NUTRACEUTICAL COFACTOR THERAPY IN MITOCHONDRIAL DISEASE
BENEFICIAL EFFECTS OF NUTRACEUTICAL COFACTOR THERAPY IN PATIENTS WITH MITOCHONDRIAL DISORDERS

By

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TITLE: Beneficial effects of nutraceutical cofactor therapy in patients with mitochondrial disorders

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Mitochondrial diseases are a group of heterogenous disorders that share common cellular consequences resulting from mitochondrial dysfunction: (i) decreased ATP production; (ii) increased reliance on alternative anaerobic energy sources; and (iii) increased production of reactive oxygen species. Objective: We evaluated the effect of a combination (COMB) therapy comprising creatine monohydrate, coenzyme Q10 and lipoic acid to target the abovementioned consequences using a randomized, double-blind, placebo-controlled, crossover study design in patients with mitochondrial cytopathies. Results: Compared with placebo, the COMB therapy resulted in lower resting plasma lactate concentrations, lower urinary 8-isoprostane excretion and attenuated the decline of peak dorsiflexion strength in all patient groups. Improved body composition was only observed in patients in the MELAS group. Interpretation: These results suggest that combination therapies targeting multiple final common pathways of mitochondrial dysfunction favorably influence surrogate markers of cellular energy dysfunction. Future therapies should be designed to target specific mitochondrial diseases to provide the greatest therapeutic benefits for those patients. In addition, future studies employing larger sample sizes in homogeneous groups of patients will be required to determine whether such combination therapies will influence function and quality of life.
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INTRODUCTION

1. Mitochondrial Disease

Mitochondrial disorders are a group of disorders that are characterized by clinical, biochemical and genetic heterogeneity and include myopathies, neuropathies, encephalopathies and cardiomyopathies (Schmeidel et al. 2003). However, tissues that have a high demand for energy, such as skeletal muscle and brain, are predominantly affected (DiMauro and Hirano 2005, Kwong et al. 2006). Despite the variability in phenotypic expression, most patients have a combination of the following clinical features: lactic acidosis, stroke and/or seizure, headaches, retinitis pigmentosa, ptosis, exercise intolerance, ophthalmoplegia, cardiomyopathy, neuropathy and hypoacusia (Schmeidel et al. 2003). The common link that all mitochondrial disorders share is a progressive decline in the ability of the mitochondria to meet the cellular energy requirements for ATP (Kwong et al. 2006, Mahoney et al. 2002, Marriage et al. 2003).

1.1 Mitochondrion: The Electron Transport Chain

The primary role of the mitochondrion is to provide ATP for the cell through oxidative phosphorylation. Oxidative phosphorylation is carried out by enzyme complexes located in the inner mitochondrial membrane that form the electron transport chain (ETC) (Figure 1): complex I (NADH:ubiquinone oxidoreductase), complex II (succinate:ubiquinone oxidoreductase), complex III (ubiquinol:ferrocytochrome c oxidoreductase), complex IV (cytochrome c oxidase) and complex V (ATP synthase) (Kwong et al. 2006, Marriage et al. 2003). Electrons are brought to complexes I and II by the reducing equivalents NADH and FADH$_2$, respectively (Baker and Tarnopolsky 2003, Mahoney et al. 2002). Complexes I and II use FMN and FAD to carry the electrons to electron shuttles such as coenzyme Q$_{10}$ (CoQ$_{10}$) and cytochrome c (Baker and Tarnopolsky 2003). As the electrons are carried through complexes I to IV, protons are pumped across the inner mitochondrial membrane at complexes I, III and IV by proton pump components (DiMauro and Hirano 2005, Enns 2003, Mahoney et al. 2002). This movement of protons creates an electrochemical gradient ($\Delta\Psi_m$) across the inner mitochondrial membrane, which is alkaline and negative on the side of the mitochondrial matrix and acidic and positive in the intermembranous space (Enns 2003). The potential energy from this gradient is used to synthesize ATP from ADP and inorganic phosphate at complex V (Baker and Tarnopolsky 2003, Enns 2003, Mahoney et al. 2002, Schmeidel et al. 2003). The adenine nucleotide translocator then exchanges the ATP formed in the mitochondria for cytosolic ADP (Enns 2003).

The ETC is the only metabolic pathway that is under control of both the mitochondrial and nuclear genomes (DiMauro 2004). Each mitochondrion contains two to 10 copies of mitochondrial DNA (mtDNA) and each cell can contain up to thousands of mitochondria, depending on the energy demands of the tissue (Enns 2003). mtDNA is a double-stranded, circular genome that is 16,596 bp in size and encodes 13 subunits of the ETC, 22 tRNAs and two rRNAs (DiMauro and Hirano 2005, DiMauro 2004, Enns 2003, Kwong et al. 2006). Nuclear DNA encodes the remaining subunits required for each complex of the ETC (Figure 1), as well as the factors that are required for the proper
Figure 1. The electron transport chain. Electrons (e⁻) are shuttled from complexes I and II by coenzyme Q₁₀ (CoQ). Complex III accepts the e⁻ from the reduced CoQ (ubiquinol). Cytochrome c transfers the e⁻ to complex IV. O₂ is reduced to form H₂O at complex IV. Electron transfer is shown in red. Protons are pumped from the matrix to the intermembranous space at complexes I, III and IV to generate an electrochemical gradient (ΔΨₘ) across the inner mitochondrial membrane. Potential energy from this gradient is used to synthesize ATP from ADP at complex V. Adapted from Baker and Tarnopolsky (2001), DiMauro (2004), DiMauro and Hirano (2005), Mahoney et al (2002).
assembly and functioning of the ETC, such as import machinery and enzymes necessary for the replication of mtDNA (DiMauro and Hirano 2005).

1.2 Mitochondrial Genetics

Mitochondrial disorders result from mutations in mtDNA or nuclear DNA (nDNA) that lead to mitochondrial dysfunction and defect(s) in oxidative phosphorylation (Mahoney et al 2002, Marriage et al 2003, Tarnopolsky and Raha 2005). Because these disorders can be due to mutations occurring in either mtDNA or nDNA, the mutation can be passed onto progeny through either maternal (mitochondrial) or Mendelian inheritance (DiMauro 2004, DiMauro and Hirano 2005, Enns 2003). Mitochondrial genetics can be distinguished from Mendelian genetics due to certain features or principles that characterize mitochondrial inheritance, such as maternal inheritance, heteroplasmy and replicative segregation (DiMauro 2004, DiMauro and Hirano 2005, Tarnopolsky and Raha 2005, Schmeidel et al 2003).

1.2.1 Maternal Inheritance

At fertilization, the oocyte provides all of the mtDNA (DiMauro 2004), thus, diseases that occur due to mutations in mtDNA are inherited from the mother.

1.2.2 Heteroplasmy and Threshold Effect

As mentioned previously, cells can contain up to thousands of mitochondria. In healthy cells and tissues, all of the mitochondria are identical (homoplasmy) (DiMauro 2004), while pathogenic mutations in mtDNA do not affect all mitochondria in a cell or tissue (heteroplasmy) (DiMauro 2004, DiMauro and Hirano 2005, Tarnopolsky and Raha 2005). The ratio of mutant to normal (or wild-type) mitochondria can range from 1 to 99, all the way to 99 to 1 (Tarnopolsky and Raha 2005). The phenotypic or clinical expression of a pathogenic mutation is determined by the ratio of mutant to wild-type mitochondria (DiMauro 2004), and it is believed that a minimum number of mutant mtDNA is required for mitochondrial dysfunction to occur within a tissue (threshold effect) (DiMauro 2004, DiMauro and Hirano 2005).

1.2.3 Replicative Segregation

During cell division, the mitochondria are split between the two daughter cells, but the ratio of mutant to wild-type mitochondria may not be maintained for the two cells. As a result, the ratios within and between tissues can shift (DiMauro 2004, Tarnopolsky and Raha 2005).

1.3 Clinical Phenotype and Mitochondrial Genetics

The features that characterize mitochondrial inheritance or genetics may explain some of the interesting aspects that are observed in mitochondrial disorders. Because the proportions of mutant mitochondria can vary between and within tissues due to replicative segregation, this may explain the different phenotypic expressions of the same pathogenic mutation among individuals, even those within the same family (Tarnopolsky and Raha 2005). In addition, because many tissues are not static but are dynamic and
continue to undergo cell division, replicative segregation may again explain how individuals with mitochondrial disease demonstrate different phenotypic expression of their disease at different points in their lifetime (DiMauro 2004), as well as the late onset of symptoms in some individuals (Schmeidel et al 2003).

