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Characterizing the Effects of Pyrroloquinoline Quinone (PQQ) Supplementation on Skeletal Muscle Mitochondrial Function and Myogenesis During Oxidative Stress and IUGR.

Allyson J. Wood, *The University of Western Ontario*

Supervisor: Regnault, Timothy RH., *The University of Western Ontario*

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Abstract

Intrauterine growth restriction (IUGR) affects 10-15% of births and is associated with placental insufficiency (PI), resulting in fetal oxidative stress (OS). This OS is a factor in the predisposition to postnatal noncommunicable disease (NCD) of which muscle mitochondrial dysfunctional is a key aspect. Pyrroloquinoline quinone (PQQ), an antioxidant-like compound, is capable of OS reduction and promotes mitochondrial function, though limited research has focused on its effects in *in utero* skeletal muscle. This study sought to investigate the impact of *in vitro* H_2O_2, a model of OS, and an *in vivo* model of OS associated IUGR, with PQQ administration, on fetal myogenesis and muscle mitochondrial function. H_2O_2, IUGR, and unexpectedly PQQ, reduced expression of myogenic and mitochondrial genes. Therefore, PQQ does not appear to attenuate OS-induced myogenic and mitochondrial dysfunction and instead negatively altered associated genes. These changes have unknown long-term consequences for altered muscle metabolism and its contribution to NCD.

Summary for Lay Audience

Babies grow quickly in the womb before birth, and when oxygen and nutrients are not properly received, these babies have been shown to have an increased risk of later life metabolic disease, such as diabetes or heart disease. This risk is specifically associated with those who experience the hypoxia-associated pregnancy complication “placental insufficiency” and endure intrauterine growth restriction (IUGR). A key reason for development of these diseases after IUGR is the characteristic reduction in skeletal muscle mass, as the organ plays an important role in whole body metabolism. The reduction is the result of diminished blood delivery, as flow is shunted to more important organs to promote survival after birth. It is predicted that early muscle mitochondrial dysfunction is the underlying pathology that leads to development of metabolic deficits and oxidative stress due to the hypoxic growth environment may be responsible for the organelle’s deterioration. This study aimed to investigate the muscle mitochondrial impairments associated with exposure to oxidative stress. Furthermore, it set out to determine if the antioxidant-like compound, pyrroloquinoline quinone (PQQ), could prevent oxidative stress from damaging mitochondria and instead promote function. A muscle cell culture model was utilized to isolate the effects

of oxidative stress and PQQ on skeletal muscle development and mitochondrial function. A second study utilized an IUGR fetal guinea pig model to determine the effects of IUGR and PQQ on skeletal muscle development, including but not limited to, muscle mitochondrial effects. Cells exposed to oxidative stress had reduced expression of genes associated with muscle development and mitochondrial function, however, the addition of PQQ did not lessen this decrease. Instead, PQQ itself decreased expression of the same genes. Similarly, IUGR animals had reduced gene expression of muscle development, mitochondrial, and metabolism markers, none of which showed increase after PQQ exposure but instead were negatively affected by exposure to the compound. Previous research has shown PQQ to have a positive effect on mitochondrial function in the liver, though this finding was not replicated within the skeletal muscle. Therefore, this study highlights the compound's opposing effects in different organ systems and underlines the need for further research.

Keywords

Skeletal Muscle, Mitochondria, Pyrroloquinoline Quinone, Intrauterine Growth Restriction, Oxidative Stress, Hydrogen Peroxide, C2C12

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List of Abbreviations

ATP	Adenosine Triphosphate
BSA	Bovine Serum Albumin
BtL	Brain-to-Liver Ratio
CAT	Catalase
COX	Cytochrome C Oxidase
CPT-1	Carnitine Palmitoyltransferase 1
CS	Citrate Synthase
CVD	Cardiovascular Disease
DOHaD	Developmental Origins of Health and Disease
ETC	Electron Transport Chain
FBS	Fetal Bovine Serum
GSH	Glutathione
H_2O_2	Hydrogen Peroxide
HS	Horse Serum
IR	Insulin Resistance
IUGR	Intrauterine Growth Restriction
LDH	Lactate Dehydrogenase
MnSOD	Mitochondrial Superoxide Dismutase
mRNA	Messenger RNA (Ribonucleic Acid)
MYF	Myogenic Factor
MYOD	Myoblast Determining Protein
MYOG	Myogenin
NADH	Reduced Nicotinamide Adenine Dinucleotide

NCD	Noncommunicable Disease
NDUFB6/8	NADH:Ubiquinone Oxidoreductase Subunit B6/8
NG	Normal Growth
NOX	NADPH-Oxidase
OS	Oxidative Stress
PAX	Paired-Box Protein
PGC-1	Peroxisome Proliferator-Activated Receptor Gamma Coactivator-1
PI	Placental Insufficiency
PQQ	Pyrroloquinoline Quinone
PPAR	Peroxisome Proliferator-Activated Receptor
RIPA	Radioimmunoprecipitation Assay Buffer
ROS	Reactive Oxygen Species
RS	Reductive Stress
RSV	Resveratrol
SGA	Small for Gestational Age
SIRT	Sirtuin
SOD	Superoxide Dismutase
TFAM	Transcription Factor A, Mitochondrial
T2DM	Type 2 Diabetes Mellitus
UCP	Uncoupling Protein
VDAC	Voltage-Dependant Anion Channel

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1 Introduction

1.1 Fetal Reprogramming During Placental Insufficiency and IUGR

Fetal reprogramming is defined as the physiological, structural, and metabolic changes that occur *in utero* after exposure to certain adverse stimuli (Kwon & Kim, 2017). Though the possible mechanisms of action behind such reprogramming remain unclear, it is evident the changes that occur remain stable after birth and influence the life-long health trajectories of the offspring (C. N. Hales et al., 1991; Marciniak et al., 2017). Central to these mechanisms is the fact that during development, the fetus is especially vulnerable to changes in placental function, including modifications to the relative rate of oxygen delivery through the organ (Longtine & Nelson, 2011).

First trimester embryogenesis is characterized by a low-oxygen environment. Maternal spiral arteries of the uterine tissue are blocked by a subpopulation of fetal endovascular trophoblasts, limiting oxygen delivery to the placenta and creating a hypoxic *in utero* growth environment (Weiss, Sundl, Glasner, Huppertz, & Moser, 2016) . This continues until the 11-14 week of pregnancy, at which time maternal blood begins to flow through the spiral arteries due to the invasion of cytotrophoblasts into the space around and inside the spiral arteries to expand the vessels and supply the placenta with an increased blood volume to support the growing demands of the fetus (Pijnenborg, Vercruysse, & Hanssens, 2006; Whitley & Cartwright, 2009). Failure of the spiral arteries to remodel in the second trimester as outlined results in an ischemic placenta (Roth et al., 2017; Trudinger & Giles, 1996) otherwise referred to as “placental insufficiency” (PI) (Chaddha, Viero, Huppertz, & Kingdom, 2004). PI is an idiopathic pregnancy complication in which the placenta fails to obtain adequate maternal blood , restricting fetal access to oxygen and nutrients. This often leads to a hypoxic growth environment and reduction in nutrient delivery, and subsequent development of fetal growth restriction (Hutter, Kingdom, & Jaeggi, 2010; Malhotra et al., 2019). Hence, PI is a known etiology of intrauterine growth restriction (IUGR) (Burton & Jauniaux, 2018), or PI-IUGR for short.

IUGR is defined as growth below the 10th percentile (Faraci et al., 2011; Peleg, Kennedy, & Hunter, 1998) and occurs within 10-15% of births worldwide (Suhag & Berghella, 2013). Compared to small-for-gestational age (SGA) infants, IUGR is pathological, in that there is an increase in incidence for both perinatal morbidity and mortality (Garite, Clark, & Thorp, 2004). Of the IUGR cases that develop due to PI, the majority are categorized as "asymmetrical" growth restriction, which make up 70-80% of all IUGR cases overall (Sharma, Shastri, & Sharma, 2016). The remaining percentage of cases are defined as symmetrical, typically the result of early pregnancy insults that result in proportional growth restriction due to sustained insult throughout gestation (Peleg et al., 1998; Sharma et al., 2016).

Compared to symmetrical IUGR, asymmetrical growth restriction occurs as a result of preferential distribution of blood flow during PI to organs vital for survival in approaching postnatal life, including the brain and heart, at the expense of other organs (Cohen, Baerts, & van Bel, 2015; Veille, Hanson, Sivakoff, Hoen, & Ben-Ami, 1993) IUGR infants are observed as having proper measuring head circumferences and femur length, compared to decreased abdominal circumference (Sharma et al., 2016). Organs such as the skeletal muscle have inhibited growth, as a result of developing under hypoxic conditions due to diminished blood flow (Rueda-Clausen et al., 2011). It is now evident this hypoxia contributes to a predisposition in IUGR offspring to developing noncommunicable diseases, such as cardiovascular disease (CVD) and type 2 diabetes mellitus (T2DM) in adulthood (Rueda-Clausen et al., 2011), in part due to fetal programming that occurs within the skeletal muscle (D T Yates et al., 2012). This correlation was first observed by David Barker, and later coined the "developmental origins of health and disease (DOHaD) hypothesis (Barker, 1998; C. N. Hales et al., 1991).

1.2 IUGR and DOHaD

In 1977, Dr. Forsdahl first documented an epidemiological correlation between early-life poverty and adulthood coronary heart disease. He speculated that a form of permanent damage occurred due to nutritional deficits that resulted in the predisposition to CVD observed (Forsdahl, 1977). Further building of Forsdahl's original findings, David Barker specifically postulated fetal adaption to a low nutrient supply programs changes to fetal organs that predispose the individual to adult-onset NCD (Barker, 1998). It was made evident by the "Barker's hypothesis" that sufficient maternal blood flow to the developing fetus was not only important for immediate fetal development but had long-term effects implications as well. Similar to low nutritional supply insult originally documented by Barker (1998), insufficient oxygen delivery to and across the placenta (I.e., hypoxia) has also been shown to elicit the same harm, like that seen in PI-IUGR specifically (Chu et al., 2019; Su, Lv, Xie, Wang, & Lin, 2013).

The role the in-utero environment plays in life-long metabolic health developmental trajectories is now increasingly relevant, as the prevalence of NCDs throughout the world continues to rise (Moore, Chaudhary, & Akinyemiju, 2017; Ranasinghe, Mathangasinghe, Jayawardena, Hills, & Misra, 2017). It appears restricted fetal growth is associated with increased incidence of insulin resistance (IR) development, the precursor for several metabolic diseases (C. Nicholas Hales & Barker, 2001). Skeletal muscle is an essential endocrine organ and the reduction in muscle mass seen in IUGR is specifically associated with said decreased insulin sensitivity (Phillips, Barker, Hales, Hirst, & Osmond, 1994; Xing et al., 2019). *In utero,* total muscle mass of an individual is determined by relative rates of myogenesis and there is a rate reduction in this process seen during IUGR (Chang et al., 2020). Importantly, this mass reduction appears to persist into adulthood (Năstase, Cretoiu, & Stoicescu, 2018). An understanding of the specific cellular physiological programming that result in and from reduced myogenesis during IUGR would therefore aid in understanding the role skeletal muscle plays in later-life risk of NCDs in IUGR offspring. More recently, IUGR diagnostic criteria has widen to include those infants who are born above the 10^{th} percentile, 2,500g threshold but may have still been unable to reach their regular developmental milestones at a molecular

level independent of birth weight (Sharma et al., 2016), highlighting that an adverse *in utero* environment that does not result in a decreased birth weight may still program metabolic disease risk at the molecular level (Morris, Say, Robson, Kleijnen, & Khan, 2012).

1.3 Hypoxia and Associated Oxidative Stress

The exact mechanisms of programming that occur during IUGR are not well defined, however it has been postulated that the oxidative stress (OS) that develops as a consequence of hypoxia is responsible (Aljunaidy, Morton, Cooke, & Davidge, 2017; Thompson & Al-Hasan, 2012). OS is defined as the imbalance between reactive oxygen species (ROS) and endogenous antioxidant defense mechanisms, primarily superoxide (SOD), catalase (CAT), and glutathione (GSH) (Birben, Sahiner, Sackesen, Erzurum, & Kalayci, 2012). Collectively, SOD and CAT aim to eliminate two potent ROS, superoxide (O^-) and hydrogen peroxide (H_2O_2), respectively (Birben et al., 2012). Similarly, GSH aids in the elimination of H_2O_2 alongside CAT (Antunes, Han, & Cadenas, 2002). Three major cellular systems appear to produce the majority of ROS including mitochondrial metabolism, NADPH oxidase (NOX), and xanthine oxidase (XO). Under physiological conditions, complex I (NADH:ubiquinone oxidoreductase) and complex III (ubiquinol:cytochrome c oxidoreductase) of the mitochondrial electron transport chain (ETC) produce endogenous oxygen free radicals due to electron leakage during energy production. Further modifications to these free radicals result in superoxide and H_2O_2 formation. Likewise, NOX produces superoxide and H_2O_2 as a by-product of its transfer of electrons across the cell membrane. Lastly, XO is a potent source of superoxide; production accompanies one of xanthine oxidase primary functions, oxidation of hypoxanthine to xanthine. In short, ROS production is inevitable under normal physiological conditions and its production alone does not definitively lead OS. It is the degree to which ROS outcompetes antioxidant activity that is fundamental to understanding OS establishment during pregnancy.

Physiological concentrations of ROS serve a critical role in pregnancy, as modulated levels are essential for effective cellular signalling during placentation and beyond

(Soares, Iqbal, & Kozai, 2017). For example, the initial hypoxic growth environment is intended to protect the placenta and early embryo against damage due to oxidative stress (OS), as neither have developed sufficient antioxidant abilities to reduce ROS concentrations. In other words, a premature influx of oxygen through the placenta, and subsequent increase in cellular metabolism in early pregnancy results in elevated production of ROS that the ill-developed SOD and CAT within the placenta and embryo cannot combat, ultimately resulting in OS (Jauniaux et al., 2000; Takehara, Yoshioka, & Sasaki, 1990). Hence, the hypoxic growth environment throughout the first ten weeks of pregnancy is critical to reach the stage of fetal development.

As fetal development progresses, the relatively hypoxic environment is meant to diminish. However, under conditions in which the placenta fails to thrive including PI-IUGR, adverse, chronic placental and fetal hypoxia develops. Unlike the beneficial effects seen during embryonic development, hypoxia due to PI is associated with OS production within the placenta and fetus (Schoots, Gordijn, Scherjon, van Goor, & Hillebrands, 2018). As previously described, blood flow is redistributed to vital organs and shunted away from the skeletal muscle as a result of the hypoxic environment of PI-IUGR. This process is conducive of OS development within the skeletal muscle itself as a result of the direct decrease in partial pressure of oxygen within the organ (Clanton, 2007). The effects of OS on myogenesis are well established and it is a known contributor to the decrease in skeletal muscle evident during IUGR. In addition to the negative effects of decreased nutritional intake of the skeletal muscle mass during IUGR (Bhasin et al., 2009; Năstase et al., 2018), OS appears to reprogram regulator factors of myogenesis to inhibit fiber development directly (Chang et al., 2020).

1.4 Myogenesis

To understand how OS affects myogenesis, physiological myogenic processes must first be described. Much of the muscle growth precedes birth, highlighting the importance of the fetal environment for adequate skeletal muscle development and growth (Yan, Zhu, Dodson, & Du, 2013). Myogenesis commences during the embryonic stages of

development, termed primary myogenesis. Early muscle cells are collected within the primitive dermomyotome and have marked expression of pair boxed transcription factors PAX3 and PAX7 (Goulding, Chalepakis, Deutsch, Erselius, & Gruss, 1991; Jostes, Walther, & Gruss, 1990). Both transcription factors regulate specification of cells to myogenic commitment, while also activating downstream myogenic regulatory genes such as myoblast determination protein 1 (MYOD1) and myogenic factor 5 (MYF5). After disintegration of the dermomyotome, $PAX3^{+}/PAX7^{+}$ committed cells align within the new myotome in which future limbs are derived and evolve to express terminal skeletal muscle specification markers, including Myod1 and Myf5 (Rudnicki et al., 1993).

The myogenic regulatory factors are highly conserved genes consistently expressed at one time during skeletal muscle development and include MYOD1, MYF5, in addition to Myogenin (MYOG), and MRF4 (Weintraub et al., 1991). MYF5 is the initial factor to be expressed, but then decreases in line with myotube fusion (Ott, Bober, Lyons, Arnold, & Buckingham, 1991; Zanou & Gailly, 2013). Confirmation of the importance of MYF5 to primary myogenesis came when mice devoid of MYF5 displayed delayed skeletal muscle development. Of note, induced gene expression of Myod1 then rescued muscle development, highlighting the importance but also redundancy of MYF5 in the presence of MYOD1 (Braun, Rudnicki, Arnold, & Jaenisch, 1992). The redundancy of both factors was further highlighted, when skeletal muscle developed adequately in MYOD1-null mice, as a result of compensatory Myf5 gene expression (Rudnicki, Braun, Hinuma, & Jaenisch, 1992), while knockout of both resulted in failure of skeletal muscle to grow (Rudnicki et al., 1993; M. Yamamoto et al., 2018), exemplifying that at least one of the two factors must stay conserved. Downstream factor MYOG is skeletal muscle specific, with early expression in primary myogenesis and continued activation until mature muscle fibers develop during secondary fetal myogenesis, which fail to form in Myog-knockout experiments, accentuating the factor's necessity (Venuti, Morris, Vivian, Olson, & Klein, 1995). MRF4 expression similarly spans embryonic and fetal myogenesis, serving as a myogenesis determination gene like Myf5 and Myod1, and continuing to be expressed throughout fetal myofiber development. Though once hypothesized that

differentiation was solely controlled my Myf5 and Myod, recent research has shown that Mrf4 expression is additionally necessary for adequate myogenesis. Knockout of either Myf5 or Myod1 in combination with Mrf4 expression, supports sufficient muscle development for postnatal survival and it is now believed MRF4 works upstream of MYOD1, similar to MYF5 (Kassar-Duchossoy et al., 2004). Of note is the importance of MRF4 in adult skeletal muscle, serving as the predominant expressed factor (Hinterberger, Sassoon, Rhodes, & Konieczny, 1991) (Figure 1.4-1).

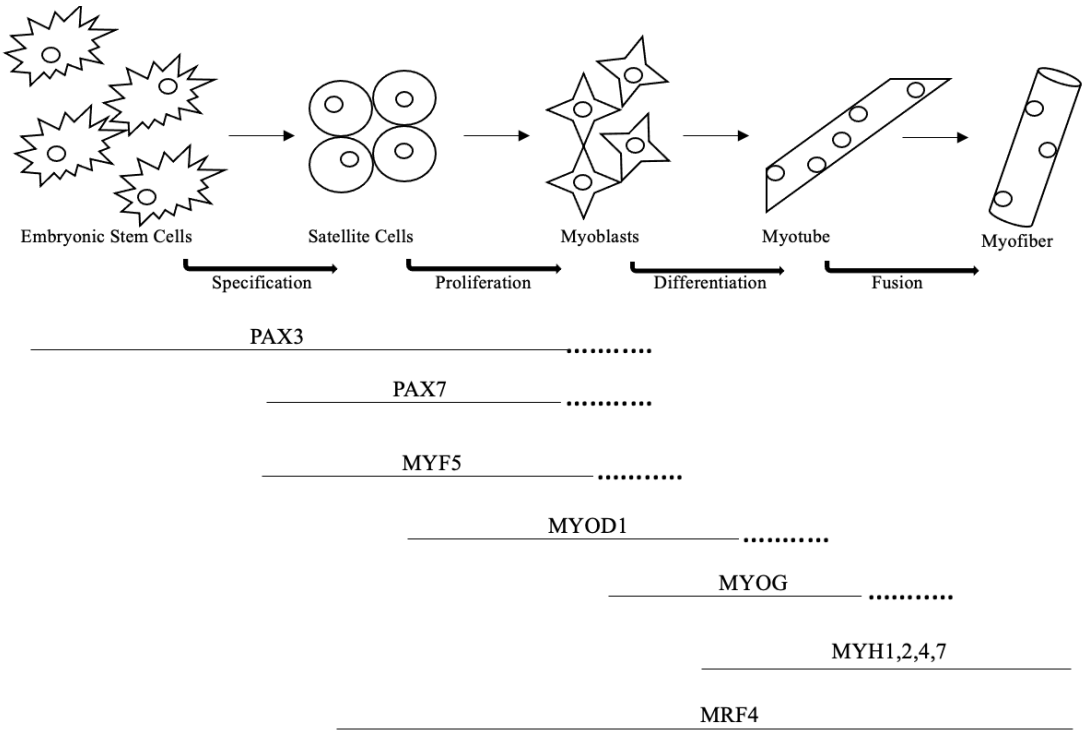


Figure 1.4.1-1 Regulation of embryonic and fetal myogenesis.

Transcription factor/gene expression is shown by a solid line and declining expression is represented by dotted lines. Figure modified from the "The Basis of Muscle Regeneration" article (Musarò, 2014).

The type of fiber that develops from primary and secondary myotubes is determined by the myosin protein composition within each. The fibers are then further classified in accordance with their relevant physiological capabilities. Simply, fibers are classified as type I (slow) or type II (fast), based on at which speed in which sarcomeres shorten (Bárány, 1967; Huxley & Niedergerke, 1954; Talbot & Maves, 2016). Myosin heavy chain isoform genes corresponding to type I fibers, including Myh7, are expressed beginning in embryonic myogenesis. Comparatively, expression of type II fiber myosin heavy chain isoform genes Myh4, Myh2, and Myh1 does not occur until the fetal stages of muscle development (Glaser & Suzuki, 2018). Fiber type is additionally influenced by myogenic regulatory factor, MYOD1, in that Myod gene expression is important for type II fiber specification and maintenance (Hughes, Koishi, Rudnicki, & Maggs, 1997; Talbot & Maves, 2016).

Lastly, the regenerative capacity of skeletal muscle, otherwise described as adulthood myogenesis, is attributed to a select population of reserved $PAX3^{+}/PAX7^{+}$ satellite cells maintained throughout primary and secondary myogenesis. PAX3 and PAX7 are redundant in that PAX7 can replace PAX3 in most functions, and PAX7 appears to be necessary for postnatal myogenesis specifically (Olguin & Olwin, 2004; Relaix, Rocancourt, Mansouri, & Buckingham, 2005; Seale et al., 2000). PAX7 expression in turn inhibits MYOD expression to arrest cells in a proliferative state by inhibiting further myogenesis induction (Olguin & Olwin, 2004). Upon skeletal muscle injury, mentioned $PAX7^{+}/MYOD^{-}$ cells regain myogenic properties and fuse to injured myofibers to allow regeneration (Moss & Leblond, 1971; Snow, 1977; Von Maltzahn, Jones, Parks, & Rudnicki, 2013). Early damage to described satellite cell pool could therefore be postulated to inhibit future regenerative capacity.

1.4.1 Hypoxia/OS and Myogenesis

As stated previously, myogenesis is altered under hypoxic growth conditions. Phenotypically, muscle mass, and subsequent myofiber area and diameter is reduced under conditions of low oxygen (Howald & Hoppeler, 2003; Scholz, Thomas, Sass, & Podzuweit, 2003). Expression of MYOD1 and subsequently MYOG, is significantly

decreased, suggesting slowed myogenesis, providing a molecular explanation for decreased skeletal muscle size under pathological hypoxic conditions (Yun, Lin, & Giaccia, 2005). It is important to note that it is under extended hypoxic conditions that such adverse effects are observed, in part due to chronic activation of HIF-1α. Acute exposure induces HIF-1α, though this short-term activation is said to be beneficial, in that studies have documented stimulation of myogenesis under these conditions (Cirillo et al., 2017). Inversely, recurrent activation of HIF-1α inhibits MYOD1 expression, therefore disrupting myogenesis (Di Carlo et al., 2004). IUGR is similarly associated with decreased MYOD1 and MYOG (Chang et al., 2020), and a reduction in the proliferative capacity of myoblasts (Dustin T Yates et al., 2014). Overexpression of HIF-1α to mimic a hypoxic growth environment is also associated with IUGR mice offspring with decreased skeletal muscle mass (Tal et al., 2010). These results together highlight that reduction of skeletal muscle mass in IUGR occurs in association hypoxia and could be explained by molecular dysregulation of myogenesis *in utero,* possibly due to OS during P-IUGR. Though PI- IUGR occurs later in pregnancy, the secondary (fetal) myogenesis occurring during this time has a larger impact on muscle size than early embryonic myogenesis. Fetal myogenesis is the process in which secondary muscle fibers form through the fusion of myoblasts to primary myotubes, and these secondary fibers account for the majority of skeletal muscle mass (Weimer & DiMario, 2016). This could explain why PI-IUGR and resulting OS has such a marked effect on the organ's size although the condition develops relatively late in gestation (Chang et al., 2020).

