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The effects of quercetin on skeletal muscle mitochondrial biogenesis and OXPHOS regulation

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THE EFFECTS OF QUERCETIN ON SKELETAL MUSCLE MITOCHONDRIAL BIOGENESIS AND OXPHOS REGULATION

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DEDICATION

This thesis is dedicated to Jeffery Lynn Cook, without whose love, support, encouragement, and voice of reason none of this would have been possible.

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ABSTRACT

A high fat diet causes a reduction in the expression of the genes of the electron transport chain, mitochondrial carrier proteins, and PGC-1α and PGC-1ß mRNA. Mitochondrial dysfunction has been linked to numerous chronic diseases. The antioxidant quercetin has been shown to protect against damage caused by exogenous and endogenous free radicals. The purpose of this study was to determine if quercetin would protect the genes of mitochondrial biogenesis (PGC-1α and PGC-1ß) and OXPHOS regulation (NDUFB5 and SDHB) from the downregulation caused by consumption of a high fat diet. The quadriceps muscle was removed from 21 male, C57BL/6J mice that had been fed a low fat (10% kcal fat), a high fat (45% kcal fat), or a high fat plus quercetin (45% kcal fat + 1.2% quercetin) diet for 3 weeks. RNA was isolated from the samples and quantitative RT-PCR was used to compare gene expression. As expected, there was a significant difference between gene expression in the low fat group compared to the high fat and high fat plus quercetin groups for all measured genes ((pgc-1α: $F = 5.76$, $df = 2$, $P = 0.011$; pgc-1ß: $F = 5.28$, $df = 2$, $P = 0.016$; NDUFB5: $F = 5.66$, $df = 2$, $P = 0.012$; SDHB: $F = 5.04$, $df = 2$, $P = 0.018$). We found no significant difference between gene expression of the high fat group compared to the high fat plus quercetin group (pgc-1α: $t = -0.71$, $df = 12$, $P = 0.49$; pgc-1ß: $t = 0.16$, $df = 12$, $P = 0.87$; NDUFB5: $t = 0.27$, $df = 12$, $P = 0.79$; SDHB; $t = -0.20$, $df = 12$, $P = 0.84$). These results indicate that this dose of quercetin is not effective in protecting the expression of genes associated with mitochondrial health from the deleterious effects cause by consumption of a high fat diet.

INTRODUCTION

Approximately two-thirds of all U.S. adults are overweight or obese (Ogden et al., 2006), thereby increasing the risk of type 2 diabetes and death (Must et al., 1999). Furthermore, type 2 diabetes mellitus has become an epidemic (Phielix & Mensink, 2008) with more than 180 million people worldwide currently afflicted expected to double by 2030 (WHO, 2006). Roughly 6% of the United States adult population, approximately 18.3 million individuals, have been diagnosed as type 2 diabetic, with another 41 million people considered pre-diabetic, or having a blood glucose level between 100 and 125 mg/dl (Qatanani & Lazar, 2007). The number one cause of death among diabetics is cardiovascular disease. CVD deaths are up to four times higher for diabetics than for non-diabetics. Hypertension, atherosclerosis, and stroke, all common among diabetics, are highly correlated with obesity (Iqbal, 2007).

A high fat diet, a known cause of obesity and type 2 diabetes (Bray & Popkin, 1998), has been linked to rapid development of insulin resistance. While the exact mechanisms of high fat induced insulin resistance have yet to be elucidated, reduced mitochondrial function and biogenesis has been implicated in both human and murine models. Specifically, Sparks et al. (2005) found a reduction in the expression of the genes of the electron transport chain, mitochondrial carrier proteins, and PGC-1α and PGC-1ß mRNA.

Peroxisome proliferator-activated receptor gamma coactivator-1 (PGC-1) is important in the development of diabetes mellitus (Sparks et al., 2005) and a master regulator of oxidative phosphorylation gene expression and mitochondrial biogenesis (Puigserver & Spiegelman, 2003). Wu et al. (1999) have suggested that the mitochondrial dysfunction associated with type 2 diabetes is due to a down-regulation of the genes that code for PGC-1α and PGC-1ß. This further suggests that decreased insulin sensitivity in humans may be due to defects in

mitochondrial function and that this dysfunction causes increased levels of intracellular fatty acid metabolites that interfere with insulin signaling within the muscle (Qatanani & Lazar, 2007).

Cardiovascular disease and diabetes, among others, have been related to mitochondrial dysfunctions and mutations in mitochondrial DNA (mtDNA) (Wallace, 1999). Reactive oxygen species (ROS) are known to target mtDNA (Knight-Lozano et al., 2002) causing a variety of effects such as mtDNA damage, decreased mtDNA-encoded mRNA transcription, and decreased ATP levels and mitochondrial redox function (Ballinger et al., 2000). In animal models of atherosclerosis, mtDNA damage is increased and this damage is shown to occur prior to development of the disease (Knight-Lozano et al., 2002).

While exercise is known to be an important factor in obesity prevention (Jakicic, 2003), individuals partaking in high fat meals may require more than exercise alone to counteract or stave off the deleterious effects of such a diet. As a result, many natural and complimentary approaches are being explored to counteract or reduce these effects. Quercetin (3.3', 4', 5, 7-pentahydroxyflavone) is a polyphenolic plant compound in the flavonol subgroup of flavonoids (Enkhmaa et al., 2005) that has been shown to protect against cellular stress in animal models (Coskun, Kanter, Korkmaz, & Oter, 2005) and improve antioxidant status in humans (Kay & Holub, 2002). High concentrations are found in apples, tea, onions, blueberries, and red wine (Williamson & Manach, 2005). Quercetin exhibits antioxidative properties that are much stronger than those of vitamin C (Heo & Lee, 2004). In apolipoprotein E–deficient mice, quercetin attenuated atherosclerotic lesion development and protected LDLs from oxidation (Hayek et al., 1997). In humans, quercetin intakes of greater than 4mg per day reduced cardiovascular disease mortality by 21% (Graf, Milbury, & Blumberg, 2005). Graf et al. (2005) found a significant inverse correlation between quercetin consumption and diabetes that was not

observed when considering total flavonoid intake. Streptozotocin (STZ) induced diabetic rats given intraparatoneal (i.p.) injections of quercetin (70 mg/kg body weight) may inhibit the tissue damage caused by hyperglycemia (Dias et al., 2005). Coskun et al. (2005) found that i.p. injections (15 mg/kg body weight) of quercetin provided a protective effect in STZ induced diabetes, possibly by preserving the integrity of pancreatic ß-cells.

Few studies have examined the effect of purified quercetin on mitochondrial function and biogenesis. A 1% quercetin dietary intervention in a rat model resulted in an up regulation of genes involved in beta oxidation and ketogenesis and a reduction in free fatty acid levels in the lungs (de Boer et al., 2006). Santos et al. (1998) found that quercetin protected rat liver mitochondrial membranes from lipid peroxidation and increased permeability caused by ROS. Another rat study involving heart mitochondria suggests that quercetin may provide a cardioprotective effect by improving mitochondrial function (Trumbeckaite et al., 2006). Our goal in this study is to determine whether the antioxidant quercetin will protect mitochondrial function against the damaging effects of a high fat diet. In order to accomplish this, we will measure PG1-α and PGC1-ß mRNA, and the expression of oxidative phosphorylation genes using real time quantitative PCR and RT-PCR. This data will support future research combining exercise and quercetin supplementation.

Specific Aims and Hypotheses

Specific Aim 1

I will examine the influence of quercetin on mitochondrial biogenesis by measuring PGC-1α and PGC-1ß mRNA, using real time quantitative PCR. I hypothesize that expression of PGC1α and PGC1ß mRNA will be greater in the high fat plus quercetin treatment group as

compared to the high fat treatment group suggesting that quercetin protected mitochondrial biogenesis from the effects of a high fat diet.

Specific Aim 2

I will examine the influence of quercetin on the expression of genes related to oxidative capacity and the electron transport chain, specifically NDUFB5 and SDHB. I hypothesize that expression of NDUFB5 and SDHB will be greater in the high fat plus quercetin treatment group as compared to the high fat treatment group suggesting that quercetin compensates for the effects of a high fat diet on oxidative capacity.

