotubes, EPS appears to increase mitochondrial content.

Effects of EPS on GLUT4 content of lean myotubes showed that 24 hr early harvest stimulation produced higher GLUT4 content. However, acute and 48 hr early harvest resulted in lower GLUT4 content. Furthermore, 48 hr late harvest stimulation resulted in higher GLUT4 content whereas acute and 48 hr did not change GLUT4 content. These results may suggest that duration of EPS promote increase in the glucose uptake, therefore having an impact on the IMCL content of the muscle, especially after 24 hr early harvest and 48 hr late harvest stimulation.

Our results also suggest that not only does the stimulation time have an effect on the lipid content of the myotubes, but also harvesting time might influence lipid and mitochondrial content. IMCL and mitochondrial content showed different results depending on harvesting time. IMCL content seemed to have an effect only after early harvest, showing higher contents for all the cohorts after 24 hr EPS. Mitochondrial content also showed different results depending on harvesting time especially in lean and T2D myotubes. Cells from lean subjects showed higher mitochondrial content after all three duration of EPS when cells were harvested immediately after the EPS. However, there was lower mitochondrial content after acute EPS when cells were harvested 24hr after the EPS (late harvest). Early harvest effects after 24 hr of stimulation in lean myotubes suggest that there is a higher complex III protein content (Figure 7C). However, the late harvest effects suggest

the opposite, and after 24 hr of stimulation the chronic effects seem to reduce complex 3 content (Figure 8C). These results suggest that early harvest effects after 24 hr or 48 hr of stimulation increase mitochondrial content (Figure 7C). Conversely, it does not appear to be the case for the late harvest effects. 24 hr late harvest stimulation effects on complex III does not seem to produce higher but rather lower mitochondrial content (Figure 8C). In addition, it appears that early and late harvest effects of stimulation have similar effect on complex V content in lean myotubes. T2D myotubes, stimulated for 24 hr and taken at early harvest showed a lower mitochondrial content, while 24 hr late harvest showed higher in mitochondrial content.

Similarly to the results of mitochondrial content, harvesting time seems to have an effect on the GLUT4 content in myotubes. Even though 24 hr early harvest EPS resulted in higher GLUT4 content, late harvest showed no change in GLUT4 content. However, this pattern is not followed by acute and 48 hr early harvest stimulation, both conditions seem to have a detrimental effect on GLUT4 content, but when both conditions are harvested late, there is an increase in GLUT4 content after 48 hr and no change after acute stimulation (Figure 9).

The results of western blotting for GLUT4 and complex V of OXPHOS suggest that stimulation and harvesting times are key factors to consider when assessing effects of EPS. As shown in Figure 7E, 8E, 9, and 10, the influence of harvesting time on OXPHOS and GLUT4 content is different depending on EPS duration. Harvesting time appears to play a key role in the metabolic pathways the myotubes undergo

after stimulation, by either allowing the cells to fully adapt to the metabolic changes, or by allowing the myotubes to replenish the depleted substrates used during EPS.

Harvesting time play a role in mitochondrial and lipid content in human myotubes. From our results, it appear that in the case of IMCL content, late harvest has a detrimental effect after 24 hr of stimulation, which may be the result of a detraining, or it may be that myotubes need some time to adapt to the effects of EPS. In the case of mitochondrial content, it appears that myotubes do not show increases immediately after the end of stimulation, but rather 24 hr after the end of stimulation, which was the case of acute stimulation of lean myotubes, acute stimulation of athlete myotubes and 24 hr stimulation T2D myotubes.

Limitations of the study include incomplete OXPHOS and GLUT4 protein data from athlete and T2D donors, due to the unfortunate degradation of proteins after performing EPS experiments and harvesting cells. Therefore data comparing the effects of EPS between those two groups could not be assessed. Other limitations include the inability to optimize the AKT and pAKT protocols; consequently AKT and pAKT data was not included in the study. Finally the age difference in T2D patients might have influenced the results, because aging has been involved in a decline in mitochondrial function (Petersen et al., 2003).