1.4.2 IUGR's Effects on Muscle Fiber Type and Mitochondrial Metabolism

Skeletal muscle fibers vary in energy production and oxidative capacity, dependant in part by their relative mitochondrial content (Crupi et al., 2018). Slow fibers are characterized by their high myoglobin and capillary content, associated with relatively increased oxidative capacity, and slow contractions allow for increased mitochondrial oxidative phosphorylation and subsequent ATP production (Glaser & Suzuki, 2018). In comparison, type II fibers are associated with anaerobic metabolism, in that they possess fewer mitochondria and rely on glycolysis for contractile ATP energy (Glaser & Suzuki, 2018). Type II fibers can be further defined as being type IIa, IIb, or IIx. Type IIx and IIb

fibers are considered glycolytic fibers, in comparison to Type IIa, commonly referred to as the “intermediate fiber”, with both glycolytic and oxidative capacity. It is important to note that Type IIb muscle fibers are only present in small mammals, and not humans (Tellis, Rosen, Thekdi, & Sciote, 2004).

Interestingly, this specification is considered plastic and fiber type “switching” is a well-documented mechanism that can occur during development and into adulthood (Buller, Eccles, & Eccles, 1960). Fiber type switching *in utero* results from changes in the metabolic environment (Bourdeau Julien, Sephton, & Dutchak, 2018), while in adulthood exercise commonly results in fiber type switches resulting in increased accumulation of fibers with relatively high oxidative capacity, like type I (S. H. Lee, Kim, Park, & Kim, 2018).

Muscle fiber type switching during IUGR has been documented, though the multiple studies conducted are contradicting to one another. Early studies reported an increased proportion of type I fibers in lower limb muscle of IUGR piglets (Bauer, Gedrange, Bauer, & Walter, 2006; Wank et al., 2000). Similarly, maternal diet restriction alone has been shown to promote a decrease in type II fiber concentration, accompanied by an increase in type I fibers within hind limb muscle of sheep (Fahey, Brameld, Parr, & Buttery, 2005). Conversely, more recent experimentation showed IUGR resulted in an increased proportion of type II fibers in sheep hind limb muscle and subsequent decrease in type I (Dustin T Yates et al., 2016). Of importance is long-term studies showing documented increases of type I fibers in IUGR sheep after 2 weeks of age, replaced by higher proportion of type II muscle fibers at 25 weeks (Daniel, Brameld, Craigon, Scollan, & Buttery, 2007). It could be postulated that early data showing type I fiber density increases is only a short-term rescuing effect (Brown, 2014). Overall, there is still speculation of the type of muscle fiber production that is favored in IUGR fetuses and offspring. In considering all the above studies, it could be theorized that *in utero* reprogramming during IUGR insult initially favors the mitochondrial-dense type I fibers to compensate for decreases in mitochondrial function, but then type I fiber concentrations are eventually decreased to reduce mitochondrial metabolism and

subsequent ROS production, in hopes of limiting the subsequent oxidative damage that occurs in association with IUGR.

The life-long effects of such switches are relatively unknown. Healthy adult humans have approximately the same number of satellite cells within type I and type II muscle fibers within the body (Kadi, Charifi, & Henriksson, 2006). Though, type II fibers do diminish faster with age (compared to type I), and therefore have diminished regenerative capacity later in life (Verdijk et al., 2014). When exposed to *in utero* stress, like IUGR, satellite cell populations are diminished (Stange, Miersch, Sponder, & Röntgen, 2020), providing the basis for the hypothesis that IUGR could inhibit previously described muscle regeneration in adulthood.

1.4.3 Relationship between mitochondrial function and myogenesis

Of note, is the role the mitochondria play in myogenesis, and vice versa. Differentiation of myoblasts is associated with an increase in oxidative phosphorylation and therefore, mitochondrial biogenesis (Wagatsuma & Sakuma, 2013). Comparably, MYOD1 was found to bind with PPAR-γ coactivator-1-β (PGC-1β), alongside other metabolically related genes and positively modulate oxidative metabolism (Shintaku et al., 2016). Similarly, muscle metabolism is highly regulated by the peroxisome proliferator-activated receptor (PPAR) family of transcription factors, in part by their regulation of fiber type distinction, in concert with PGC-1α (Schuler et al., 2006). PGC-1α- induced skeletal muscle remodelling is associated with conversion of glycolytic fibers types to those that favour oxidative metabolism (Mortensen, Frandsen, Schjerling, Nishimura, & Grunnet, 2006). Hence, adequate myogenesis is responsible for proportion of mitochondrial metabolism and subsequent function, while mitochondrial function is also important for the growth of skeletal muscle. Interestingly, skeletal muscle mitochondrial dysfunction specifically is evident in IUGR offspring, in both fetal and postnatal studies (K. Cheng et al., 2020; Pendleton et al., 2020), and it is postulated that both findings are the result of OS (J. A. Kim, Wei, & Sowers, 2008; Rodríguez-Rodríguez et al., 2018). It is important to note that mitochondrial dysfunction has been investigated as a key contributor to the susceptibility of metabolic disease observed within IUGR offspring

(Liu et al., 2012; Pendleton et al., 2020). Therefore, it appears OS programs both inhibitions and myogenesis and downstream mitochondrial function directly, that likely contribute to NCD risk.

1.4.4 Muscle Mitochondrial Dysfunction

Increased ROS production in part due to mitochondrial electron leakage during OS can damage the organelle's proteins, enzymes, and lipids directly resulting in increased electron leakage and further free radical production. Hence, mitochondrial dysfunction ultimately facilitates more ROS generation and sustains OS through a positive feedback loop of damage. Additionally, continued OS exposure to the organelle results in mitochondrial DNA damage, leading to mutations in the mitochondrial genome that compound existing dysfunction as a result of previously mentioned OS damage (Hollensworth et al., 2000). Of note is the specific decrease in expression of Pgc-1α in IUGR offspring, that both contributes to OS and to further mitochondrial dysfunction (Pendleton et al., 2020; Zeng, Gu, Liu, & Huang, 2013).

Though commonly referred to in the context of mitochondrial biogenesis, the increase of mitochondrial mass, protein peroxisome proliferator-activated receptor-gamma coactivator (PGC)-1α is also a regulator of the mitochondrial antioxidant defense system that works to mitigate OS and prevent the described mitochondrial damage. In environments of elevated hypoxia and OS, induction of PGC-1α is evident (Zhu et al., 2010) and overexpression of the coactivator is associated with decreased ROS accumulation and relative reduction of mitochondrial apoptosis (Valle, Álvarez-Barrientos, Arza, Lamas, & Monsalve, 2005). Additionally, mitochondrial SOD (MnSOD/SOD2) levels are increased upon similar overexpression (St-Pierre et al., 2003). Overall, PGC-1α's protective effects within the mitochondria appear to mediated in-part due to a PGC-1α – MnSOD dependent pathway through an interaction with the mitochondrial deacetylase, Sirtuin 3 (SIRT3) (Kong et al., 2010; Wang et al., 2015). SIRT3 is a known regulator of mitochondrial function and energy homeostasis (Ahn et al., 2008), as a downstream target of PGC-1α (Kong et al., 2010). It is through activation of SIRT3 that increased PGC-1α expression elicits increased MnSOD levels. SIRT3 can

additionally feedback to PGC-1α itself to promote mitochondrial biogenesis and overall function when expressed to aid in prevention of the organelle's demise due to OS (Jornayvaz & Shulman, 2010; J. Zheng et al., 2018). Under physiological stress, like OS, PGC-1α interacts with transcriptional factors involved in mitochondrial biogenesis, including nuclear receptor factors (NRF) 1 and 2, and transcription factor A, mitochondrial (TFAM). Increased expression of PGC-1α induces the transcription of NRF-1 and 2, which in turn increase expression of TFAM (Taherzadeh-Fard et al., 2011). TFAM is essential for oxidative phosphorylation, encoding 13 components of the ETC (Bonawitz, Clayton, & Shadel, 2006; Falkenberg, Larsson, & Gustafsson, 2007), in addition to driving mitochondrial biogenesis through regulation of gene transcription and replication of mitochondrial DNA (Ngo, Lovely, Phillips, & Chan, 2014). In short, the decrease in PGC-1α evident during IUGR is likely a large contributing factor to the mitochondrial dysfunction evident in IUGR due to decreases in mitochondrial biogenesis but may also contribute to further mitochondrial damage by OS because of reduced expression of downstream MnSOD.

1.5 Antioxidant Therapy

As a result of the host of diseases that have been attributed in some part to OS, the potential for exogenous antioxidant therapies to prevent and treat said disorders is notable. Exogenous antioxidant consumption within our regular diets complements existing endogenous antioxidant defense mechanisms. These groups of dietary antioxidants include ascorbic acid (vitamin C) and tocopherol (vitamin E), carotenoids, and polyphenols, and can be classified as "bioactive". A bioactive compound is one in which, after consumption, can elicit an effect on the body. This effect can be positive or negative, and effective dosing of said compounds is critical in preventing negative consequences.

1.5.1 Early Studies of Antioxidant Treatment Options

The emergence of OS as a contributing factor to preeclampsia in early research prompted studies utilizing vitamin C and E supplementation to attempt to reduce occurrence of the disorder. Similarly, early studies on pregnancy loss found an association between miscarriage as well as IUGR and decreased maternal vitamin C and E serum

concentrations. Vitamin C acts a scavenger for ROS, eliminating them to minimize OS. Vitamin E is similar in function, as it scavenges lipid peroxyl radicals of lipid peroxidation to prevent ROS-mediated cellular damage and more recently has been documented to be an inhibitor of NADPH oxidase activity, a previously mentioned contributor to OS. Initial studies found vitamin C and E supplementation decreased expression of markers of preeclampsia, specifically the ratio of PAI-1 to PAI-2, which is a common clinical measure for the disorder. Incidence of preeclampsia was also significantly reduced in those who were diagnosed with predisposition to preeclampsia after taking both vitamins. These findings encouraged further research, with several others trying to replicate the positive results, including in models of IUGR. Unfortunately, reproducibility of the positive effects on preeclampsia occurrence was low, and high doses of each vitamin were needed to elicit positive effects in pregnancy. Concerningly, the high dosages experimented with in animals would pose health risks to humans (D.-H. Lee, Folsom, Harnack, Halliwell, & Jacobs, 2004). Lastly, several studies showed vitamin C and E supplementation during pregnancy increased the risk of premature rupture of membranes (PROM) (Spinnato et al., 2008). In 2019, the World Health Organization (WHO) concluded vitamin C and E is not recommended to improve maternal and perinatal outcomes during pregnancy (WHO, n.d.).

1.5.2 Mitochondria-Targeted Therapies

Mitochondrial dysfunction is evident during several other adverse pregnancy conditions linked to OS like PI-IUGR, in addition to the role it plays in metabolic disease (Bhatti, Bhatti, & Reddy, 2017). As such, recent research into treatments for such disorders has focused on those that target the organelle and promote functioning.

Resveratrol (RSV) is a bioactive polyphenol compound found in plants synthesized to protect the organism against injury. It is found in a variety of food and drink products but is most widely recognized for its existence in wine. It initially became popular as a potential beneficial supplement when studies showed cardiovascular health benefits after regular and moderate wine drinking as a result of RSV consumption. Several studies have shown the compound to act as an antioxidant, though it is not a strong free radical

scavenger itself. RSV primarily elicits its antioxidant effects by activating transcription factors important for redox balance within the body, including the activation of mitochondrial-bound antioxidant pathways through increased Sirt1 expression to diminish ROS production by the organelle (Ungvari, Sonntag, De Cabo, Baur, & Csiszar, 2011). RSV additionally directly promotes proper mitochondrial biogenesis through this same pathway (Ungvari et al., 2011). Compared to vitamins C and E, RSV's effects on pregnancy have not been thoroughly studied in humans It has however been shown to cross the placenta in several animal studies and increase uterine blood flow, thereby increasing oxygen delivery and indirectly decreasing hypoxia-induced ROS in the fetus. In a rodent model of preeclampsia, antioxidant enzyme SOD was upregulated in placental trophoblasts following maternal consumption of RSV, and oxidative stress, defined as relative malondialdehyde (MDA) content, was reduced to a level that prevented apoptosis of in the same placental cells during pregnancy (Zou et al., 2014). It is important to note that RSV bioavailability appears to be low due to rapid metabolism within the body (Sergides, Chirilă, Silvestro, Pitta, & Pittas, 2016). It is therefore likely that continued supplementation throughout pregnancy would be needed to elicit an effect on the mother and fetus during pregnancy (S. Zheng, Feng, Cheng, & Zheng, 2018) and overall, the safety and efficacy of such treatment is not yet well understood, though preliminary data shows RSV exposure throughout gestation may inhibit fetal pancreas development (Roberts et al., 2014).

MitoQ is an antioxidant compound specifically designed to mimic the endogenous mitochondrial antioxidant coenzyme Q10 (CoQ10) (Tauskela, 2007). CoQ10 serves as a cofactor to the ETC to promote energy production and is a significant lipid antioxidant that simply reduces production of ROS (Saini, 2011). MitoQ was designed to specifically accumulate within the mitochondria and augment the action of existing CoQ10 (Gottwald et al., 2018). Human studies of MitoQ's effects in pregnancy are limited and the manufacturer currently recommends it not be taken while pregnant. Early research showed that in rodent models of placental insufficiency, MitoQ does protect against placental hypoxia and subsequent OS by increasing placental maternal blood space volume and surface area (Nuzzo et al., 2018). Unfortunately, MitoQ uptake within the

developing fetus was low, and it was concluded the beneficial effects of MitoQ will likely only be seen at the placental level (Nuzzo et al., 2018), inferring that administration of MitoQ after onset of placental dysfunction would not help prevent negative fetal changes that may have already occured. The compound's specific effects in incidences of preeclampsia and IUGR are relatively unknown. Though, one recent study using a mouse model of reduced placental perfusion concernedly found that early pregnancy MitoQ supplementation exacerbated placental dysfunction by inhibiting placenta development and that it can also increase the risk of preeclampsia development (Yang et al., 2021). It has been concluded since that with early pregnancy administration of antioxidants, and the role ROS play as signaling molecules that promote placental development needs to be considered in future research (Yang et al., 2021).

Of particular interest is the novel bioactive antioxidant-like compound pyrroloquinoline quinone (PQQ), found in commonly consumed food items, especially kiwi, parsley, and soybeans (Kumazawa, Sato, Seno, Ishii, & Suzuki, 1995). It is also found in breast milk, highlighting the plausible importance of the compound in early postnatal life (Mitchell, Jones, Mercer, & Rucker, 1999). Like both MitoQ and RSV, it has positive effects on mitochondrial function, including in vitro stimulation of biogenesis within the liver through activation of PGC-1α (Chowanadisai et al., 2009). As previously described, increased PGC-1α expression is associated with increased activity of mitochondrial antioxidants (Kong et al., 2010; Wang et al., 2015), which is in part how PQQ additionally reduces ROS production and OS. PQQ also directly acts as a ROS scavenger itself (Misra et al., 2004). Overall, PQQ has been shown to be 100-1000 times more effective than other antioxidants at reducing concentrations of ROS within the body (T. E. Stites, Mitchell, & Rucker, 2000). Lastly, it is effective at microgram quantities (Harris et al., 2013) unlike vitamin C and RSV, and therefore diminishing the risk that it will elicit harmful effects in pregnancy like those seen with high-dose vitamin C and E supplementation.

PQQ’s effects during pregnancy are not well studied; many of its known positive effects were studied in a postnatal or adult environment. It has however known to increase

placental surface area and weight (Jonscher, Stewart, Alfonso-Garcia, DeFelice, Wang, Luo, Levi, Heerwagen, Janssen, De La Houssaye, et al., 2017), as well as significantly increase the number of viable offspring and expression of antioxidant genes within the placenta when given throughout pregnancy in sows (B. Zhang et al., 2019). Similarly, Jonscher et al. (2017) additionally found prenatal PQQ treatment decreased indices of liver OS, induced by maternal obesity and a high fat diet, in offspring of mice fed PQQ throughout gestation. In regard to IUGR, PQQ shows promise in that it positively acts on PGC-1α (Chowanadisai et al., 2010) which was previously stated to be downregulated in IUGR (Zeng et al., 2013) and other adverse metabolic conditions (Riehle & Abel, 2012; Son et al., 2020). Overall, direct studies of the effects of PQQ supplementation during PI-IUGR are lacking, and its effects on skeletal muscle development *in utero* are unknown.

1.6 Thesis Rationale and Objectives

1.6.1 Rationale

The prevalence of noncommunicable diseases continues to increase throughout the world (Moore et al., 2017; Ranasinghe et al., 2017). An adverse in utero environment during development increases the risk of developing these disorders in adulthood (Dunlop, Cedrone, Staples, & Regnault, 2015) including the relatively hypoxic environment associated with PI-IUGR (Rueda-Clausen et al., 2011). As blood flow is diverted to the brain and heart, which are more critical for long-term survival, diminished concentrations of oxygen and nutrients reach the developing skeletal muscle and growth is impeded. The reduction in growth is associated with changes to mitochondrial gene and protein expression and subsequent alterations in mitochondrial function. This phenomenon results in increased incidences of IR, a key precursor to NCD (J. A. Kim et al., 2008; Simmons, Suponitsky-Kroyter, & Selak, 2005; H. Zhang, Li, Hou, Zhang, & Wang, 2016). Skeletal muscle is the principal organ responsible for glucose uptake, and alterations to mitochondrial gene expression impede this process, contributing to the generation of IR and subsequent NCD. In short, the adverse *in utero* environment is

proposed to reprogram key muscle mitochondrial genes, contributing to the predisposition to metabolic disease observed in IUGR offspring.

PI-IUGR is a form of placental failure that results in the development of fetal OS (Guvendag Guven, Karcaaltincaba, Kandemir, Kiykac, & Mentese, 2013). As blood oxygen concentrations decrease during IUGR progression, an increased concentration of ROS is produced (Rashid, Bansal, & Simmons, 2018). This increase in ROS cannot be subdued by endogenous antioxidant mechanisms, and hence forth, there is development of OS. Mitochondrial dysfunction is often defined as a reduction in ETC complex abilities and overall energy production. It is widely observed that OS and mitochondrial function have an inverse relationship, in that increased OS can damage the organelle (Hollensworth et al., 2000) and the mitochondria themselves can contribute to OS production under adverse conditions (Taddei et al., 2012). Gene and associated protein expression related to mitochondrial function such as those of the ETC have been reported to be significantly decreased in *in vivo* and *in vitro* models of OS (Guitart-Mampel et al., 2019; Pendleton et al., 2020). Of note, OS is associated with epigenetic modulating effects (Campos et al., 2007), and it has been postulated that this the mechanism behind decreased expression of key mitochondrial function genes observed in IUGR.

Given that OS is associated with several adverse pregnancy conditions in addition to PI–IUGR, exogenous antioxidant therapies to treat these conditions has and continues to be explored. Initial research examined the use of vitamin C and E as treatment options for the OS-associated pregnancy conditions such as preeclampsia (Shennan & Duckworth, 2010). Data showed no benefit, and in some cases, more harm including increased incidence of premature rupture of membranes (PROM), as a result of vitamin C and E exposure together during pregnancy (Spinnato et al., 2008). Acknowledging the importance of mitochondrial dysfunction in OS progression and furthermore the association between disease states and mitochondrial dysfunction, recent research has focused on compounds specifically targeting the organelle. Resveratrol, a bioactive polyphenol, has been shown to increase mitochondrial function, including ETC complex activity (Gueguen et al., 2015), but its low bioavailability suggests the need for continued

maternal consumption through pregnancy to elicit effects, which may harm development of the fetus (Roberts et al., 2014). MitoQ, a compound designed to mimic an endogenous mitochondrial antioxidant, but early gestation supplementation was shown to inhibit placental development (Yang et al., 2021).

A possibly more effective supplement is the compound pyrroloquinoline quinone (PQQ). PQQ is a bioactive, antioxidant-like compound shown to promote mitochondrial biogenesis alongside decreasing OS (Chowanadisai et al., 2010; Nunome, Miyazaki, Nakano, Iguchi-Ariga, & Ariga, 2008). PQQ is active at only milligram quantities (T. Stites et al., 2006) and participates in redox cycling to recycle its antioxidant abilities (He, Nukada, Urakami, & Murphy, 2003). The ability for it to function at small quantities in comparison to other conventional antioxidant suggests PQQ can act without the adverse effects reported in other studies, like those observed with Vitamin C (D.-H. Lee et al., 2004). For reference, humans consume an average of 0.1-1.0 mg per day in their diet (Harris et al., 2013).

Harris et al. (2013) found that at consumption of 0.2 - 0.3 mg PQQ/kg/day for 2-3 days resulted in a significant increase to antioxidant activity, and a reduction in indices of inflammation within collected plasma. PQQ with the same scaled doses, accounting for heightened metabolism in mice, found similar results, in that postnatal offspring were protected from hepatic lipotoxicity and inflammation if exposed to PQQ *in utero* and in early life (Jonscher, Stewart, Alfonso-Garcia, DeFelice, Wang, Luo, Levi, Heerwagen, Janssen, de la Houssaye, et al., 2017). Overall, though the positive effects of PQQ are evident in postnatal studies, its effects in a *in utero* setting are relatively unknown. Additionally, limited studies have investigated the effects of PQQ on skeletal muscle development.

1.6.2 Objectives and Hypotheses

The first objective was to determine the effects of short and long-term PQQ treatment on muscle cell differentiation and markers of mitochondrial function in a cell culture model

of fetal skeletal muscle development, when challenged with H_2O_2 mimicking an OS environment. It was postulated that H_2O_2 would negatively alter skeletal myogenesis pathways, and that PQQ treatments would mitigate OS and rescue myogenesis and markers of mitochondrial function.

The second objective was to study the effects of maternal PQQ administration during pregnancy on normal growth and IUGR fetal skeletal muscle myogenesis and markers of mitochondrial function. It was postulated that IUGR would be associated with altered myogenesis and markers of decreased mitochondrial function while PQQ consumption during pregnancy would reduce measures of OS, in conjunction with the promotion of myogenesis and muscle mitochondrial function in both normal and IUGR fetuses.

2 Methods and Materials

2.1 C2C12 Cells: H_2O_2 and PQQ Supplementation

The C2C12 immortalized *Mus muculus* (i.e. mouse) myoblast cell line was utilized for *in vitro* experimentation. The cell line is a subclone of myoblasts originating from the thigh muscle of an adult female C3H mouse (Yaffe & Saxel, 1977), which is capable of rapid differentiation and has contractile abilities similar to that of developing skeletal muscle (Nedachi, Fujita, & Kanzaki, 2008). It is commonly utilized to mimic developing *in utero* skeletal muscle due to similarities in gene expression and regulation of myogenic processes between the cells and *in vivo* muscle (Burattini et al., 2004; Rajan et al., 2012).

C2C12 myoblasts (Group A), from passage 2 to 20, were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin until ~70-80% confluency was reached. Cells where then induced to differentiate for 7 days into myotubes by supplementing media with 2% horse serum (HS) and 1% penicillin/streptomycin instead of FBS. An additional subset of cells was cultured with the addition of 110 mg/L sodium pyruvate in both the FBS and HS DMEM media (Group B). All cells were incubated at 21% O_2 and 5% CO_2 (with N_2 balance) at 37℃. After seven days of differentiation, cells were serum starved for five hours to induce cell cycle synchronization (M. Chen et al., 2012). Cells and culture media were then collected for mRNA, protein, and activity analysis.

A subset of cells was incubated with either 1uM or 10uM of PQQ (Sigma-Aldrich; CAS number 72909-34-3) for the total seven days, final 48 hours, or final 5 hours of differentiation. A further subset was exposed to 750uM or 1mM H_2O_2 (to simulate *in utero* oxidative stress) (Figure 2.1-1)

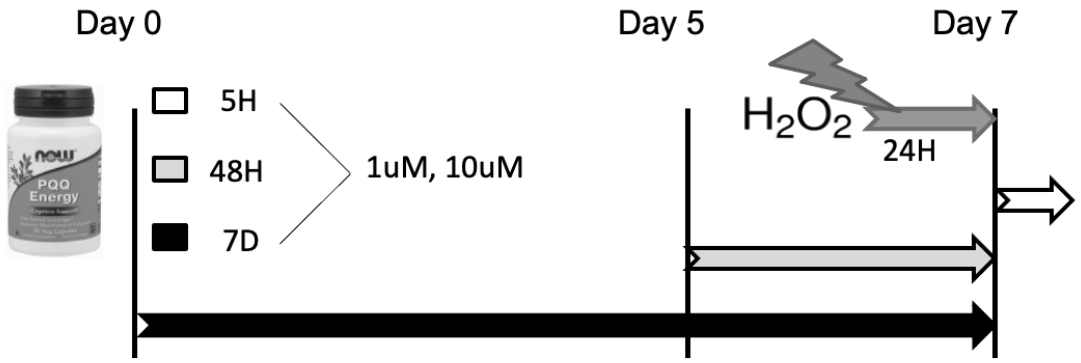


Figure 1.6.2-1 Cell Culture Model.

C2C12 myoblasts were differentiated for seven days to form myotubes. 1uM or 10uM of PQQ was administered for all 7 days of differentiation, or the final 5H or 48H of differentiation. 750uM or 1mM H_2O_2 was administered for the final 24H of differentiation to induce oxidative stress.