Mitochondrial disorders are classified genetically as they are caused by pathogenic mutations in either mtDNA or nDNA. The majority of mitochondrial disorders that have been identified to date are those that result from mutations in mtDNA; although, the identification of nDNA mutations leading to mitochondrial dysfunction is rapidly increasing (DiMauro 2004, DiMauro and Hirano 2005, Suomalainen and Kaukonen 2001).

1.4 Consequences of Mitochondrial Disorders

Regardless of whether the pathogenic mutation leading to the mitochondrial dysfunction resides in the mtDNA or nDNA, the consequences are the same: (i) decreased ATP production; (ii) an increased reliance on alternative anaerobic energy sources; and (iii) increased production of reactive oxygen species (ROS) (Mahoney et al 2002, Tarnopolsky and Raha 2005). All three consequences of mitochondrial dysfunction are intertwined, in that a defect in oxidative phosphorylation will disrupt the transport of electrons along the ETC (either through the primary defect/mutation of the mitochondrial disorder or due to ROS), resulting in a decreased production of ATP. Therefore, the cell must use alternative sources of energy that do not rely on the mitochondria, such as the phosphocreatine (PCr) system, adenylate kinase/AMP deaminase pathway or glycolysis/glycogenolysis (Tarnopolsky et al 1997). However, lactic acidosis and a reduction in PCr stores can result from this increased reliance on alternative energy stores (Mahoney et al 2002). During normal oxidative phosphorylation, the terminal electron acceptor is molecular oxygen, which is normally converted to water at complex IV (DiMauro 2004, DiMauro and Hirano 2005); however, it is widely accepted that at rest in ‘healthy’ mitochondria, approximately 1% to 4% of the molecular oxygen is converted to ROS (Enns et al 2003, MacKenzie et al 2004, Mahoney et al 2002). Complexes I and III have been commonly thought to be the two major sites of ROS generation in the ETC (MacKenzie et al 2004); however, Liu et al (2002) showed that the flavin mononucleotide (FMN) group of complex I is a physiologically relevant source of ROS generation in the ETC through reversed electron transfer from succinate, not the ubiquinone site of complex III. This production of ROS may be increased with ETC dysfunction (Enns 2003); in fact, it has been suggested that a ‘vicious cycle’ occurs in mitochondrial disease (Kwong et al 2006, Mahoney et al 2002). This vicious cycle is a positive feedback loop where the initial mutation in oxidative phosphorylation results in increased production of ROS. The ROS then attack iron-sulphur centres (4Fe-4S) of complex I (Sadek et al 2004) and enzymes of the tricarboxylic acid cycle, such as aconitase (Flint et al 1993, Gardner et al 1995) and fumarase (Flint et al 1993), which results in their inactivation. Disruption of the ETC, such as by inactivation of complex I, may lead to the formation of more ROS (Enns 2003). Chronic exposure to these ROS can result in oxidative damage to DNA, proteins and lipids (Enns 2003) – mtDNA is particularly susceptible to oxidative damage as it is in the mitochondria, the site of ROS generation. This can result in mutations in
mtDNA, which can exacerbate the defect in oxidative phosphorylation, which leads to the generation of more ROS (Kwong et al 2006, Mahoney et al 2002).

1.6 Therapy for Mitochondrial Disease

To date, there is no curative treatment for mitochondrial disease; as a result, the use of nutritionally based strategies or nutraceutical therapies that target the three consequences of mitochondrial dysfunction have been explored as a potential therapy for mitochondrial disease (Mahoney et al 2002, Marriage et al 2003). The ultimate goal of nutritionally based therapy is to increase ATP production and to reduce the accumulation of toxic metabolites (ROS) to slow or arrest the progression of clinical symptoms (Marriage et al 2003) and improve patients’ quality of life until curative interventions (such as gene therapy) are available (Baker and Tarnopolsky 2003).

2. Creatine

Creatine is a guanidino compound that is endogenously synthesized in the liver, the kidneys and pancreas from the amino acids arginine, glycine and methionine (Wyss and Kaddurah-Daouk 2000). Creatine is synthesized in two steps. An amidino group is transferred from arginine to glycine, generating guanidinoacetate and L-ornithine; this is catalyzed by the rate-limiting enzyme L-arginine:glycine amidinotransferase (Wyss and Kaddurah-Daouk 2000). The amidino group of guanidinoacetate is then methylated by S-adenosyl-L-methionine:N-guanidinoacetate methyltransferase using a methyl group (Wyss and Kaddurah-Daouk 2000), which is provided by methionine (Baker and Tarnopolsky 2003, Tarnopolsky and Beal 2001). It is widely accepted that, in mammals, guanidinoacetate is formed in the kidney and then methylated (Wyss and Kaddurah-Daouk 2000). Endogenous production provides approximately 1 to 2 g/day of creatine (Pearlman et al 2006). The pool of creatine (creatine and PCr) in the body is degraded within tissues in an unregulated and nonenzymatic process to form creatinine (Snow and Murphy 2003, Wyss and Kaddurah-Daouk 2000), which is then excreted from the body via the kidney (Bemben and Lament 2005, Wyss and Kaddurah-Daouk 2000). In vivo, the conversion to creatinine is an irreversible process (Wyss and Kaddurah-Daouk 2000). Approximately 2 g of creatine (2% of total creatine) is degraded and excreted from the body per day as urinary creatinine (Wyss and Kaddurah-Daouk 2000). To maintain body stores, creatine can also be supplied exogenously through the diet from meat-containing products (Marriage et al 2003, Tarnopolsky and Beal 2001, Wyss and Kaddurah-Daouk 2000). An omnivorous diet supplies approximately 1 to 2 g/day of creatine (Pearlman et al 2006, Snow and Murphy 2003).

The bloodstream transports both endogenous (from the liver, kidneys and pancreas) and exogenous creatine to the tissues with high-energy demands, such as skeletal muscle, brain and heart (Snow and Murphy 2003, Wyss and Kaddurah-Daouk 2000). Creatine uptake into cells occurs via a sodium-dependent transporter, creatine transporter-1 (CreaT) (Snow and Murphy 2003) against a large concentration gradient (Snow and Murphy 2003, Wyss and Kaddurah-Daouk 2003).
2.1 Role of Creatine in the Cell

The majority of creatine (over 90%) is stored in skeletal muscle (Wyss and Kaddurah-Daouk 2000, Snow and Murphy 2003) and exists as free creatine (40%) and PCr (60%) within the cell (Bemben and Lamont 2005). Within tissues, PCr acts as a temporal energy buffer: ADP is rephosphorylated to produce ATP during periods of high-energy demand by creatine kinase (CK) (Wyss and Kaddurah-Daouk 2000, Walliman et al 1998, Schlattner et al 2006) (Figure 2).

![Diagram of the PCr-Cr shuttle and the microcompartments of mitochondrial creatine kinase (mtCK). Octameric mtCK binds to the inner and outer mitochondrial membranes, forming complexes with the adenine nucleotide translocator (ANT) and voltage-dependent anion channel (VDAC or porin). The complexes allow for the exchange of mtCK substrates. ANT exchanges the ATP formed in the mitochondria for ADP. mtCK then phosphorylates creatine (Cr) to form phosphocreatine (PCr). PCr then enters the cytosol through VDAC, and is then reconverted to ATP by cytosolic CK (not shown). Adapted from Schlattner et al (2006).](image)

Figure 2. The PCr-Cr shuttle and the microcompartments of mitochondrial creatine kinase (mtCK). Octameric mtCK binds to the inner and outer mitochondrial membranes, forming complexes with the adenine nucleotide translocator (ANT) and voltage-dependent anion channel (VDAC or porin). The complexes allow for the exchange of mtCK substrates. ANT exchanges the ATP formed in the mitochondria for ADP. mtCK then phosphorylates creatine (Cr) to form phosphocreatine (PCr). PCr then enters the cytosol through VDAC, and is then reconverted to ATP by cytosolic CK (not shown). Adapted from Schlattner et al (2006).
CK exists as three different isoforms, two homodimers, one found in the muscle (CK-MM) and the other in the brain (CK-BB), as well as a heterodimer (CK-MB) (Wyss and Kaddurah-Daouk 2000). During the first 10 seconds of muscle contraction, the breakdown of PCr in muscle provides approximately 50% of the energy (Bogdanis et al 1996).