In summary, lipid and mitochondrial content of sedentary lean subjects myotubes followed the expected adaptations showed in previous research (Bajpeyi et al., 2011; Moro et al., 2009). However, athletes and T2D myotubes show very different adaptations that might be due in part because of their metabolic differences: T2D patients' cells have a reduced oxidative capacity and increased

IMCL content (Moro et al., 2009), while endurance training athletes have a greater oxidative capacity and greater mitochondrial function (Bajpeyi et al., 2014). Stimulation time and harvesting time are key factors that lead to different metabolic adaptations. Special attention must be paid when referring to chronic EPS, as demonstrated by the results, different stimulation times result in different adaptations. Furthermore, harvesting time is a major factor influencing the metabolic adaptations of myotubes, as the results demonstrate, acute and chronic effects of stimulations are different, and do not follow a defined pattern.

Future studies should aim to investigate the effect of a high fat growth medium in the mitochondrial and lipid content of cultured human myotubes. Furthermore, studies should also focus on the effects of EPS on lipid metabolities including diacylglycerols and ceramides.

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Appendix A

Supplementary Tables and Figures

Table 1. Summary of significant differences in lipid and mitochondrial content after early and late harvest EPS in different cohorts p<0.05 vs. control, N= 3.

		Lipid Content		Mitochondrial Content	
		Early	Late	Early	Late
Lean	Acute	↔	↔	↑	↓
	24 hr	↑	↔	↑	↔
	48 hr	↔	↔	↑	↔
Athlete	Acute	↑	↔	↔	↑
	24 hr	↑	↔	↑	↓
	48 hr	↓	↔	↑	↔
T2D	Acute	↔	↔	↓	↓
	24 hr	↑	↔	↓	↑
	48 hr	↔	↔	↔	↓

Table 2. Differences in OXPHOS complex III content after early and late EPS

	Complex III	
	Early	Late
Acute	↓	↑
24 hr	↑	↓
48 hr	↑	↑

Table 3. Differences in OXPHOS complex V content after early and late harvest EPS

	Complex V	
	Early	Late
Acute	↓	↓
24 hr	↑	↑
48 hr	↓	↑

Table 4. Differences in GLUT4 content after early and late harvest EPS

	GLUT 4	
	Early	Late
Acute	↓	↑
24 hr	↑	↔
48 hr	↓	↑

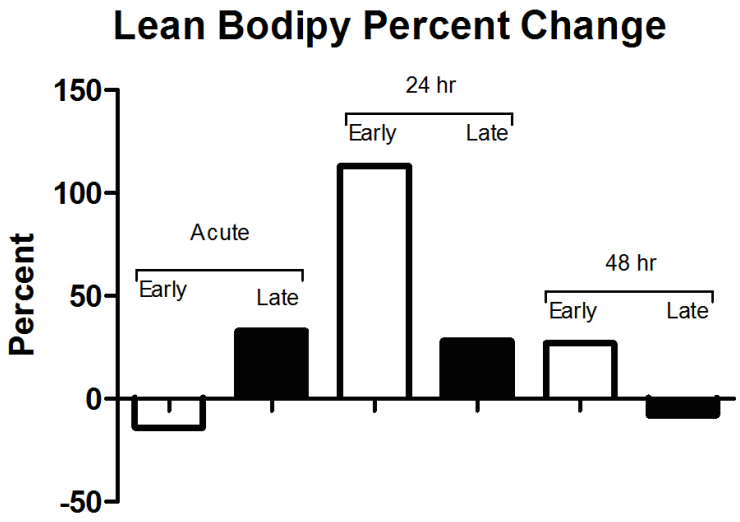

Figure 11. Lipid content in myotubes from sedentary lean individuals showed the greatest percentage change after 24 hr early harvest EPS. A 110% increase was experienced after 24 hr early harvest stimulation.

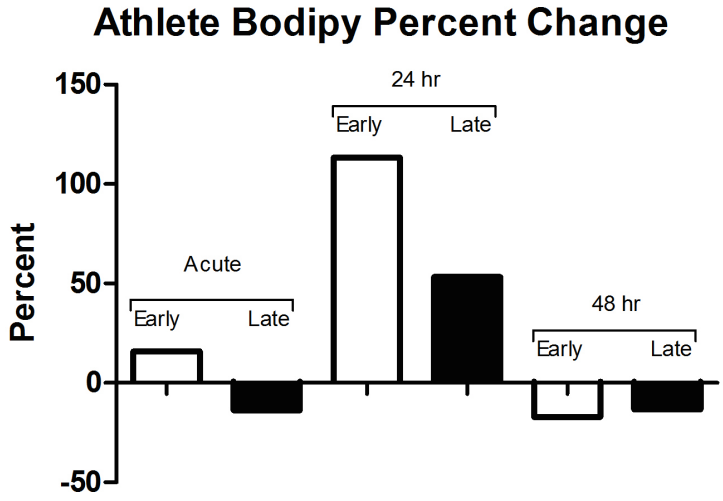

Figure 12. Athlete's lipid content had a 50% increase after 24 hr late harvest EPS and a 100% increase after 24 hr early harvest EPS.