2.2 Guinea Pig Model of Spontaneous IUGR

All animal procedures were conducted in accordance with the Canadian Council of Animal Care guidelines. The Animal Use Protocol (AUP #2018-110) was approved by the Western University Animal Care Committee. Time-mated pregnant Dunkin-Hartley guinea pigs (Charles River Laboratories, Wilmington, MA) were housed on-site in separate enclosures. Guinea pigs are an adequate model for human pregnancy due to several anatomical and physiological similarities present throughout gestation. These similarities including comparable progesterone levels, as well as sharing a hemomonochorial placental structure which except the same mechanisms of spiral artery remodeling. Finally, guinea pigs have longer pregnancies compared to other rodent species, resulting in more mature neonates like humans (Harrell et al., 2017)

The animals were housed in a temperature and humidity-controlled environment, with a 12:12-hour light-dark cycle. Food (Guinea Pig Diet 5025; Lab Diet, St. Louis, MO) and water were provided *ad libitum*. Food consumption, water consumption, and body weight were measured daily, in addition to measuring litter size (Table 1).

At mid-gestation (37 days; term ~69 days), dams were randomly assigned to either the PQQ or control groups. For dams in the PQQ group, PQQ was administered in drinking water a concentration of 1.5 mg/L. Given an average water consumption of 125 mL/kg/day, this concentration corresponds with an average dose of 0.18 mg PQQ/day/kg/, a scaled equivalent of doses administered in other studies in mice and humans (Harris et al., 2013; Jonscher, Stewart, Alfonso-Garcia, DeFelice, Wang, Luo, Levi, Heerwagen, Janssen, de la Houssaye, et al., 2017). As stated, initial human studies utilizing doses ranging from 0.2 – 0.3 mg PQQ/kg/day found that consumption of the compound had a positive effect on reducing markers of inflammation and positively promoting mitochondrial metabolism (Harris et al., 2013). Maternal PQQ consumption did not significantly affect fetal or maternal parameters measured (Table 1 and 2).

Dams were euthanized by CO_2 inhalation on gestational day 65. Following euthanasia, fetuses were dissected from damns, weighed, and sex was determined. Brain-to-liver weight ratio (BtL) was calculated to determine if a fetus was growth restricted or not. Guinea pig pregnancies demonstrate spontaneous IUGR as a result of large litter sizes and therefore no *in utero* manipulation was needed to induce said pregnancy complication. Fetuses were classified as IUGR if BtL was >0.65 and fetal weight was <80g, with fetuses outside both thresholds classified as normal growth (NG) (Table 2). Placental weight and ratio to fetal weight was also measured (Table 2). Fetal male gastrocnemius tissue was flash frozen in liquid N_2 and stored at -80℃ for future analyses. Gastrocnemius muscle was selected as it has both type I oxidative and type II glycolytic fibers versus the relatively more oxidative muscle, the soleus.

Table 1 Maternal Guinea Pig Characteristics

Group	Litter Size	Weight (g)	Food Consumption	Water Consumption
Control Water	4.667 ± 0.8819	1158 ± 198.7	51.73 ± 4.901	121.3 ± 25.47
PQQ Water	5.500 ± 0.2887	1350 ± 54.51	48.28 ± 3.054	107.6 ± 26.54

Data presented as mean ± SEM. Significance determined by two-way ANOVA; no significant changes were observed.

Table 2 Fetal Guinea Pig Characteristics

Group	Fetal Weight (g)	Brain-to-Liver Ratio	Placental Weight (g)	Placental – to – Fetal Weight
Normal Growth (NG)	102.641 ± 3.373	0.490 ± 0.040	4.991 ± 0.298	0.049 ± 0.002
Intrauterine Growth Restriction (IUGR)	***67.479 ± 4.288*** ***	***0.848 ± 0.117*** ***	***4.240 ± 0.636*** *	***0.062 ± 0.004*** **
Normal Growth + Maternal PQQ Consumption (NG/PQQ)	93.227 ± 4.987	0.535 ± 0.023	4.697 ± 0.339	0.049 ± 0.002
Intrauterine Growth Restriction/Maternal PQQ Consumption (IUGR/PQQ)	***71.883 ± 7.820*** ***	***0.778 ± 0.068*** ***	***3.813 ± 0.228*** *	***0.053 ± 0.003*** **

Data presented as mean ± SEM. Significance determined by two-way ANOVA, *p<0.05, **p<0.01, ***p<0.001.

2.3 Rt-qPCR

Adherent C2C12 cells (Group A) were collected in Trizol reagent and stored at -80℃ before RNA isolation. Guinea pig gastrocnemius muscle was powered using a mortar and pestle in liquid N_2 and stored in Trizol reagent (Invitrogen) at -80℃. Both cell and muscle samples were thawed, and 200 microliters of chloroform was added for every 1mL used during initial collection. Samples were briefly gently shaken by hand to combine contents and then centrifuged for 15 minutes at 12000 x rcf at 4℃. The mixture separated into a lower red phenol-chloroform phase, a middle interphase, and a colourless upper aqueous phase. 400uL of the upper phase was removed and put into a new tube, and then 500uL of isopropanol was additional added for every 1mL of Trizol used initially. This new mixture was briefly vortexed prior to centrifugation at 12000 x rcf at 4℃ for 15 minutes. The supernatant was removed, leaving only the RNA pellet. The pellet was washed by adding 1mL of 75% ethanol per 1mL of initial Trizol, then centrifuged at 7500 x rcf for 10 minutes at 4℃. Samples were then stored at -20℃ overnight. The next day, the ethanol was removed, being careful not to disrupt the pellet, and 1mL of new ethanol was added before centrifugation at 7500 x rcf for 10 minutes at 4℃. This step was repeated once more before removing all ethanol. The pellet was then air-dried and dissolved in PCR-grade water. Samples were stored at -20℃ until further analysis.

The quality and quantity of RNA yield was determined by the NanoDrop 2000 spectrophotometer (Thermo-Scientific). Quality was specifically measured using the A260/280 ratio (≥1.8). To further ensure RNA quality, samples were separated on a 1.5% agarose gel stained with RedSafe in a formaldehyde buffer at 100V for 30 minutes. Samples were screened for degradation by visualization using the Bio-Rad ChemiDoc Imager of the 28S and 18S bands. Only samples without degradation (i.e., clear 28S and 18S bands) were used for future experiments.

Three micrograms of total RNA were used for reverse transcription using M-MLV Reverse Transciptase (200U/uL) (ThermoFisher #28025013) in a 20-uL reaction volume. In a nuclease-free microcentrifuge tube, 1uL of Oligo (dT)21 (40uM), 0.5uL of random

primers (100uM), 1uL dNTP mix (10mM each dATP, dGTP, dCTP, and dTTP at neutral pH) and 3 ug of total RNA were combined. Mixture was adjusted to a final volume of 13uL with RNase-free water and heated at 65℃ for 5 minutes before being chilled on ice. Contents from tube were collected by brief centrifugation and 4uL of 5X First-Strand Buffer and 2uL of DTT (0.1M) was added. Contents were gently mixed before incubation at 37℃ for 2 minutes, after which 1uL of M-MLV RT (200 units) was added. A final incubation at for 100 minutes at 37℃ occurred, and then the reaction was inactivated by heating at 70℃ for a final 15 minutes.

Primer sets were designed using the NCBI Primer-Blast tool based on published *Mus musculus* and *Cavia porcellus* sequences (Table 1 and 2). Primer fidelity was assessed by performing a quantitative PCR (qPCR) reaction on a pooled cDNA sample using SensiFastTM SYBR® No-ROX kit (FroggaBio) with Bio-Rad CFX384 detection system, at 60℃ (primers were designed for a Tm of 60℃. DNA products were electrophoresed on a 2% agarose gel stained with RedSafe dye in a TAE running buffer at 100V for 30 minutes. DNA products were visualized using Bio-Rad ChemiDoc Imager and only primers with products of the predicted size were used. Primer efficiencies were measured and only those with efficiency >90% were used for qPCR analysis.

qPCR was performed using the SensiFastTM SYBR® No-ROX kit (FroggaBio) on the CFX 384 real-time PCR detection system (Bio-Rad) at a denaturing temperature of 95℃, annealing temperature of 60℃, and elongation temperature of 75℃ for 40 cycles. A total volume of 12uL in each well contained 2uL of diluted cDNA, 0.048uL of primer mix, 6uL of SYBR Green, and the remaining volume was filled with PCR-grade water. The data was analyzed using the $2^{-\Delta\Delta ct}$ method (Livak and Schmittgen, 2001) using Hrpt, Rsp12, Tubb, or Rpl13 as housekeeping genes (Masilamani, Loiselle, & Sutherland, 2014).

Table 3 Mouse (*Mus musculus)* Primer Pairs

Gene	NCBI Accession #	Strand	Sequence (5' → 3')
Cox7a1	NM_009944.3	Forward	TCTTCCAGGCCGACAATGAC
		Reverse	GCCCAGCCCAAGCAGTATAA
Hprt	NM_013556.2	Forward	ATGGACTGATTATGGACAGGACTG
		Reverse	TCCAGCAGGTCAGCAAAGAAC
Myod1	NM_010866.2	Forward	TGCTCTGATGGCATGATGGATTA
		Reverse	AGATGCGCTCCACTATGCTG
Myog	NM_031189.2	Forward	GAAGCGCAGGCTCAAGAAAG
		Reverse	CGCGAGCAAATGATCTCCTG
Ndufb6	NM_001033305.3	Forward	CGTACCGCTCCAGTCTCTTC
		Reverse	CCCTTAAGAGGGATGCTGCC
Pax7	NM_011039.2	Forward	CGATTAGCCGAGTGTCTCAGA
		Reverse	TCCAGACGGTTCCCTTTGTC
Pgc-1α	NM_008904.2	Forward	AAGGATGCGCTCTCGTTCAA
		Reverse	CATAGCTGTCGTACCTGGGC
Rps12	NM_011295.6	Forward	AAGGCATAGCTGCTGGAGGTGTAA
		Reverse	AGTTGATGCGAGCACACACAGAT
Tfam	NM_009360.4	Forward	TAGGCACCGTATTGCGTGAG
		Reverse	GACAAGACTGATAGACGAGGGG

Table 4 Guinea Pig (*Cavia porcellus)* Primer Pairs

Gene	NCBI Accession #	Strand	Sequence (5' → 3')
Atp5a1	XM_003474067.4	Forward Reverse	GCTGCCCAAACTAGGGCTAT GAGTGGCAGCATCGAGATCA
Atp5pb	XM_003479227.4	Forward Reverse	ACCCTACATGCTTGGAACTGG CGCTTCTGAACAAGTGCCTG
β-Tubulin/ Tubb	XM_003460999.4	Forward Reverse	TGGTCGGCCTCTCAGAATCTT TTATCACCTCCCAGAACTTGGC
Cox4i2	XM_003476668.3	Forward Reverse	CCACCAAATCAGCAAAGCCG CATGCCGGATGAGCCTTTCT
Cox7a1	XM_003467228.4	Forward Reverse	CAGTGTGTGTCCTTGTCCGA GGAGGTCATTGTTCTCCTTGGAA
CS	XM_003475969.4	Forward Reverse	GGCATATGCAGAGGGTGTCA CCGATACTACTGCCCTCACG
Mhy1	XM_003466219.2	Forward Reverse	GTGGACAAACTGCAAGCCAA ATGTCTTTGGTCACTTTCCTGCAT
Mhy2	XM_003466220.3	Forward Reverse	GGAGGAGGCTGAGGAACAAT GTCTTGCTCTGGTCATTCCACA
Myh4	XM_013148710.1	Forward Reverse	GTAATTGCCTGCTTTGAGCCTG TAGCTCCGCCTTCTGTCTTG
Myh7	XM_003474485.3	Forward Reverse	TTCCGAAAGGTGCAGCATGA TTCCTCCCAAGGGGCTGTTA

Myod1	XM_003465725.2	Forward	TCCGACGGCATGATGGACTA
		Reverse	GCTGTAATAGGCACCGTCGT
Myog	XM_003474821.2	Forward	CCCAAGGTAGAGATCCTGCG
		Reverse	TACATTCACTGGGCACCGTAG
Ndufb6	XM_003470823.4	Forward	GCCCCCGAATATTCCCAGGTG
		Reverse	CATGGCTTAAAAGCCTTTGACATTT
Pax7	XM_003471164.2	Forward	GTGCCCTCAGTGAGTTCGAT
		Reverse	GTCCAGTCGGTTCCCTTTGT
Pgc-1α	XM_003467408.4	Forward	CCCAAGGGTTCCCCATTTGA
		Reverse	CCCAAGGGTTCCCCATTTGA
Ppar- α	NM_001173004.1	Forward	AAGGCCTCAGGCTACCACTA
		Reverse	CAGGTGAGGACTTCGGCTTT
Ppar-δ	XM_013156813.2	Forward	CCAGCAGTTACACAGACCTCC
		Reverse	CCGTAGTGGAAGCCAGACG
Ppar-γ	XM_003462736.4	Forward	CAATAGGCCTCACGAGGAGC
		Reverse	ATCCGCCCAAACCTGATGG
Rpl13	XM_003461190.4	Forward	TCAATCAGCCAGCTCGGAAG
		Reverse	GTGAATACCAGCCACCCGAA
Sirt1	XM_013156679.2	Forward	TTGCAACTGCATCTTGCCTG
		Reverse	TCATGGGGTATGGAACTTG
Sirt3	XM_004999545.3	Forward	TCCCCTGGAGGTGGAACCTTTT
		Reverse	CCAGCTGGAACAGAGAAGA
Tfam	XM_003473570.4	Forward	TGCGCTCACCTTTCAGTTTTG
		Reverse	TGTGCCAAAACTGCTCTCTCA
Ucp2	XM_013145311.2	Forward	CGACGTGGTCAAGACGAG
		Reverse	AGGAGGGCATGAACCCTTTG

2.4 Western Blots

2.4.1 Total Protein Extraction and Immunoblotting

Adherent C2C12 cells (Group A) were washed with cold PBS in the dish. Cold radioimmunoprecipitation assay (RIPA) buffer, supplemented with protease and phosphatase (1ug/mL leupeptin and aprotinin, 1mM PMSF, 1mM sodium orthovanadate, 2mM sodium fluoride) inhibitors, was utilized to extract total proteins from the cells; cells were left in the buffer for 5 minutes before being scraped off plates with a plastic policeman and collected. Cells were mixed by vortexing for 5 seconds and incubated on ice for 5 minutes. Cells were further mixed by pipetting up and down 10 times then sonicated at an amplitude of 30 for 5 seconds. Samples were lastly centrifuged at 16000 x rcf at 4℃ for 10 minutes and supernatants were collected and stored at -20℃ for future experiments.

Protein quantity was measured using the Pierce™ BCA Protein Assay Reagent Kit (ThermoFisher) and a microplate reader. 30ug of total protein was mixed with 5x Laemmli sample buffer with 100uL beta-mercaptoethanol (BME), boiled for 5 minutes, and loaded onto a hand-cast SDS-PAGE gel (4% stacking layer, 10-15% separating layer). Gel electrophoresis was conducted at 70-75V until samples migrated through the wells, then ran at 120-30V for 60-90 minutes in 1X running buffer. After proteins were sufficiently separated, they were transferred from the SDS-PAGE gradient gels onto polyvinylidene fluoride (PVDF) membranes (Amersham™ Hybond™ P 0.2 um PVDF) for 90-120 minutes at 100V using 1X transfer buffer. The membranes were Ponceau stained, followed by a methanol rinse, then imaged with the Bio-Rad ChemiDoc Imager to show efficient transfer, equal loading of protein, and allow for total protein normalization (Figure 2.4.1-1).

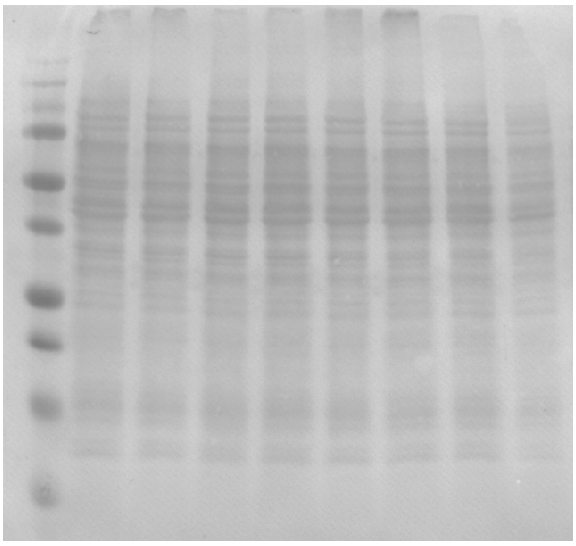


Figure 2.4.1-1 Representative Ponceau S Image

Ponceau S staining binds to protein transferred to PVDF membranes. Staining allows for the determination the extent and success of the protein transfer that occurred, and visible protein lanes can then be utilized for total protein normalization (Goldman, Harper, & Speicher, 2016).

Following several washes in Tris-buffered saline with Tween 20 (TBS-T) to remove Ponceau S stain, membranes were blocked in 5% milk or bovine serum albumin (BSA) in TBS-T for 1 hour at room temperature. They were then incubated with primary antibodies of 1:500-1:1000 dilutions in 5% milk or BSA at least overnight at 4℃ (Table 3). Following this incubation, the membranes were washed for 10 minutes, 3 separate times, in TBS-T before incubation with secondary antibodies (mouse or rabbit; Table 3) for one hour at room temperature

Following secondary antibody incubation, membranes were again washed in TBS-T three times for 10 minutes. Using the electrochemiluminescent substrate Clarity Max™ (BioRad), total protein abundance was then visualized using the ChemiDoc Imaging System (BioRad). Image Lab Software (BioRad) was employed to quantify protein bands.

Table 4 Western Blot Antibody List

Antibody	Brand and Catalog #	Dilution
LDHA (C4B5)	Cell Signaling; #3582	1:1000
OXPHOS (ETC Complexes)	Abcam; ab110413	1:1000
PGC-1α	ThermoFisher; PA5-38021	1:500
SIRT3 (D22A3)	Cell Signaling; #5490	1:1000
SOD2 (D9V9C)	Cell Signaling; #13194	1:1000
VDAC1/Porin [20B12AF2]	Abcam; ab14734	1:5000
Rabbit Secondary (Anti-rabbit IgG, HRP-linked)	Cell Signaling; #7074	1:10000
Mouse Secondary (Anti-mouse IgG, HRP-linked)	Cell Signaling; #7076	1:10000

2.5 Activity Assays

2.5.1 Lactate Dehydrogenase Activity

Cell culture media was collected from PQQ and H_2O_2 treated cells from Group B after 19 hours of 1mM H_2O_2 exposure. Media samples were frozen at -80℃ until utilized. Lactate dehydrogenase (LDH) activity within the samples was then measured using the colorimetric LDH Assay Kit (Abcam; CAT ab102526). In brief, 40uL of media was mixed with 10uL of assay buffer and plated in duplicate. A 1.25mM reduced nicotinamide adenine dinucleotide (NADH) standard curve dilution was also prepared according to the manufacturer's instructions to generate a curve composed of 0, 2.5, 5,

7.5, 10, and 12.5 nmol/well, plated in duplicate. 50uL of reaction mix (48uL of LDH Assay Buffer, 2uL LDH substrate mix) was added to each well with sample and standard, then the plate was mixed well. Using a microplate reader, the OD at 450nm was measured kinetically every 2-3 minutes for 60 minutes at 37℃, protected from light. Total protein concentration within the media was also measured and used to normalize the LDH concentration within each sample. Total LDH activity was expressed in mU/mL relative to total protein.

2.5.2 Superoxide Dismutase Activity

Adherent C2C12 (Group B) were washed with cold PBS before being scraped off the plate using a plastic policeman. Collected cells were centrifuged at 1000 x g for 10 minutes at 4°C. The cell pellet was then homogenized in a cold 20mM HEPES buffer, pH 7.2 (containing 1mM EGTA, 210mM mannitol, 70mM sucrose) before being centrifuged at 1500 x g for 5 minutes at 4°C. The supernatant containing total SOD (cytosolic and mitochondrial) was collected and stored at -80°C or centrifuged at 10000 x g for 15 minutes at 4°C to separate cytosolic SOD from mitochondrial SOD. The supernatant containing cytosolic SOD was then removed and stored at -80°C for future analysis. The remaining mitochondrial SOD pellet was homogenized in the cold buffer (20mM HEPES buffer, pH 7.2, containing 1mM EGTA, 210mM mannitol, 70mM sucrose) and stored at -80°C for future analysis.

SOD activity within the samples was then measured using the colorimetric SOD Assay Kit (Cayman Chemical; Item No. 706002) and protocol. In brief, 10uL of sample, plated in triplicate, was mixed with 200uL of diluted Radical Detector (Cayman Chemical; Item No. 706004) in 96-well plate. 20uL of SOD Standard (Cayman Chemical; Item No. 706005) with 1.98mL of dilute Sample Buffer (Cayman Chemical; Item No. 706003) was mixed to produce a 0 to 0.050 U/mL final SOD activity standard curve. Reaction was initiated by adding 20uL of diluted Xanthine Oxidase to all wells in use. The addition of potassium cyanide to a final concentration of 1-3mM to wells containing mitochondrial SOD samples to inhibit any residual cytosolic SOD. After mixing, the plate was incubated for 30 minutes at room temperature then the absorbance was read at 440-

460nm using a plate reader. Protein concentration of the samples was tested and used as an internal control to normalize the SOD concentration in the samples. Total SOD activity was expressed as U/mL relative to total protein.

2.5.3 Catalase Activity

Adherent C2C12 (Group B) were washed with cold PBS before being scraped off the plate using a plastic policeman. Collected cells were centrifuged at 1000 x g for 10 minutes at 4°C. The cell pellet was then homogenized in a cold 50mM potassium phosphate buffer, pH 7.0 (containing 1mM EDTA) before being centrifuged at 10000 x g for 15 minutes at 4°C. The supernatant was collected and stored at -80°C for future analysis.

CAT activity within the sample was then measured using the colorimetric Catalase Assay Kit (Cayman Chemical; Item No. 707002) and protocol. In brief, 10uL of Catalase Formaldehyde Standard (Cayman Chemical; Item No. 707014) with 9.99 mL of diluted Sample Buffer (Cayman Chemical; Item No. 707012) to obtain a 4.25 formaldehyde stock solution. This solution was further diluted to produce a standard curve with final concentration ranging from 0-75uM formaldehyde, plated in duplicate. Each standard well contained 100uL of diluted Assay Buffer (Cayman Chemical; Item No. 707010), 30uL of methanol, and 20uL of standard. Sample wells contained 100uL of the same diluted Assay Buffer, 30uL of methanol, and 20uL of sample, plated in duplicate. To initiate the reaction, 20uL of diluted hydrogen peroxide was added to all utilized wells, and then the plate was covered and shaken for 20 minutes at room temperature. 30uL of potassium hydroxide was then added to each well to terminate the reaction, followed by 30uL of Catalase Purpald (Cayman Chemical; Item No. 707017). The plate was again covered and shaken for 10 minutes at room temperature before addition of 10uL of potassium periodate (Cayman Chemical; Item No. 707018). The plate was covered and shaken one final time for 5 minutes at room temperature before the absorbance was read at 540 nm. Protein concentration of the samples was tested and used as an internal control to normalize the CAT concentration in the samples. Total CAT activity was expressed as nmol/min/mL relative to total protein.

2.6 Statistics

GraphPad Prism 9 was used for all statistical analyses. All data is presented in box-whisker plots, representing the median 25th and 75th quartiles, and minimum/maximum values. *In vitro* (I.e., in C2C12 myoblasts) enzyme activity, as well protein and gene expression were analyzed using a one-way ANOVA with a Dunnett's multiple comparisons test. *In vivo* (I.e., in fetal guinea pig muscle) gene expression was analyzed by two-way ANOVA and Tukey's multiple comparisons test. A p value of less than 0.05 was considered significant. Values were determined to be outliers if they were more than two standard deviations away from the mean.

3 Results

3.1 Effects of H_2O_2 and PQQ on LDH, CAT, and SOD activity and total protein abundance

3.1.1 LDH Activity and Total Protein Abundance

LDH activity within the cell culture media of developing *in vitro* myotubes was not significantly affected by acute 1mM H_2O_2 exposure. Similarly, 1uM and 10uM PQQ exposure alone did not significantly affect LDH activity within the media (Figure 3.1.1-1). Lastly, 1um and 10uM PQQ alone did not affect total LDHA protein abundance at any time point (Figure 3.1.1-2).

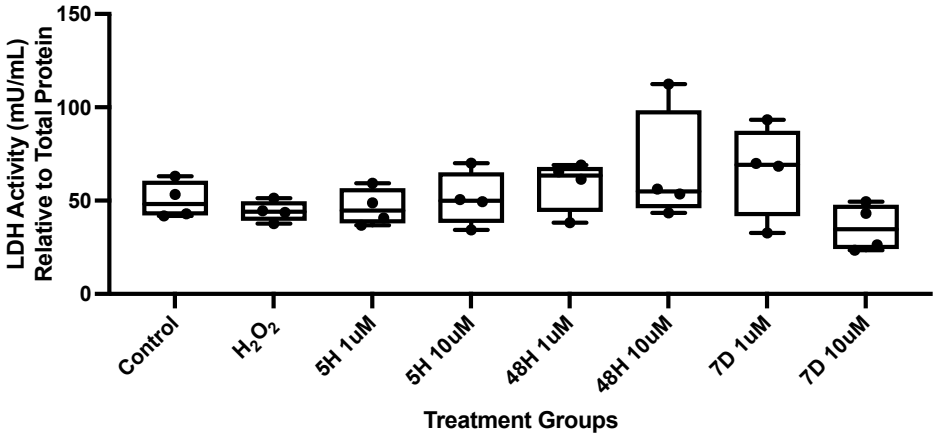


Figure 3.1.1-1 LDH Activity with the cell media of developing *in vitro* myotubes.