In addition, PCr acts as a spatial energy buffer between the sites of energy production and utilization (the mitochondria and the cytosol, respectively) using a mitochondrial isoform of CK, mtCK, which is located in the intermembranous space of the mitochondria (Wyss and Kaddurah-Daouk 2000, Walliman et al 1998, Schlattner et al 2006, Dolder et al 2001). In vivo, mtCK exists in equilibrium between octameric and dimeric forms (Schlegel et al 1988). The octameric form of mtCK aids the functional coupling between a voltage-dependent anion channel (VDAC or porin), located on the outer mitochondrial membrane, and adenine nucleotide translocase (ANT), located on the inner mitochondrial membrane (Walliman et al 1998, Schlattner et al 2006, Dolder et al 2001) (Figure 2). It has been suggested that mtCK, ANT and VDAC form a functional microcompartment (Dolder et al 2001). Speers et al (2005) demonstrated that octameric mtCK induces contact sites between the inner and outer mitochondrial membrane. The authors found that the number of contact sites increased three-fold in mitochondria isolated from transgenic mice expressing ubiquitous mtCK compared with control mice. In the microcompartment, ANT supplies the ATP that is used by mtCK to form PCr, which is then delivered to the cytosol by VDAC (Dolder et al 2001, Schlattner et al 2006, Walliman et al 1998). When mtCK is in its octameric form and associated with ANT, a direct protective effect is observed on the mitochondrial permeability transition (MPT), formed by VDAC and ANT (Schlattner et al 2006, Walliman et al 1998). The dimerization of mtCK by free radical (or ROS) attack leads to the detachment of mtCK from membranes, resulting in the opening of the MPT, which can impair aerobic respiration (Schlattner et al 2006, Dolder et al 2001). Soboll et al (1999) showed that the addition of peroxynitrite to pure mtCK resulted in the dimerization of 66% of the mtCK octamers.

2.2 Creatine Supplementation

In addition to consuming meat-containing products, exogenous creatine can also be supplied as a supplement – creatine monohydrate (CrM). CrM is a tasteless white powder that is poorly water-soluble and widely available across North America (Tarnopolsky and Beal 2001). In healthy humans, the average skeletal muscle store of total creatine (creatinine + PCr) is approximately 125 mmol/kg dry weight (Wyss and Kaddurah-Daouk 2000), although it can range from 90 to 160 mmol/kg dry weight (Snow and Murphy 2003). CrM supplementation has been shown to increase skeletal muscle total creatine by 20% (Harris et al 1992, Hultman et al 1996), and by as much as 50% in some individuals depending on their initial muscle total creatine concentrations (Harris et al 1992). Typical short-term supplementation strategies comprise a loading period of four to five days, during which 20g of CrM are consumed per day in four separate doses (5 g each) (Bemben and Lamont 2003, Tarnopolsky and Beal 2001). This period of loading has been shown to result in an increase of approximately 20% of total creatine (Harris et al 1992, Hultman et al 1996). Following the loading phase, Hultman et al (1996) showed that 30 days of 2 g/day of CrM maintains the increase in muscle total creatine. The dose
during the maintenance phase of creatine supplementation can range from 2 to 5 g/day (Wyss and Kaddurah-Daouk 2000). Doses of 2 to 3 g/day of CrM have been suggested for longer periods of supplementation (longer than one month) (Bemben and Lamont 2005, Baker and Tarnopolsky 2003).

2.3 Proposed Benefits of CrM Supplementation

2.3.1 Alternative energy source

In mitochondrial disorders, there is a progressive decline in the ability of the mitochondria to aerobically generate ATP (Marriage et al, Mahoney et al 2002). Therefore, the cell must use alternative energy sources that do not rely on the mitochondria, such as the PCr system (Tarnopolsky et al 1997), which can result in a reduction in PCr stores. Low levels of total creatine (Tarnopolsky and Parise 1999) and PCr (Kornblum et al 2005, Tarnopolsky and Parise 1999) have been observed in the muscle from patients with mitochondrial disease. Harris et al (1992) demonstrated that, in healthy individuals, increases in muscle total creatine in response to loading were greatest in those individuals with low resting total creatine levels. These results suggest that patients with mitochondrial disease would likely benefit most from a therapy that would include CrM supplementation due to lower endogenous stores. Restoring or enhancing intracellular creatine homeostasis and preventing ATP depletion with CrM supplementation in patients with mitochondrial disease may ameliorate symptoms of their disease, such as lactic acidosis and exercise intolerance. For example, lactic acidosis may be ameliorated due to an increased reliance on PCr, which may decrease anaerobic glycolysis, resulting in lower production of lactate.

2.3.2 Antioxidant properties

In addition to its well-established and researched role as an alternative energy source, creatine has also been shown to have direct antioxidant properties (Lawler et al 2001, Sestili et al 2006). Lawler et al (2001) used a cell-free, in vitro system to determine the effect of creatine on five ROS systems. They showed that creatine acts as a direct antioxidant, but that its antioxidant properties are selective (Lawler et al 2001). Creatine was shown to have the ability to quench ROS, such as superoxide (O$_2^-$) and peroxynitrite (ONOO$^-$), but not noncharged nonradical hydroperoxides, such as hydrogen peroxide (H$_2$O$_2$) (Lawler et al 2001). Sestili et al (2006) examined the ability of creatine to provide cytoprotection in cultured mammalian cells. The authors showed that when cells were oxidatively injured through exposure to different oxidizing agents, preincubation with creatine provided a protective effect in a concentration-dependent manner (Sestili et al 2006). Together, these preliminary results suggest that creatine may have the ability to act as a direct free radical scavenger in vivo.

2.4 Effects of CrM Supplementation

2.4.1 Improvements in Strength

There has been a prolific amount of research that has examined the effect of CrM supplementation – this previous research has demonstrated that, in the majority of cases, CrM leads to increases in muscle strength and positive changes in body composition, alone or when combined with strength training. For example, Bemben et al (2001)
assessed the effects of nine weeks of CrM supplementation combined with a resistance training and conditioning program in young, college football players using a double-blind, randomized, placebo-controlled study design. The authors found that resistance training combined with CrM supplementation resulted in increased strength as measured by bench press, power clean and squat compared with resistance training alone (Bemben et al 2001). Similar results were observed following a double-blind, placebo-controlled, six-week resistance training program in young, healthy resistance-trained males (Becque et al 1999). Becque et al (1999) found that when combined with resistance training, CrM increased the arm flexor strength to a greater degree than did placebo. The benefits of CrM supplementation, alone or combined with resistance training, have also been observed in other studies in young, healthy adults (Tarnopolsky and MacLennan 2000, Maganaris and Maughan 1998, Volek et al 1997), as well as healthy older adults (Chrusch et al 2001, Brose et al 2003), patients with neuromuscular disease, such as muscular dystrophy (Tarnopolsky et al 2004, Walter et al 2000, Louis et al 2003) and mitochondrial disorders (Tarnopolsky et al 1997, Tarnopolsky and Martin 1999, Komura et al 2003). In a randomized, double-blind, controlled trial, Tarnopolsky et al (1997) observed an increase in handgrip strength and isometric dorsiflexion torque in seven patients with mitochondrial cytopathies following three weeks of CrM supplementation. However, not all studies performed to date have found that CrM supplementation leads to increased muscle strength. Contrasting results have been demonstrated in young healthy participants (Wilder et al 2001), patients with mitochondrial disease (Klopstock et al 2000) as well as patients with other neuromuscular diseases, such as Charcot-Marie-Tooth disease (Chetlin et al 2004) or myotonic dystrophy (Walter et al 2002, Tarnopolsky et al 2004).

2.4.1.1 Proposed Mechanism

The improvements in strength observed with CrM supplementation may be explained by the role of PCr within the cell. PCr provides energy for the cell through its role as a temporal and spatial energy buffer (Schlattner et al 2006, Walliman et al 1998, Wyss and Kaddurah-Daouk 2000). It has been shown that PCr provides approximately 50% of the energy required for short, high-intensity muscle contraction (Bogdanis et al 1996). Increases in intramuscular PCr with CrM supplementation can prevent ATP depletion during muscle contraction, as more PCr is available to aid in the generation of ATP from ADP via the CK reaction, which can improve muscle performance. In addition, during high-intensity muscle contraction, there is an increase in intracellular acidosis due to glyco- gen breakdown that provides a portion of the ATP required for this action. The generation of ATP from PCr through the CK reaction buffers acidosis by utilizing the H+ ions that cause the acidosis (Pearlman et al 2006; Figure 2). By providing increased amounts of PCr, more H+ ions will be removed from the cell through the CK reaction, which may improve intracellular acidosis and cellular homeostasis (Pearlman et al 2006). Together, this can lead to an improved environment for the generation of ATP, which can result in an improvement in repetitive, high intensity muscle contractions.