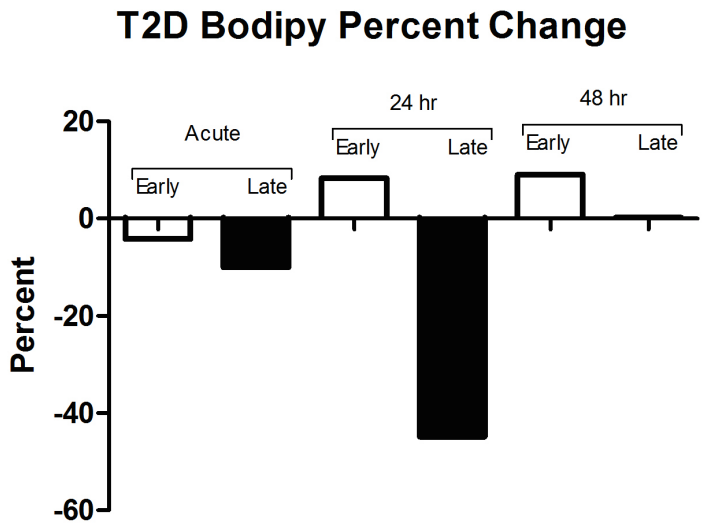

Figure 13. 24 hr late harvest EPS showed an average 50% reduction in lipid content of T2D myotubes.

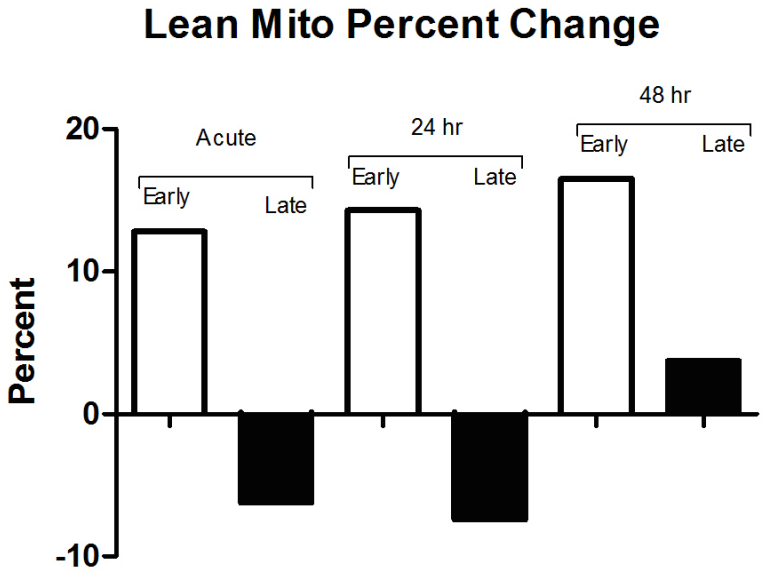

Figure 14. Mitochondrial content in sedentary lean myotubes showed an average 10% increase after acute, 24 hr and 48 hr early harvest EPS. Late harvest EPS showed a small increase after 48 hr, and an average 5% decrease after acute and 24 hr EPS.

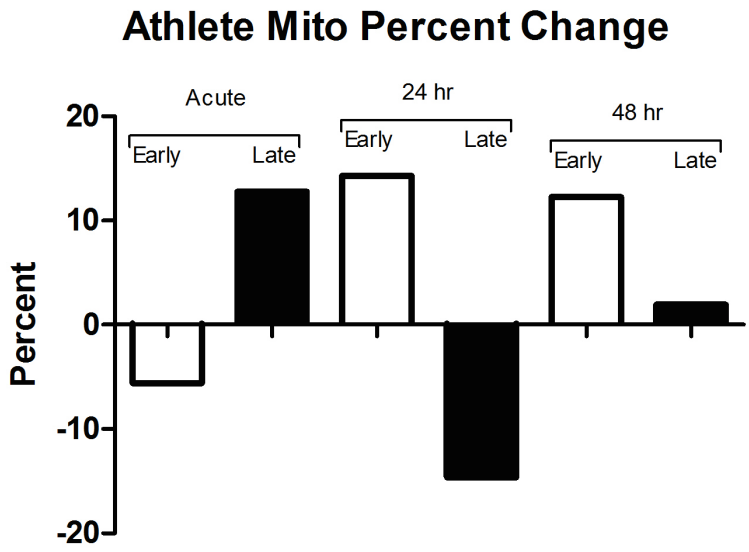

Figure 15. Mitochondrial content in athlete's myotubes showed an average 15% increase after 24 hr and 48 hr of early harvest stimulation and acute late harvest stimulation. There was also an average 15% decrease after 24 hr late harvest stimulation.