LDH activity is a measure of the relative cytotoxicity that has occurred within the cells. Relative to control, 19H 1mM H_2O_2 exposure before collection did not affect LDH activity. Similarly, short-term, and long-term exposure of either low dose 1uM or high dose 10uM PQQ alone did not affect LDH activity relative to control. N=3-4. Data presented as means ± SEM (significance determined by one-way ANOVA and Dunnett's multiple comparisons test, no significant differences were observed).

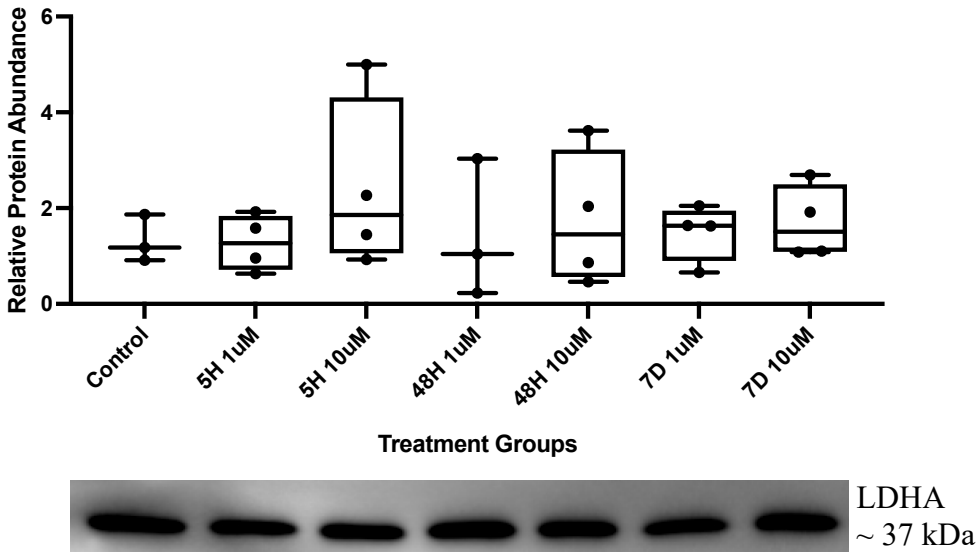


Figure 3.1.1-2 Relative total protein abundance of LDHA in developing *in vitro* myotubes.

LDHA is an enzyme involved in anaerobic metabolism. 1uM and 10uM PQQ for a short-term or long-term exposure time in developing myotubes did not affect total protein abundance of LDHA. N=3-4. Data presented as means ± SEM (significance determined by one-way ANOVA and Dunnett's multiple comparisons test, no significant differences were observed).

3.1.2 SOD Activity

Total SOD activity within developing *in vitro* myotubes was not significantly affected by acute 1mM H_2O_2 exposure nor 1mM H_2O_2 in combination with PQQ (Figure 3.1.2-1A). PQQ alone did not result in a change in activity relative to control (Figure 3.1.2-1B).

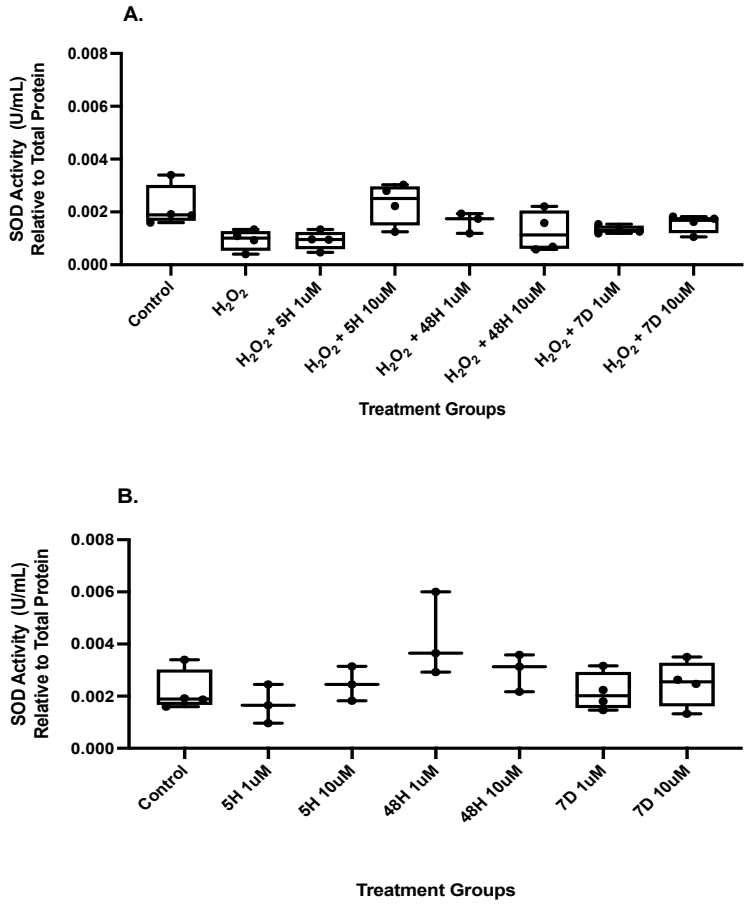


Figure 3.1.2-1 Relative total SOD activity in developing *in vitro* myotubes.
SOD activity is associated with dismutation of superoxide. (A) Relative to control, 24H 1mM H_2O_2 exposure did not significantly affect SOD activity, including PQQ + H_2O_2 exposure (B) Short term and long-term exposure of either 1uM or 10uM PQQ alone did not affect activity relative to control. N=3-4. Data presented as means ± SEM (significance determined by one-way ANOVA and Dunnett's multiple comparisons test, no significant differences were observed).

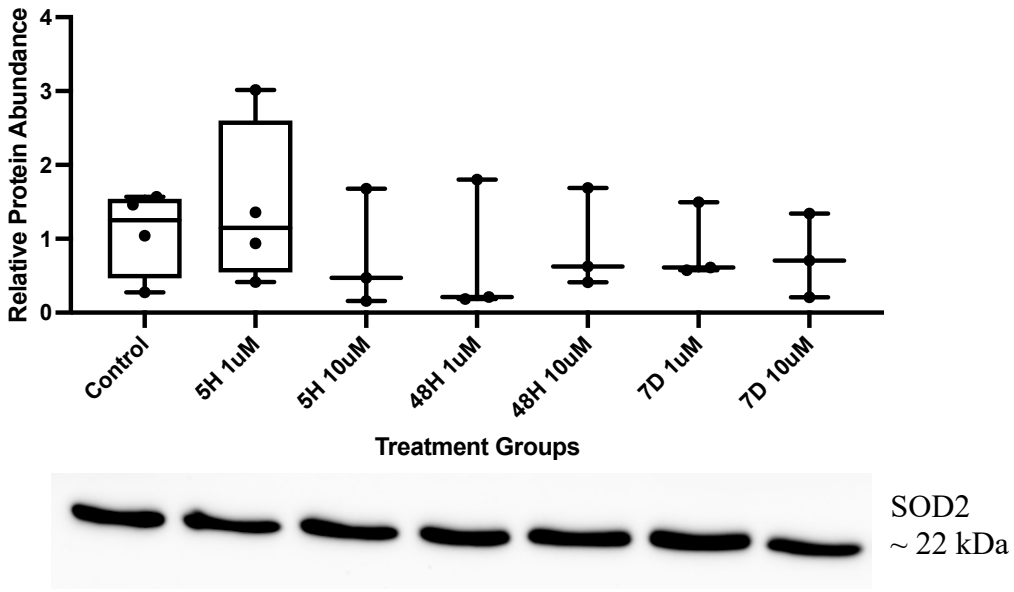


Figure 3.1.2-2 Relative total protein abundance of SOD2 in developing *in vitro* myotubes.

SOD2 is the mitochondrial SOD (also referred to as MnSOD) antioxidant responsible for the dismutation of H_2O_2. 1uM and 10uM PQQ for short-term or long-term exposure in developing myotubes did not affect total protein abundance of SOD2. N=3-4. Data presented as means ± SEM (significance determined by one-way ANOVA and Dunnett's multiple comparisons test, no significant differences were observed).

3.1.3 CAT Activity

Total CAT activity within developing *in vitro* myotubes was not significantly affected by acute 1mM H_2O_2 exposure nor by 1mM H_2O_2 in combination with any exposure length or dose of PQQ (Figure 3.1.3-1A). Similarly, 1uM and 10uM PQQ exposure alone did not significantly affect total CAT activity relative to control (Figure 3.1.3-1B).

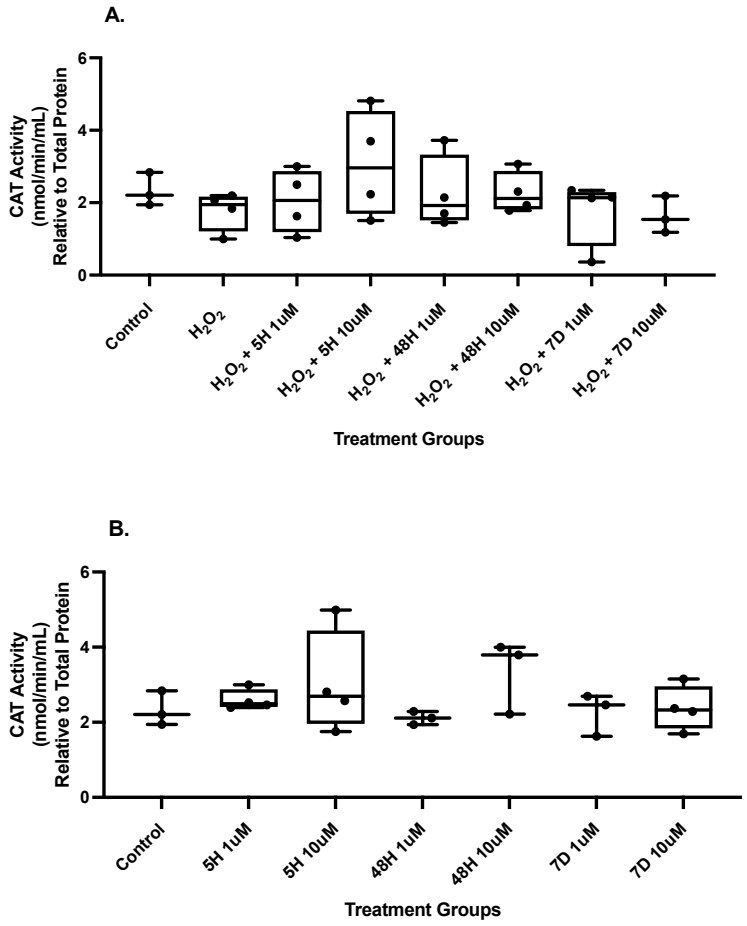


Figure 3.1.3-1 Relative CAT activity in developing *in vitro* myotubes.

CAT activity is associated with the breakdown of H_2O_2. (A) Relative to control, 24H 1mM H_2O_2 exposure did not significantly affect SOD activity, including PQQ + H_2O_2 exposure. (B) Short term and long-term exposure of either 1uM or 10uM PQQ alone did not affect CAT activity relative to control. N=3-4. Data presented as means ± SEM (significance determined by one-way ANOVA and Dunnett's multiple comparisons test, no significant differences were observed).

3.2 Choosing an Effective Dose of H_2O_2 in Group A Cells

Concentrations ranging from 200uM to 1mM of H2O2 have been shown to induce OS within C2C12 cells (Li et al., 2020). After exposure of Group A cultured myoblasts to H_2O_2 concentrations ranging from 250uM to 1mM for 24 hours, qualitative analysis of cell morphology was conducted. 1mM H2O2 significantly altered the number of living cells, compared to control and other concentrations of H_2O_2, so the concentration was deemed too cytotoxic to be utilized in future experiments. The next highest concentration 750uM was therefore chosen to induce OS in subsequent experiments with Group A cells (Figure 3.1.3-1).

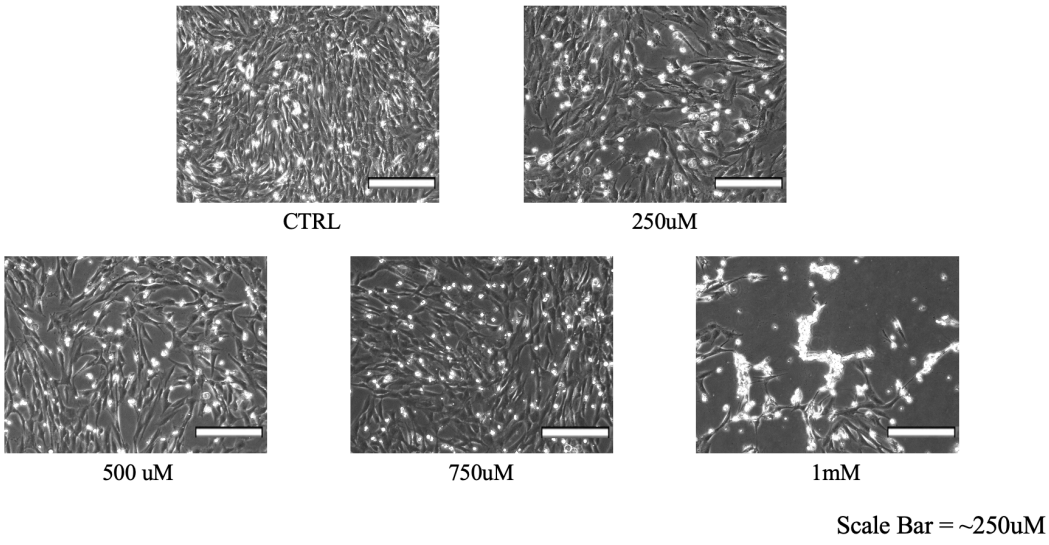


Figure 3.1.3-1 Relative cytotoxicity of varying concentrations of H_2O_2 in differentiating *in vitro* myoblasts.

Within cultured C2C12 cells, concentrations ranging from 200uM to 1mM of H_2O_2 have been shown to induce OS within the developing myotubes. After 24H exposure to 1mM H_2O_2, 1mM was determined qualitatively to be too cytotoxic and resulted in significant cell death. The next highest concentration, 750uM, which did not appear to be significantly cytotoxic to the cells, was chosen to induce OS in differentiating myotubes in subsequent experiments.

3.3 H_2O_2 and PQQ negatively affected gene expression of markers of mitochondrial function and myogenesis but not total protein abundance

3.3.1 Mitochondrial Function

Ndufb6, Cox7a1, and Tfam expression within *in vitro* developing myotubes was significantly decreased ($p<0.05$) as a result of 24H 750uM H_2O_2 exposure (Figure 3.3.1-1A, Figure 3.3.1-2A, and Figure 3.3.1-4A), while relative Pgc-1α was not affected, compared to control (Figure 3.3.1-3A). Next, both 1uM and 10uM PQQ supplementation at all timepoints (5H, 48H, 7D) did not rescue expression when paired with H_2O_2 exposure, as a significant decrease ($p<0.001$) in Ndufb6 and Cox7a1 expression continued to be evident (Figure 3.3.1-1A and Figure 3.3.1-2A), whereas no combination of exposure length and PQQ doses with H_2O_2 significantly changed PGC-1α or Tfam expression (Figure 3.3.1-3A and Figure 3.3.1-4A). Similar to the effects of H_2O_2 exposure, 1uM and 10uM PQQ alone at the 5H, and 48H timepoints significantly decreased ($p<0.01$) expression of Ndufb6 (Figure 3.3.1-1B), and both concentrations at the 7D timepoint significantly decreased ($p<0.01$) expression of both Ndufb6 and Cox7a1 (Figure 3.3.1-1B and Figure 3.3.1-2B), while overall PQQ alone did not affect Pgc-1α or Tfam expression (Figure 3.3.1-3B and Figure 3.3.1-4B). Finally, no dose or length of exposure of PQQ or H_2O_2, nor the combination of the two, affected the total protein abundance of complexes I, II, III, and V (Figure 3.3.1-5A-H), and PQQ alone did not affect VDAC1 (Figure 3.3.1-6), PGC-1α (Figure 3.3.1-7A) or SIRT3 (Figure 3.3.1-7B) total protein abundance within the myotubes.

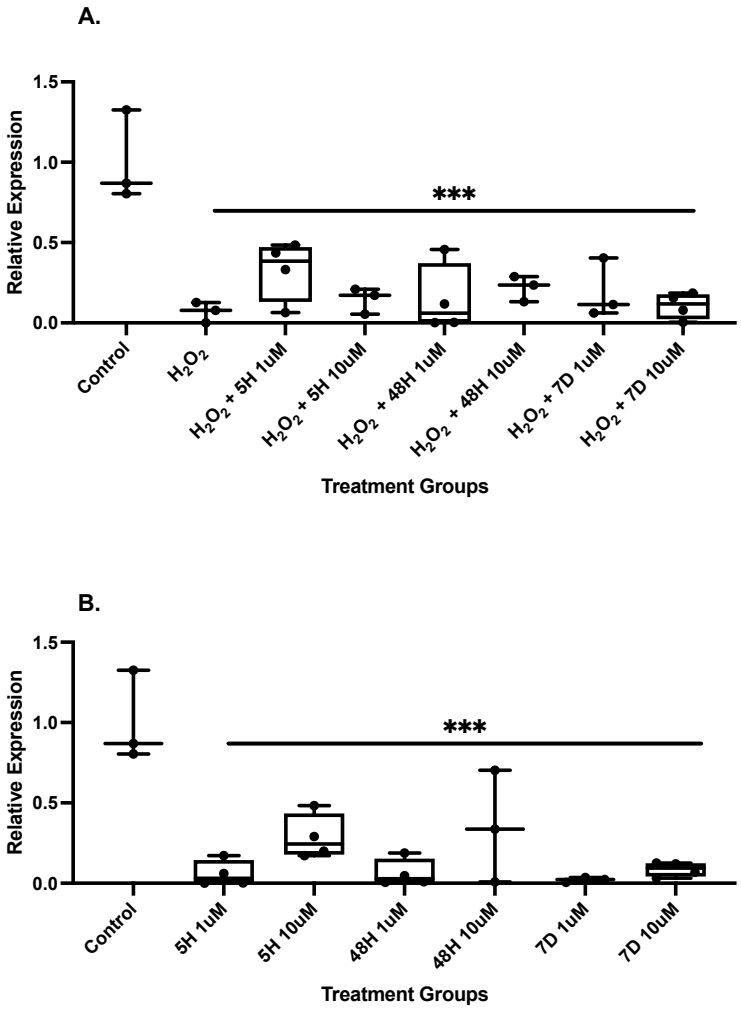


Figure 3.3.1-1 Relative mRNA expression of Ndufb6 in developing *in vitro* myotubes.

NADH:ubiquinone oxidoreductase (complex I) of the ETC expresses Ndufb6. (A) Relative to control, 24H 750uM H_2O_2 exposure significantly decreased Ndufb6 expression and neither 1uM nor 10uM PQQ exposure rescued expression at any timepoint. (B) Short-term and long-term exposure of either 1uM or 10uM PQQ alone significantly decreased expression of Ndufb6, relative to control. N=3-4. Data presented as means ± SEM (significance determined by one-way ANOVA and Dunnett's multiple comparisons test, ***$p<0.001$).

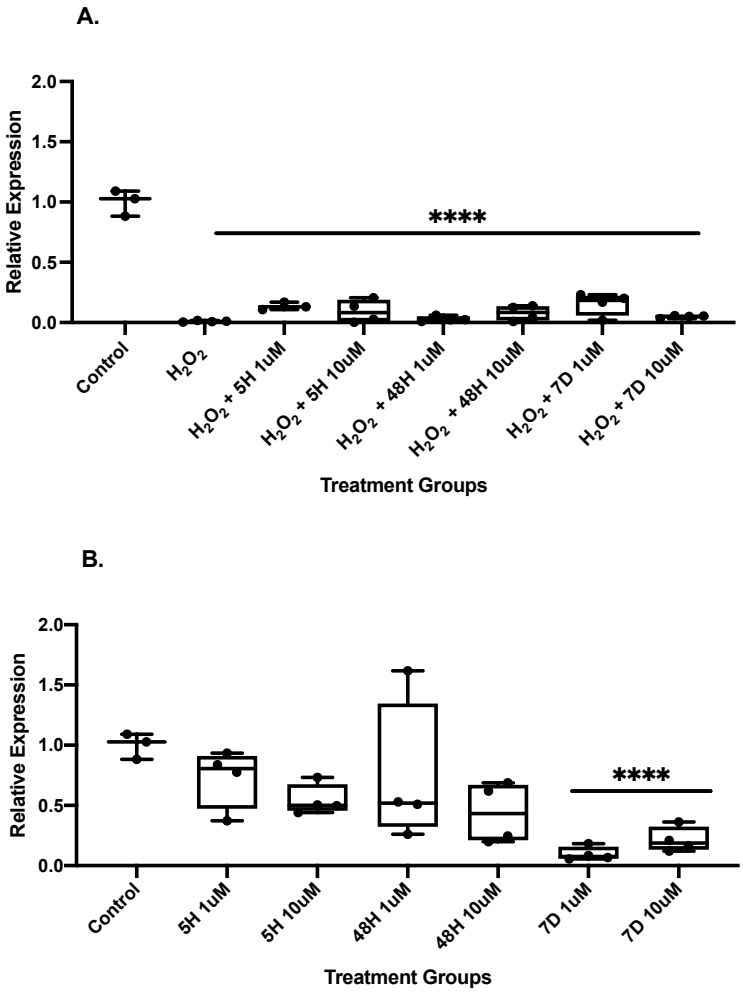


Figure 3.3.1-2 Relative mRNA expression of Cox7a1 in developing *in vitro* myotubes.

Cytochrome c oxidase (complex IV) of the ETC expresses Cox7a1. (A) Relative to control, 24H 750uM H_2O_2 significantly decreased Cox7a1 expression and neither 1uM or 10uM PQQ exposure rescued expression at any timepoint. (B) Long-term exposure of either 1uM or 10uM PQQ alone significantly decreased expression of Cox7a1, relative to control. N=3-4. Data presented as means ± SEM (significance determined by one-way ANOVA and Dunnett's multiple comparisons test, **** $p<0.0001$).

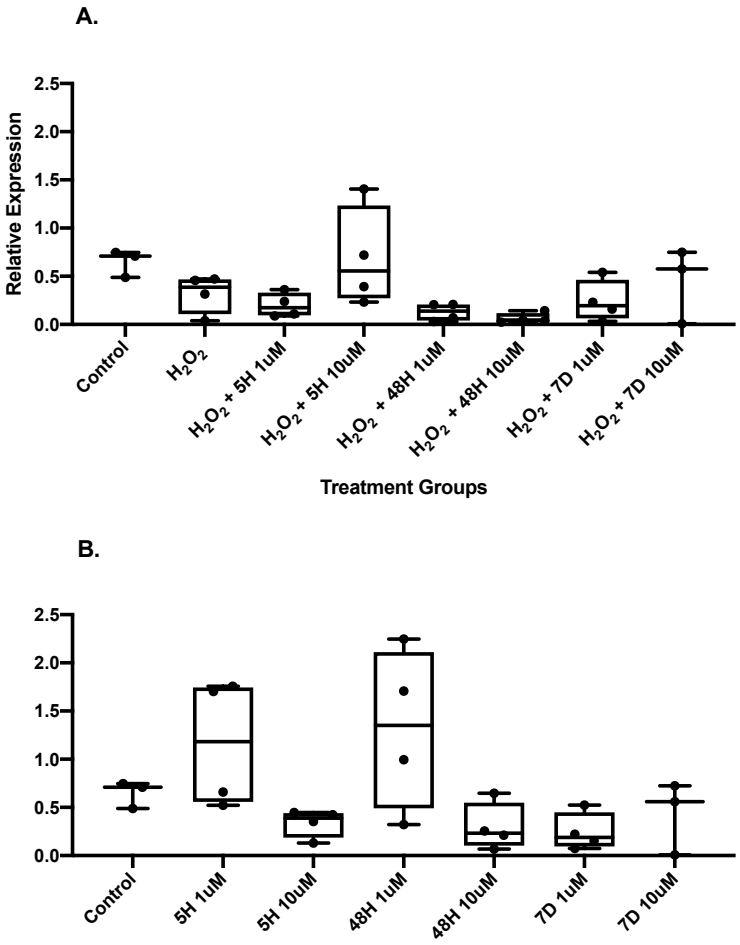


Figure 3.3.1-3 Relative mRNA expression of Pgc-1α in developing *in vitro* myotubes.

Pgc-1α expression is associated with mitochondrial biogenesis. (A) H_2O_2 alone nor in combination with PQQ at any timepoint or dose affected expression of Pgc-1α, relative to control. (B) No dose or time-point of PQQ exposure alone resulted in a significant effect to Pgc-1α expression, relative to control. N=3-4. Data presented as means $\pm$ SEM (significance determined by one-way ANOVA and Dunnett's multiple comparisons test; no significant changes were observed).