In addition, in a recent randomized, double-blind study, Smith et al (2006) found that CrM supplementation resulted in a change in the composition of the vastus lateralis
muscle in patients with Charcot-Marie-Tooth (CMT) disease. They observed a significant decrease (approximately 30%) in type I myosin heavy chain (MHC) and a nonsignificant increase (approximately 23%) in type IIa MHC following a 12-week resistance training program in patients with CMT who were given CrM compared with those given placebo. This change in type IIa MHC content was correlated with chair rise time (ie, higher type IIa MHC content led to faster [lower] chair rise times), suggesting improvements in muscle function occurred. These recent results from Smith et al (2006) suggest that CrM supplementation alters the MHC content of the muscle in patients with neuromuscular disease (CMT), resulting in improvements in muscle power (thus, high-intensity muscle contraction).

2.4.2 Improvements in Body Composition

Improvements in body composition – increases in FFM and total body water – have also been observed following supplementation with CrM. These results have been observed across different study populations using a variety of methods to assess body composition. In healthy young participants, significant increases in FFM, as measured by hydrostatic weighing, have been observed following CrM supplementation when compared with placebo (Becque et al 1999, Bemben et al 2001). In addition, Bemben et al (2001) observed increases in total body water following CrM supplementation when measured by bioelectrical impedance. Increases in FFM have also been observed in healthy older adults as measured by dual energy X-ray absorptiometry (Brose et al 2003, Chrusch et al 2001). Similar beneficial effects have been observed in patients with neuromuscular disease, such as Duchenne muscular dystrophy (Tarnopolsky et al 2004). However, not all studies have shown these improvements in body composition. Despite demonstrating improvements in strength, Tarnopolsky et al (1997) did not observe increases in FFM (using dual energy X-ray absorptiometry) in seven patients with mitochondrial disease during the CrM phase of their double-blind, placebo-controlled, cross-over study.

2.4.2.1 Proposed Mechanism

It has been suggested that the improvements in body composition observed with CrM supplementation may be due to increases in total body water, particularly intracellular increases, such as in skeletal muscle cells (Bemben and Lamont 2005, Wyss and Kaddurah-Daouk 2000). The proposed mechanism for this increase in intracellular water is an increased osmotic load that is associated with increases in intracellular concentrations of creatine (Bemben and Lamont 2005). Bemben et al (2001) showed that combined with training in healthy young participants, CrM supplementation resulted in significant increases in total body water and intracellular fluid compared with the placebo and control groups. No differences were observed in the amount of extracellular fluid among the three groups (Bemben et al 2001). It has been suggested that the intracellular swelling caused by CrM supplementation can serve as a signal for myofibrillar proliferation and muscle hypertrophy. Satellite cells play an important role in muscle hypertrophy; they are quiescent stem cells that are located between the sarcolemma and basal lamina of the muscle fibre (Olsen et al 2006, Vierck et al 2003). When satellite cells are activated, they proliferate, differentiate and fuse as myonuclei with muscle fibres,
leading to growth in postnatal skeletal muscle (Olsen et al 2006, Vierck et al 2003). Creatine has been shown to activate satellite cells (Olsen et al 2006, Vierck et al 2003), perhaps through an intracellular swelling mechanism. In in vitro cell cultures, the addition of CrM to the medium resulted in significantly increased satellite cell differentiation (Vierck et al 2003). In addition, a recent study by Olsen et al (2006) demonstrated that when combined with strength training in healthy young participants, CrM increased the proportion of satellite cells and myonuclei per fibre to a greater extent than did strength training alone. Although, the exact manner in which CrM causes satellite cell activation, myofibrillar proliferation and hypertrophy is not clear (through intracellular swelling or another unknown mechanism), CrM supplementation has improved body composition in a variety of healthy and disease populations.

3. **CoQ₁₀**

CoQ₁₀ (or 2,3 demethoxy-5-methyl-6decaprenyl benzoquinone) is a fat-soluble quinone (Bonakdar and Guarneri 2005) that is comprised of two functional groups (Baker and Tarnopolsky 2003). One component is a hydrophobic side chain that is formed by 10 isoprenoid units which allows movement of CoQ₁₀ within the inner mitochondrial membrane (Baker and Tarnopolsky 2003, Geromel et al 2002). The second is a benzoquinone center that ultimately produces ubiquinol (CoQ₁₀H₂) through two sequential steps of single electron reductions (Baker and Tarnopolsky 2003). The primary role of CoQ₁₀ within the cell is to act an essential cofactor of the ETC within the mitochondria. CoQ₁₀ accepts electrons from complexes I and II, and subsequently transfers each electron to complex III (Maione et al 2002, Tarnopolsky and Beal 2001).

### 3.1 CoQ₁₀ Biosynthesis

CoQ₁₀ synthesis involves the synthesis of quinone ring and isoprenoid tail (Baker and Tarnopolsky 2003, Geromel et al 2002). Phenylalanine or tyrosine yield a 4-hydroxybenzoate, which then undergoes condensation by the action of polyprenyl transferase to yield decaprenyl hydroxybenzoate (Geromel et al 2002). This prenylated parahydrobenzoate is then modified by a variety of steps that involve hydroxylation, methylation and decarboxylation (Geromel et al 2002). The isoprenoid tail is synthesized from three acetyl coenzyme A molecules to yield 3-hydroxy-3-methyl glutaryl coenzyme A (HMG-CoA) via condensation reactions (Baker and Tarnopolsky 2003). HMG-CoA is then modified by HMG-CoA reductase to ultimately yield farnesyl-pyrophosphate (PP) through a series of steps (Baker and Tarnopolsky 2003). Farnesyl-PP contains three isoprene units and is elongated through the addition of isopentenyl-PP by trans-polyisoprenyl transferase, which adds one isoprene unit (Baker and Tarnopolsky 2003, Geromel et al 2002). CoQ₁₀ biosynthesis also requires a number of cofactors, including vitamins B₂, B₆, B₁₂ and C, folic acid, niacinamide and pantothenic acid (Baker and Tarnopolsky 2003). To date, the cellular location for all of the steps of CoQ₁₀ biosynthesis is still unknown in humans (Geromel et al 2002).
3.2 Proposed Mechanisms of Action of CoQ₁₀

3.2.1 Restoring electron flow

Mitochondrial disorders are caused by mitochondrial dysfunction and defect(s) in oxidative phosphorylation (Schmeidel et al. 2003, Tamopolsky and Raha 2005) that can be caused by missing or defective components in the ETC. It is widely believed that CoQ₁₀ supplementation can act as a bypass agent to circumvent the missing or defective components to restore electron flow in the ETC (Geromel et al. 2002, Mahoney et al. 2002) and thus, ultimately increase the production of ATP through oxidative phosphorylation (Marriage et al. 2003). Chan et al. (2002) induced cytotoxicity in *in vitro* hepatocyte cell suspensions by using rotenone to block complex I, which resulted in a decrease in mitochondrial potential and ATP depletion. The authors demonstrated that when CoQ was given with rotenone, it acted as a bypass agent for complex I of the ETC and prevented the depletion in ATP, the decrease in mitochondrial potential and cytotoxicity, and restored hepatocyte respiration (Chan et al. 2002). In addition, Marriage et al. (2004) also demonstrated that lymphocytes from healthy individuals incubated with CoQ₁₀ showed significant increases in mitochondrial ATP synthesis, and that the increase occurred in a dose-dependent manner.

3.2.2 Antioxidant effects

When in its reduced form, CoQ₁₀ acts as an antioxidant *in vitro* (Chan et al. 2002, Tomasetti et al. 2001) and *in vivo* (Migliore et al. 2004). In an *in vitro* study, Tomasetti et al. (2001) exposed lymphocytes to atmospheric O₂, which resulted in oxidative damage to DNA as measured by strand breaks. Incubation with CoQ₁₀ prevented these strand breaks to the DNA, resulting in less DNA oxidative damage (Tomasetti et al. 2001). Further *in vitro* evidence from Chan et al. (2002) supports this finding. Chan et al. (2002) found that incubation with CoQ₁₀ at higher concentrations prevented ROS formation in cultured hepatocytes incubated with an inhibitor of complex I activity. In addition, when lymphocytes taken from subjects who consumed CoQ₁₀ for two weeks were exposed to oxygen, the CoQ₁₀-enriched lymphocytes showed significantly fewer DNA strand breaks than did native lymphocytes (Tomasetti et al. 2001). DNA strand break formation was also found to be inversely related to plasma concentrations of CoQ₁₀ (Tomasetti et al. 2001). CoQ₁₀ supplementation has also been shown to decrease markers of oxidative stress in patients with mitochondrial disease. Migliore et al. (2004) demonstrated a decreased frequency of cells with nuclear DNA damage, evidenced by chromosome breakage or chromosome loss (micronucleated cells), in patients with mitochondrial disease following two weeks of ubidecarenone supplementation, a CoQ₁₀ analogue.