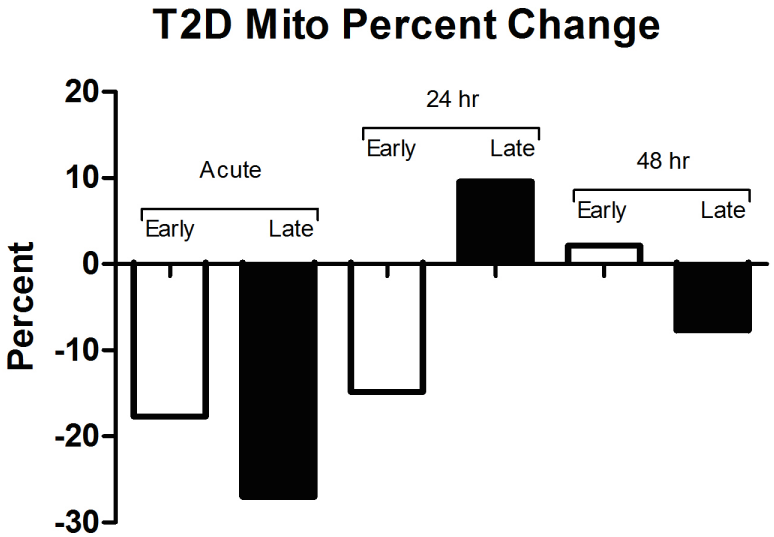

Figure 16. Acute late harvest EPS showed an average 25% decrease in mitochondrial content. Acute and 24 hr early harvest EPS showed an average 15% decrease in mitochondrial content. 24 hr late harvest showed a 10% increase in mitochondrial content.