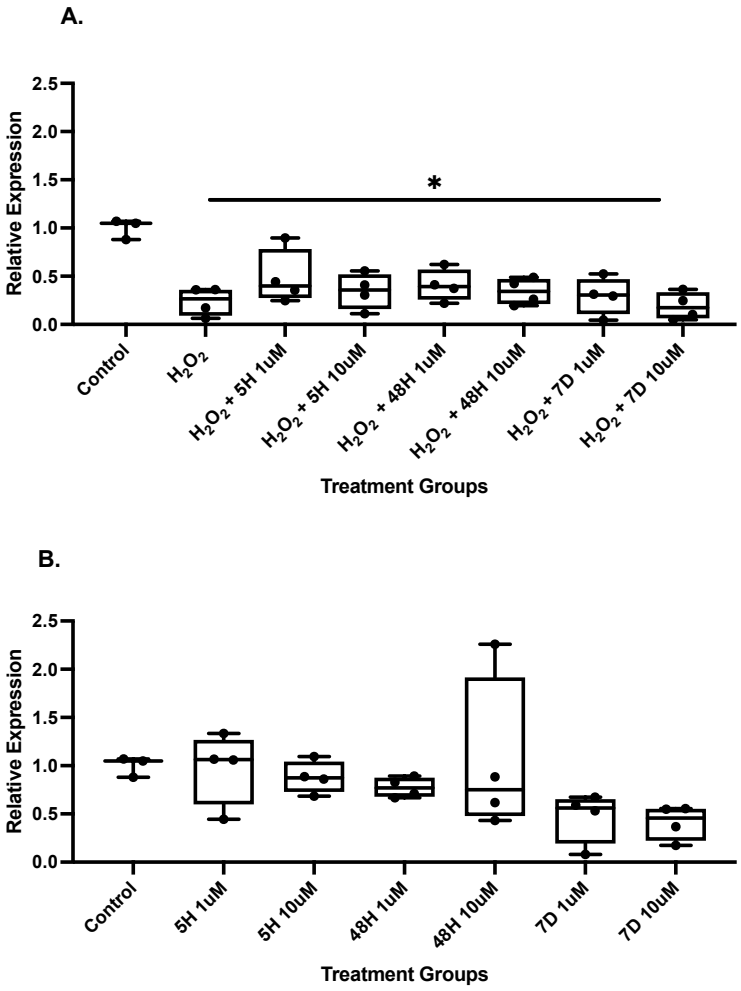


Figure 3.3.1-4 Relative mRNA expression of Tfam in developing *in vitro* myotubes.

Tfam expression induces mitochondrial DNA transcription. (A) Relative to control, 24H 750uM H_2O_2 exposure significantly decreased Tfam expression and neither 1uM nor 10uM PQQ exposure rescued expression at any timepoint. (B) No concentration or time-point of PQQ exposure alone resulted in a significant effect to Tfam expression, relative to control. N=3-4. Data presented as means ± SEM (significance determined by one-way ANOVA and Dunnett's multiple comparisons test; *$p<0.05$).

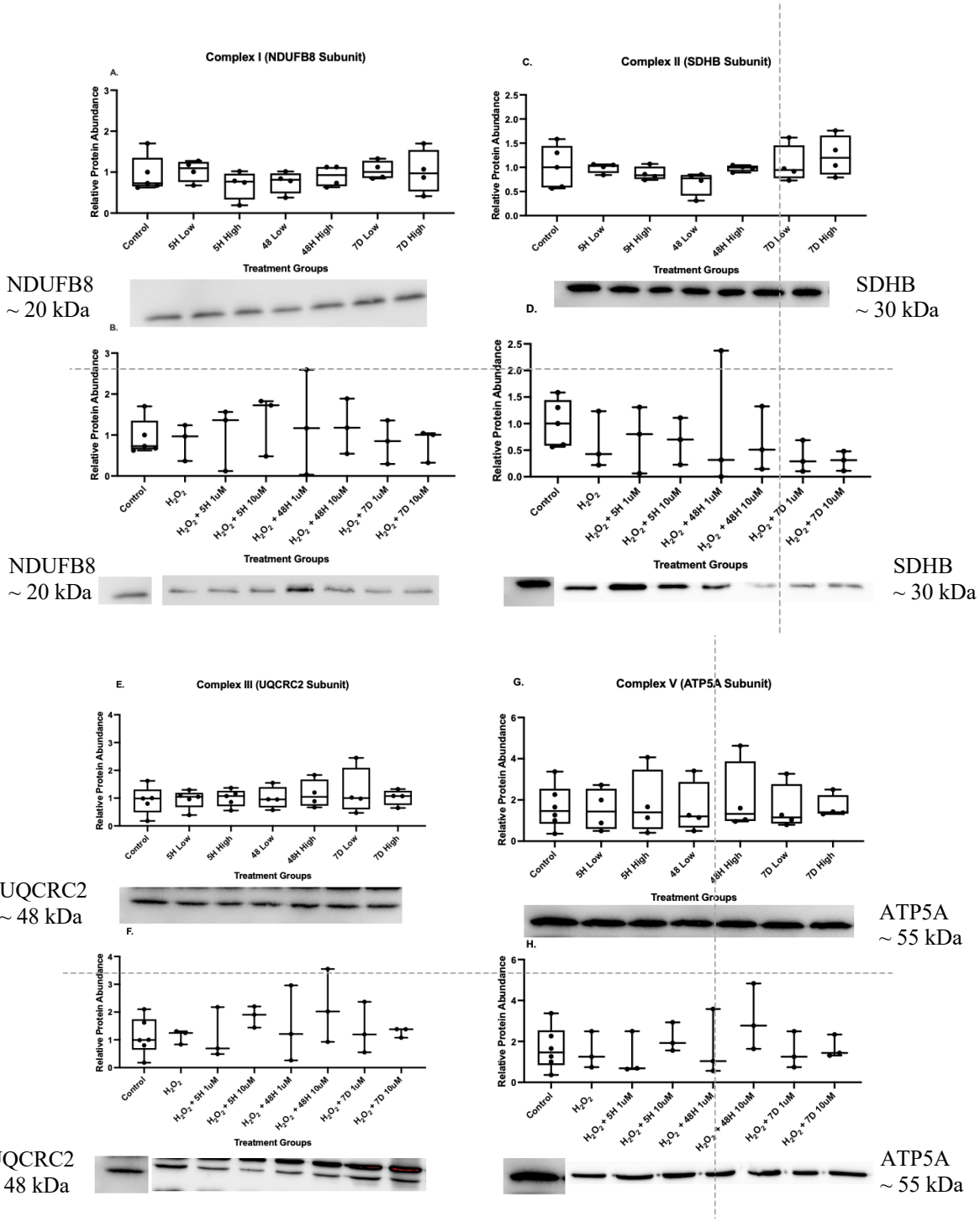


Figure 3.3.1-5 Total protein abundance of ETC complexes (I, II, III, V) in developing *in vitro* myotubes.

NADH: ubiquinone oxidoreductase (complex I) expresses NDUFB8, succinate dehydrogenase (complex II) expresses SDHB, coenzyme Q: cytochrome c reductase

(complex III) expresses UQCRC2, and ATP Synthase (complex V) expresses ATP5A. (A-D) Neither 24H 750UM H_2O_2 or 1uM/10uM PQQ exposure alone, nor H_2O_2 in combination with PQQ, at any timepoint or concentration, affected total protein abundance of (A,B) NDUFB8, (C,D) SDHB, (E,F) UQCRC2, or (G,H) ATP5A, relative to control. N=3-6. Data presented as means ± SEM (significance determined by one-way ANOVA and Dunnett's multiple comparisons test, no significant changes were observed).

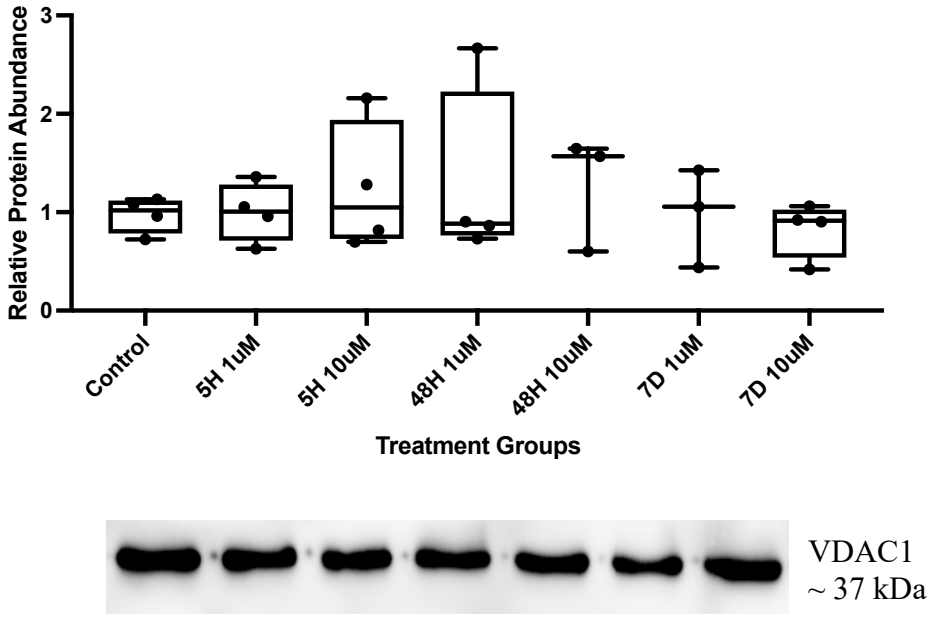


Figure 3.3.1-6 Total protein abundance of VDAC1 in developing *in vitro* myotubes.

VDAC1 expression is associated with apoptosis regulation within the mitochondria and is commonly utilized as a marker of mitochondria number. Neither 24H 750uM H_2O_2 nor H_2O_2 in combination with PQQ, at any timepoint or concentration, affected total protein abundance of VDAC1 relative to control. PQQ alone did not affect VDAC1 compared to control. N=3-4. Data presented as means ± SEM (significance determined by one-way ANOVA and Dunnett's multiple comparisons test, no significant changes were observed).

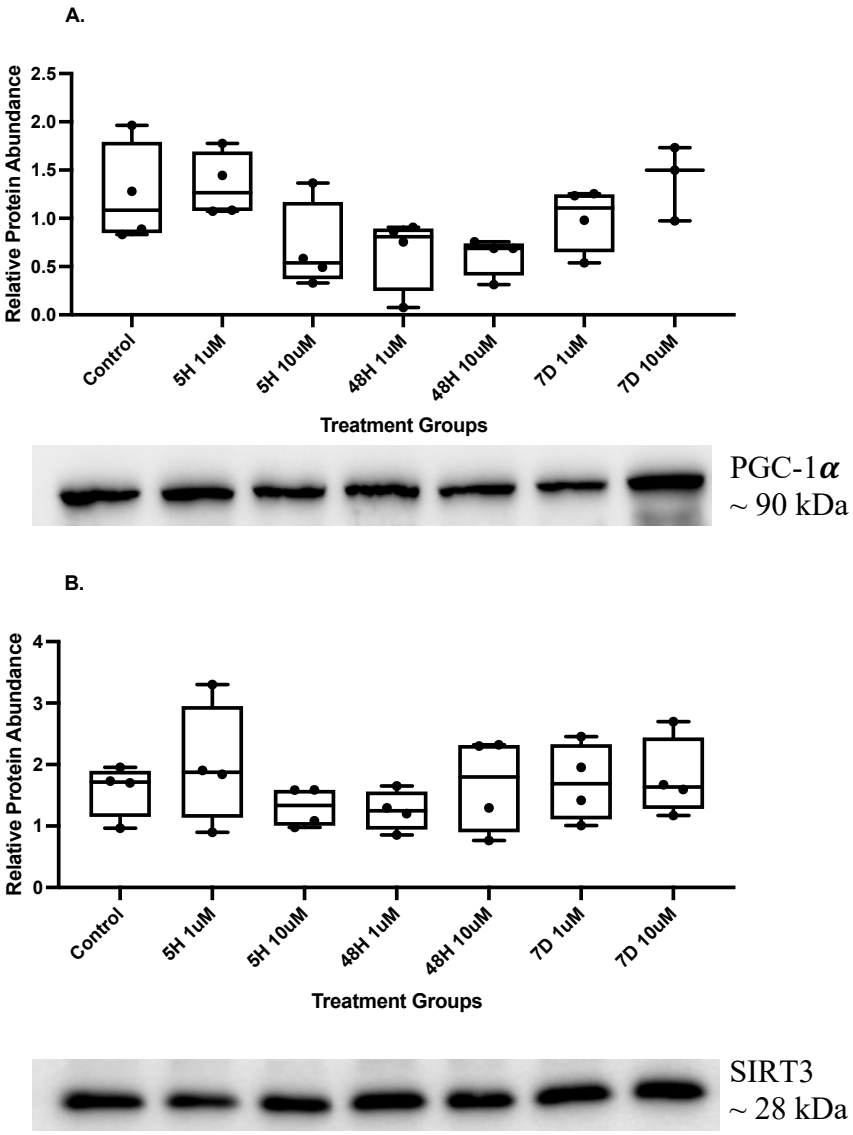


Figure 3.3.1-7 Total protein abundance of PGC-1α and SIRT3 in developing *in vitro* myotubes.

PGC-1α and SIRT3 expression are associated with mitochondrial biogenesis. (A) PQQ alone did not affect PGC-1α compared to control. (B) PQQ alone did not affect SIRT3 compared to control. N=3-4. Data presented as means $\pm$ SEM (significance determined by one-way ANOVA and Dunnett's multiple comparisons test, no significant changes were observed).

3.3.2 Myogenesis

In vitro myotube Pax7 expression was not significantly affected by 24H 750uM H_2O_2 exposure, nor any combination of H_2O_2 and PQQ during differentiation, relative to control (Figure 3.3.2-1A and Figure 3.3.2-1B). In comparison, myotube expression of Myod1 and Myog was significantly decreased ($p< 0.001$) as a result of H_2O_2 exposure, and all combinations of PQQ and H_2O_2, relative to control (Figure 3.3.2-2A and Figure 3.3.2-3A). Pax7 expression was significantly affected by PQQ, in that all doses and exposure timepoints (except 5H 1uM and 7D 1uM) significantly decreased ($p<0.05$) expression relative to control (Figure 3.3.2-1B). Lastly, 1uM and 10uM PQQ alone for 7D significantly decreased ($p<0.05$) expression of Myod1 (Figure 3.3.2-2B), while all timepoints and doses studied did not significantly affect Myog expression within the myotubes (Figure 3.3.2-3B).

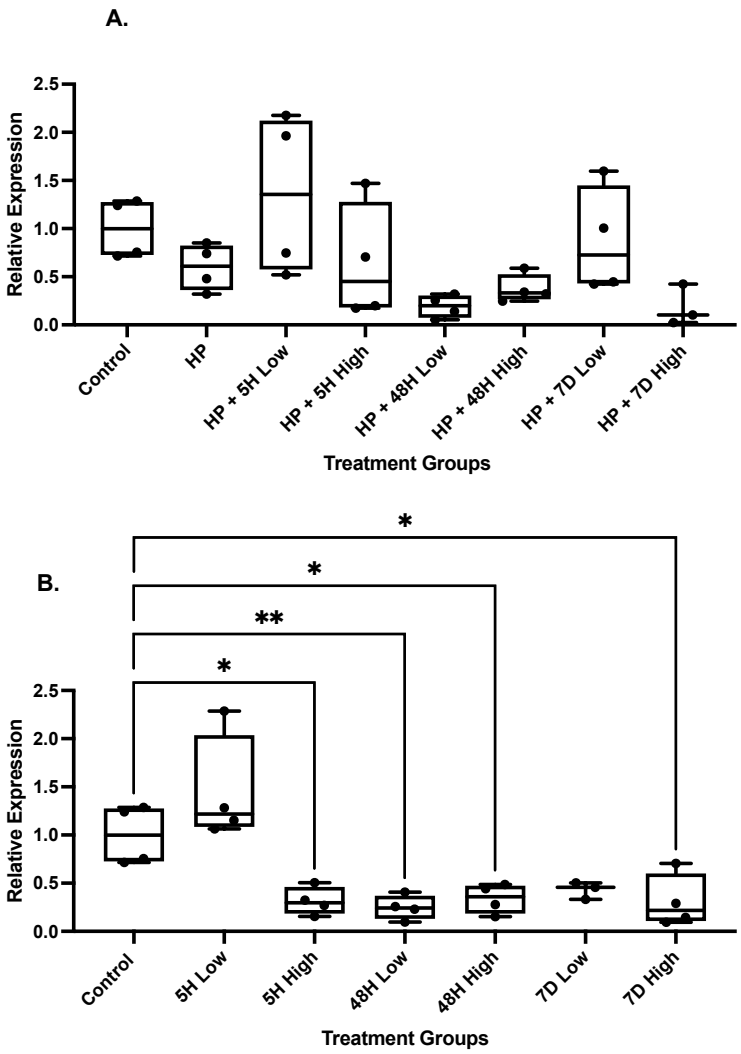


Figure 3.3.2-1 Relative mRNA expression of Pax7 in developing *in vitro* myotubes.

Pax7 expression is associated with proliferation during myogenesis and overall regenerative capacity of the skeletal muscle. (A) 24H 750uM H_2O_2 alone nor in combination with low and high dose PQQ at any timepoint affected expression of Pax7, relative to control. (B) Exposure to 1uM and 10uM PQQ alone, for short-term and long-term durations, significantly decreased expression, relative to control. N=3-4. Data presented as means ± SEM (significance determined by one-way ANOVA and Dunnett's multiple comparisons test; *p<0.05, **p<0.01).

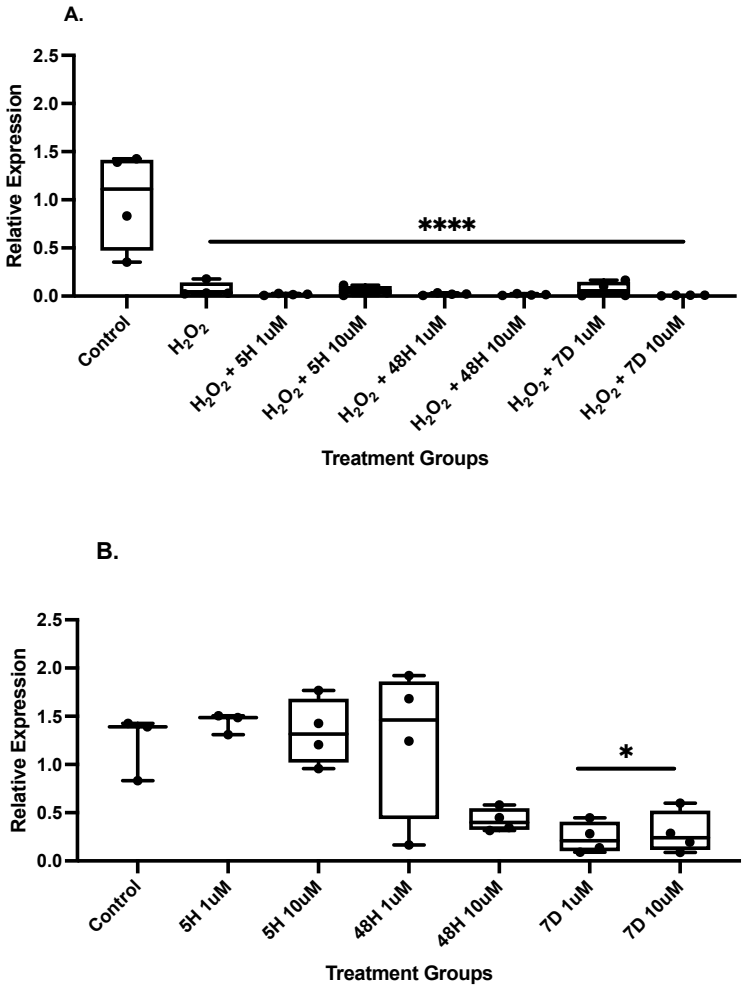


Figure 3.3.2-2 Relative mRNA expression of Myod1 in developing *in vitro* myotubes.

Myod1 expression is associated with differentiation of myofibers during myogenesis. (A) Relative to control, 24H 750uM H_2O_2 significantly decreased Myod1 expression and neither 1uM nor 10uM PQQ exposure rescued expression at any timepoint. (B) Long-term 1uM and 10uM exposure to PQQ alone significantly decreased expression of Myod1 relative to control in the developing myotubes. N=3-4. Data presented as means ± SEM (significance determined by one-way ANOVA and Dunnett's multiple comparisons test; *$p<0.05$, ****$p<0.0001$).

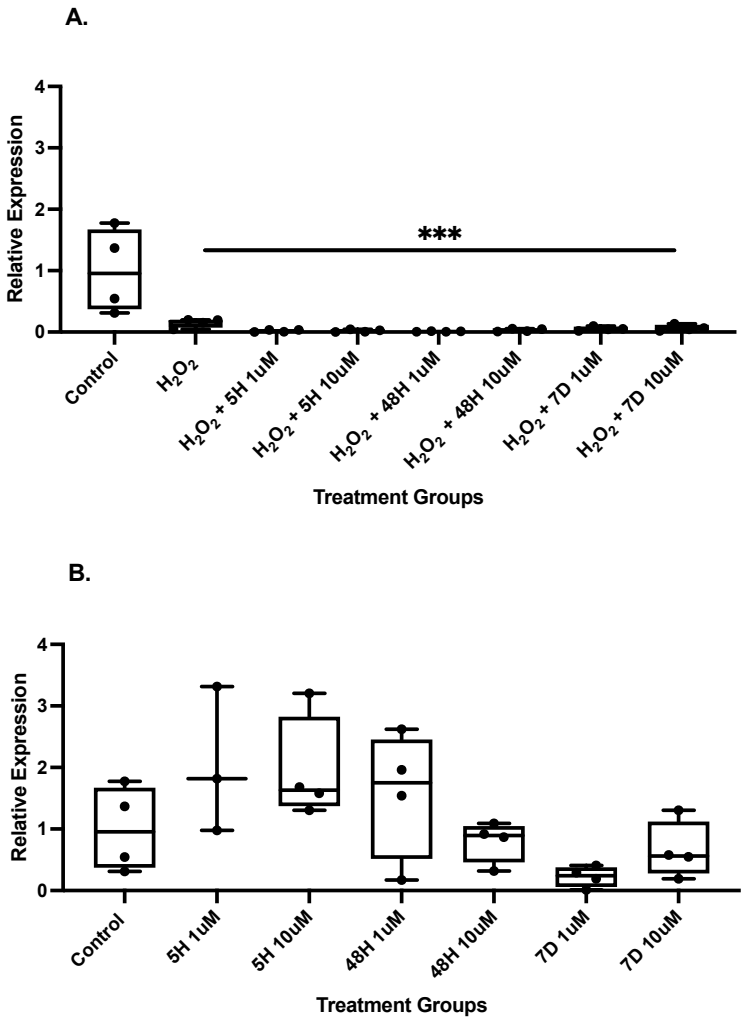


Figure 3.3.2-3 Relative mRNA expression of Myog in developing *in vitro* myotubes.

Myog expression is associated with differentiation during myogenesis. (A) Relative to control, 24H 750uM H_2O_2 significantly decreased myotube Myog expression and neither 1uM nor 10uM PQQ exposure significantly rescued expression at any timepoint. (B) Exposure to PQQ alone did not affect expression of Myog, relative to control. N=3-4. Data presented as means ± SEM (significance determined by one-way ANOVA and Dunnett's multiple comparisons test; ***$p<0.001$).

3.4 IUGR and Maternal PQQ Exposure Significantly Impacted Gastrocnemius mRNA Expression

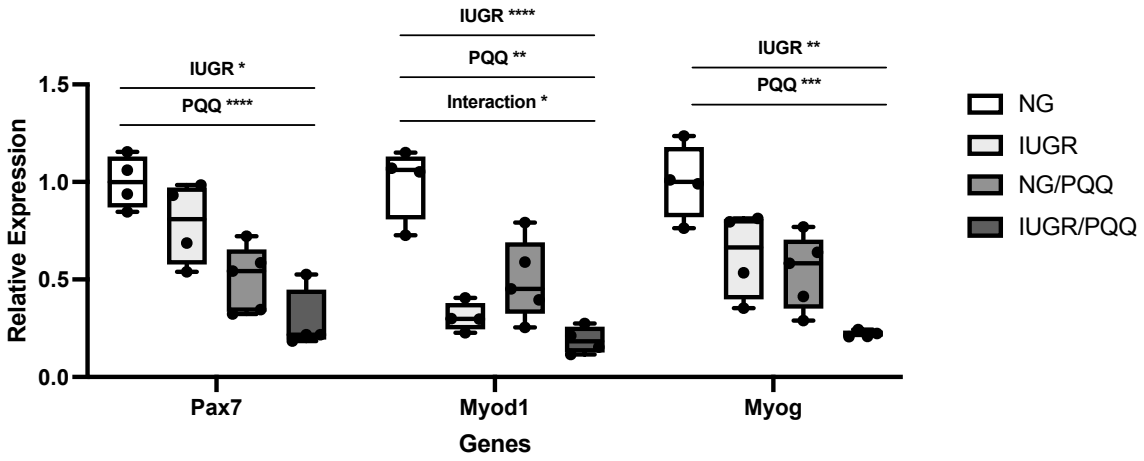
3.4.1 Myogenesis

Expression of fetal gastrocnemius muscle Pax7 mRNA was significantly decreased in association with IUGR ($F_{1,13} = 6.799$, $p<0.05$) and maternal PQQ consumption ($F_{1,13} = 36.03$, $p<0.01$); expression was significantly decreased in NG/PQQ and IUGR/PQQ animals, compared to NG water control animals. Myod1 expression was significantly decreased in association with IUGR ($F_{1,13} = 45.27$, $p<0.0001$) and maternal PQQ consumption ($F_{1,13} = 17.50$, $p<0.01$). A significant interaction between variables was also observed ($F_{1,13} = 6.722$, $p<0.05$) and IUGR ($p<0.001$), NG/PQQ ($p<0.01$), and IUGR/PQQ animals ($p<0.0001$) had variable decreases in expression compared to NG water control animals. IUGR/PQQ ($p<0.05$) was also associated with a significant decrease in Myod1 expression, compared to NG/PQQ animals. Lastly, Myog expression within the muscle was decreased in association with IUGR ($F_{1,13} = 16.37$, $p<0.01$) and maternal PQQ consumption ($F_{1,13} = 25.34$, $p<0.001$), with IUGR, NG/PQQ, and IUGR/PQQ animals having lower expression compared to NG water control animals as well (Figure 3.4.2-1).