3.3 Clinical Benefit of CoQ₁₀ Supplementation

Due to evidence from previous research showing CoQ₁₀ acts as a bypass agent and as an antioxidant, the effects of CoQ₁₀ supplementation have been examined in patients with a variety of neuromuscular diseases. Many studies have demonstrated clinical improvements in patients with a variety of mitochondrial disorders who were supplemented with CoQ₁₀ (Abe et al. 1999, Berbel-Garcia 2004, Bresolin et al. 1990, Chen et al. 1997, Goda et al. 1987, Ogasahara et al. 1986, Remes et al. 2002, Shinkai et al. 2000),
as well as other neuromuscular disorders (Folkers and Simonsen 1995). Berbel-Garcia et al (2004) found that following therapy with 300 mg/day of CoQ₁₀, a patient with MELAS (mitochondrial encephalopathy, lactic acidosis and stroke-like episodes) demonstrated improvements in exercise intolerance, metabolic acidosis and serum CK levels. Other case reports have observed similar results in patients with MELAS treated with CoQ₁₀ (Goda et al 1987, Shinkai et al 2000).

Studies in larger populations of patients have shown comparable results (Bresolin et al 1990, Chen et al 1997, Folkers and Simonsen 1995). Folkers and Simonsen (1995) conducted two double-blind studies, both of which were followed by an open phase, of CoQ₁₀ (100 mg/day) in patients with various forms of dystrophy, Charcot-Marie-Tooth disease and Welander disease. The authors observed that the patients supplemented with CoQ₁₀ reported subjective improvements in muscular and exercise performance; although, it is unclear if these improvements were observed during the double-blind or open phase of the study. Bresolin et al (1990) found that following a 6-month open trial of ubidecarenone supplementation, patients with mitochondrial myopathies demonstrated significant decreases in post-exercise lactate concentrations. The authors also observed significant increases in Medical Research Council index score, an index of global muscle strength, following supplementation. Following the 6-month open phase of their trial of ubidecarenone supplementation, Bresolin et al (1990) selected patients who showed at least a 25% in post-exercise lactate concentrations ("responders") and conducted a double-blind phase that was 3 months in duration. Those patients who continued taking the active therapy maintained the decrease in postexercise serum lactate concentrations; however, for those patients who were treated with placebo, this decrease lost statistical significance. In addition, Chen et al (1997) also observed significant increases in the Medical Research Council index score and a trend toward decreased serum lactate concentrations following exercise in patients with a variety mitochondrial disorders.

4. α-Lipoic acid

α-Lipoic acid (or 1,2-dithiolane-3-pentanoic acid) is a mitochondrial dithiol compound that occurs naturally; it is also often referred to as thioctic acid (Packer et al 1995, Wollin and Jones 2003). Lipoic acid is both water- and fat-soluble, and is widely distributed within the cytosol as well as cellular membranes (Wollin and Jones 2003). Lipoic acid plays a critical role in energy metabolism by acting as a necessary cofactor for pyruvate dehydrogenase and α-ketoglutarate dehydrogenase, both of which are enzyme components of the tricarboxylic acid cycle in the mitochondrial matrix (Packer et al 1997, Smith et al 2004, Turnbull and Beal 2001). Lipoic acid has a high reduction potential (Smith et al 2004), and exists within the cell as lipoic acid and dihydrolipoic acid (DHLA), its reduced form (Figure 3) (Smith et al 2004, Wollin and Jones 2003).
4.1 Supplementation and Bioavailability of Lipoic Acid

Lipoic acid is abundant in tissues that have high metabolic activity, such as heart, liver and kidney (Packer et al 2001, Wollin and Jones 2003). The synthetic pathway for lipoic acid is not well understood (Packer et al 2001); however, it is known that lipoic acid is synthesized within the mitochondria by lipoic acid synthase from octanoic acid and an unknown sulfur-containing molecule (Packer et al 2001). The source of the two sulfhydryl components is currently not known, but it has been suggested that the sulfur may be donated from the enzyme itself (Smith et al 2004). In support of this, Ollagnier-de Choudens and Fontecave (1999) demonstrated that lipoic acid synthase of Escherichia coli contains an iron-sulfur centre. Lipoic acid can also be supplied from exogenous dietary sources, such as spinach, broccoli and brussel sprouts (Wollin and Jones 2003).

Under normal post-fed conditions, unbound lipoic acid is almost undetectable (Breithaupt-Grogle et al 1999, Teichert et al 1998), and it has been suggested that lipoic acid only reaches therapeutic levels through supplementation (Wollin and Jones 2003). Supplementation with lipoic acid has been shown to transiently increase plasma concentrations of free lipoic acid (Breithaupt-Gropler et al 1999, Teichert et al 1998). Teichert et al (1998) demonstrated that following an oral dose of lipoic acid (200 mg), the mean time to peak plasma concentration was 80 min, while Breithaupt-Gropler et al (1999) observed a mean time of 30 min following the same dose. The bioavailability varies from approximately 20% to 40% following an oral dose of lipoic acid (Biewenga et al 1997, Smith et al 2004, Wollin and Jones 2003). Tiechert et al (1998) found that following a 200 mg oral dose of lipoic acid, the average absolute bioavailability was 29.1%. The variability in the bioavailability of lipoic acid is affected by the dose given and whether it is consumed with food, as simultaneous food intake has been shown to decrease the appearance of lipoic acid in plasma (Gleiter et al 1996). On absorption into the cell, lipoic acid is reduced to DHLA (Smith et al 2004, Wollins and Jones 2003) by mitochondrial lipoamide dehydrogenases or the thioredoxin/thioredoxin reductase system.
Lipoic acid is then rapidly cleared from the body and excreted as in the urine as lipoic acid and its metabolites (Packer et al 2001, Smith et al 2004).

4.2 Proposed Mechanisms of Action of Lipoic Acid

The chemical reactivity of lipoic acid is related to its chemical structure (Smith et al 2004). Lipoic acid contains a dithiolane ring that other sulfhydryl-containing molecules, eg, glutathione, do not contain and it is this component that gives lipoic acid its unique properties (Figure 3) (Smith et al 2004). The location of the two sulfur atoms on the ring leads to a high electron density and the torsional strain distorts the ring, leading to the reactivity of the thiol groups (Smith et al 2004). Together these features make lipoic acid a highly reactive molecule – under physiological conditions, only NAD(P)H/NAD(P)+ has a higher redox potential than DHLA/lipoic acid (Packer et al 1995, Smith et al 2004). These properties indicate that DHLA/lipoic acid has the ability to reduce oxidized compounds and function as an antioxidant (Smith et al 2004, Wollin and Jones 2003).

4.2.1 Antioxidant properties

As discussed above, the dithiolane ring gives lipoid acid a high redox potential that allows it to reduce oxidized molecules, including a variety of ROS, such as the hydroxyl radical (Scott et al 1994), as well as reactive nitrogen species (Trujillo and Radi 2002). Given that DHLA is the reduced form of lipoic acid, it is not surprising that it has been shown to be a potent scavenger of ROS in in vitro studies (Scott et al 1994, Suzuki et al 1991, Trujillo and Radi 2002); however, despite the oxidized state of its sulfhydryl groups, lipoic acid has also demonstrated that it is capable of scavenging a variety of ROS in vitro (Scott et al 1994, Suzuki et al 1991, Trujillo and Radi 2002). Between lipoic acid and DHLA, many ROS and reactive nitrogen species-induced reactions can be terminated (Table 1) (Scott et al 1994, Suzuki et al 1991, Trujillo and Radi 2002).

In addition to its abilities as a free radical scavenger, lipoic acid/DHLA have also been shown to regenerate endogenous antioxidants that have been oxidized, such as vitamins C and E (Kagan et al 1992, Serbinova et al 1992), as well as CoQ10 (Kozlov et al 1999, Nohl et al 1997). Vitamin E, or α-tocopherol, is a lipid-soluble antioxidant that plays an important role in protecting cell membranes against lipid peroxidation and damage (Packer et al 1995). There is evidence that DHLA regenerates vitamin E directly or indirectly via vitamin C (Kagan et al 1992, Serbinova et al 1992). Following 6 weeks of lipoic acid supplementation (1.65 g/kg), Serbinova et al (1992) demonstrated it prevented the loss of vitamin E while protecting against ischemia/reperfusion-induced injury in the Langendorff isolated rat hearts. Thus, lipoic acid/DHLA are key to for the redox capacity of the cell by keeping other endogenous antioxidants in their functional, reduced state (Smith et al 2004).