LITERATURE REVIEW

Over 2 billion adults are overweight worldwide. Of those, 300 million are clinically obese. (Li, Bowerman, & Heber, 2005) making obesity a global epidemic (Zimmermann-Belsing & Feldt-Rasmussen, 2004). Two-thirds of the population of the United States is overweight or obese (Ogden et al., 2006). Over 9% of all health care dollars, approximately 93 billion dollars annually, can be attributed to obesity (Finkelstein, Fiebelkorn, & Wang, 2003). Persons who are overweight or obese are at an increased risk of type 2 diabetes, cardiovascular disease, hypertension, stroke, some cancers, and death (Must et al., 1999), however, it has been well established that even a small reduction (10%) in body weight can ameliorate, or even reverse, these conditions (Pi-Sunyer, 1996).

The incidence of type 2 diabetes mellitus is rapidly increasing (Phielix & Mensink, 2008). More than 180 million people worldwide are afflicted with type 2 diabetes mellitus and this number is expected to double by 2030 (WHO, 2006). Roughly 6% of the United States adult population, approximately 18.3 million individuals, has been diagnosed as type 2 diabetic, with another 41 million considered pre-diabetic (Qatanani & Lazar, 2007), defined as having a fasting blood glucose level between 100 and 125 mg/dl.

Disease States And Mitochondrial Function

Insulin resistance is defined as the inability of a normal amount of insulin to elicit a response in organs such as muscle, fat, and liver that results in an increase in circulating levels of glucose (Ranheim, Dumke, Schueler, Cartee, & Attie, 1997). Increased glucose concentrations lead to increased insulin production (Smith, 1994). Excessive fat ingestion causes an increase in circulating triacylglycerides and free fatty acids (FFA) (Montecucco, Steffens, & Mach, 2008). FFA increase the activation of protein kinase C θ, which, in turn, inhibits the activation of the

insulin receptor substrate preventing the uptake of glucose into the cell (Montecucco et al., 2008).

A HFD has been implicated in obesity and type 2 diabetes development (Bray & Popkin, 1998). Increased lipid oxidation is associated with a HFD (Storlien et al., 1991). Kim et al. (2000) demonstrated that rats fed a high fat diet for three weeks developed insulin resistance, possibly through a decrease in glycogen synthase activity in skeletal muscle caused by increased levels of oxidized lipids.

Skeletal muscle is one of the most important tissues affected by DM2 (Phielix & Mensink, 2008). Skeletal muscle contains large quantities of mitochondria and relies heavily on the energy produced by oxidative phosphorylation (Kelley, He, Menshikova, & Ritov, 2002). Obese and type 2 diabetics have been shown to have smaller mitochondria than their lean, healthy counterparts, however Kelley et al. have also demonstrated that the skeletal muscle mitochondria of type 2 diabetics may also be enlarged and damaged (Kelley et al., 2002).

Reduced mitochondrial biogenesis and oxidative phosphorylation have been implicated in the pathogenesis of type 2 diabetes (Boirie, 2003). Reduced expression of the genes involved with oxidative phosphorylation as well as mitochondrial biogenesis has been associated with ingestion of a high fat diet in both humans and mice (Sparks et al., 2005). *In vivo*, impaired mitochondrial function is involved in the early pathogenesis of type 2 diabetes (Phielix & Mensink, 2008). Mitochondrial function has been shown to be impaired even after normalization for mitochondrial content (Ritov et al., 2005). In other words, the reduction in mitochondrial number, as demonstrated by mitochondrial DNA quantities, was not great enough to account on its own for the reduction in electron transport chain activity.

It has been hypothesized that impaired mitochondrial function may contribute to the development of skeletal muscle insulin resistance (Simoneau, Veerkamp, Turcotte, & Kelley, 1999). Insulin resistant individuals have been shown to have significantly higher triglyceride concentrations in muscle which is accompanied by decreased mitochondrial function as evidenced by a reduction in mitochondrial oxidative capacity and ATP production (Petersen et al., 2003). Increased lipid accumulation in skeletal muscle is associated with a reduced oxidative capacity in type 2 diabetics and the obese.

Increased lipid accumulation is also associated with insulin resistance. Impaired mitochondrial function, as measured by the rates of incomplete fatty acid oxidation and the accumulation of beta oxidative intermediates, contributes to the development of human insulin resistance (Lane, 2006). Incomplete fatty acid oxidation is increased in the muscle tissue of high-fat diet induced insulin resistance. 3-hydroxy-butyryl carnitine, a marker of incomplete fat oxidation, is increased in the muscle tissue during high-fat diet induced insulin resistance in rodents (Koves et al., 2005) . When mitochondrial function is recovered, circulating levels of insulin and levels of incomplete fat oxidation, as measured by 3-hydroxy-butyryl carnitine, decrease and insulin sensitivity is returns (An et al., 2004; Boucher et al., 2004).

Type 2 diabetics have been reported to have an increased number of mitochondrial DNA (mtDNA) mutations. Mitochondrial DNA is more susceptible to ROS and NOS damage than nuclear DNA (nDNA) and has a repair mechanism that is less efficient increasing the likelihood of developing acquired mutations (Liang, Hughes, & Fukagawa, 1997). Diabetes and cardiovascular disease have both been linked to mtDNA mutations and mitochondrial dysfunction(Wallace, 1999). Nishikawa et al. (2000) have shown an increased level of free radical damage to mtDNA of diabetic patients.

PGC-1 is important in the development of diabetes mellitus (Sparks et al., 2005). PGC-1 α has been demonstrated to be a master regulator of oxidative phosphorylation gene expression and mitochondrial biogenesis (Puigserver & Spiegelman, 2003). Wu et al. (1999) have suggested that the mitochondrial dysfunction associated with type 2 diabetes is due to a down-regulation of the genes that code for PGC-1 α and PGC-1 ß. PGC-1 α and PGC-1 ß are both down-regulated in overweight non-diabetic and obese diabetic Mexican Americans (Patti et al., 2003) while obese Caucasians with type 2 diabetes have down-regulated PGC-1-responsive genes (Mootha et al., 2003). This further suggests that decreased insulin sensitivity in humans may be due to defects in mitochondria function and that this dysfunction causes increased levels of intracellular fatty acid metabolites that interfere with insulin signaling within the muscle (Qatanani & Lazar, 2007).

The electron transport chain of oxidative phosphorylation involves five enzyme complexes, each comprised of multiple subunits. While complex II is encoded completely by nDNA, the subunits of complexes I, III, IV, and V are encoded by both nDNA and mtDNA (Ton, Hwang, Dempsey, & Liew, 1997).

Complex I catalyzes the transfer of two electrons from NADH to coenzyme Q, which, in turn, transfers them to complex III. Complex I is the least understood of the complexes, mostly because of its large size and number of subcomplexes. The NADH dehydrogenase (ubiquinone) 1B gene is encoded by mtDNA and codes for a protein subcomplex of complex I (Ton et al., 1997). Defects leading to reduced or altered production of the subunits of complex I will reduce oxidative capacity causing an increase in free radical production and a reduction in ATP production (Ugalde, Janssen, van den Heuvel, Smeitink, & Nijtmans, 2004).

Complex II is the smallest of the electron transport complexes. It accepts electrons from succinate through FAD and transfers them to complex III via ubiquinone. The nDNA gene of the same name encodes its subcomplex succinate dehydrogenase complex, subunit B (SDHB). Any reduction in the ability of SDHB to transfer electrons results in decreased ATP production as well as increased oxidative stress from both the respiratory chain and exogenous sources (Rustin, Munnich, & Rotig, 2002).

Lifestyle Interventions

Weight loss resulted in an increase of oxidative capacity in skeletal muscle (Kern, Simsolo, & Fournier, 1999). Weight loss also improved insulin sensitivity in all obese individuals, diabetic and non-diabetic (Friedman et al., 1992). The expression of oxidative enzymes is enhanced with physical activity (Papa, 1996). Physical activity also causes an increase in mitochondrial biogenesis as represented by an increase in the expression of the enzymes succinate dehydrogenase and NADH dehydrogenase among others, while inactivity causes a reduction in biogenesis (Essig, 1996). Endurance training causes up to a 10-fold increase in PGC-1 mRNA. The increased expression of PGC-1α may lead to increased levels of GLUT4 that could explain the improved mitochondrial biogenesis seen with exercise (Baar, 2004).