3.4.2 Fiber Type Composition

Expression of Myh7 (type 1a; slow oxidative) was significantly decreased in association with IUGR ($F_{1,13} = 4.880$, $p <0.05$) and maternal PQQ consumption ($F_{1,13} = 7.267$, $p<0.05$); expression was lower in IUGR/PQQ animals compared to NG water control animals. Expression of Myh2 (type 2a; fast oxidative)was significantly decreased in association with only maternal PQQ consumption ($F_{1,13} = 12.89$, $p<0.01$) with expression being lower in IUGR/PQQ animals compared to NG water control animals. Expression of Myh4 (type 2b; fast glycolytic) was significantly decreased in association with both IUGR ($F_{1,13} = 22.67$, $p<0.001$) and maternal PQQ consumption ($F_{1,13} = 7.755$, $p<0.05$), with IUGR, NG/PQQ, and IUGR/PQQ animals having decreased expression compared to NG water control animals. Lastly, expression of Myh1 (type 2x; fast oxidative) was

significantly decreased in conjunction with IUGR ($F_{1,13} = 20.44$, p<0.001), as expression was lower in IUGR and IUGR/PQQ animals compared NG water control animals (Figure 3.4.2-2).



3Figure 3.4.2-1 Relative mRNA expression of Pax7, Myod1, and Myog in fetal gastrocnemius tissue.

Relative mRNA expression of myogenesis markers in NG or IUGR fetal guinea pig gastrocnemius muscle exposed to control or PQQ-addition maternal water consumption. Proliferating myoblasts express Pax7, while differentiated myofibers express Myod1 and Myog. IUGR alone negatively affected Pax7, Myod1 and Myog expression, relative to control. PQQ negatively affected Pax7, Myod1, and Myog expression as well. An interaction effect on Myod1 was also observed. N=4-5. Data presented as means ± SEM (significance determined by two-way ANOVA and Tukey's multiple comparisons test, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).

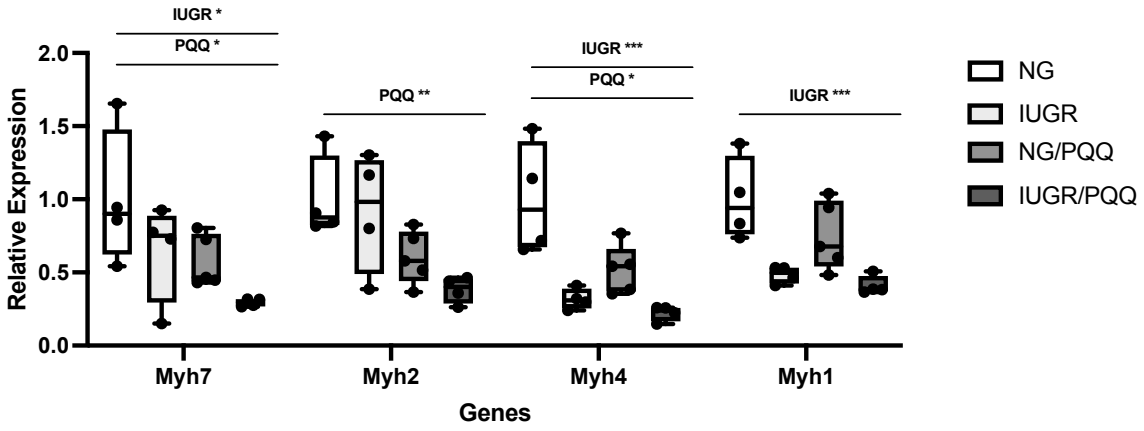


Figure 3.4.2-2 Relative mRNA expression of Myh7, Myh2, Myh4, and Myh1 in fetal gastrocnemius tissue.

Relative mRNA expression of myosin heavy chain genes (MHC) in NG or IUGR fetal guinea pigs exposed to control or PQQ-addition maternal water consumption is shown. Slow oxidative fibers express Myh7, while fast oxidative fibers, types IIa and IIx, express Myh2 and Myh1, respectively. Fast glycolytic fibers, type IIb express Myh4. IUGR negatively affected Myf7, Myf4, and Myf1 expression. PQQ negatively affect Myf7, Myf2, and Myf4 expression. N=4-5. Data presented as means ± SEM (significance determined by two-way ANOVA and Tukey's multiple comparisons test, *$p<0.05$, **$p<0.01$, ***$p<0.001$).

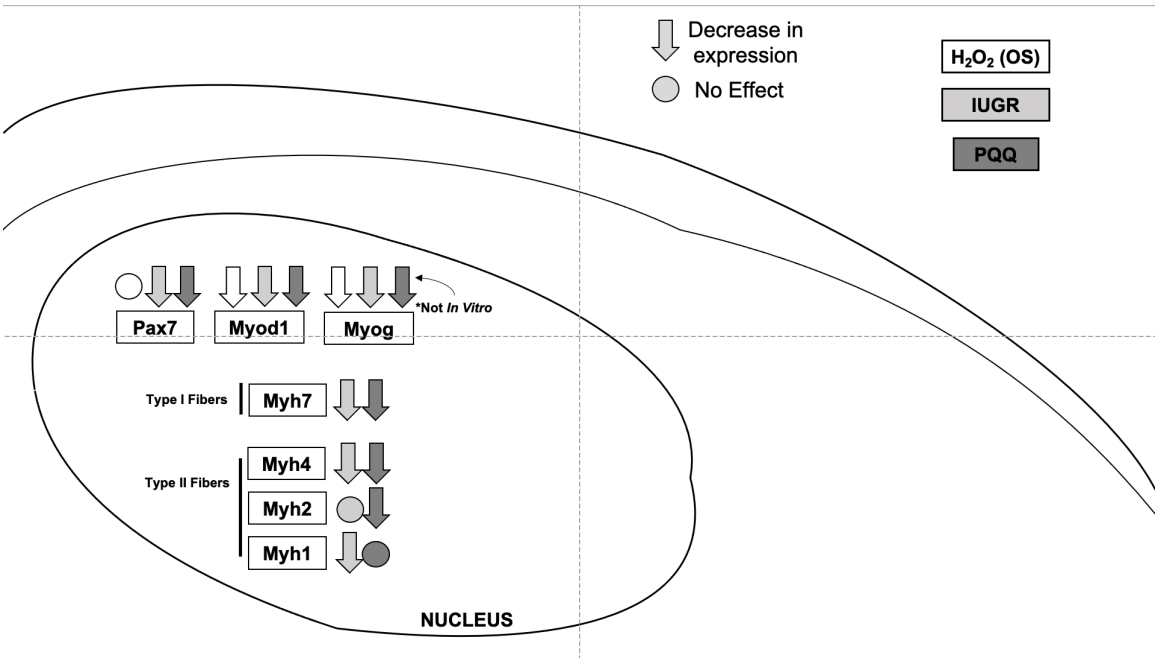


Figure 3.4.2-3 Summary of the relative mRNA expression of myogenic genes in developing *in vitro* myotubes and fetal gastrocnemius muscle.

Based on treatment group, the downward arrows represent a significant decrease in relative mRNA expression, while the circles represents that no significant change in expression was observed for each corresponding myogenic gene.

3.4.3 Electron Transport Chain

Ndufb6 expression was significantly decreased in association with IUGR ($F_{1,13} = 38.87$, $p \leq 0.0001$), as expression in IUGR and IUGR/PQQ animals was decreased relative to NG water control animals. Similarly, Cox7a1 and Cox4i2 (complex IV subunits) expression was significantly decreased in association with IUGR ($F_{1,13} = 68.92$, $p<0.0001$; $F_{1,13} = 49.34$, $p<0.0001$); IUGR/CTRL and IUGR/PQQ animals had lower expression of both genes, compared to NG water control animals. As well, Atp5a1 (complex V subunit) expression and Atp5pb (complex V subunit) expression had significantly decreased expression in conjunction with both IUGR (($F_{1,13} = 63.95$, $p<0.0001$; $F_{1,13} = 45.33$, $p<0.0001$), with decreased expression in IUGR and IUGR/PQQ animals relative to NG water control animals too. Lastly, CS expression was significantly decreased in association with IUGR ($F_{1,13} = 39.73$, $p<0.0001$) and maternal consumption of PQQ (($F_{1,13} = 5.453$, $p<0.05$), with decreased expression observed in IUGR, NG/PQQ, and IUGR/PQQ animals, compared to NG water control animals (Figure 3.4.4-1).

3.4.4 Mitochondrial Biogenesis and Fatty Acid Oxidation

Pgc-1α expression was significantly decreased in association with IUGR ($F_{1,13} = 25.20$, $p<0.001$), and maternal PQQ consumption ($F_{1,13} = 12.46$, $p<0.01$), in addition to an observed significant interaction effect ($F_{1,13} = 5.616$, $p<0.05$), with IUGR ($p<0.01$), NG/PQQ ($p<0.01$), and IUGR/PQQ (0.001) animals exhibited varying decreases in expression, relative to NG water control animals. Sirt1 expression was significantly decreased in association with both IUGR ($F_{1,13} = 12.21$, $p<0.01$) and maternal PQQ consumption ($F_{1,13} = 8.489$, $p<0.05$); IUGR and IUGR/PQQ animals had lower expression compared to NG water control animals. Related Sirt3 was also significantly decreased in association with IUGR ($F_{1,13} = 68.11$, $p<0.0001$), and PQQ ($F_{1,13} = 20.57$, $p<0.001$), in addition to an observed significant interaction ($F_{1,13} = 4.689$, $p<0.05$), with IUGR ($p<0.001$), NG/PQQ ($p<0.01$), and IUGR/PQQ ($p<0.0001$) animals demonstrating varying decreases in expression relative to NG water control animals. A significant decrease was also observed in Sirt3 expression IUGR/PQQ animals ($p<0.01$), compared to NG/PQQ animals. Tfam expression was significantly decreased by IUGR ($F_{1,13} =$

54.80, p<0.0001) only; expression was decreased in IUGR and IUGR/PQQ animals, compared to NG water control animals and NG/PQQ animals. Cpt-1β expression was significantly decreased in conjunction with IUGR ($F_{1,13}$ = 24.29, p<0.001) and maternal PQQ consumption ($F_{1,13}$ = 4.849, p<0.05), with IUGR and IUGR /PQQ animals showing lower expression relative to NG water control animals (Figure 3.3.4-1). Lastly, Ucp2 expression was significantly decreased in association with IUGR ($F_{1,13}$ = 30.06, p<0.001) and maternal PQQ consumption ($F_{1,13}$ = 6.122, p<0.05); only IUGR and IUGR/PQQ animals show decreased expression compared to NG water control animals, and IUGR/PQQ was decreased relative to NG/PQQ animals too (Figure 3.4.4-2).

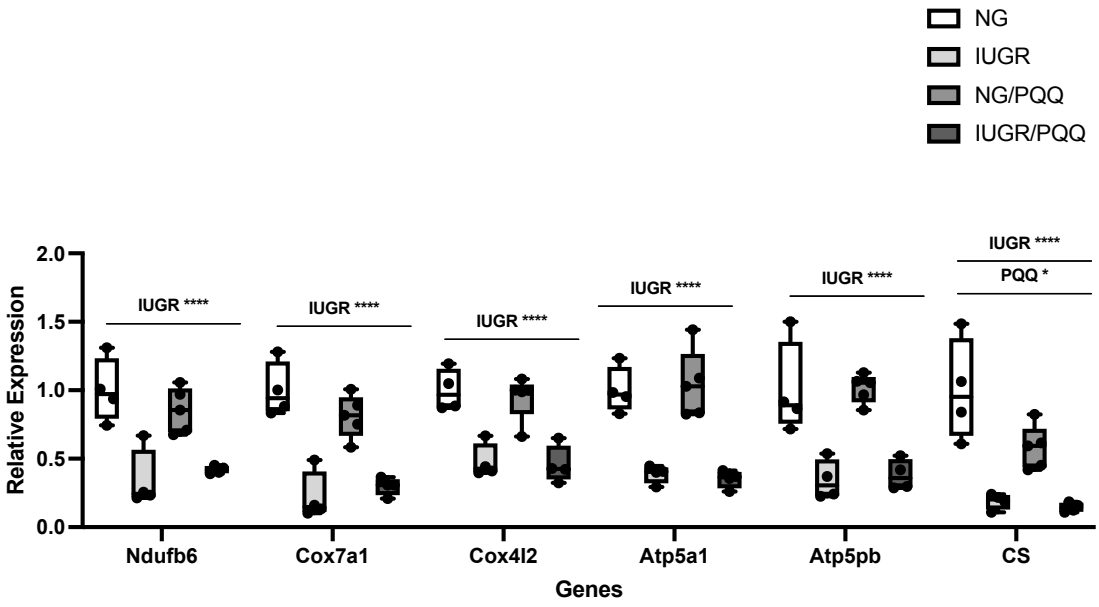


Figure 3.4.4-1 Relative mRNA expression of ETC complexes and citrate synthase in fetal gastrocnemius muscle.

Relative mRNA expression of electron transport chain (ETC) complexes and citrate synthase in NG or IUGR fetal guinea pigs exposed to control or PQQ-addition maternal water consumption is shown. NADH: ubiquinone oxidoreductase (complex I) expresses Ndufb6, cytochrome c oxidase (complex IV) expresses Cox7a1 and Cox4i2, and ATP Synthase (complex V) expresses Atp5a1 and Atp5pb. Citrate synthases expresses CS. IUGR negatively affected all genes studied, while CS expression was also affected by PQQ. N=4-5. Data presented as means ± SEM (significance determined by two-way ANOVA and Tukey's multiple comparisons test, *p<0.05, ****p<0.0001).

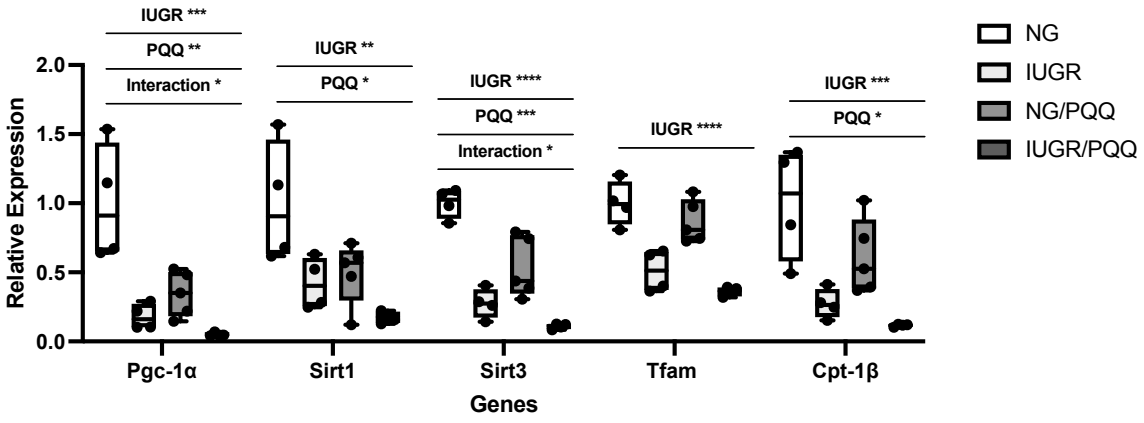


Figure 3.4.4-2 Relative mRNA expression of markers of mitochondrial metabolism and fatty acid oxidation in fetal gastrocnemius muscle.

Relative mRNA expression of mitochondrial metabolism and fatty acid oxidation markers in NG or IUGR fetal guinea pigs exposed to control or PQQ-addition maternal water consumption are shown. Pgc-1α, Sirt1, and Sirt3 are expressed during regulation of cellular metabolism involving the mitochondria. Tfam expression induces mitochondrial DNA transcription. Cpt-1β expression increases during fatty-acid oxidation in skeletal muscle. IUGR and PQQ negatively affected all genes studied, compared to control, except for Tfam expression, which was only negatively affected in association with IUGR. N=4-5. Data presented as means $\pm$ SEM (significance determined by two-way ANOVA and Tukey's multiple comparisons test, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).

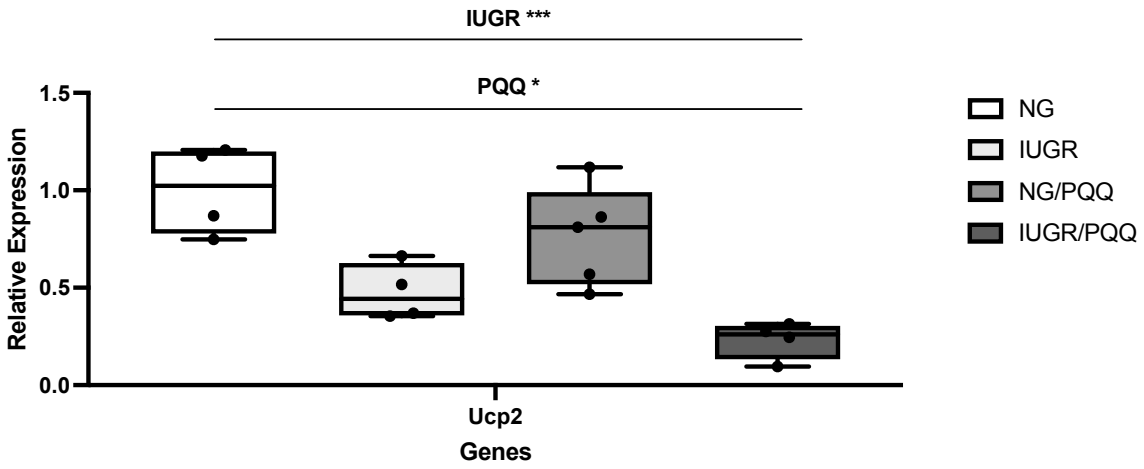


Figure 3.4.4-3 Relative mRNA expression of Ucp2 in fetal gastrocnemius muscle.

Relative mRNA expression of Ucp2 in NG or IUGR fetal guinea pigs exposed to control or PQQ-addition maternal water consumption is shown. Ucp2 uncouples oxygen from mitochondrial ATP synthesis and is associated with the reduction of OS in the mitochondria. IUGR and PQQ negatively affected expression. N=4-5. Data presented as means ± SEM (significance determined by two-way ANOVA and Tukey's multiple comparisons test, *$p<0.05$, ***$p<0.001$).

3.4.5 Peroxisome Proliferator-Activated Receptors

Ppar-α was significantly decreased by IUGR ($F_{1,13}$ = 58.26, p<0.0001), maternal PQQ intake ($F_{1,13}$ = 23.64, p<0.001), and a significant interaction was evident ($F_{1,13}$ = 8.448, p<0.05), as decreased expression was evident in IUGR (p<0.0001), NG/PQQ (p<0.001), and IUGR/PQQ animals (p<0.0001), compared to NG water control animals. Additionally, a significant decrease in IUGR/PQQ (p<0.05) animals compared to NG/PQQ animals was observed too. Ppar-δ expression was significantly decreased in association IUGR ($F_{1,13}$ = 16.45, p<0.01) and maternal PQQ consumption ($F_{1,13}$ = 13.22, p<0.01) too; IUGR, NG/PQQ, and IUGR/PQQ animals all demonstrating decreased expression, relative to NG water control animals. Finally, Ppar-γ expression was significantly decreased in association with IUGR ($F_{1,13}$ = 14.98, p<0.01) and maternal PQQ consumption ($F_{1,13}$ = 8.671, p<0.05), and only IUGR/CTRL and IUGR/PQQ animals show decreased expression compared to NG water control animals (Figure 3.4.5-1).

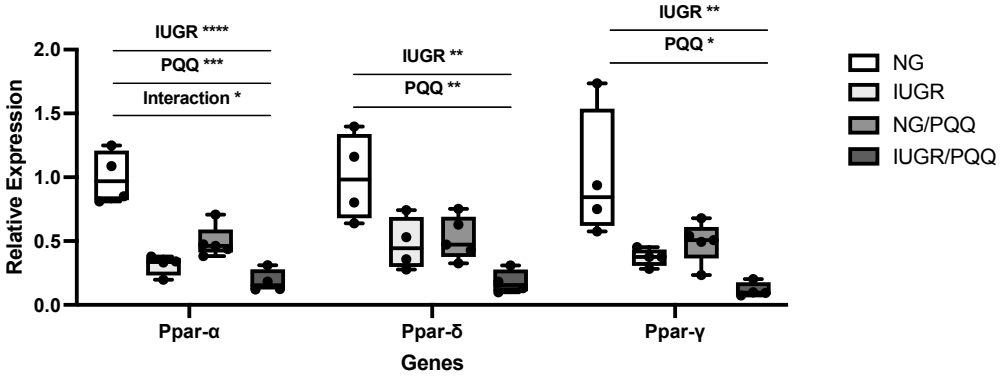


Figure 3.4.5-1 Relative mRNA expression of Ppar-α,δ, and γ in fetal gastrocnemius muscle.

Relative mRNA expression of PPARs in NG or IUGR fetal guinea pigs exposed to control or PQQ-addition maternal water consumption is shown. Ppar-α expression regulates overall energy homeostasis, while Ppar-γ expression is involved in glucose metabolism. Ppar-δ expression is important for fatty acid metabolism. IUGR and PQQ as negatively affected all 3 genes, compared to control. N=4-5. Data presented as means $\pm$ SEM (significance determined by two-way ANOVA and Tukey's multiple comparisons test, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).

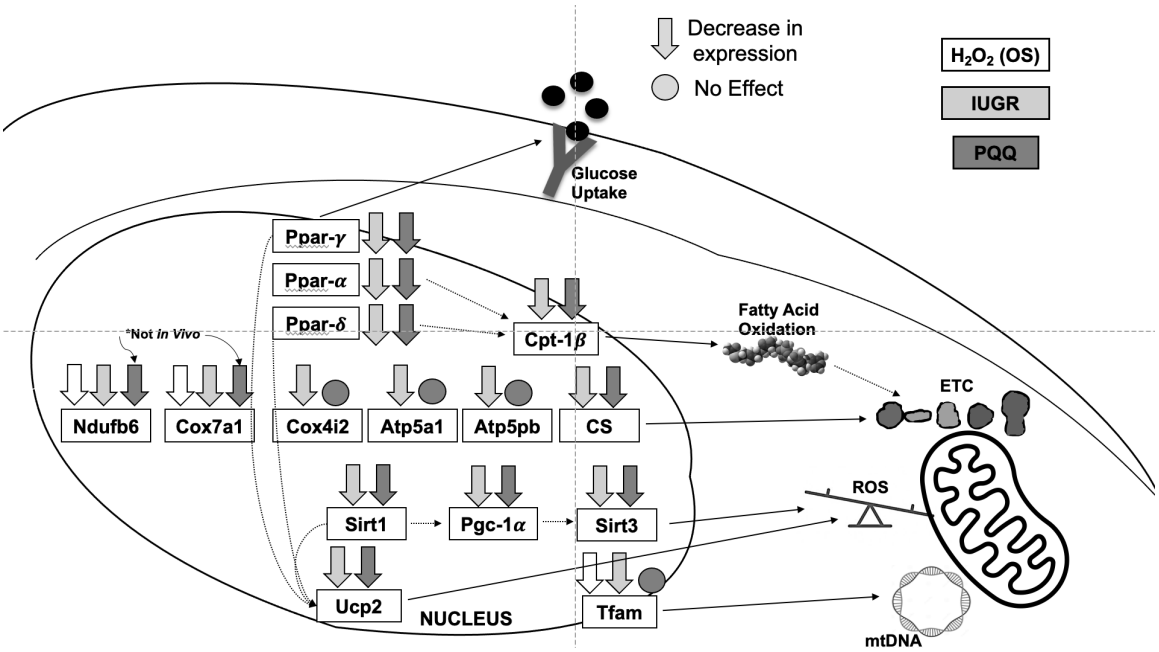


Figure 3.4.5-2 Summary of the relative mRNA expression of genes involved in cellular metabolism in developing *in vitro* myotubes and fetal gastrocnemius muscle.

Based on treatment group, the downward arrows represent a significant decrease in relative mRNA expression, while the circles represents that no significant change in expression was observed for each corresponding metabolic gene.