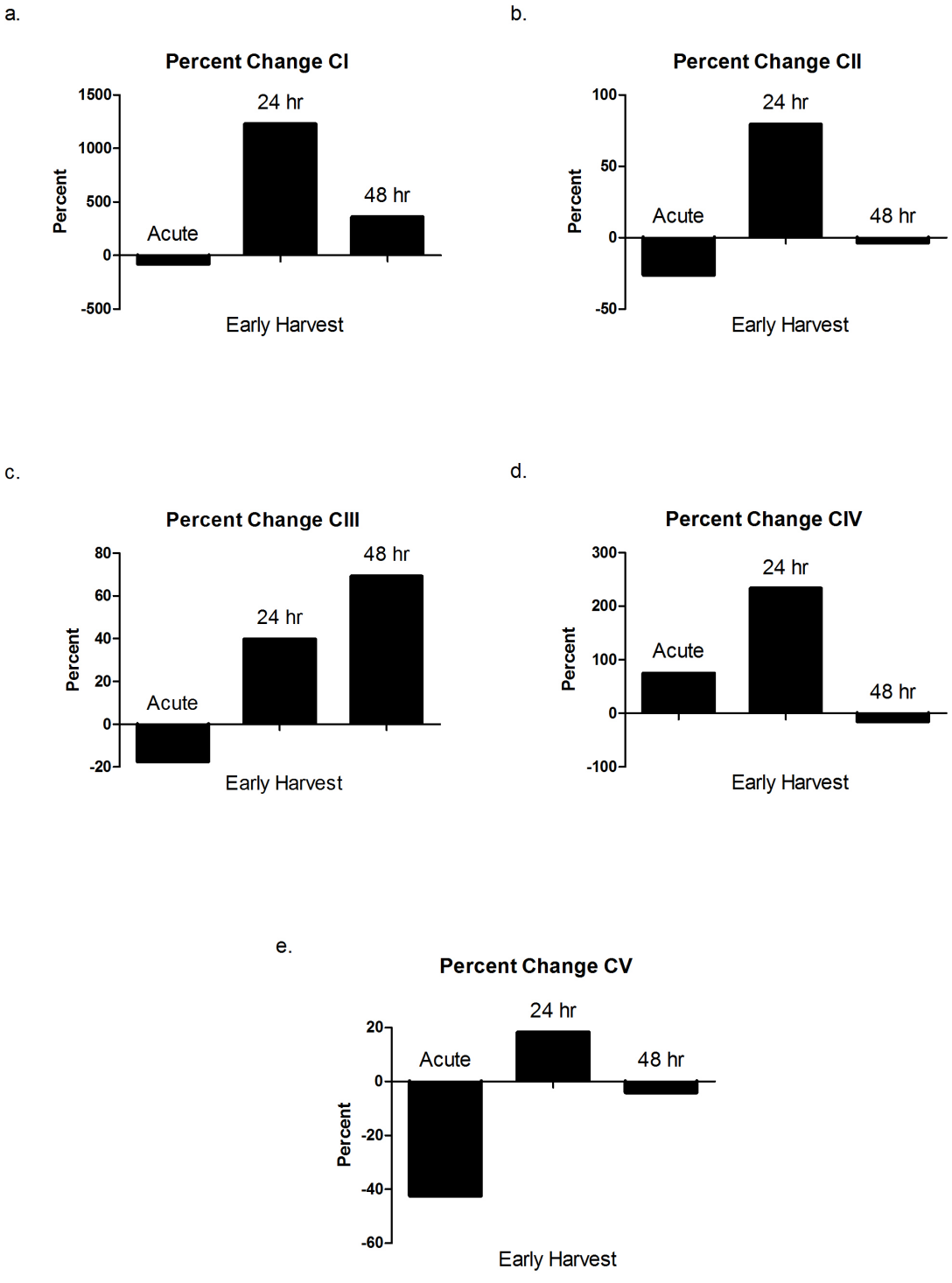

Figure 17. Mitochondrial content represented by complex III increased 40% after 24 hr early harvest EPS and 65% after 48 hr early harvest stimulation. ATP production represented by complex V increased 20% after 24 early harvest EPS and decreased 40% after acute early harvest stimulation.