Despite the evidence of the benefits physical activity and weight loss, lifestyle interventions are difficult to achieve or maintain (Qatanani & Lazar, 2007). The need for pharmaceutical or naturaceutical treatments to this pandemic seems evident.

Free Radical Activity

The reactivity of free radicals comes from the presence of at least one unpaired electron. They are formed as mediators or by-products of processes such as neurotransmission

inflammatory reactions (Boots, Haenen, & Bast, 2008). Free radicals are involved in the pathology of a number of conditions. Oxidized LDL is involved in the development of atherosclerosis. Free radicals play several roles in the development of cancer. They contribute to the development of diabetes by playing a role in the autoimmune destruction of ß cells while also impairing the action of insulin. Free radicals have even been implicated in asthma, rheumatoid arthritis, and cataracts (Knekt et al., 2002).

ROS react easily with and damage many biological molecules including DNA, RNA, lipids, carbohydrates, and proteins (Diplock et al., 1998) ROS cause damage to cells and tissues through lipid peroxidation, DNA breakage processes, and protein cross-linkage (Chow, Shen, Huan, Lin, & Chen, 2005). The free radical oxidizes the attacked molecule by removing an electron or hydrogen atom. For example, when ROS oxidize unsaturated fatty acids, a hydrogen atom is removed and an unpaired electron remains on the fat molecule. The lipid radical reacts with oxygen to form a peroxyl radical. The peroxyl radical removes a hydrogen atom from a second fatty acid forming a lipid hydroperoxide and a new lipid radical. A by-product of this chain reaction is malondialdehyde (MDA) (Halliwell & Chirico, 1993). MDA has the ability to cause mutagenic lesions by reacting with DNA bases (Spiteller, 2001). The damage caused by ROS can also cause loss of enzyme function, disturbed cell signaling, increased cell permeability, and cell death (Boots et al., 2008). These damages are thought to be involved in the etiology of diseases such as diabetes (Mehta, Rasouli, Sinha, & Molavi, 2006), stroke, coronary artery disease (Prior, 2003) and cancer (Valko, Rhodes, Moncol, Izakovic, & Mazur, 2006). When the production of reactive species surpasses the body's endogenous antioxidant network's ability to provide protection, a state of "oxidative stress" occurs (Boots et al., 2008).

Oxidative phosphorylation is the primary source of endogenous ROS. Almost 90% of consumed oxygen goes to the mitochondria where it is reduced to H_2O creating a proton electrochemical gradient in the inner mitochondrial membrane necessary for the production of ATP. During oxidative phosphorylation a small fraction of that oxygen (approximately 3%) is only partially reduced resulting in the formation of reactive oxygen species (Magder, 2006). ROS, such as hydrogen peroxide (H2O2), singlet oxygen ($^1O_2$), superoxide radicals ($O_2.^-$), and hydroxyl radicals (OH•) are the most reactive species derived from aerobic metabolism (Gutteridge, 1994).

Various types of cells, such as endothelial cells, cells of the nervous system, and macrophages, produce nitric oxide through the enzyme activity of nitric oxide synthase (NOS). Neuronal (nNOS) and endothelial NOS (eNOS) produce nitric oxide important for the regulation of vascular function and nerve cell communication (Huk et al., 1998). Activated macrophages increase production of nitric oxide as well as the free radical superoxide. The inducible NOS (iNOS) produced by macrophages leads to much larger nitric oxide concentrations. When NO and the free radicals react, peroxynitrite is formed. Peroxynitrite has the ability to directly oxidize the LDL leading to irreversible cell membrane damage.

Antioxidant Activity

Halliwell and Seis (1995; Sies, 1993) define an antioxidant as "any substance that, when present in low concentrations compared to that of an oxidizable substrate, significantly delays or inhibits the oxidation of that substrate." Conditions associated with high levels of oxidative stress could see halted progression or even a reversal as a result of antioxidant supplementation (Boots et al., 2008). Dietary intake of naturally occurring antioxidants can possibly prevent the development of diabetes and atherosclerosis (Wang et al., 2006) by reducing the state of

oxidative stress and damage (Halliwell, 1996). It is this possible use of antioxidants as treatment and prevention that has spurred much of the recent interest in them (Vertuani, Angusti, & Manfredini, 2004).

Flavonoids

Flavonoids act as potent free radical scavengers (Kandaswami & Middleton, 1994). It is well established that flavonoids, polyphenols found in plants, have antioxidant, anti-inflammatory, anticarcinogenic, antiviral, and antithrombotic properties (Nijveldt et al., 2001). The antioxidant activity of flavonoids is related to molecular structure. Flavonoids are composed of three six-membered rings (Peluso, 2006) As the number of hydroxyl groups attached to the flavonoid increases, so does antioxidant capacity (Behling et al., 2006). The hydroxyl groups of flavonoids are highly reactive. As a result, free radicals readily oxidize the flavonoid resulting in a less reactive and more stable radical (Korkina & Afanas'ev, 1997). Current data points to the idea that it is this antioxidant activity that provides the health benefits linked to dietary vegetables, fruits, and red wine (Rahman, Biswas, & Kirkham, 2006).

The flavonoids are subdivided, based on molecular structure, into 4 main classes: flavones, flavanones, catechins, and anthrocyanins (Nijveldt et al., 2001). Flavones are found in celery and parsley and have anti-cancer properties. Citrus fruits are good sources of flavanones (Peluso, 2006). Catechins are found in teas, chocolate, wine and many other plant sources and have been shown to help prevent the development of the metabolic syndrome, possibly by increasing lipid metabolism (Ikeda, 2008). Anthrocyanins sp? provide the red or purple pigments to many plants, fruits and vegetables. They provide a protective effect against cancer, inflammation and diabetes (Mazza, 2007).

It has been suggested that flavonoids may provide effective treatments for obesity as well as cardiovascular disease and type 2 diabetes mellitus (Peluso, 2006). More specifically, flavonoids have an effect on energy metabolism. In animal models, green tea, which contains the polyphenol epigallocatechin gallate (EGCG), is associated with a reduction in adipose tissue mass that was not accompanied by a reduction in energy intake (Choo, 2003). Furthermore, a diet containing caffeine-free green tea extract, known to be rich in polyphenols, stimulated lipid metabolism in mice (Murase, Haramizu, Shimotoyodome, Nagasawa, & Tokimitsu, 2005). Kampferol and quercetin increased the oxygen consumption of human skeletal muscle cells by as much as 30%, thereby increasing cellular energy expenditure. It is believed that this is accomplished through an upregulation of type 2 deiodinase which, when in circulation, causes an increase in the production of the thyroid hormonone triiodothyronin (T3) (da-Silva et al., 2007). This, in turn, causes an increase in energy expenditure (Silva, 2006).

Quercetin

Quercetin is the strongest of the flavonoid free radical scavengers (it contains five hydroxyl groups) and it is suggested that quercetin has the ability to contribute to the total plasma antioxidant capacity more than 6 times that of vitamin C and urate combined (Arts, Sebastiaan Dallinga, Voss, Haenen, & Bast, 2004). Quercetin (3.3', 4', 5, 7-pentahydroxyflavone) is a polyphenolic compound in the flavonol subgroup of flavones, along with kampferol and myricetin (Enkhmaa et al., 2005). Quercetin also has the ability to boost the endogenous antioxidant system. Treatment with quercetin (25, 50 and 75 mg/kg body weight) reduced the amount of alcohol damage to the antioxidant enzymes superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase, and the nonenzymatic endogenous antioxidant glutathione (Molina, Sanchez-Reus, Iglesias, & Benedi, 2003).