4 Discussion

4.1 Impact of Hydrogen Peroxide and PQQ on Antioxidant Enzymes' Total Protein Abundance and Activity

4.1.1 Studying OS and PQQ using H_2O_2 and LDH Activity/Total Protein Abundance

Exogenous H_2O_2 is utilized to induce OS within cell culture systems to study the insult's negative effects, as well as possible preventative treatments (Bosutti & Degens, 2015; Konyalioglu, Armagan, Yalcin, Atalayin, & Dagci, 2013). OS promotes the degradation of cellular membranes, resulting in the release of lactate dehydrogenase (LDH) in the extracellular space (Jovanović et al., 2010). Measuring the LDH activity within cell culture media, including that of C2C12 cells (Lin Tan, Shavlakadze, Grounds, & Arthur, 2015), can therefore can be utilized to determine the relative level of cytotoxicity induced by models of OS, as a proxy measurement of membrane damage (Kaja, Payne, Naumchuk, & Koulen, 2017).

Acute H_2O_2 exposure at concentrations as little as 100 uM has been shown to increase LDH activity within cell culture media (Hong & Liu, 2004; Wijeratne, Cuppett, & Schlegel, 2005), but this result was not replicated in the present study. Instead, 19H exposure to 1mM of H_2O_2 did not significantly affect LDH within the media of the differentiated myotubes. More recent research provides a possible explanation for this finding, as it was shown that 1mM H_2O_2 can actually inhibit the activity of LDH isolated from rabbit muscle (suspended in PBS) after only 1H of direct exposure (Kendig & Tarloff, 2007). It could therefore be postulated that even if OS was present within the cells and LDH was released into the media, that enzyme activity would not be increased due to presence of H_2O_2 within the media as well.

Secondly, PQQ at concentrations below 30uM has been shown to function as an antioxidant, while concentrations above 30uM have been associated with pro-oxidant effects in several cell types including those derived from the liver and adrenal glands (He

et al., 2003; J. Kim, Harada, Kobayashi, Kobayashi, & Sode, 2010; Wu, Pan, Shen, & Xing, 2018). In support of these studies, neither 1uM nor 10uM PQQ at any timepoint studied (5H, 48H, 7D) resulted in a significant change in LDH activity within collected media, similar to control and H_2O_2 treatments. Though, it is again important to consider the possible inhibition of LDH by H_2O_2 when interpreting these results, as PQQ has been shown to interact with culture media and produce H_2O_2, though the production mechanisms are unclear (He et al., 2003). It has been suggested that oxidation of reduced PQQ is the mechanisms associated with downstream H_2O_2 production (Mukai, Ouchi, Nagaoka, Nakano, & Ikemoto, 2016).

Furthermore, more recent research has suggested PQQ itself can inhibit LDH activity by directly binding to the enzyme, specifically LDHA of the skeletal muscle, and catalyzing the oxidation of NADH to NAD+ to promote the formation of pyruvate instead (Akagawa et al., 2016). Akagawa et al. (2016) suggest PQQ does not impact LDHA protein abundance, which this current study's data supports. However, activity studies would be needed to further confirm Akagawa et al.'s (2016) findings. Increased pyruvate production facilitated by PQQ-bound LDH implies increased energy production, though no functional test was conducted during this study to confirm.

4.1.2 CAT Activity

Though extracellular LDH activity proved to be an ineffective method to measure OS, the relative abundance and activity of relevant endogenous antioxidants is can also be used as proxy measurement, as decreased activity abundance and activity infers increased ROS accumulation (Ighodaro & Akinloye, 2018). Catalase (CAT) is among the most important antioxidant mechanisms when it comes to decreasing H_2O_2 directly, as its activity is increased during cellular ROS production to decompose H_2O_2 into water and oxygen to prevent damage (Ighodaro & Akinloye, 2018). Several contradicting studies have been published regarding the activity of CAT during incidences of OS specifically, including in skeletal muscle directly. Many of muscle-focused studies examined the activity of CAT in response to exercise or aging, both known promoters of ROS production and OS, and CAT has been shown to be both increased and decreased, as well as not effected

under such conditions (Berzosa et al., 2011; Ji, Dillon, & Wu, 1990; Lauer et al., 2005; Sullivan-Gunn & Lewandowski, 2013). Of note however, a recent postnatal IUGR pig model showed growth restriction to be associated with a significant decrease in CAT activity within the skeletal muscle, alongside decreased mRNA expression (L. Zhang et al., 2020). Overall, it has been concluded that OS is associated with decreased CAT activity, contributing to the pathological concentrations of OS (Ahmed Amar, Eryilmaz, Demir, Aykan, & Demir, 2019), while exercise is more commonly associated with increased CAT activity, as ROS remains at physiological concentrations (Dao et al., 2011).

It was hypothesized that a decrease in CAT activity would be evident after acute H_2O_2 exposure in the developing myotubes in line with the past OS research; it appears relatively high levels of H_2O_2 can outcompete cellular mechanisms that promote CAT production, and subsequently prevent the antioxidant's induction in *in vitro* settings (Martins & English, 2014). . Conversely, lower doses of H_2O_2 have been shown to induce CAT activity and protect against oxidative damage (Martins & English, 2014). In the current setting, there was no change in CAT activity observed in this study. In support of this finding though, one hour exposure of 1mM of H_2O_2 to *in vitro* yeast cells, in which CAT is highly abundant, surprisingly resulted in weak CAT activity stimulation and a significant decrease in cell viability (Martins & English, 2014). In short, this could be why an increase in CAT activity was not observed in this study, but further supports the study's assumption that 1mM H_2O_2 could model OS within the developing myotubes.

In addition to studies showing supplementation of other antioxidant-like compounds has the same positive effect in several organs, including postnatal IUGR skeletal muscle (Santos, Freitas, Xavier, Saldanha, & Freitas, 2008; L. Zhang et al., 2020), two recent studies have shown PQQ alone to increase *in vitro* neuronal and *in vivo* adult renal CAT activity in the presence of OS (Guan et al., 2015; Kumar & Kar, 2014). Interestingly, the present study did not observe the same increase. It is important to note that the increases in CAT activity observed in the Guan et al. (2015) and Kumar and Kar (2014) studies were only significantly increased compared to samples with decreased CAT activity as a result of cellular damage associated with OS, not compared to control samples. As this

study did not observe elevated LDH release from cells after H2O2 exposure, it can be concluded severe OS and associated damage was not present, unlike the previous studies in which a change in CAT was observed after PQQ exposure. Therefore, it is not surprising a significant increase in CAT activity after PQQ supplementation did not occur.

4.1.3 SOD Activity and Total Protein Abundance

There is an observed correlation between increased total SOD activity and elevated H_2O_2 concentrations, as the enzyme aids in the dismutation of superoxide into H_2O_2, which is observed under physiological conditions to prevent cellular damage (Younus, 2018). Conversely, exogenous H_2O_2 exposure directly has been shown to inhibit SOD3 (I.e., extracellular SOD) activity specifically (Casano ', Gomez, Lascano, Gonzalez, & Trippi, 1997; Wedgwood et al., 2011), through peroxidase activity (Jewett, Rocklin, Ghanevati, Abel, & Marach, 1999; Wedgwood et al., 2011). IUGR is also associated with a significant decrease in total SOD activity (H. Zhang, Li, & Wang, 2015). In contrast to these observations, 12H 500uM/L H_2O_2 exposure to developing C2C12 myoblasts resulted in decrease of total SOD activity that was nonsignificant (Li et al., 2020). Similarly, the response the cells had to H_2O_2 exposure in this study regarding total SOD activity, was decreased but not significantly in comparison to control. Therefore, the current study supports the findings of Li et al. (2020).

Not unlike CAT, PQQ, has been shown to increase SOD activity during insult (Kumar & Kar, 2014). Interestingly, however other studies have shown the opposite, in which the enzyme's activity is decreased due to PQQ supplementation after a maternal high-fat diet insult (Jonscher, Stewart, Alfonso-Garcia, DeFelice, Wang, Luo, Levi, Heerwagen, Janssen, De La Houssaye, et al., 2017). Of note, the study in which PQQ upregulated SOD activity were conducted in adult mice (Guan et al., 2015), while PQQ exposure *in utero* resulted in the significant decrease to SOD1 activity within developing fetal mouse liver in the Jonscher et al. (2017) study after insult. It is important to note the same study

showed that relative to control, SOD activity was unaltered by PQQ exposure alone. In alignment with the Jonscher et al. (2017) study, in the current study, PQQ exposure alone did not increase total SOD activity within the developing myotubes compared to control, nor was total protein abundance of SOD2 affected. Conversely, the same significant decrease observed in SOD activity after insult in the Jonscher et al. (2017) study was not observed, in samples exposed to PQQ and H_2O_2 in combination. Though, as stated regarding CAT activity above too, LDH activity in culture media in this study was not elevated after H2O2 exposure, and therefore it can be concluded severe OS and associated damage was not present. Thus, a significant insult was likely not present in this study's *in vitro* model, unlike the insult present in Jonscher et al. (2017) experiments, so PQQ was unlikely then to decrease SOD experience when paired with the H_2O_2 exposure.

4.1.4 H_2O_2 and Sodium Pyruvate

It is important to note the activity assays conducted during this study were completed with cells cultured with media supplemented with sodium pyruvate. The addition of sodium pyruvate to cell culture media has been shown to promote cell survival, hence why it was initially added to the study's culture system as per many established culture protocols (Alvarez-Elizondo, Barenholz-Cohen, & Weihs, 2019; Bergemann, Rebl, Otto, Matschke, & Nebe, 2019). Unfortunately, it has also been shown to suppress pathways in which PQQ is known to act on, including PGC-1α and its downstream mitochondrial targets within the same cell type (Philp, Perez-Schindler, Green, Hamilton, & Baar, 2010). More importantly, it has been shown itself to quench H_2O_2 within cell culture media, thereby inhibiting the ability of H_2O_2 to induce OS within the cells (Kelts, Cali, Duellman, & Shultz, 2015). It is therefore likely that the addition of sodium pyruvate to the cell culture media before the conduction of the studies' activity assays likely affected the results, in that OS was likely not fully induced in the cells, compared to other studies utilizing H_2O_2 to induce oxidative damage.

4.2 Expression of myogenesis regulators is negatively impacted by H_2O_2, IUGR, and PQQ

The mechanisms by which IUGR specifically effects myogenesis are well-defined (Chang et al., 2020), except for the specific role OS plays in said pathology, which remains unclear. Similarly, the effects of PQQ on fetal skeletal muscle development are relatively unknown, as its effects on the organ have been primarily studied in adult muscle samples (Kuo, Shih, Kao, Yeh, & Lee, 2015; Ma et al., 2019), or have focused on mitochondrial function in fetal skeletal muscle (Pendleton et al., 2020). Studies conducted using *in vitro* models of myotubes have additionally not measured markers of myogenesis directly, and instead have focused on the compound's antioxidant and metabolic effects (Supruniuk, Miklosz, & Chabowski, 2020; Xu et al., 2018). Hence, this study aimed to fill these gaps in research.

First, it was observed that acute *in vitro* H_2O_2 exposure significant decreased the expression of Myod and its downstream target, Myog. As stated previously, both these genes promote muscle differentiation, and the decreased expression seen as a result of H_2O_2 would infer decreased development of myotubes. Furthermore, IUGR was also associated with decreased expression of both genes (Chang et al., 2020). Studies have shown an association between IUGR and OS (Rashid et al., 2018), and so it could be inferred by these results that the OS the occurs during IUGR is at least partly responsible for the decreased myogenesis and subsequent diminished skeletal muscle mass. The fact that in this study's culture system, OS negatively impacted myogenesis markers, is strong evidence that this system may be in operation in developing fetal skeletal muscle as well. Additionally, this conclusion is supported by previous research aimed at understanding the mechanism underlying the actions of OS on myogenesis, in which OS has been shown to increase Nf-κB acitivity, which in turn inhibits myogenesis and decreases expression of Myod (Kozakowska, Pietraszek-Gremplewicz, Jozkowicz, & Dulak, 2015). In another model of IUGR, fetal gene expression of Pax7 did not change within semitendinosus muscle, but IUGR was still associated with impaired proliferation of Pax7+ myoblasts (Dustin T Yates et al., 2014), demonstrated by reduced incorporation of 5-Bromo-2'-deoxyuridine (BrdU) in cells isolated from IUGR fetuses (Dustin T Yates et

al., 2014). In support of this study, Pax7 expression was not affected by H_2O_2, though expression was decreased in association with IUGR.

It was initially hypothesized that PQQ would reduce OS, and subsequently, protect myogenic regulator factors from the downregulation seen in IUGR (Dustin T Yates et al., 2014). Surprisingly, low and high dose PQQ exposure throughout the 7 days of myotube differentiation *in vitro* resulted in a significant decrease in Myod1, and the compound did significantly decreased expression of Pax7 at all time-points explored. Similarly, the *in vivo* PQQ and IUGR/PQQ muscle tissue, had decreased Pax7, Myod1, and Myog mRNA expression. The negative effects of PQQ alone on Myod1 were only evident specifically after long-term PQQ exposure *in vivo* and *in vitro* during early development, hence the inferred long-term reduction in ROS production by PQQ in early development appeared to be detrimental to myogenesis progression. As referenced earlier, this phenomenon has been seen in placental development, in that early supplementation of mitochondrial antioxidant, MitoQ, inhibited growth of the organ (Yang et al., 2021).

Though proliferation is supported by a relative decrease in ROS production, physiological concentrations of ROS are important for skeletal muscle differentiation, and there is a documented decrease in expression of endogenous antioxidant enzymes in line with increases in Myod and Myog expression (Kozakowska et al., 2015). In developing C2C12 myoblasts specifically, an artificial increase in antioxidant mechanisms was shown inhibit Pax7 and Myod expression, shown to be the result of reductive stress (RS) (Rajasekaran, Shelar, Jones, & Hoidal, 2020). RS can be considered the opposite of OS, though still pathological, in which cells become more reduced relative to a normal state. This overexpression reduces physiological ROS levels, in turn inhibiting ROS cell signaling that would otherwise promote muscle development (Pérez-Torres, Guarner-Lans, & Rubio-Ruiz, 2017), which is further supported by the decreased of Pax7 and Myod1 observed by Rajasekaran et al. (2020). It could therefore be inferred that PQQ exposure induced RS within the developing *in vitro* myotubes and *in vivo* muscle, resulted in inhibited myogenesis, represented by the observed decrease in expression of Pax7, Myod1, and Myog in both models.

4.3 IUGR and PQQ Independently Altered Gastrocnemius Fiber Type

The observed changes in expression of myosin heavy chain isoforms indicates that IUGR and maternal PQQ consumption both influenced fetal skeletal muscle fiber type. The gastrocnemius of the fetal and adult guinea pig is composed of both slow oxidative type I and fast oxidative or glycolytic type II fibers (Aquin, Sillau, Lechner, & Banchero, 1980). Myh7 is expressed in type I fibers (Stuart et al., 2016), while fast oxidative fibers, types IIa and IIx, express Myh2 and Myh1 respectively (Tonge, Jones, Bardsley, & Parr, 2010). Type 2b fast glycolytic fibers express Myh4 (Tonge et al., 2010), though this fiber type is absent in humans (Harridge, 2007). IUGR was associated with a higher proportion of type I oxidative fibers, seen in early life piglets, in which there was a significant increase in type I fibers compared to type II (Bauer et al., 2006). In partial support of this study, the observed decrease in Myh4 and Myh1 in control diet IUGR animals suggests the adverse IUGR environment alone promotes processed associated with oxidative metabolism and increased type I fiber numbers, though Myh7 expression was also decreased, and IUGR alone did not affect Myh2 expression. It is important to note that the results of this study are limited in that the concentration of each fiber type was not directly measured, as done by Bauer et al. (2006), and further research examining the developmental consequences of the observed mRNA expression changes on fiber type composition need to be conducted.

This decreased expression of myosin heavy chain isoforms associated with type II fibers, seen in association with IUGR, could also be the result of increased oxidative damage to the more susceptible fiber type. Increased ROS production is associated with IUGR (Rashid et al., 2018), but the impact of ROS on muscle fiber composition in IUGR are ill-defined. Though, exercise does result in an increased production of ROS too within the skeletal muscle and the body appears to favor the switch to type I fibers due to their better ROS scavenging abilities (Powers, Ji, Kavazis, & Jackson, 2011; Schantz & Dhoot, 1987). Compared to type I fibers , type II fibers are more prone to oxidative damage (Schantz & Dhoot, 1987), which has also been specifically observed within the chosen muscle type of this study, the gastrocnemius muscle (Koutakis et al., 2014).

Similarly, maternal PQQ consumption alone specifically decreased Myh4, again suggesting a shift towards more oxidative metabolism. This result along with the decreased Myf4 expression observed due to IUGR, highlight a potential adverse effect of maternal PQQ supplementation and IUGR on early muscle development. Studies have shown that in regard to later life metabolic insults, sufficient type II fibers are important for adequate energy production using fatty acid oxidation (Izumiya et al., 2008). In short, the reduction in Myh4 observed in this study could indicate IUGR and surprisingly PQQ predisposes individuals to diminishing capacity to combat metabolic disease. In addition, decreased Myod1 expression is associated with inhibited type II fiber development, as Myod1 expression is relatively higher in type II fibers, compared to type I (Talbot & Maves, 2016). The results of this study further support this finding, as both Myod1 and Myf4 gene expression were both decreased in IUGR and PQQ exposed fetal muscle. Again, the current study was conducted at the mRNA expression level, and further studies are needed to confirm if the reduction in Myh4 expression translates to diminished type II fiber growth.

Similar to the prediction that increased ROS resulted in diminished expression of type II fiber myosin heavy chain isoforms in IUGR, early PQQ exposure itself could be promoting oxidative damage within developing skeletal muscle. The potential cytotoxicity of PQQ within the organ should therefore be studied more thoroughly. *In vivo* fibroblast growth studies in the context of cancer progression have shown that PQQ promotes apoptosis and increases intracellular ROS concentrations under conditions of high concentration PQQ exposure (Min et al., 2014). Our maternal PQQ dose was equivalent to supplement doses available on the market (I.e., 10-20 mg), though compared to the average daily consumption of PQQ through food human's intake, 0.1-1.0 mg per day (Harris et al., 2013), it was relatively high and was administered for an extended period of time during fetal development. In comparison, previous human studies that saw a positive effect only administered equivalent doses of PQQ for 48-76 hours (Harris et al., 2013). These factors combined could have resulted in the same negative effects accumulating over time within the fetal muscle before sample collection, as seen with the previously mentioned repeated fetal exposure to similar compound, MitoQ, in which development of the pancreas was inhibited (Roberts et al., 2014).

When IUGR occurred in conjunction with maternal PQQ intake, fiber type changes as identified above as occurring in IUGR or PQQ alone cohorts appeared to be more apparent, in that IUGR/PQQ animals displayed significantly decreased expression of all studied MHCs, though the mechanisms behind this finding are unknown. These results suggest inhibited development of all fiber types, though the physiological implications of this are not well defined. As this data is too at the mRNA level, further examination is needed for a more sufficient idea of IUGR and PQQ's combined effects on fiber type proportions and function.

Lastly, the Pgc-1α-Nrf-Tfam pathway, is considered the main regulatory pathway associated with fiber type adaptions to ROS associated with exercise (Wright et al., 2007), as the scavenging ability of skeletal muscle is partly dictated by its relative function and mitochondrial number (A. Cheng et al., 2016). Increased expression of Pgc-1α and Tfam is often associated as with increased mitochondrial biogenesis and concentration respectively, specifically in regard to skeletal muscle mitochondria (Baar et al., 2002; Gordon, Rungi, Inagaki, & Hood, 2001), and expression of both is increased as a result of exercise to combat potential oxidative stress (Jung & Kim, 2014; Theilen, Kunkel, & Tyagi, 2017). In regard to fiber type specifically, increased expression of PGC-1α is associated with a higher proportion of oxidative muscle fiber types in adult mice (Lin et al., 2002), which as stated previously help protect the organ from oxidative damage. Interestingly, in this current study, IUGR and PQQ exposed gastrocnemius muscle had decreased relative Pgc-1a mRNA expression, as well as Tfam expression that was decreased. This would primarily suggest a decrease in type I fiber production, but as mentioned, no decrease in Myh7 was observed. Compellingly, more recent studies found Pgc-1α to be dispensable in fiber type switching, in that knockout of the mitochondrial gene did not or only moderately decreased the number of type I fibers present (Handschin et al., 2007; Zechner et al., 2010). Overall, though the decreases in Pgc-1α and downstream Tfam may mean decreases in muscle mitochondrial function, the role these changes play in muscle fiber determination appear to be limited in the current study and others.

4.4 The impact of H_2O_2, IUGR, and PQQ on Markers of Mitochondrial Metabolism

As previously stated, IUGR is associated with muscle mitochondrial dysfunction (Rashid et al., 2018), though the specific genes affected during said dysfunction is not clear. For one, contradicting studies have been published in regard to Pgc-1α mRNA expression in relation to IUGR, with some showing an increase while others have documented a significant decrease dependent on the organ studied (Jones et al., 2019; Liu et al., 2012). Of interest, however is the significant decrease in Pgc-1α gene expression evident in adult skeletal muscle after IUGR specifically (Liu et al., 2012; Zeng et al., 2013), as the current study also identified the same decreased in fetal IUGR muscle. PQQ has been shown to significantly increase Pgc-1α expression directly to promote mitochondrial biogenesis with the liver (Chowanadisai et al., 2009), and therefore was proposed to be a plausible treatment for IUGR-associated mitochondrial dysfunction in this muscle study. PQQ did not have the same effect in both *in vitro* myotubes, in which expression was unaffected, and *in vivo* skeletal muscle, as PQQ significantly decreased expression of Pgc-1α alone. Similarly, total protein abundance of PGC-1α was unaffected in developing myotubes exposed to PQQ. It is important to acknowledge that myotubes were only allowed to differentiate for 7 days in culture, and hence the *in vitro* experiment could be considered "short-term" in comparison to the *in vivo* studies in which fetal muscle was exposed to PQQ for weeks. This could explain discrepancies between Pgc-1α's lack of change in total protein abundance, in comparison to the significant decrease observed in fetal tissue. PGC-1α protein has a longer half-life then other mitochondrial proteins (Wright et al., 2007), and therefore the expression decrease seen in IUGR fetal tissue may have been evident in the cells if culture experiments were able to be carried out for an extended period of time.

Several up and downstream genes of Pgc-1α were also studied. Sirt1 and Sirt3 both increasing expression of the gene, in addition to Pgc-1α also increasing Sirt3 expression in a positive feedback loop style, and therefore are both Sirt1 and 3 are critical components cellular metabolism (H. Yamamoto, Schoonjans, & Auwerx, 2007). A previous study had shown PQQ to increase the expression of both Sirt1 and 3 in *in vitro*

liver (Jian Zhang et al., 2015), though like the previously mentioned effects on Pgc-1α, PQQ supplementation in fetal skeletal muscle resulted in significantly decreased expression of Sirt3 and Sirt1 expression was decreased though not significantly. PQQ supplementation also did not affect total protein abundance of SIRT3 in the *in vitro* myotubes, which was surprising due to SIRT3's relatively short-half (Iwahara, Bonasio, Narendra, & Reinberg, 2012). It is evident that PQQ likely has organ-specific effects on mitochondrial gene expression, and likely subsequent function, though protein studies do not correlate with mRNA effects and activity assay was not conducted to test for functional changes. The same significant decreases were seen in fetal IUGR samples, those this was in support of recent research in which SIRT3 protein expression was downregulated in placental samples (Naha et al., 2020), and research showing Sirt1 mRNA expression to be significantly decreased in the brains of IUGR offspring (Caprau et al., 2007). Tfam, a regulator of mitochondrial DNA transcription, is also downstream of Pgc-1α, and is associated with decreased expression during IUGR, specifically evident in skeletal muscle (Liu et al., 2012); the results of the current study support this finding. It is important to note that though early research implied that decreased Tfam expression coincided with decreased mitochondrial biogenesis and subsequent organelle dysfunction, this is not always the case. Functional tests should be conducted to determine the significance of changes in Tfam expression specifically within the IUGR model to confirm mitochondrial dysfunction (Kozhukhar & Alexeyev, 2019). PQQ has been shown to have the opposite effect, in that short-term exposure induced Tfam expression in developing liver cells (Chowanadisai et al., 2010). In this study, PQQ alone did not affect Tfam expression in fetal skeletal muscle, though this is supported by another study in which continuous administration of PQQ to hindlimb skeletal muscle did not increase TFAM protein expression significantly (Kuo et al., 2015).

Another mitochondrial gene known to be negatively affected in IUGR is that of the enzyme carnitine palmitoyltransferase-1 (CPT-1), which is associated with fatty acid oxidation within skeletal muscle (Sebastián et al., 2009); mRNA expression of Cpt-1 has been shown to be significantly reduced in postnatal brains of IUGR rat offspring (Puglianiello, Germani, Antignani, Tomba, & Cianfarani, 2007). In support of this earlier study, Cpt-1β (I.e., the skeletal muscle isoform of Cpt-1) was documented in this study to

be significantly decreased as a result of IUGR. The effects of PQQ alone on Cpt-1β are unknown, and therefore this study presents a novel finding that the compound significantly decreases expression. It is however known the Pgc-1α regulates the functionality of Cpt-1β within the skeletal muscle (Lane et al., 2003), and therefore, the observed decreased in Pgc-1α mRNA expression could be the cause. Again, the functional effects of this decrease, along with the other identified, were not studied. Therefore, the consequences of the relative decreases in mRNA are unknown at this time, though it is inferred that these findings imply mitochondrial dysfunction.