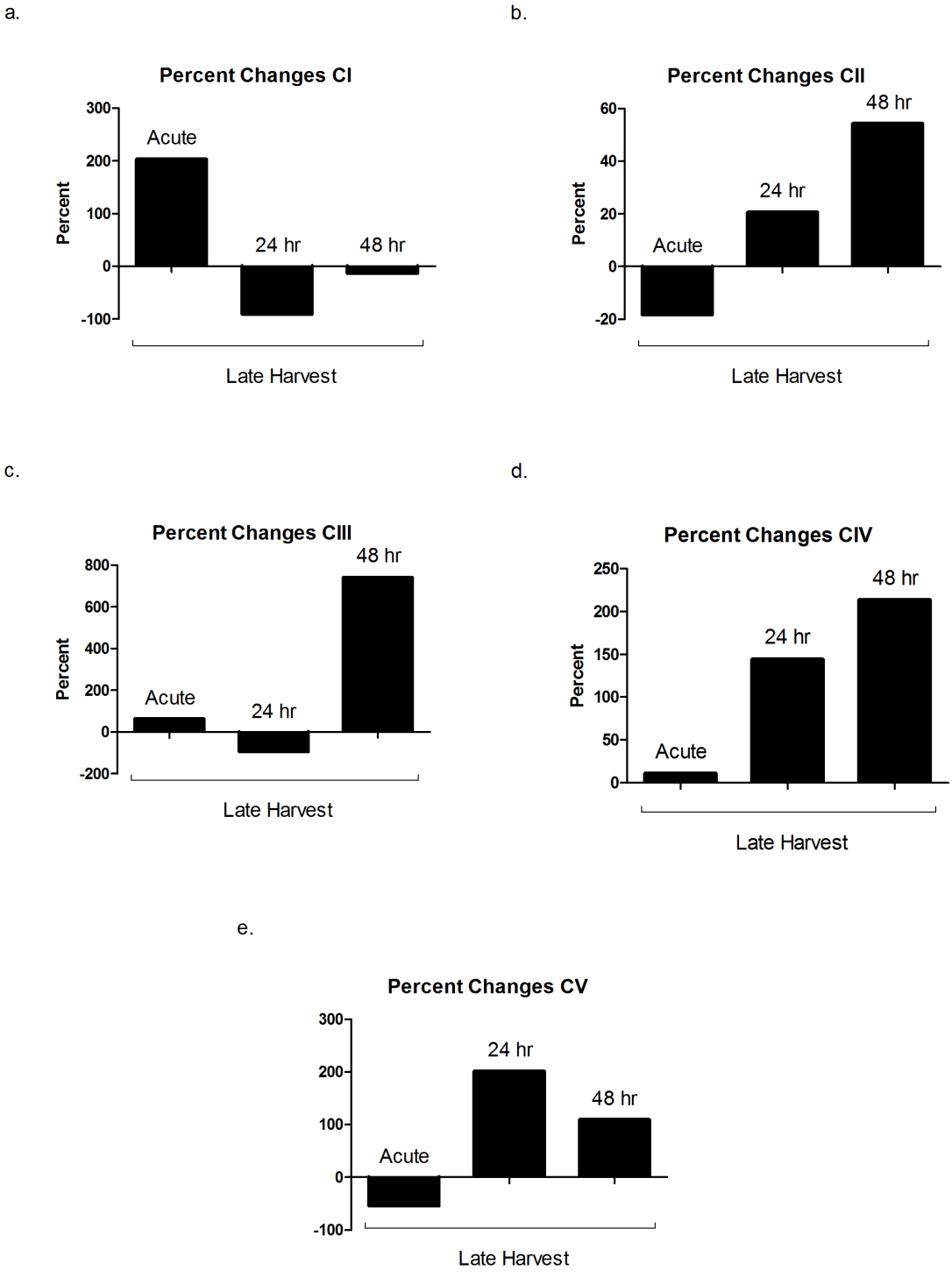

Figure 18. Mitochondrial content represented by complex III increased 700 % after 48 hr late harvest EPS. Mitochondrial content represented by complex V increased 200% after 24 hr late harvest stimulation and 110% after 48 hr late harvest stimulation.

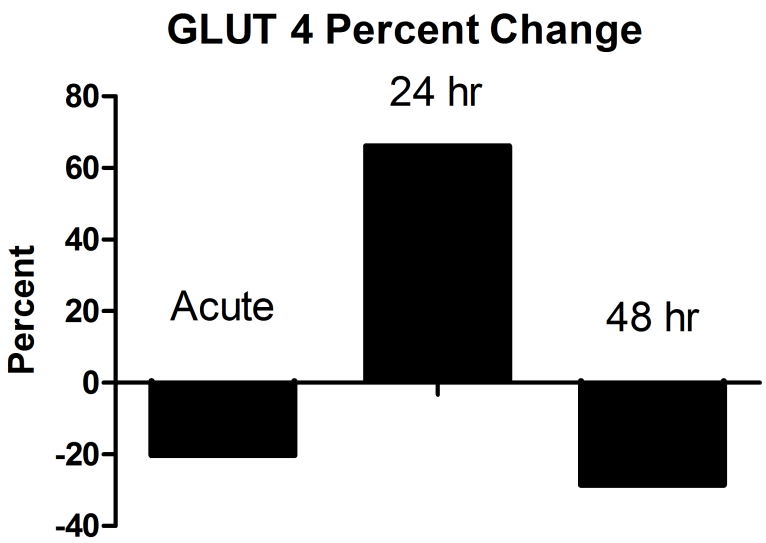

Figure 19. GLUT4 protein content increased 65% after 24 hr early harvest EPS and decreased 20% after acute early harvest stimulation and 30% after 48 hr early harvest stimulation.