Interest in quercetin within the scientific community (Pawlikowska-Pawlega et al., 2007) and the supplements industry has increased in recent years due to an increase in the popularization of quercetin's health benefits (Weldin, Jack, Dugaw, & Kapur, 2003). Even though quercetin is the major bioflavonoid in the human diet, (Lamson & Brignall, 2000) U.S. consumption of dietary quercetin is only about 30 mg/day (Weldin et al., 2003). High concentrations are found in apples, red grapes, tea, onions, berries, leafy green vegetables, and red wine (J. C. Chen et al., 2005; Williamson & Manach, 2005). Absorption occurs within 30 minutes of ingestion (Lesser, Cermak, & Wolffram, 2004).

Quercetin is taken up in the small intestine (de Boer et al., 2006), although the exact mechanism of absorption has yet to be elucidated (Murota & Terao, 2003). It is immediately metabolized by epithelial cell enzymes and then further metabolized in the liver resulting in the quercetin metabolites isorhamnetin (3'OCH_3-quercetin) and tamarixetin (43'OCH_3-quercetin). Quercetin metabolites accumulate in the blood stream (Murota & Terao, 2003). Quercetin and its metabolites were found in all tissues analyzed after treating rats for 11 weeks with a 1000 or 10,000 mg quercetin per kg diet. The highest concentrations were found in lung tissue; the lowest in white fat and brain. Within analyzed quadriceps muscles, 69% was in the isorhamnetin form, 19% as tamarixetin, 11% as quercetin (de Boer et al., 2005). While quercetin is conjugated during absorption, this does not have a detrimental effect on its antioxidant capacity (Juzwiak et al., 2005; Morand et al., 1998). Because quercetin accumulates in the system, quercetin's effects last for an extended period of time (Castilla et al., 2006).

Fat content of the quercetin-enriched diet seems to be important for bioavailability. Bioavailability in pigs fed quercetin enriched diets (approximately 9mg/kg body weight) was significantly higher with a diet containing 17% fat compared to a standard pig diet containing

3% fat (Lesser et al., 2004). In humans, the food source is also important. Even after adjusted for intake, the bioavailability of quercetin from onions was twice that of the bioavailability from tea. This is probably due to the difference in the quercetin conjugates within the two foods. The quercetin glucoside in the onions is likely absorbed more readily than the quercetin rutinoside found in the tea (de Vries et al., 1998). The ingestion of 11mg per day of quercetin by healthy volunteers caused an increase in both plasma quercetin concentration and total plasma antioxidant capacity (Wilms, Hollman, Boots, & Kleinjans, 2005). In humans, quercetin's plasma half-life is between 17 and 28 hours (de Boer et al., 2006). Ingestion of 50 mg quercetin will lead to plasma concentrations of approximately 0.75-1.5 μmol/L (Rowland et al., 2003; Scalbert & Williamson, 2000).

The maximum tolerable dose has been established to be at least 2 doses of 2500 mg/kg taken 24 hours apart (J. da Silva et al., 2002). This is the maximum tested dosage and no adverse effects were observed. This is much higher than the common dose of quercetin used for supplemental or medicinal purposes (100 – 500 mg/day) (Goodman & Gilman, 1970). Quercetin was not carcinogenic at 10,000 40,000 or 100,000 mg quercetin per kg diet (Morino et al., 1982) and has been demonstrated to be nonmutagenic in doses as high 1000 mg/kg body weight (Aeschbacher, Meier, & Ruch, 1982).

Quercetin is involved in various biological actions including antiviral, anticarcinogenic, and anti-inflammatory actions (Mi, Zhang, & Taya, 2007). It prevents the oxidation of lipids both *in vivo* and *in vitro* (Ferry et al., 1996; Fiorani et al., 2001). It is even suggested that he antioxidant effects of quercetin could protect neuronal cells against oxidative-stress induced neurotoxicity such as the type that causes Alzheimer's disease (Heo & Lee, 2004). These actions are believed to be related to the antioxidant properties of quercetin, such as its ability to diminish

reactive oxygen species, inhibit lipid peroxidation, scavenge free radicals, and inhibit the damage hydrogen peroxide causes to DNA (E. L. da Silva, Piskula, Yamamoto, Moon, & Terao, 1998; Fiorani et al., 2001; Johnson & Loo, 2000; Mi et al., 2007).

Quercetin has also been shown to have an effect on athletic performance. Elite cyclists given 300 mg quercetin twice per day in an antioxidant supplementation beverage experienced a 3.1% decrease in a 30 km time trial performance with no change to diet or training regimen as compared to baseline and the control group who received the antioxidant beverage without the quercetin. This is practically significant as the difference between first and fifth place in the 2004 men's Olympic 50km road time trial was 1%; the difference between first and ninth was 3%. The decrease in time is attributed to an increase in power output, although a mechanism for this increase has yet to be determined (MacRae & Mefferd, 2006). A repeat of this study is necessary to look at dose as well as absorption, preferably using a quercetin supplementation alone as opposed to general antioxidant supplementation. Trained cyclists ingesting 500 mg pure quercetin twice daily (mixed into Tang beverage) for 3 weeks had 9 times the plasma quercetin levels compared to the placebo group. This group also had a significantly reduced incidence rate of upper respiratory tract infection during the two weeks following a 3-day period of intense exercise, despite no changes in immune function (Nieman et al., 2007).

A 21% decrease in cardiovascular disease mortality is seen in humans ingesting 4 mg or more of quercetin per day (Graf et al., 2005). A study of the dietary habits of over 10,000 Finnish participants revealed an association between higher levels quercetin intake and reduced ischemic heart disease mortality (Knekt et al., 2002). A separate study with the same participants revealed that those individuals with higher flavoniod intakes also had smaller common carotid artery intima-media thickness (CCA-IMT) (Mursu et al., 2007). This finding is important as

CCA-IMT is an independent predictor of cardiovascular disease (Lorenz, Markus, Bots, Rosvall, & Sitzer, 2007). Graf et al. (2005) found a significant inverse correlation between quercetin consumption and diabetes by extrapolating the data collected by the Knekt group (2002). This correlation was not seen when looking at total flavonoid intake.

In vitro, 2 – 30 μmol/L of quercetin prevented the oxidation of LDL cells (O'Reilly, Sanders, & Wiseman, 2000). Quercetin ingestion levels were negatively correlated with plasma total and LDL cholesterol levels (Arai et al., 2000). A reduced progression of atherosclerosis through reduced LDL oxidation and aggregation, and decreased foam cell production was seen in apo E-null mice that were given 50 μg quercetin per day (Hayek et al., 1997). An increased resistance to LDL oxidation was seen in humans after 14 days of supplementation with 30 mg quercetin per day (Chopra, Fitzsimons, Strain, Thurnham, & Howard, 2000). Red grape juice, of which quercetin is a major polyphenol, decreases blood plasma levels of the inflammatory biomarker MCP-1, LDL and oxidized LDL while simultaneously increasing HDL levels (Castilla et al., 2006). Mulberry leaves prevented development of atherosclerotic lesions in LDL receptor deficient mice by protecting the LDL from oxidation provided by the very high levels of quercetin aglycone in the leaves (250 mg aglycone per 100 g fresh weight) that equaled approximately 0.02 mg/kg body weight (Enkhmaa et al., 2005). These leaves are also known to provide an antihyperglycemic effect (F. Chen, Nakashima, Kimura, & Kimura, 1995). They have been extensively used in this manner as a naturaceutical treatment for diabetes in Chile, Korea, and Japan (Lemus, Garcia, Delvillar, & Knop, 1999).

Stage 1 hypertensive patients saw a significant drop in systolic, diastolic, and mean arterial pressure after ingesting 730 mg quercetin per day for four weeks. The same was not seen in pre-hypertensive patients, suggesting that while quercetin may provide a protective effect, it

will only bring about improvements when used to treat an existing disease state (Edwards et al., 2007). The reduction seen was similar to that seen with lifestyle modifications such as increased physical activity, weight loss, or decreased sodium or alcohol intake (Whelton et al., 2002). Interestingly quercetin supplementation did not cause a decrease in systemic oxidative stress markers (Edwards et al., 2007).