Lastly, relative uncoupling protein 2 (Ucp2) expression was measured as a marker of mitochondrial function, as the protein serves to regulate oxidative phosphorylation (Tian, Ma, Tse, Wong, & Huang, 2018). Importantly, Ucp2 also serves to attenuate ROS production and protect the mitochondria from oxidative damage (Brand & Esteves, 2005). Though Ucp2 mRNA expression has been shown to be decreased in fetal IUGR sheep islets (Kelly et al., 2017), it has also been shown to be increased in the postnatal IUGR skeletal muscle (Lane et al., 2003) and unaffected in postnatal IUGR piglet liver (Ferenc et al., 2018). This study observed a significant decrease in Ucp2 in IUGR and even more so IUGR/PQQ in the fetal skeletal muscle. It is evident that Ucp2 mRNA expression during IUGR is largely dependent on the organ studied and the relative stage of development in which sample collection occurs; gene expression *in utero* and postnatal within the same organ appears to able to vary greatly, prompting the need for longitudinal studies. Even so, the decrease in Ucp2 gene expression in this current study implies increased OS stress within the two sample groups. It is also important to note that postnatal OS has been shown to induce Pgc-1α expression for downstream induction of endogenous antioxidant mechanisms, including Ucp2, in the response to ischemic brains to prevent damage (S. Der Chen et al., 2011), but this protective mechanism appears to be lost in this study's IUGR and PQQ exposed muscle, denoted by the decreased Pgc-1α and Ucp2 observed.

4.5 Negative Impact of IUGR and PQQ on PPAR expression

PPAR expression in muscle, including during *in utero* muscle growth, has not been well studied. The majority of studies have focused on the importance of PPARs within the liver and adipose tissue, due to their relatively high expression within these organs (Kersten, Desvergne, & Wahli, 2000). Past research highlighted a plausible relationship between decreased PPAR-γ and enhanced growth restriction under hypoxic conditions (Julian et al., 2014), as IUGR development is associated with a hypoxic growth environment as previously described (Ream, Ray, Chandra, & Chikaraishi, 2008). In support of this, a significant decrease of relative mRNA expression of PPAR-α, δ, and γ in all IUGR animals was found in this study.

Independent of IUGR, PQQ administration alone was also associated with significant decreases in muscle PPAR- α, and δ mRNA in normal growth animals. Within the postnatal liver, prolonged PQQ exposure has little effect on relative PPAR-α mRNA expression (Bauerly et al., 2011). In comparison, there was a significant decrease observed in this study. Of note, a decrease in PPAR- γ mRNA in the same animals was trending towards significance. These results suggest both fetal growth restriction and exposure to PQQ during development negatively affect key regulators of energy homeostasis within the skeletal muscle. It is important to highlight that Bauerly and colleagues (2011) study was conducted with adult rats, versus the fetal tissue employed in this study; fetal and adult tissue have been shown to have varying expression of each of the PPARs (Abbott, Wood, Watkins, Das, & Lau, 2010). Though, it is now evident that PQQ likely has organ-specific effects on PPAR expression. These studies together highlight possible variabilities in gene expression regulation during versus after the critical *in utero* developmental window that are important to consider when interpreting results. In more detail, PPAR expression *in utero* appears to be more susceptible to epigenetic alteration. The expression of each is easily affected by many early life insults including adaptions to changes in maternal diet in an attempt to adapt to the insult and promote organ function postnatally. *In utero* epigenetic changes to PPAR-α specifically have been shown to occur and be stable long-term (Lillycrop, Phillips, Jackson, Hanson,

& Burdge, 2005). These stable adaptions of PPARs as a result of *in utero* exposures could predispose the developing fetus to metabolic irregularities later in life, when ultimately, their diet and environment is likely more sufficient (Lian, Deng, Chen, & Deng, 2018; Rees, McNeil, & Maloney, 2008). Therefore, IUGR and PQQ's independent effects on PPAR expression warrant further, long-term investigation.

Modifications to PPAR expression in turn can lead to changes to the mitochondria. Reduction in PPAR-β/δ expression in the skeletal muscle of adult mice results in decreased mitochondrial oxidative capacity, and related fiber type switching to increased type II fiber concentrations (Schuler et al., 2006). Interestingly, the data of this study does not support the Schuler et al. (2006), as both a decrease in all PPARs and Myf4 was observed. Conversely, activation of PPAR-γ promoted mitochondrial biogenesis in part to due to downstream upregulation of Pgc-1a and Tfam, though this effect was not studied in skeletal muscle (Miglio et al., 2009). It could then be inferred from presented data that the IUGR and PQQ fetal animals independently will have associated mitochondrial dysfunction due to changes in PPAR expression, as result of the presented decreased expression of each PPAR, in addition to the decreases observed in Pgc-1α, and Tfam expression described. This appears to be the first study of this nature.

4.6 The ETC of skeletal muscle is negatively affected by H_2O_2, IUGR, and PQQ

Hypermethylation of ETC complex I subunit Ndufb6 and complex IV subunit Cox7a1 has been observed in the muscle of those with T2DM phenotypes (Zhou, Sun, Li, & Zhu, 2018), and since IUGR is associated with increased incident of said metabolic disorder, it is therefore likely changes to these genes contributed to the mitochondrial dysfunction evident in these offspring too (Mandò et al., 2014; Pendleton et al., 2020). Though the mechanisms of said hypermethylation are not well known, oxidative stress induced epigenetic regulation has been proposed (Strakovsky & Pan, 2012). In support of these hypotheses, it was observed in this study that acute hydrogen peroxide exposure to developing myotubes resulted in significantly decreased Ndufb6 and Cox7a1 mRNA expression. Similarly, relative expression of both markers was significantly decreased in the gastrocnemius of fetal IUGR animals. However, it cannot be confirmed that oxidative

stress was present and responsible, as no markers of said stress were examined in the guinea pig model. Though, Complex I and Complex IV dysregulation is associated with wide-scale mitochondrial dysfunction resulting in increased oxidative stress and it can therefore be inferred that oxidative stress is present within our IUGR model due to diminished Ndufb6 and Cox7a1 expression. Relative mRNA expression of Atp5a1 and Atp5pb, both subunits of complex V, was also significantly decreased in IUGR animals, suggesting further impairment to oxidative phosphorylation and long-term mitochondrial defects, including further oxidative damage (Jonckheere et al., 2013; Lebiedzinska et al., 2013).

Oxygen consumption is significantly decreased in the skeletal muscle, associated with reduced complex I activity in IUGR fetal sheep skeletal muscle, which is postulated to lower potential oxidative damage to the organ (Pendleton et al., 2020). The data from the described study supports this finding, as the decrease in Ndufb6 in fetal IUGR animals could be associated with decreased complex I activity. It is important to note that another study found complex I activity to be unaffected in skeletal muscle of older IUGR neonatal piglets with no change in expression of complex I subunits (K. Cheng et al., 2020), highlighting a need for further research of the stability of changes in fetal gene expression. Similarly, preliminary unpublished data in continuation of the present study found Ndufb6 expression to be unchanged in IUGR guinea pig offspring at 4 months of age, supporting the Cheng et al. (2020) study. It could be inferred the decrease in complex I seen in fetal skeletal muscle is a compensatory change that is no longer needed to prevent oxidative damage in a relative normoxic postnatal environment and hence is reversed. Comparatively, there were no observed protein abundance changes of the complexes within this study as a result of acute hydrogen peroxide exposure, though this is supported by other literature which suggests posttranscriptional regulatory events are present in IUGR that result in discrepancies between mRNA expression and protein concentration, specifically in regard to the complexes (Mandò et al., 2014; Pendleton et al., 2020).

Lastly, limited data has been published that suggests PQQ can induce complex I activity in livers in vitro (T. Stites et al., 2006). Likewise, low dose RSV has also been shown to

directly bind to complex I and increase its activity within the brain (Gueguen et al., 2015). As a result of these described studies, it was predicted PQQ would increase mitochondrial function by affecting complex I as well. The opposite was observed in this study's cell culture model, as PQQ resulted in significantly decreased Ndufb6 expression, though functional tests were not conducted to confirm changes in activity. The same gene was unaffected by maternal PQQ intake in the *in vivo* model. Comparatively, long-term PQQ exposure decreased Cox7a1 expression in developing myotubes, while expression was unchanged in maternal PQQ exposed fetal animals. In comparison to other studies, developing liver exposed to PQQ has been shown to exhibit increased Complex IV activity (Chowanadisai et al., 2010). Lastly, PQQ did not affect protein abundance of the complexes *in vitro.* In short, the effects of PQQ on the ETC complexes in skeletal muscle are still unclear. It is important to note that all described studies focused on the effects off PQQ and RSV in *in vitro* and postnatal liver and brain, while this study examined PQQ's effects in fetal skeletal muscle development. The organ differences between studies could possibly explaining the discrepancies seen.

4.7 Limitations

Firstly, the cell culture model utilized has several limitations to acknowledge. As previously referenced, the C2C12 cell line is commonly utilized to mimic *in utero* skeletal muscle growth (Jing Zhang et al., 2019), but it is important to recognize that it is transformed adult mouse cells and hence may have altered processes related to myogenesis and mitochondrial function (Abdelmoez et al., 2020). Secondly, the utilization of H_2O_2 to mimic oxidative stress within the cells did not allow for longitudinal studies, as H_2O_2 is quite detrimental to C2C12 survival after 24H exposure and is not commonly utilized past this time point (Gülden, Jess, Kammann, Maser, & Seibert, 2010; Siu, Wang, & Alway, 2009). Since oxidative stress present in IUGR occurs for an extended period of time (Potdar et al., 2009), the acute H_2O_2 exposure utilized is hence limited in how effectively it can be compared to any adverse pregnancy condition. Media with a high glucose concentration (25mM) is also utilized to proliferate and differentiate the cells and it could therefore be said that the cells were not grown in a physiologically relevant environment. Similarly, cells were grown in 21% O_2, whereas

fetal development occurs in relative hypoxia at 5% O_2. Lastly, the sex of the C2C12 cells is female, while *in vivo* studies were conducted in male tissue, and therefore comparisons between models may be flawed, as IUGR has been shown to have sex-specific effects, in that males are more greatly affected (Lane et al., 2003). Despite these limitations, the study did replicate findings of other similar fetal IUGR experiments. It also appears this is the first study of its kind utilizing C2C12 to study PQQ's effects on skeletal muscle development these in vitro studies allowed specific mechanistic pathways to be examined. Regarding the guinea pig model utilized, the presented data only included male animals; subsequent research should include female data as sex-specific changes are prevalent in other studies of IUGR. Positively, this IUGR model is comparable to idiopathic PI-IUGR in humans, as guinea pigs IUGR is spontaneous, and studies required no *in utero* manipulation, as described in the methods.

Interpretation of the studies' results is also limited by the prevalent use of qPCR, and subsequent lack of protein data collected from fetal guinea pig muscle, though many genes were examined, investigating multiple pathways of interest, which assisted in determining future directions. As reported in this study's cell culture experiments and confirmed by other studies, changes in mRNA expression and protein abundance are not always correlated. Hence, by not examining protein data in the fetal samples, the study missed collection of relevant information to make further conclusions. In addition, protein data presented from cell culture studies was limited in its own way, as minimal post-translational modifications were studied, and activity assays are lacking that would give the best overall picture of functional effects of changes in gene expression.

4.8 Future Directions

The presented data provides evidence which supports that both a H_2O_2 insult and an adverse *in utero* environment negatively affect skeletal muscle development and key readouts of muscle mitochondrial metabolism. Additionally, evidence is presented that PQQ supplementation during *in vitro* myotube development inhibits myogenesis and key readouts of mitochondrial function, and the same results were observed after maternal PQQ supplementation during the key fetal muscle developmental (I.e., myogenic)

windows. A logical progression would then be to identify if gene expression changes found, result in relevant functional consequences. With functional in situ experiments such as glucose uptake assays for example, the relative mitochondrial and metabolic consequences of the changes in gene expression could be observed.

Regarding mitochondrial function specifically, H_2O_2, and PQQ *in vitro* each independently decreased gene expression of complex I and IV subunits of the mitochondrial ETC. Conversely, protein abundance of both complexes was unaffected, highlighting a potential compensatory increased translational ability that may or may not result in maintenance of ETC function. To confirm PQQ does in fact negatively affect the ETC, activity assays for each complex should be undertaken and total mitochondrial respiration could be measured as a representation of overall mitochondrial function after supplementation of the compound. Similarly, protein abundance nor activity of the ETC complexes was measured in fetal gastrocnemius muscle and should be conducted to further substantiate relative mRNA data presented.

Furthermore, evidence is presented that OS is present in fetal IUGR development, represented by relevant decreases in expression of key mitochondrial genes shown to be negatively affected by the stressor in previous studies and by the relative decrease in Ucp2 gene expression observed. Though, more direct markers of OS were not measured within the gastrocnemius, thus should be to validate its presence. Of interest is the p53-p66shc-Pin1 pathway, including the role the genes play in ROS regulation during oxidative stress. PQQ's effects on this pathway are also relatively unknown, and it remains speculative if these genes play a relevant role in IUGR mitochondrial dysfunction. On the other hand, to protect against oxidative stress three major endogenous antioxidants exist to diminish ROS concentrations: SOD, catalase, and glutathione (Birben et al., 2012). Relative activity of each is commonly used as a measure of oxidative stress, with decreased activity corresponding with increased oxidative stress (Ighodaro & Akinloye, 2018; Michiels, Raes, Toussaint, & Remacle, 1994). *In vivo* muscle studies studying these antioxidants remains incomplete, and unfortunately, *in vitro* activity assays showed no decrease in SOD or CAT activity after H_2O_2 exposure

suggesting OS was not induced. Though, mitigating factors such as sodium pyruvate addition to cell culture media and PQQ inhibitory effects of LDH activity should also be considered when interpreting these *in vitro* results.

Lastly, several gene expression changes previously shown to be associated with mitochondrial dysfunction during IUGR have been proposed to be the result of epigenetic down-regulation of gene expression including increased DNA methylation as a result of hypoxia-induced OS (Campos et al., 2007; Roifman et al., 2016). In short, it is postulated that OS-induced DNA methylation is the mechanism responsible for the observed mitochondrial gene expression changes in IUGR offspring and further studies should be conducted to confirm. In support of this proposition, several genes previously associated with mitochondrial dysfunction, including Ndufb6, Cox7a1, Cpt-1, and Pgc-1α had decreased expression within both our model of OS, and IUGR. Overall, understanding the epigenetic effects induced by OS would be a useful further study to understand upstream regulators of gene expression changes that result in later life NCD in IUGR offspring.

4.9 Conclusion

In conclusion, the initial hypothesis proved to be true, in that H_2O_2 and IUGR did negatively affect markers of muscle development and mitochondrial function. Firstly, both H_2O_2 and IUGR resulted in the reduction of Ndufb6 and Cox7a1 expression (Figures 3.3.1-1,2; Figure 3.4.4-1), and the observed decreases in Pgc-1α and its downstream counterpart's mRNA expression in IUGR fetuses provides further context for this finding (Figure 3.4.4-2). Though, it is of note that total protein abundance of NDUFB8 was unaffected by acute H_2O_2 exposure (Figure 3.3.1-5), nor was Pgc-1α gene or protein expression significantly (Figures 3.3.1-3,7). Even so, together these results support the theory that *in utero* OS exposure as a response to a hypoxic growth environment results in the mitochondrial dysfunction evident in IUGR. This muscle mitochondria dysfunction is contributing factor to the increased risk of metabolic disease in adulthood observed in IUGR offspring, including but not limited to dysfunction associated with decreased expression of Ndufb6, Cox7a1, Pgc-1α, and Cpt-1. In

summary, the findings of this study provide evidence that OS is a likely contributor to the observed association between IUGR and adulthood metabolic disease, including CVD and T2DM (Zhou et al., 2018).

Secondly, in reference to myogenesis, Myod, and Myog expression were decreased as a result of IUGR (Figure 3.4.2-1), and H_2O_2 (Figure 3.3.2-2,3). Not unlike the mitochondrial function markers previously discussed, these results suggest the OS present *in utero* during IUGR contributes to the reduction seen in skeletal muscle mass by downregulating myogenesis. Furthermore, IUGR was associated with a significant decrease in fetal muscle expression of Myf4 and Myf1 (Figure 3.4.2-2), the myosin heavy chain isoform genes associated with type 2b and type 2x fibers, respectively. Evidently, all type 2 fibers are the most susceptible to oxidative damage (Schantz & Dhoot, 1987) and hence this study again suggests *in* utero OS plays a role changes to fiber development seen during IUGR. Whether these changes to Myf4 and Myf1 gene expression translate to switches in fiber type directly is unknown. Overall, these results are supported by previous studies in which IUGR skeletal muscle growth reduction was associated with decreased rates of myogenesis, but not increased muscle apoptosis as a result of the hypoxic growth environment (Chang et al., 2020). Though Pax7 expression was not affected by either H_2O_2 (Figure 3.3.2-1) nor IUGR (Figure 3.4.2-1), other studies have shown that intact expression Pax7 does not necessarily infer functional proliferation and myogenesis was still ultimately diminished in IUGR groups (D. T. Yates et al., 2012).

In contrast to the original hypothesis, PQQ did not increase expression of key markers of mitochondrial function. For example, previous studies have shown PQQ to increase expression of Pgc-1α, but this study observed no increase within *in vitro* muscle cells (Figure 3.3.1-3) while *in vivo* muscle had decreased expression (Figure 3.4.4-2). Similarly, markers downstream of Pgc-1α, including Sirt3 and Cpt-1β, showed decreased expression in PQQ-exposed fetal muscle (Figure 3.4.4-2) Lastly, Ndufb6 and Cox7a1 expression was also decreased by PQQ exposure in cell culture (Figure 3.3.1-1,2), though fetal muscle samples did not show the same negative effect. Simply put, these results do suggest that though PQQ has been shown to be beneficial to mitochondrial function in

postnatal liver and brain, its effect *in utero* skeletal muscle differ and the long-term effects of PQQ administration during gestation on mitochondrial function should be explored. It is important to consider that PQQ administration did not affect total protein abundance of any mitochondrial marker in culture, suggesting the observed decreases in gene expression may take time to manifest in protein abundance or may not translate to changes in protein abundance at all. Thus, the relative functional effects of documented changes in mitochondrial gene expression as a result of PQQ should be examined.

Secondly, PQQ did not protect against damage to myogenesis regulation and instead appeared to negatively affect genes involved in the process, in opposition to the original hypothesis again. PQQ alone decreased expression of both *in vitro* and *in vivo* muscle Myod and Myog expression (Figures 3.2.2-2,3; Figure 3.4.2-1). Furthermore, PQQ alone significant decreased Pax7 expression in fetal muscle, unlike IUGR (Figure 3.4.2-1). Overall, these results provide evidence that *in utero* PQQ exposure may dysregulate myogenesis, and this could be the result of its antioxidant properties. It is known that physiological levels of ROS promote differentiation of skeletal muscle, and similar placental research showed *in utero* exposure to antioxidants in can inhibit said signalling and inhibit development.

Finally, PQQ had varying effects on the expression of genes associated with skeletal muscle fiber type. Similar to IUGR, PQQ exposure alone was associated with decreased expression of Myf4 (Figure 3.4.2-2), though the functional significance and mechanisms behind this decrease are relatively unknown. Additionally, the combination of IUGR and PQQ exposure in fetal muscle appeared to augment the negative effects of IUGR on myogenesis, in that the reductions seen in Pax7, Myod, and Myog were all greater in IUGR/PQQ fetal tissue than the decreases seen as a result of PQQ and/or IUGR alone (Figure 3.4.2-1). IUGR/PQQ fetal samples also had decreased expression of all fiber type genes examined (Figure 3.4.2-2). The mechanisms behind these elevated negative changes and the physiological relevance of these decreases in gene expression are unknown, though the results do suggest widespread inhibition of the development all fiber types in IUGR/PQQ fetuses and likely a greater decrease skeletal muscle mass than

that associated with IUGR alone. The same augmentation was observed in genes associated with mitochondrial function, including Pgc-1α, Sirt1, and Tfam. Again, the mechanisms behind this combined negative effect on gene expression have yet to be explored, nor have their functional effects.

All together the results of this study suggest the OS during IUGR is a potential mechanism for the muscle mitochondrial dysfunction observed in IUGR offspring. This study additionally identified that PQQ exposure during skeletal muscle development may be detrimental to mitochondrial function and myogenesis progression and amplify to the adverse effects of IUGR.

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Appendix

Animal Use Protocol:

AUP Number: 2018-110

AUP Title: Modulating the in utero environment to prevent later life insulin resistance

Yearly Renewal Date: 10/01/2021

The YEARLY RENEWAL to Animal Use Protocol (AUP) 2018-110 has been approved by the Animal Care Committee (ACC), and will be approved through to the above review date.

Please at this time review your AUP with your research team to ensure full understanding by everyone listed within this AUP.

As per your declaration within this approved AUP, you are obligated to ensure that:

1) Animals used in this research project will be cared for in alignment with:

a) Western's Senate MAPPs 7.12, 7.10, and 7.15

http://www.uwo.ca/univsec/policies_procedures/research.html

b) University Council on Animal Care Policies and related Animal Care Committee procedures

http://uwo.ca/research/services/animalethics/animal_care_and_use_policies.html

2) As per UCAC's Animal Use Protocols Policy,

a) this AUP accurately represents intended animal use;

b) external approvals associated with this AUP, including permits and scientific/departmental peer approvals, are complete and accurate;

c) any divergence from this AUP will not be undertaken until the related Protocol Modification is approved by the ACC; and

d) AUP form submissions - Annual Protocol Renewals and Full AUP Renewals - will be submitted and attended to within timeframes outlined by the ACC.

http://uwo.ca/research/services/animalethics/animal_use_protocols.html

3) As per MAPP 7.10 all individuals listed within this AUP as having any hands-on animal contact will

a) be made familiar with and have direct access to this AUP;

b) complete all required CCAC mandatory training (training@uwo.ca); and

c) be overseen by me to ensure appropriate care and use of animals.

4) As per MAPP 7.15,

a) Practice will align with approved AUP elements;

b) Unrestricted access to all animal areas will be given to ACVS Veterinarians and ACC Leaders;

c) UCAC policies and related ACC procedures will be followed, including but not limited to:

i) Research Animal Procurement

ii) Animal Care and Use Records

iii) Sick Animal Response

iv) Continuing Care Visits

5) As per institutional OH&S policies, all individuals listed within this AUP who will be using or potentially exposed to hazardous materials will have completed in advance the

appropriate institutional OH&S training, facility-level training, and reviewed related (M)SDS Sheets, http://www.uwo.ca/hr/learning/required/index.html

Submitted by: Copeman, Laura

on behalf of the Animal Care Committee

University Council on Animal Care

Curriculum Vitae

Name:	Allyson Wood
Post-secondary Education and Degrees:	Trent University Peterborough, Ontario, Canada 2014-2018 B.Sc. Western University London, Ontario, Canada 2018-2021 M.Sc.
Honours and Awards:	Honour Roll, Trent University 2017, 2018 14th Annual Department of Physiology and Pharmacology Research Day Poster Award 2020
Related Work Experience	Teaching Assistant Western University 2018-2021

Research Presentations:

Allyson Wood, Christina Vanderboor, Timothy Regnault. Novel antioxidant-like compound pyrroloquinoline quinone's (PQQ) effects on mitochondrial function in differentiating mouse muscle tubules. 13th Annual Department of Physiology and Pharmacology Research Day, London, ON, November 5th, 2019.

Allyson Wood, Timothy Regnault. Pyrroloquinoline quinone's (PQQ) effects on oxidative stress and mitochondrial function in developing myotubes in a placental insufficiency IUGR environment. 14th Annual Department of Physiology and Pharmacology Research Day, Virtual, November 3rd, 2020.

Allyson Wood, Lin Zhao, Timothy Regnault. Pyrroloquinoline quinone's (PQQ) effects on oxidative stress and mitochondrial function in developing myotubes in a placental insufficiency IUGR environment. 8th Annual Canadian National Perinatal Research Meeting (CNPRM), Virtual, February 8th-12th 2021.

Allyson Wood, Lin Zhao, Timothy Regnault. Pyrroloquinoline Quinone's (PQQ) effects on skeletal muscle gene expression during oxidative stress/fetal growth restriction. 19th Annual Paul Harding Research Day, Virtual, April 28th, 2021.

Allyson Wood, Lin Zhao, Timothy Regnault. Pyrroloquinoline Quinone (PQQ) Supplementation Negatively Alters Skeletal Muscle Gene Expression in Addition to Oxidative Stress/IUGR's Effects. London Health Research Day (LHRD), Virtual, May 11th, 2021.

Allyson Wood, Lin Zhao, Timothy Regnault. Pyrroloquinoline Quinone (PQQ) Exposure Negatively Alters Skeletal Muscle Gene Expression in Addition to Oxidative Stress/Fetal Growth Restriction Effects. DOHaD Canada Webinar 2021, Virtual, May 31st – June 2nd, 2021.

Allyson Wood, Lin Zhao, Timothy Regnault. Pyrroloquinoline Quinone (PQQ) Supplementation Negatively Alters Skeletal Muscle Gene Expression in Addition to Oxidative Stress/IUGR's Effects. 68th Annual Meeting of the Society for Reproductive Investigation (SRI), Boston, MA, USA July 6-9th, 2021.