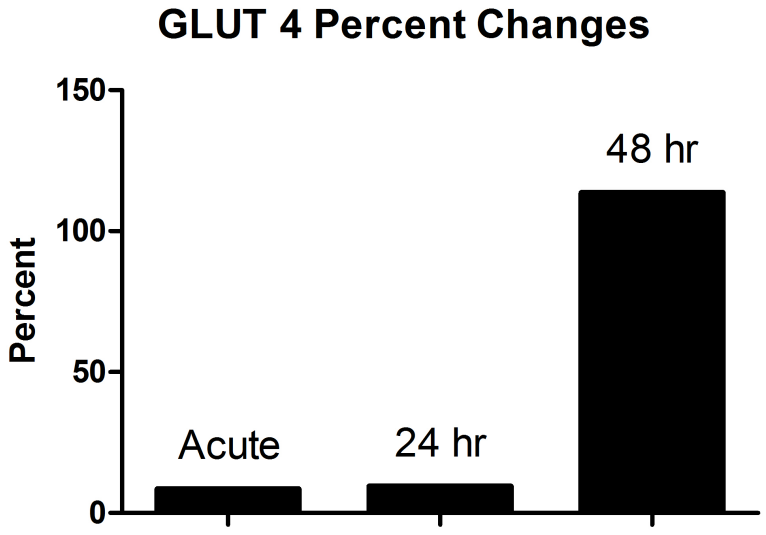

Figure 20. GLUT4 content increased by 100% after 48 hr late harvest EPS, there was a 5% increase after acute and 24 hr late harvest stimulation.

Appendix B

Confocal Microscope Images

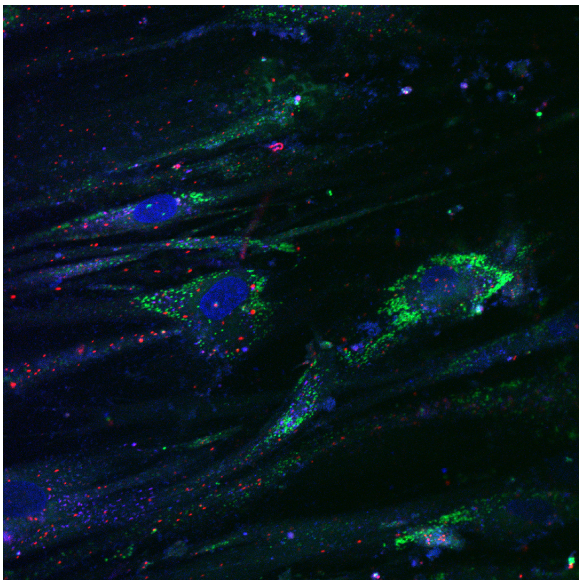

Figure 21 Confocal image of 24 hr early harvest control sedentary lean subjects. Nucleus blue, lipid green and mitochondria red

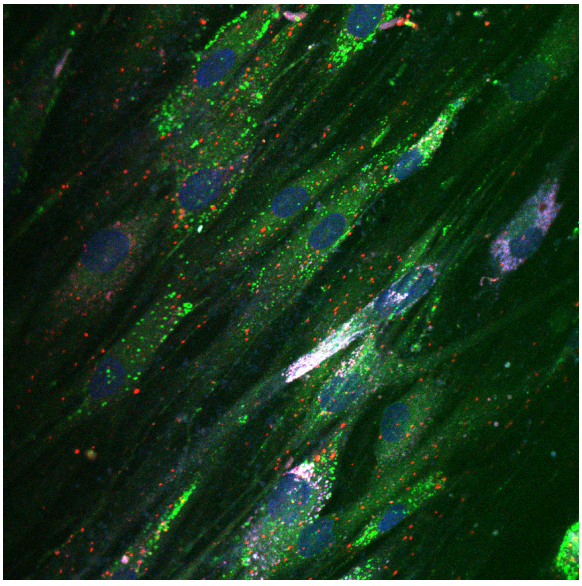

Figure 22 Confocal image of 24 hr early harvest EPS sedentary lean subjects. Nucleus blue, lipid green and mitochondria red

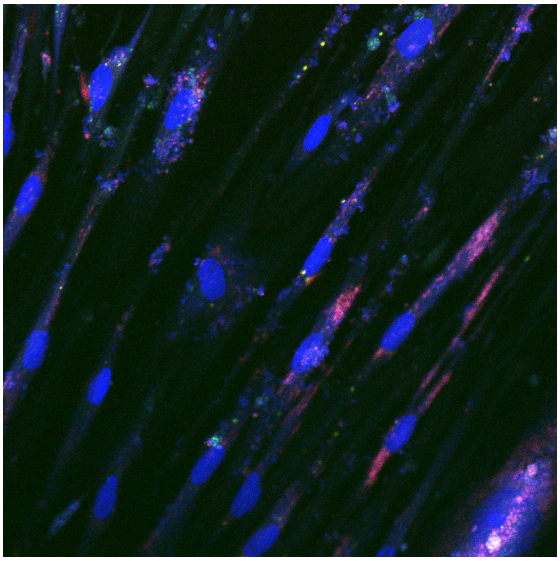

Figure 23. Confocal image of 24 hr early harvest control athletes. Nucleus blue, lipid green and mitochondria red