This is contrary to results seen with daily quercetin ingestion (10 mg/kg body weight) by spontaneously hypertensive rats. Duarte et al. (2001) found a reduction in oxidative state as well as decreases in elevated blood pressure and cardiac and renal hypertrophy compared with healthy Wistar Kyoto rats. There was no change in body weight in either group of rats (Duarte et al., 2001). Carlstrom et (2007) saw similar effects, but only after administering quercetin by gavage for 4 days. When the rats were given a diet enriched with 1500 mg quercetin per kg diet, there were no differences between the SHR and SHR+Q groups in hypertension, cardiac hypertrophy, or vascular dysfunction (Carlstrom et al., 2007).

When Sprague-Dawley rats were fed a high-fat, high-sucrose (HFS) diet supplemented with 200, 700, 2000, or 5000 mg per kg diet for four weeks, there was a suppression of blood pressure elevation compared to those fed a HFS diet with no added quercetin. Quercetin also reduced lipid peroxidation and reduced the decrease in plasma and urine nitric oxide levels as well as vascular tissue NOS activity. The increased availability of nitric oxide, due do increased NOS activity, coupled with quercetin's antioxidant activity is believed to be the reason behind the decrease in blood pressure, although more research needs to be done in order to determine an exact mechanism (Yamamoto & Oue, 2006).

Mitochondrial membrane permeability transition (MMPT) is a condition that occurs during a pathological build-up of free radicals in which the mitochondrial membrane

permeability increases due to lipid peroxidation. Damage caused by MMPT is key in cellular damage as well as apoptosis. Of the flavonoids tested, quercetin was found to be the most potent inhibitor of lipid peroxidation and MMPT, as measured by mitochondrial swelling in rat liver (Santos et al., 1998).

Our lab has observed an increase in energy expenditure in C57BL/6J mice given free access to a high fat diet supplemented with 800 mg quercetin per kg diet for 3 weeks compared to those given a high fat control diet (Stewart et al., 2008). Quercetin has been shown to increase lypolysis in rat adipocytes through the inhibition of PDE3 (Kuppusamy & Das, 1992). Quercetin also has the ability to increase fatty acid catabolism thereby modifying the metabolism of fatty acids. The 41 week, long-term intervention demonstrated the ability of a 1% quercetin diet (approximately 500 mg quercetin per kg body weight per day) to effectively reduce circulating concentrations of free fatty acids in Fisher 344 rats as well as upregulate genes involved in the fatty acid catabolism pathways in rat lung tissue. Similar effects were not seen with a 0.1% quercetin diet (approximately 50 mg quercetin per kg body weight per day) (de Boer et al., 2006). When 1.08 µg/L quercetin were was combined with rutin and hyperoside, Trumbeckaite et al. (2006) saw a decrease in state 3 respiration rate in rat heart mitochondria, suggesting that the quercetin was involved in the protection of mitochondrial function. This is important given the difficulty in finding molecules that increase energy expenditure (da-Silva et al., 2007). Combined, these data point to the possibility that quercetin may have an influential effect on mitochondrial health.

Animal Model

Within 6W of being placed on a high fat (35.8% kcal) diet, C57BL/6J mice develop abdominal obesity and type 2 diabetes (Surwit, Kuhn, Cochrane, McCubbin, & Feinglos, 1988).

Previous work has shown that skeletal muscle contributes to the development of insulin resistance in C57BL/6J mice after 3 weeks of being fed a high-fat diet (55% kcal fat) (Park et al., 2005). It has also been established that C57BL/6J fed a high fat diet for 3 weeks will experience a downregulation of necessary OXPHOS and mitochondrial biogenesis genes (Sparks et al., 2005).

MATERIALS AND METHODS

Animals

Male, C57BL/6J mice (N = 80) (Jackson Laboratory, Bar Harbor, ME) were weaned onto a low fat diet (10% kcal fat, Research Diets, New Brunswick, NJ). At 6 weeks of age, the mice were allowed free access to one of three diets (Research Diets): 1. high fat (HF) (45% kcal fat) (D12451), 2. high fat + quercetin (HF + Q) (45% kcal fat + 1.2 % quercetin diet) (D06081502), 3. Low fat diet (10% kcal fat) (D12450B) (table 1). Five cohorts of animals were used in this study. Energy expenditure was measured in cohort A at the 3-week timepoint in order to determine the zenith and nadir of the metabolic cycle. Food consumption and body composition were measured in cohorts B and C. Cohorts B and C were then sacrificed during zenith or nadir at the 3-week timepoint. All mice were individually housed in shoebox cages with corncob bedding in controlled environmental conditions (22°C) on a 12 hour light: dark cycle. All experiments were reviewed and approved by the Pennington Biomedical Research Center Institutional Animal Care and Use Committee (Protocol # 297).

Diets And Food Consumption

HF + Q diets were cold processed by Research Diets using quercetin ≥ 98% purity (HPLC) (Q0125) purchased from Sigma Aldrich (St. Louis, MO). All diets were stored at 4˚C in airtight, light protected refrigerators. Mass spectrometry was used at the start of the study and again on a sample stored in the same conditions for 8 weeks to evaluate the stability of the diet during storage as well as the final concentration of quercetin within the diet. Every 3 days all food was removed from cages and replaced. A 48-hour food consumption mea sure was preformed every week by weighing the food before and after the measurement period. Mice were given water *ad libitum*.

Blood Plasma Quercetin Analysis

Two hundred μL blood plasma samples were diluted with 200μl water and 500μl of sodium acetate buffer (100mM, pH 5). β-Glucuronidase type HP-2 (20014 units) (Sigma-St. Louis, MO.) was added, vortexed, and incubated for 30 minutes at 37°C. Samples were then added to glass tubes with 1ml of water. These were partitioned once with 2ml hexane to defat. The samples were sonicated at 50°C for 15 minutes then centrifuged for 6 minutes at 5000 rpm after which the hexane fraction was discarded. Samples were then partitioned 3 times with ethyl acetate (2ml), vortexed, and placed back in the sonicator again at 50˚C for another 15 minutes. The samples were re-centrifuge at 5000 rpm for 6 minutes. Ethyl acetate partitions were SpeedVac dried then resuspended in 95% ethanol (125 μL) in a sonicator for 10 minutes at 50°C. After a brief centrifugation, the samples were transferred into autosampler vials and analyzed with a liquid chromatography-mass spectrometry LC/MS system consisting of a Waters (Milford, MA) LC-MS Integrity™ unit with a W616 pump and W600S controller, W717plus auto-sampler, W996 PDA detector and a Varian 1200L (Varian Inc., Palo Alto, CA) triple quadrupole mass detector with electrospray ionization interface (ESI), operated in either positive, or negative ionization mode. The electrospray voltage was -4.5 kV, heated capillary temperature was 240^{o} C, sheath gas air for the negative mode, and electrospray voltage 5 kV and sheath gas nitrogen for the positive ionization mode; mass detector scanning from 110 to 1400 atomic mass units. Data from the Varian 1200L mass detector was collected and compiled using Varian's MS Workstation, v. 6.41, SP2.

A standard curve for quercetin was made from solutions at concentrations of 0.1 μg/ml, 1.0μg/ml and 10μg/ml in 95% ethanol to quantify the abundance of quercetin measured in

plasma which can be used to estimate quercetin concentrations in the blood of the animals. Naïve high fat plasma samples where spiked with either 500ng or 5000ng of quercetin in order to determine the percent recovery of quercetin during sample preparation. The mouse plasma samples and standards were prepared for analysis by LC-MS as described above. The recovery of the quercetin from the spiked samples was at 22% for low and high concentrations, suggesting predictably higher actual concentrations of quercetin in the blood of the treated animals than reported.

Analysis Of Dietary Quercetin

A mortar and pestle were used to powder 1-gram samples of murine high fat diet formulated with 1.2% quercetin. The samples were extracted with 5 mL of 95% ethanol. We simultaneously processed 1-gram high fat diet samples with 12 mg of quercetin. Solids were removed using centrifugation. The extract was speedvac dried, resuspended in 1 mL of water and partitioned 3 times with 1 mL of hexane. The water was then partitioned 3 times with 1 mL of ethyl acetate, dried, and resuspended in 500 µl ethanol. This was then analyzed using LC-MS analysis.