In agreement with its ability to directly scavenge ROS and indirectly regenerate other antioxidants, lipoic acid has been shown to decrease oxidative stress in vivo in animal models (Hagen et al 1999) and in humans (Marangon et al 1999). Old rats showed
Table 1. Reactive oxygen and nitrogen species scavenged by lipoic acid and dihydrolipoic acid (Packer et al 2001, Smith et al 2004)

<table>
<thead>
<tr>
<th>Reactive oxygen species</th>
<th>Lipoic acid</th>
<th>Dihydrolipoic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydroxyl radical</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Singlet oxygen</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Peroxyl radical</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Superoxide</td>
<td>No</td>
<td>Yes</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Reactive nitrogen species</th>
<th>Lipoic acid</th>
<th>Dihydrolipoic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peroxynitrite</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Nitric oxide radical</td>
<td>No</td>
<td>Yes</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Other</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydrogen peroxide</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Hypochlorous acid</td>
<td>Yes</td>
<td>Yes</td>
</tr>
</tbody>
</table>

significantly lower levels of oxidative stress compared with control rats following two weeks of supplementation with lipoic acid (Hagen et al 1999). Similarly, Marangon et al (1999) supplemented healthy adults with lipoic acid (600 mg/day) for two months and showed that lipoic acid decreased several markers of oxidative stress.

4.2.2 Enhancer of CrM Uptake

Burke et al (2003) examined the effect of lipoic acid on creatine uptake into muscle following supplementation with CrM. Young, healthy males (n=16) were given CrM, CrM + sucrose, or CrM + sucrose + lipoic acid for five days. The authors showed that when supplemented with CrM and lipoic acid, increases in muscle PCr and total creatine were significantly higher than when the participants were given CrM or CrM + sucrose (Burke et al 2003).

4.3 Clinical Benefits of Lipoic Acid Supplementation

Due to the strong evidence from previous in vitro and in vivo research showing the potent antioxidant effects of lipoic acid/DHLA, the effects of lipoic acid supplementation have been investigated in a variety of diseases in which ROS and oxidative stress have been implicated in their etiology, such as diabetic neuropathy (Packer et al 2001, Tankova and Koev 2005, Ziegler et al 1995), and to a lesser extent, neuromuscular disease (Barbiroli et al 1995, Craigen 1996). Supplementation with lipoic acid has resulted in improvements in diabetic neuropathy (Tankova and Koev 2005). Following two months of lipoic acid supplementation (600 mg/day: 10 days of intravenous supplementation, followed by 60 days of oral supplementation), 74% of patients...
demonstrated full recovery of the symptoms of partial ocular nerve paralysis, as well as a significant decrease in foot pain (Tankova and Koev 2005). The ALADIN (Alpha-Lipoic Acid in Diabetic Neuropathy) study also demonstrated improvements in the Total Symptom Score and Neuropathy Disability Score following three weeks of lipoic acid supplementation (Zeigler et al 1995).

Surprisingly, in contrast to the abundant research examining the effect of lipoic acid in diabetes mellitus, including randomized clinical trials, there is a paucity of research examining the possible effects of lipoic acid in neuromuscular diseases, such as mitochondrial disorders, despite the numerous studies implicating oxidative stress and ROS in the etiology of this group of diseases. A transient improvement in lactate levels (decrease of approximately 30%) was reported in a patient with Leigh disease following supplementation with lipoic acid (Craigen 1996). Barbiroli et al (1995) reported more robust results; they showed that one month of treatment with lipoic acid (600 mg/day) resulted in a 55% increase in brain PCr and a 72% increase in phosphorylation potential in a patient with CPEO (chronic progressive external ophthalmoplegia).

5. Combination Therapies

Theoretically, because there a number of consequences resulting from defects in mitochondrial dysfunction - decreased ATP production; increased reliance on alternative anaerobic energy sources; and increased production of ROS (Mahoney et al 2002, Tarnopolsky and R1ha 2005) – a nutraceutical therapy that targets all three consequences simultaneously is likely the best approach to improve biochemical and clinical variables in patients with mitochondrial disorders (Mahoney et al 2002). Thus, most therapeutic strategies for mitochondrial disorders have moved from single-compound therapies to combination therapies (or therapeutic “cocktails”). Although trials in patients with mitochondrial disorders is difficult due to the rarity of the disorders, as well as due to their clinical and genetic heterogeneity, studies have evaluated the efficacy of combination therapies targeting more than one of the three processes described previously. These include case reports (Cacic et al 2001, Chariot et al 1999, Napolitano et al 2000), open trials (Artuch et al 1998, Marriage et al 2004, Matthews et al 1993, Peterson 1995, Tanaka et al 1997) and a retrospective study (Panetta et al 2004) (Table 2).

Table 2 illustrates the heterogeneity of previous research using nutraceutical cocktails in patients with mitochondrial disorders: variability in (i) patient numbers; (ii) periods of follow-up; (iii) populations of mitochondrial disease; (iv) compounds included in the therapeutic strategy; (v) outcome measures; and (vi) results. These factors make the task of concluding which therapeutic cocktail (if any) as the most efficacious inherently difficult, particularly when combined with the weak study designs employed by these studies. A more rigorous study design, such as a randomized, double-blind, placebo-controlled crossover trial, would remove many of the questions clouding the decision for many researchers, and perhaps, clinicians as well.
Table 2. Nutraceutical cocktails in the treatment of neuromuscular disease

<table>
<thead>
<tr>
<th>Study Design</th>
<th>Sample size</th>
<th>Disorder(s)</th>
<th>Nutraceutical Components</th>
<th>Effects</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Case report</td>
<td>1</td>
<td>Leigh disease</td>
<td>CrM, lipoic acid, thiamine [levodopa]</td>
<td>Condition stabilized; less pronounced extrapyrimidal symptoms</td>
<td>Cacic et al (2001)</td>
</tr>
<tr>
<td>Open label, Prospective 18 months</td>
<td>13</td>
<td>KSS, CPEO, mitochondrial cytopathy</td>
<td>Vitamin E, carnitine, ubiquinone, riboflavin, vitamin C, vitamin K$_3$</td>
<td>No changes observed in lactate, plasma alanine; 4 patients showed improvement in their clinical course, 5 stabilized and 4 demonstrated a worsening in their clinical status</td>
<td>Artuch et al (1998)</td>
</tr>
<tr>
<td>Study Design</td>
<td>Sample size</td>
<td>Disorder(s)</td>
<td>Nutraceutical Components</td>
<td>Effects</td>
<td>Reference</td>
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<tr>
<td>Open label Prospective</td>
<td>12</td>
<td>LHON, CPEO, MELAS, NARP, COX deficiency</td>
<td>CoQ₁₀, carnitine, vitamin C, vitamin K₁, vitamin B complex (thiamine, riboflavin, niacinamide, pyridoxine, pantothenic acid, biotin, cyanocobalamin, folic acid)</td>
<td>ATP synthesis improved in patient lymphocytes; three patients (LHON, CPEO, MELAS) reported improved exercise tolerance</td>
<td>Marriage et al (2004)</td>
</tr>
<tr>
<td>Open label Prospective</td>
<td>16</td>
<td>KSS, MERRF, myopathy, + demyelinating neuropathy</td>
<td>CoQ₁₀, vitamin K₃, vitamin C, multivitamin tablet (vitamins B₁, B₂, niacinamide, thiamine, vitamin C)</td>
<td>No changes observed in resting lactate, urine lactate:creatine ratio, P₁: ATP, P₇: P₁ or in any clinical symptoms</td>
<td>Matthews et al (1993)</td>
</tr>
<tr>
<td>Open label Prospective Unknown</td>
<td>16</td>
<td>mitochondrial myopathy, KSS, MELAS, MERRF</td>
<td>Vitamin K₃, vitamin C, vitamin E, CoQ₁₀ [methylprednisolone]</td>
<td>No acute changes as measured by ³¹P-NMR spectroscopy; patients appeared to survive longer with less functional disability and medical complications</td>
<td>Peterson (1995)</td>
</tr>
<tr>
<td>Study Design</td>
<td>Sample size</td>
<td>Disorder(s)</td>
<td>Nutraceutical Components</td>
<td>Effects</td>
<td>Reference</td>
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</tr>
<tr>
<td>Open label, Prospective 6 to 42 months</td>
<td>9</td>
<td>MELAS, KSS, MERRF, COX deficiency</td>
<td>Cytochrome c, flavin mononucleotide, thiamine diphosphate</td>
<td>Improvements in clinical symptoms reported by 8 of 9 patients – improvements in ADL, decrease in easy fatigability, decreased severity and duration of stroke-like episodes (MELAS patients)</td>
<td>Tanaka et al (1997)</td>
</tr>
<tr>
<td>Retrospective 3 days to 7 years</td>
<td>15</td>
<td>MELAS, KSS, complex I or IV, or III + IV deficiency</td>
<td>Differing combinations of: CoQ10, vitamin B10, vitamin B2, vitamin C and vitamin K; as well as a high-fat diet for patients with complex I deficiency</td>
<td>Improvement observed in clinical symptoms in 9 patients, including fewer seizures, increased energy, general well-being, improved alertness and/or fewer migraines</td>
<td>Panetta et al (2004)</td>
</tr>
</tbody>
</table>