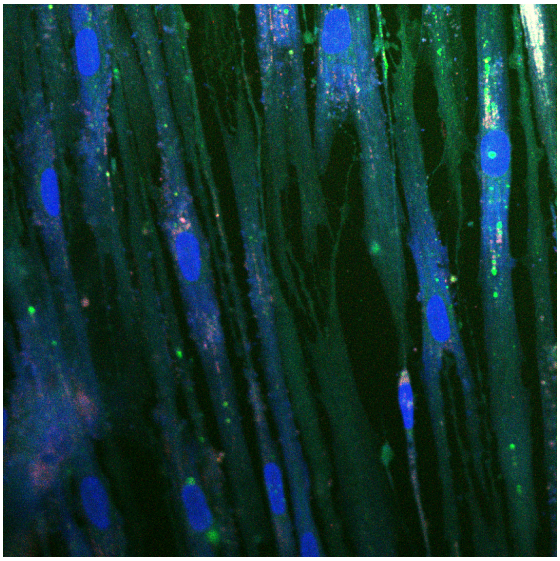

Figure 24. Confocal image of 24 hr early harvest EPS athletes. Nucleus blue, lipid green and mitochondria red

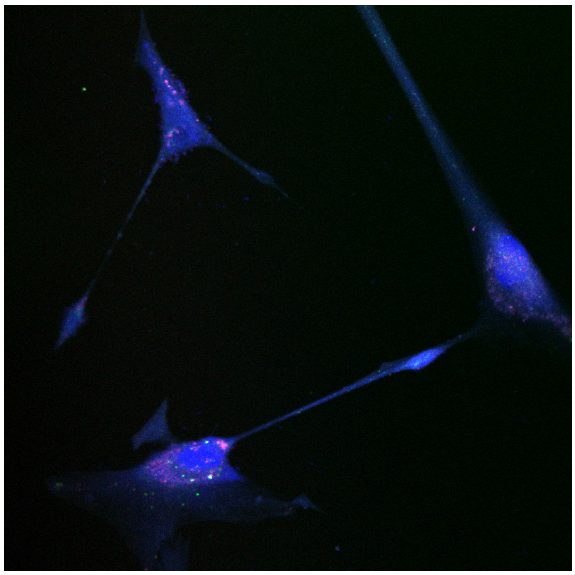

Figure 25. Confocal image of 24 hr early harvest control T2D. Nucleus blue, lipid green and mitochondria red

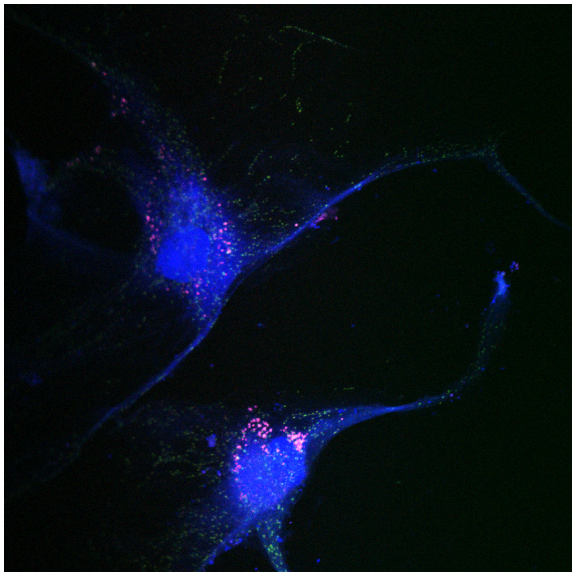

Figure 26. Confocal image of 24 hr early harvest EPS T2D. Nucleus blue, lipid green and mitochondria red

Curriculum Vita

Daniel Conde was born in Léon, Guanajuato, México. The first son of Marcela Iwashige. He graduated from Instituto Jassa A.C., Léon, Guanajuato, México, in the Spring of 2003. He entered The University of Texas at El Paso in Spring 2004 where he graduated with a Bachelor in Science of Kinesiology. In Spring 2013 he started his masters in Science of Kinesiology. While working in his masters degree he worked as a Teaching and Research assistant for the department of Kinesiology. He has been a guest lecturer for both undergraduate and graduate exercise physiology courses. In spring 2014 he was an oral poster presenter at the ACSM Texas chapter conference in Fort Worth, Texas. In Summer 2014 he was an oral poster presenter at the ACSM National Conference in Orlando, Florida.

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