The ratio of the quercetin formulated food and the quercetin-spiked food was used to estimate any potential degradation of quercetin in the diet after formulation at the beginning of the study prior to animal treatment and after the study was completed. The initial ratio was 67% and the final ratio was 66% suggesting some degradation of the quercetin during the diet formulation but little degradation over the time course of the study. The high fat diet without quercetin as well as the low fat diet did not contain any detectable amounts of quercetin using these sensitive methods.

Body Weight And Composition

Body weight and composition (NMR Bruker Minispec, Billerica, MA) were measured every week in 2 cohorts (n=8 mice/treatment/time point) for the duration of the study. Body weight was determined by weighing the mouse in an NMR tube using a laboratory scale tared to the weight of the tube for every measurement. A mouse NMR (Bruker, Minispec) was used to measure body composition. Fat free mass (FFM) was determined by subtracting fat mass (FM) from total body weight. FFM or FM were divided by total body weight and multiplied by one hundred in order to determine percent FFM and percent FM.

RNA Isolation And Quantification

RNA from skeletal muscle samples will be isolated using the TRIzol reagent method (Chomczynski, 1993). Mitochondrial DNA and genomic DNA primers and probes were purchased from Integrated DNA Technologies (Coralville, Iowa). Real-time reverse transcriptase PCR were carried out using the SmartCycler thermal cycler (Cephein, Sunnyvale, CA).

Statistical Analysis

Measured genes were quantified using the $\Delta\Delta$ C_t method in which the C_t values of the experimental groups are compared to those of the control groups using the following formula: $\Delta\Delta\ C_t = \Delta C_{t,\ treatment} - \Delta C_{t,\ control}$. Statistical analysis was performed using an unpaired Student's t-test for high fat diet $\Delta\Delta\ C_t$ and high fat plus quercetin $\Delta\Delta\ C_t$. Validation of the effects of the high fat diet was performed using a 1-way analysis of variance (ANOVA) to examine the difference between the ΔC_t of the low fat group and that of the high fat and high fat plus quercetin groups followed by Tukey's post hoc test. A P value < 0.05 was used to determine significance.

RESULTS

According to mass spectrometry, at the start of the intervention the high fat plus quercetin diet contained 0.8% (8000 mg/kg diet) quercetin. High fat plus quercetin samples stored for the study duration in the diet storage area contained 0.79% (7900 mg/kg diet) indicating a 1% decrease in the quercetin during storage. Quercetin was found in neither the low fat nor the high fat diets.

Plasma quercetin content was measured at the zenith (1:00 a.m., night) and nadir (1:00 p.m., day) of the metabolic cycle. Mice are nocturnal and consume the majority of their diet during their waking hours. Nighttime plasma quercetin concentration was 1.5 times that of daytime concentration.

Food consumption averaged 5.00 ± 0.08 (high fat) and 4.90 ± 0.05 (high fat plus quercetin per 48-hour period for the 3-week intervention period. Food consumption was not significantly different between groups throughout the duration of the study and consumption did not change during the study (Fig. 1).

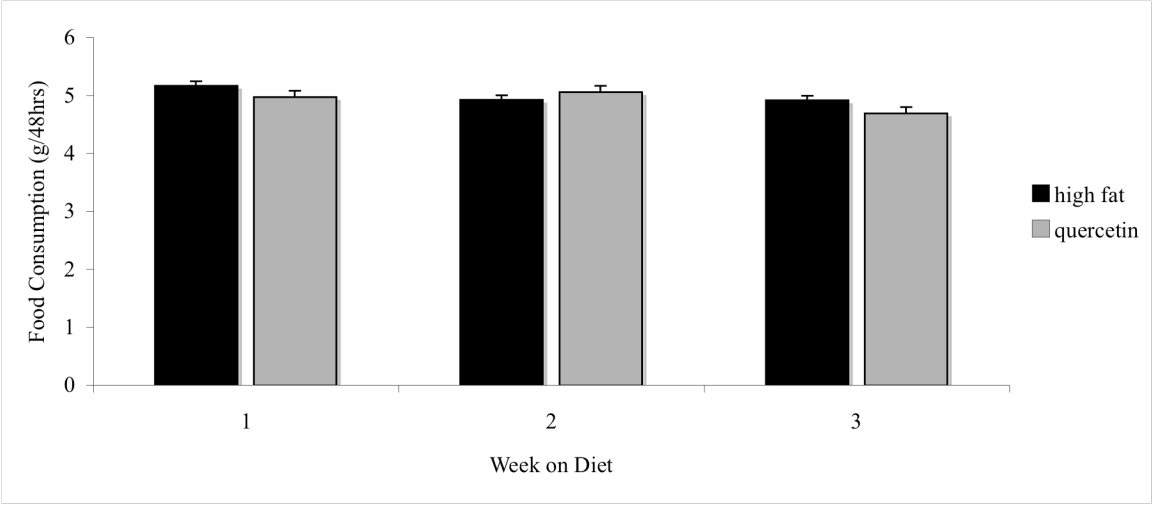

Figure 1: Weekly average food consumption. There was no difference in food consumption between groups at any point during the study.

Body weight and body composition were measured weekly during the intervention period. There was no significant difference in body weight between the high fat and the high fat plus quercetin groups at any point during the study (Fig. 2). Average body weight throughout the intervention was 21.88 ± 0.23g for the high fat group and 21.77 ± 0.15g for the high fat plus quercetin group with an average weight gain over the 3 weeks of 3.2g and 3.4g respectively.

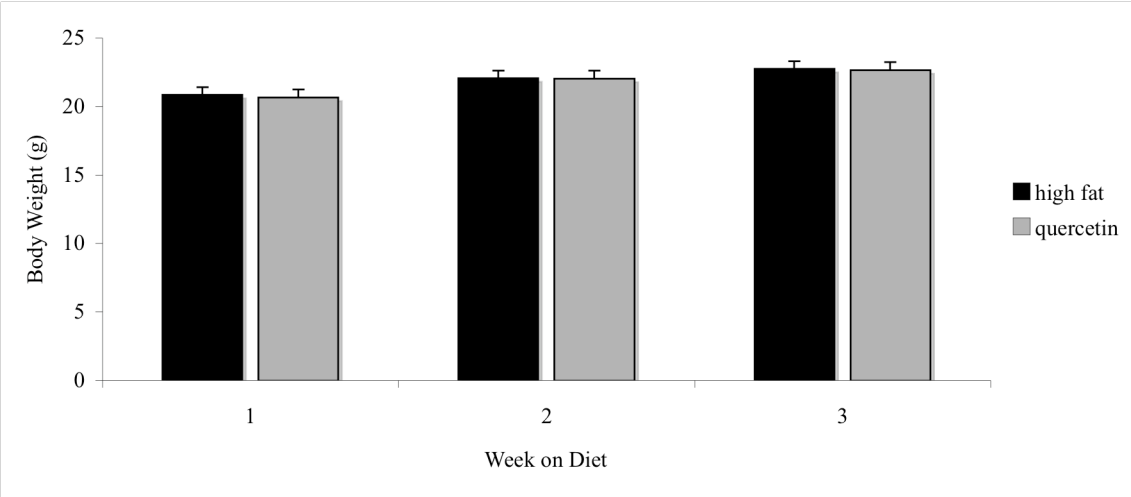

Figure 2: Weekly average body weight. There were no significant differences at any point during the study period.

Body composition was determined as the NMR measure of fat mass divided by body weight, multiplied by 100. Percent fat mass did not differ significantly between the high fat and the high fat plus quercetin groups during the entirety of the study (Fig 3).