31P- NMR Phosphorous-31 nuclear magnetic resonance; ADL Activities of daily living; CoQ10 Coenzyme Q10; COX Cytochrome c oxidase; CPEO Chronic progressive external ophthalmoplegia; CrM Creatine monohydrate; KSS Kearns-Sayre syndrome; LHON Leber hereditary optic neuropathy; MELAS Mitochondrial encephalopathy, lactic acidosis and stroke-like episodes; MERRF Myoclonic epilepsy and ragged-red fibre syndrome; NARP Neuropathy, ataxia, retinitis pigmentosa; PCr Phosphocreatine; P1 Inorganic phosphate
RATIONALE FOR PRESENT TRIAL

1. Rationale

Based on the evidence presented that illustrates the potential efficacy of the proposed compounds from in vitro studies and in vivo trials in humans demonstrating their ability to mitigate against one or more of the final common pathways of mitochondrial dysfunction, the potential therapeutic efficacy of a combination of the following compounds was evaluated: (i) CrM (alternative energy source [Tarnopolsky and Parise 1999] and antioxidant [Sestili et al 2006]); (ii) α-lipoic acid (antioxidant [Scott et al 1994] and to enhance CrM uptake [Burke et al 2003]); and (iii) CoQ₁₀ (as an antioxidant [Migliore et al 2004] and bypass of complex I of the electron transport chain [Marriage et al 2004]). A randomized, double-blind, placebo-controlled, crossover study design was used to examine the therapeutic efficacy of this combined nutraceutical therapy.

2. Rationale for outcome variables measured

2.1 Lactate

Mitochondrial disease results from a mutation that leads to a defect in oxidative phosphorylation (Tarnopolsky and Raha 2005), which results in decreased mitochondrial ATP production (Marriage et al 2004) and an increased reliance on alternative, anaerobic energy sources that do not require the mitochondria, such as glycolysis/glycogenolysis, which can lead to increased levels of lactate. Thus, not surprisingly, an elevated plasma lactate concentration/lactic acidosis is a common clinical feature in mitochondrial disease (Schmeidel et al 2003).

The majority of previous reports examining the effectiveness of a therapeutic intervention in patients with mitochondrial disease have measured blood lactate concentrations (Ab et al 1999, Artuch et al 1998, Berbel-Garcia et al 2004, Bresolin et al 1990, Chen et al 1995, Craigen 1996, Klopstock et al 2000, Komura et al 2003, Marriage et al 2004, Matthews et al 1993, Ogasahara et al 1986, Remes et al 2002, Tanaka et al 1997, Tarnopolsky et al 1997). Plasma lactate levels can act as an indirect marker showing changes in cellular energy utilization – decreases in plasma lactate concentrations may indicate that either an improvement in mitochondrial ATP production occurred or there was a change in the alternative energy source used, i.e., a decrease in glycogen utilization and/or an increase in PCr utilization. Therefore, we measured plasma lactate concentrations to indirectly show the effect of the combination therapy on cellular energy utilization in patients with mitochondrial disease.

2.2 Oxidative stress markers

As previously discussed, increased levels of ROS and oxidative stress have been implicated in the etiology of mitochondrial disease. Both Migliore et al (2004) and Yen et al (2004) demonstrated that patients with mitochondrial disease had higher levels of oxidative stress compared with controls using different biomarkers of oxidative stress. Canter et al (2005) showed that patients who had higher degrees of heteroplasmy for a mitochondrial DNA mutation had higher levels of oxidative stress as measured by blood
isoprostane concentrations. In addition, antioxidant supplementation has been demonstrated
to decrease levels of oxidative in patients with a variety of mitochondrial diseases
(Migliore et al 2004). All three compounds in the combination therapy given in the present
study have been shown to have properties that would result in decreased oxidative stress.

2.2.1 8-Isoprostane

Isoprostanes are prostaglandin-like compounds formed by the peroxidation of
Isoprostanes share a variety of characteristics that have led some to consider them to be
the most reliable marker to assess oxidative stress in vivo (Milne et al 2005, Montuschi et
al 2004). Among their other qualities, isoprostanes (i) are chemically stable; (ii) are a
peroxidation-specific product; (iii) are formed in vivo; (iv) are detectable in their steady-
state levels in a variety of human tissues and fluids; and (v) may be biochemically
sensitive for dose-finding studies using antioxidants (Montuschi et al 2004). In addition,
Milne et al (2005) has suggested that the measurement of isoprostanes in urine can be
used an index of systemic oxidative stress over time. Lower levels of urinary isoprostanes
were found in healthy volunteers following supplementation with the antioxidant, lipoic
acid (Marangon et al 1999). When combining the positive attributes of isoprostanes – (i)
considered by some authors as the most reliable marker of oxidative stress in vivo (Milne
et al 2005, Montuschi et al 2004); (ii) isoprostane can be measured in urine (Marangon et
al 1999), which provides a noninvasive method to measure lipid peroxidation; and (iii)
there are commercially available enzyme-linked immunosorbent assays (ELISA) (eg,
MediCorp Inc, Montreal, Quebec) that measure 8-isoprostane – isoprostanes are an
appealing biomarker to be used to measure oxidative stress.

2.2.2 8-hydroxy-2'-deoxyguanosine

8-hydroxy-2' deoxyguanosine (8-OHdG) is formed by the oxidation of guanosine
residues in DNA and can be used to indicate the extent of oxidative damage to DNA (Wu
et al 2004). Following repair of oxidized DNA bases, 8-OHdG is then excreted in
the urine without further metabolism (Wu et al 2004). 8-OHdG has been used as an indirect
measure of oxidative damage to DNA in different populations (Rodriguez and
LHON (Leber’s hereditary optic neuropathy), a mitochondrial disease, showed higher
levels of leukocyte 8-OHdG compared with healthy controls. 8-OHdG is also a useful
and attractive biomarker that can be used to measure oxidative stress in vivo for two
reasons: (i) similar to 8-isoprostane, 8-OHdG is also excreted in urine (Wu et al 2004),
offering a noninvasive manner in which to measure levels of oxidative DNA damage and
(ii) a reliable and accurate high performance liquid chromatography method has been
developed to measure urinary 8-OHdG (Bogdanov et al 1999).

2.3 Measures of strength and fatigue

Myopathy is a clinical feature characteristic to mitochondrial disease (Schmeidel
et al 2003), with muscle weakness often reported as a symptom in many mitochondrial
disorders (DiMauro 2004, Schmeidel et al 2003). As such, previous studies have often
used measures of muscle strength and/or fatigue to determine if their therapeutic intervention led to improvements in strength and/or fatigue in this patient population, eg, increases in strength or an attenuation in the loss of strength (Bresolin et al 1990, Chen et al 1997, Klopstock et al 2000, Matthews et al 1993, Tarnopolsky et al 1997, Tarnopolsky and Martin 1999). Changes in muscular strength and/or fatigue in different muscle groups have been measured using the Muscle Research Council scale, which measures the strength in 32 muscles (Bresolin et al 1990, Chen et al 1997, Klopstock et al 2000), as well as isometric knee extension (Klopstock et al 2000, Tarnopolsky and Martin 1999), handgrip strength (Tarnopolsky and Martin 1999, Tarnopolsky et al 1997) and/or ankle dorsiflexion fatigue (Tarnopolsky et al 1997, Tarnopolsky and Martin 1999). Previous work from our laboratory has shown that ankle dorsiflexion strength (Tarnopolsky et al 1997, Tarnopolsky and Martin 1999) and fatigue (Tarnopolsky and Martin 1999), as well peak handgrip (Tarnopolsky et al 1997, Tarnopolsky and Martin 1999) and knee extension strength (Tarnopolsky and Martin 1999) improved with CrM supplementation in patients with mitochondrial disease. As these measures may reflect changes in strength caused by a nutraceutical intervention, combined with the fact that our laboratory is experienced in using these measures, handgrip and ankle dorsiflexion peak strength and fatigue, and peak knee extension strength were measured in the patients in the present study.