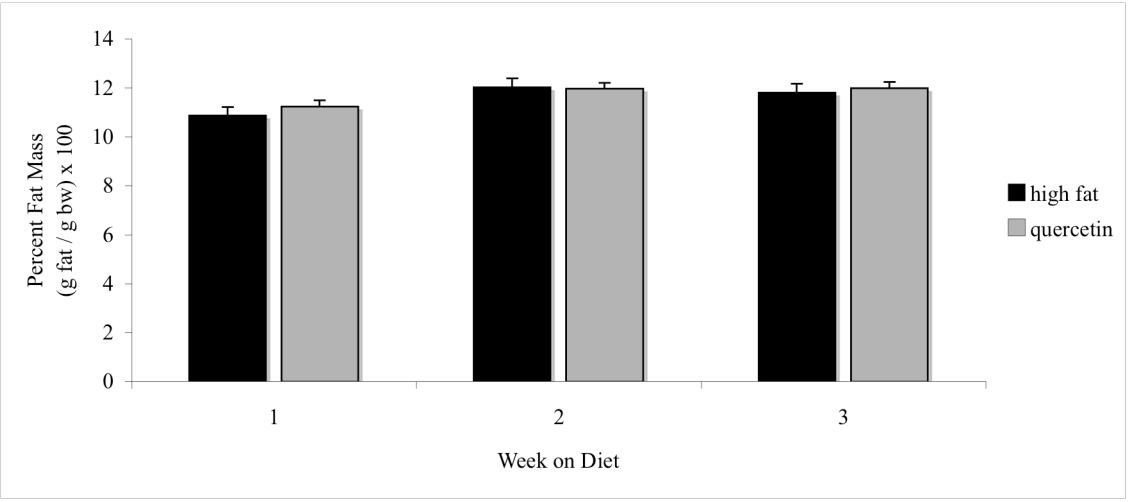

Figure 3: Weekly average percent fat mass. There were no significant differences at any point during the study period.

Mice fed a high fat diet had a reduced expression of the genes involved in mitochondrial biogenesis (pgc-1α: F = 5.76, df = 2, $P = 0.011$; pgc-1ß: F = 5.28, df = 2, $P = 0.016$) as well as the genes involved in the regulation of complex I (NDUFB5: F = 5.66, df = 2, $P = 0.012$) and complex II (SDHB: F = 5.04, df = 2, $P = 0.018$) of the electron transport chain as compared to those fed a low fat diet, as previously demonstrated by Sparks et al. (2005).

There was no significant difference between the high fat $\Delta\Delta C_t$ and the high fat plus quercetin $\Delta\Delta C_t$ for the expression of genes tested for mitochondrial biogenesis (pgc-1α: $t = -0.71$, df = 12, $P = 0.49$; pgc-1ß: $t = 0.16$, df = 12, $P = 0.87$) (Fig. 4). There was also no significant difference for the high fat $\Delta\Delta C_t$ and the high fat plus quercetin $\Delta\Delta C_t$ for the expression of genes from complex I (NDUFB5: $t = 0.27$, df = 12, $P = 0.79$) and complex II (SDHB; $t = -0.20$, df = 12, $P = 0.84$) of the electron transport chain as a measure of oxidative capacity (Fig 5).

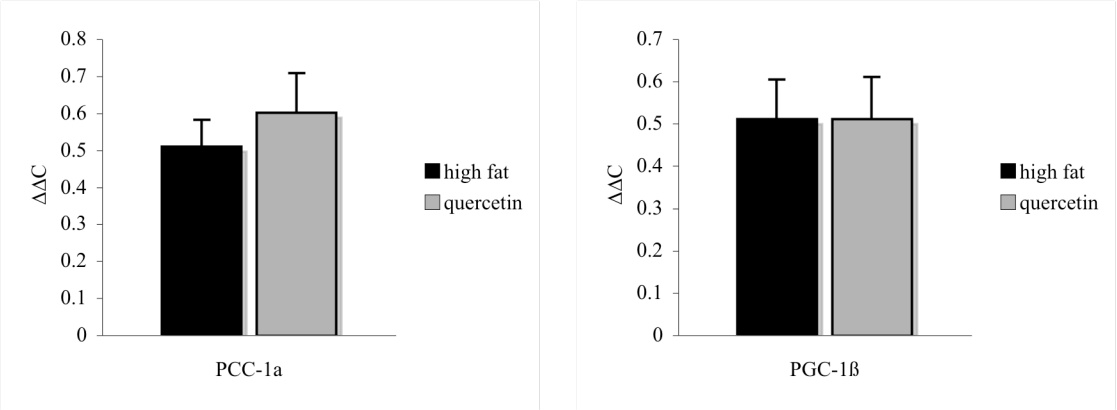

Figure 4: $\Delta\Delta C_t$ values for PGC-1α and PGC-1ß, markers of mitochondrial biogenesis.
PGC-1 = peroxisome proliferator-activated receptor gamma coactivator-1

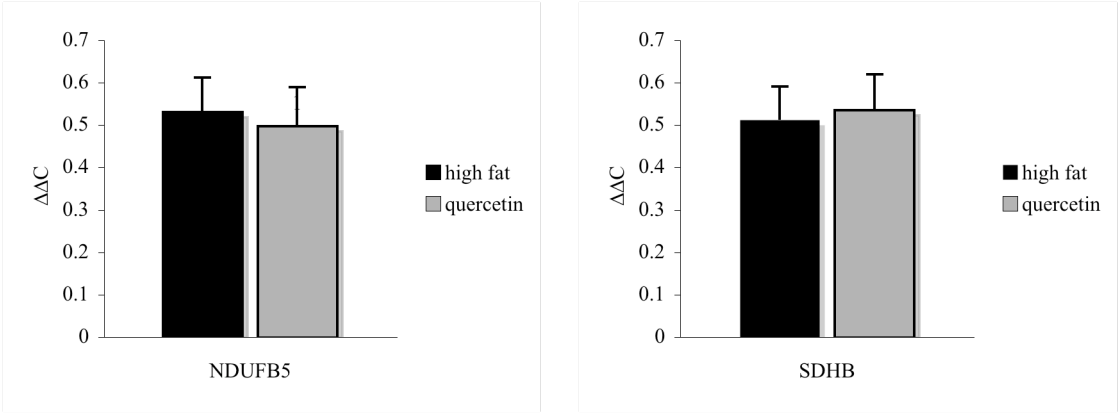

Figure 5: $\Delta\Delta C_t$ values for NDUFB5 of complex I and SDHB of complex II of the electron transport chain.
NDUFB5 = NADH dehydrogenase (ubiquinone) one ß subcomplex 5

DISCUSSION

The purpose of this study was to examine the effects of the antioxidant flavonoid quercetin on mitochondrial health. Specifically, we measured the expression of genes involved in mitochondrial biogenesis and oxidative capacity of skeletal muscle mitochondria in a murine model known to exhibit a downregulation of genes required for oxidative phosphorylation in skeletal muscle (Sparks et al., 2005) when fed a high fat diet. This same diet is also known to cause this model to develop obesity and type 2 diabetes mellitus (Petro et al., 2004). Reactive oxygen species (ROS) have been implicated in the development of diabetes as well as the pathology of a number of other conditions such as cancers, and atherosclerosis (Knekt et al., 2002). As a result of the high reactivity of ROS with molecules such as DNA and RNA, ROS are capable of causing genetic mutations leading to a loss of cell function or cell death (Spiteller, 2001). ROS have also been shown to target mtDNA (Knight-Lozano et al., 2002) causing mtDNA damage, decreased mtDNA-encoded mRNA transcription, and decreased ATP levels and mitochondrial redox function (Ballinger et al., 2000). As oxidative phosphorylation is the primary source of endogenous ROS (Magder, 2006), studies examining the effects of flavonoids, such as quercetin, on mitochondrial health are of particular importance and yet are underrepresented in the literature (Trumbeckaite et al., 2006).

The quadriceps muscle was removed from 21 male, C57BL/6J mice that had been fed a low fat (10% kcal fat), a high fat (45% kcal fat), or a high fat plus quercetin (45% kcal fat + 1.2% quercetin) diet for 3 weeks. RNA was isolated from the samples and quantitative RT-PCR was used to compare the expression of select genes of mitochondrial biogenesis and oxidative phosphorylation.

In accordance with the findings of Sparks et al. (2005), expression of genes involved in mitochondrial biogenesis (PGC-1α: $F = 5.76$, $df = 2$, $P = 0.011$; PGC-1ß: $F = 5.28$, $df = 2$, $P = 0.016$) and OXPHOS genes (NDUFB5: $F = 5.66$, $df = 2$, $P = 0.012$, SDHB: $F = 5.04$, $df = 2$, $P = 0.018$) was reduced in mice fed a high fat diet compared to those fed a low fat diet. PGC-1α and PGC-1ß are responsible for mitochondrial biogenesis activation as well as increases in the expression of the OXPHOS genes. Increases in expression of PGC-1α may cause an increase in OXPHOS gene expression by the transcription factors necessary for the replication of mitochondrial DNA (Sparks et al., 2005). There was not a significant difference in the expression of PGC-1α and PGC-1ß in mice fed a high fat diet supplemented with quercetin compared to those fed a high fat diet (pgc-1α: $t = -0.71$, $df = 12$, $P = 0.49$; pgc-1ß: $t = 0.16$, $df = 12$, $P = 0.87$) (Fig. 1), indicating that the quercetin did not provide a protective effect against the damage consumption of a high fat diet has on the mitochondrial biogenesis.

The NDUFB5 gene is located in the mitochondrial genome. It codes for a protein subunit of complex I of the electron transport chain. Complex I is responsible for the transfer of an electron from NADH to complex III through ubiquinone. SDHB codes for a subunit of complex II of the electron transport chain, however it is part of the nuclear genome. Complex II also transports electrons to complex III through ubiquinone, however it receives them from FADH. No significant difference occurred in the gene expression of NDUFB5 ($t = 0.27$, $df = 12$, $P = 0.79$) or SDHB ($t = -0.20$, $df = 12$, $P = 0.84$) (Fig. 2) in mice fed a high fat diet as opposed to those fed a high fat diet supplemented with quercetin. Again, this suggests that quercetin was unable to compensate for the effects of a high fat diet on oxidative capacity.

The lack of quercetin's effect on mitochondrial function is contrary to what has been seen by other researchers. Quercetin upregulated beta oxidation and ketogenesis in rats (de Boer et

al., 2006), prevented lipid peroxidation increased membrane of rat liver mitochondrial membranes by scavenging free radicals (Santos et al., 1998), and increased state 2 respiration in rat heart mitochondria (Trumbeckaite et al., 2006).

The lack of a difference in food consumption between the high fat and the high fat plus quercetin groups (Fig 1) coupled with the increase in energy expenditure at the 3-week timepoint previously discovered (Stewart et al., 2008) in the high fat plus quercetin group led us to expect decreased fat deposition. We, however, found no differences in either body weight (Fig. 2) or body composition (Fig. 3) suggesting that there was no shift in substrate utilization. These results are not without precedence. Yamamoto et al. (2006) found no difference in weight gain or epididymal fat weight in spontaneously hypertensive rats fed a high fat, high sucrose diet containing 0, 0.02%, 0.07%, or 0.2% quercetin. There were no differences in weigh gain, final weight, or organ weight between healthy Swiss mice fed a standard commercial diet for 4 weeks compared to those fed the same diet supplemented with 0.5% quercetin (Barrenetxe et al., 2006), suggesting quercetin had no effect on growth rate or metabolism. However, neither of these studies measured energy expenditure or body composition.

One possible explanation for this is the very large dose of quercetin fed to the mice. In this study, 1.2% quercetin supplementation translated into a murine consumption of approximately 30mg/day or 1600mg quercetin/kg body weight/day during the 3-week intervention period. This is 10,000 times the average U.S. daily quercetin consumption of approximately 30mg/day or between 0.35 and 0.41mg/kg body weight (CDC, 2008; Weldin et al., 2003). Even when accounting for the quercetin degradation that occurred during diet formulation, the dose is still very large at just over 1000mg/kg body weight/day. While doses as high as 10% quercetin supplementation in an oral diet have shown no carcinogenicity (Morino et

al., 1982), other aspects of toxicity and mutagenicity have not been adequately tested at the dose administered in the current study. The researchers who demonstrated increased state 2 mitochondrial respiration also saw that at high concentrations (1.08 ng / ml and higher) quercetin caused a decrease in state 3 respiration thereby reducing the ability of the mitochondria to convert ADP to ATP, possibly through the inhibition of the complexes of the electron transport chain (Trumbeckaite et al., 2006). Mattarei et al. (2008) also found that quercetin had the ability to reduce ATP production by inhibiting ATP synthase suggesting that flavonoid concentration may play a role in this (Kanadzu, Lu, & Morimoto, 2006).

It has also been demonstrated that at high concentrations quercetin can become a pro-oxidant, generating ROS through autoxidation and redox-cycling (Chang, Chi, & Wang, 2006). Quercetin mediated by H_2O_2 has also been shown to cause DNA cleavage and fragmentation (Kawanishi, Oikawa, & Murata, 2005). While 10 – 25 µmol/L of quercetin protected against DNA strand breaks, H_2O_2 induced cell toxicity, and apoptosis, 50 – 250 µmol/L of quercetin these as well as capsase activation and oligonucleosomal DNA fragmentation (Watjen et al., 2005). Yamashita et al. (1999) found that when quercetin reacted with DNA bound copper (II), it resulted in extensive oxidative DNA damage. It is thought that the same is possible with other metal ions, such as iron (III) (Oikawa, 2008). Lipid bound copper (II) is also a target for pro-oxidant quercetin, leading to the oxidation of LDL. Interestingly, when another pro-oxidant is available, quercetin will preferably react with it in place of the copper (Filipe et al., 2004).

The production of ROS by quercetin is also possible without a transition metal. Oxidized quercetin can react with the endogenous antioxidant glutathione (GSH) forming a thiyl radical. The thiyl radical then reacts with another GSH to form $GSSG^{\bullet-}$, a radical disulfide anion, which rapidly reacts with oxygen to form GSSG and superoxide (Galati, Chan, Wu, & O'Brien, 1999).

According to Boots et al. (2007), the oxidized quercetin may cause a reaction with the sulfhydryl group of the enzyme calcium-ATPase, impairing its ability to contribute to cellular calcium balance maintenance (Boots, Haenen, den Hartog, & Bast, 2002). The elevated cytosolic calcium will reduce mitochondrial efficiency by increasing the permeability of the mitochondrial membrane allowing the escape of cytochrome-c (Orrenius, Zhivotovsky, & Nicotera, 2003), the protein responsible for the transport of electrons between electron transport chain complexes III and IV.

Mattarei et al. (2008) also suggest that an alternative possible mechanism may be the ability of oxidized quercetin to form covalent bonds directly with DNA and cellular proteins. This has been seen *in vitro* in human intestinal and hepatic cells with binding to protein occurring at a greater rate than binding to DNA, however both occur simultaneously (Walle, Vincent, & Walle, 2003). While there is evidence that quercetin DNA adducts are transient (van der Woude et al., 2005; Walle et al., 2003), this was determined through examination of a single quercetin treatment. Quercetin DNA adduct formation during chronic exposure to high levels of quercetin has not been examined. It had been suggested that *in vivo* methylation of quercetin may protect DNA, however it has recently been demonstrated that adduct formation does occur, only in reduced levels (van der Woude, Boersma, Alink, Vervoort, & Rietjens, 2006) suggesting that chronic exposure to dietary quercetin may contain DNA toxicity risks. More research in this area, especially in an *in vivo*, model is necessary and may shed light on the results of the current study.

Our lab group is currently reproducing the current study with three much smaller doses of quercetin (0.05, 0.24, 0.5 mg/kg body weight/day) that more accurately reflect feasible human consumption. It will be possible, through this new study, to determine if smaller doses of

quercetin may protect the expression of the genes associated with mitochondrial biogenesis and oxidative phosphorylation regulation from the damaging effects of a high fat diet. Measuring the expression of these genes in mice that remained on the quercetin diet for a longer period of time could provide greater insight into the role quercetin may have played. If after 8 weeks of quercetin supplementation, gene expression were lower in the high fat plus quercetin group as compared to the high fat group, it would suggest that this dose of quercetin may have compounded the damaging effects of the high fat diet. Examining the effects of such a high dose of quercetin on the mitochondrial function in a murine model fed a low fat diet would also be of interest, as it would provide further and more definitive evidence that the dose we used did, indeed, become toxic.

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