2.4 Body Composition

Previous studies have shown that CrM supplementation results in improvements in body composition, including increases in FFM and total body water (Becque et al 1999, Bemben et al 2001, Brose et al 2003, Chrusch et al 2001, Tarnopolsky et al 2004). Increases in FFM may have important therapeutic implications for disease groups in which myopathy and muscle weakness are characteristic, such as mitochondrial disorders. These improvements have been observed across different study populations using a variety of methods to assess body composition. Changes in body composition following CrM supplementation have been previously measured using hydrostatic weighing (Becque et al 1999, Bemben et al 2001), dual energy X-ray absorptiometry (DEXA) (Brose et al 2003, Chrusch et al 2001, Tarnopolsky et al 1997, Tarnopolsky et al 2004a, Tarnopolsky et al 2004b), and bioelectrical impedance (BIA) (Bemben et al 2001, Walter et al 2002). The majority of studies that used hydrostatic weighing or DEXA were conducted in healthy participants. Thus, BIA was used to assess body composition in the present study as (i) the patient experiences no discomfort when it is used; (ii) it is transportable and can be brought to the patient eliminating any inconvenience caused to the patient; and (iii) it is inexpensive compared with hydrostatic weighing and DEXA.

3. Hypothesis

We hypothesized that following supplementation with the combination therapy, patients with mitochondrial disease would demonstrate increases in pulmonary function; increased peak strength; decreased muscle fatigue; improvements in body composition; decreased oxidative stress; and improvements in biochemical clinical markers of disease (eg, lactate) compared with placebo.
Beneficial effects of creatine, CoQ$_{10}$ and lipoic acid in mitochondrial disorders

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Abstract

Mitochondrial disorders share common cellular consequences: (i) decreased ATP production; (ii) increased reliance on alternative anaerobic energy sources; and (iii) increased production of reactive oxygen species. The purpose of the present study was to determine the effect of a combination therapy (creatine monohydrate, coenzyme Q₁₀ and lipoic acid to target the abovementioned cellular consequences) on several outcome variables using a randomized, double-blind, placebo-controlled, crossover study design in patients with mitochondrial cytopathies (MELAS n=3; deletions n=4; other n=9). The combination therapy resulted in lower resting plasma lactate and urinary 8-isoprostanes, as well as an attenuation in the decline of peak ankle dorsiflexion strength in all patient groups; whereas higher fat-free mass was observed only in the MELAS group. Together, these results suggest that combination therapies targeting multiple final common pathways of mitochondrial dysfunction favorably influence surrogate markers of cellular energy dysfunction. Future studies with larger sample sizes in relatively homogeneous groups will be required to determine whether such combination therapies will influence function and quality of life.

Key words: Creatine, coenzyme Q₁₀, lipoic acid, mitochondrial cytopathy, randomized trial
Introduction
Mitochondrial diseases represent a group of disorders affecting mitochondrial energy transduction and are characterized by clinical, biochemical and genetic heterogeneity (Marriage et al 2003). In spite of great variability in phenotypic expression, most patients have a combination of the following clinical features: lactic acidosis, stroke and/or seizure, headaches, retinitis pigmentosa, ptosis, exercise intolerance, ophthalmoplegia, cardiomyopathy, neuropathy and hypoacusia (Mahoney et al 2002, Schmeid et al 2003, Tarnopolsky et al 1997).

Mitochondrial dysfunction results in a number of cellular consequences including (i) decreased ATP production; (ii) increased reliance on alternative anaerobic energy sources; and (iii) increased production of reactive oxygen species (ROS) (Mahoney et al 2002, Tarnopolsky and Raha 2005). To date, there is no curative treatment for mitochondrial disease, and most strategies have been designed to mitigate against the aforementioned cellular consequences (Mahoney et al 2002, Marriage et al 2003). Previous reports of therapeutic strategies in patients with mitochondrial disease have examined the effects of a single compound, such as coenzyme Q_{10} (CoQ_{10}) (Berbel-Garcia et al 20034, Bresolin et al 1990, Migliore et al 2004) or creatine monohydrate (CrM) (Komura et al 2003, Kornblum et al 2005, Tarnopolsky et al 1997). Based on the concept that mitochondrial dysfunction leads to several cellular pathophysiological consequences (Tarnopolsky and Beal 2001), most therapeutic strategies for mitochondrial disorders have used combination therapies (or a therapeutic “cocktail”) as opposed to monotherapy. Studies have evaluated the efficacy of combination therapies targeting more than one of the three processes described above; however, these were either case reports (Chariot et al 1999, Napolitano et al 2000), open trials (Artuch et al 1998, Marriage et al 2004, Matthews et al 1993, Peterson 1995, Tanaka et al 1997) or a retrospective study (Panetta et al 2004). Based on evidence of potential efficacy in human trials with mitochondrial disease and/or evidence from human trials or in vitro studies showing the proposed compound could mitigate against one or more of the final common pathways of mitochondrial dysfunction, we proposed to evaluate the potential therapeutic efficacy of a combination of the following compounds: (i) CrM (alternative energy source [Tarnopolsky and Parise 1999] and antioxidant [Sestili et al 2006]); (ii) α-lipoic acid (antioxidant [Marangon et al 1999] and to enhance CrM uptake [Burke et al 2003]); and (iii) CoQ_{10} (as an antioxidant [Migliore et al 2004] and bypass of complex I of the electron transport chain [ETC] [Marriage et al 2004]). We report here the results from the first randomized, double-blind, placebo-controlled, crossover trial examining the effects of a targeted combination therapeutic “cocktail” combining CrM, CoQ_{10} and α-lipoic acid in patients with mitochondrial cytopathy.

Materials and Methods
Patients. Seventeen patients with definite or probable mitochondrial disease were recruited from the Neuromuscular and Neurometabolic Clinic at McMaster University in Hamilton, Ontario. Diagnosis was confirmed using a combination of clinical symptoms, fasting serum lactate concentration, muscle biopsy findings (ragged red fibers and/or cytochrome c oxidase negative fibers), and mitochondrial DNA (mtDNA) analysis. One
patient did not complete one arm of the study for personal reasons; consequently, their
data were excluded from the analysis. The final analysis was based on the 16 patients (10
women and six men) who were divided into three different groups based on their
diagnosis. The characteristics of the patient population are shown in Table 3. The first
group comprised three patients with mitochondrial encephalopathy, lactic acidosis and
stroke-like episodes; (MELAS) (patients 1 to 3) (MELAS group). The second group
consisted of three patients diagnosed with chronic progressive external ophthalmoplegia
(CPEO) (patients 4 to 6) and one patient diagnosed with Kearns-Sayre Syndrome (KSS)
(patient 7), all of whom had a deletion(s) detected in muscle-derived mtDNA
(CPEO/KSS group). The third group comprised patients with a variety of mitochondrial
disorders: six patients with mitochondrial cytopathy (patients 8 to 13), two patients with
Leber’s hereditary optic neuropathy (LHON) (patients 14 and 15) and one patient with
mitochondrial neurogastrointestinal encephalopathy (MNGIE) (patient 16) (Other group).
The study received ethical approval from the McMaster Research Ethics Board and all
patients provided informed, written consent.

**Design/Intervention.** The patients participated in a randomized, double-blind, placebo-
controlled, crossover study, in which each participant received both the treatment and
placebo for two months, with a five-week washout period between trials. The treatment
phase comprised 3 g of CrM + 2 g dextrose + flavoring (Neotine®, Avicena, Palo Alto,
CA), 300 mg of α-lipoic acid (Tishcon, Westbury, NY) and 120 mg of CoQ10 (Qgel®,
Tishcon, Westbury, NY) taken daily at both 0900h and 2100h. The placebo phase
comprised identical appearing and tasting powder (5 g dextrose + flavoring, Avicena,
Palo Alto, CA) and gel capsule (soybean oil, Tishcon, Westbury, NY) placebos.

Following a 4 h fast, patients completed testing before and after each intervention
phase at approximately the same time of day (within a 2 h to 3 h window) for both trials.

**Measurements.** The height and weight of the participants were recorded at their first
visit only. All other outcome measures were taken at all visits. The participants
underwent handgrip, ankle dorsiflexion (at a joint angle of 90°) and knee extension
strength testing using a custom-made force transducer device with the data fed directly
into a computer containing data acquisition and analysis software, as previously
described (Tarnopolsky et al 1997). For all strength measures, the participants were tested
on their right side with the settings individualized to hand size and held constant among
visits. For peak strength, the participants performed three 5 s trials separated by
approximately 30 s. The trial with the best result was taken as the peak handgrip, ankle
dorsiflexion or knee extension strength. The participants also performed a 1 min
isometric handgrip and ankle dorsiflexion fatigue test (9 s duty: 1 s rest cycle).
Pulmonary function testing, comprised of forced vital capacity (FVC) and forced
expiratory volume in 1 s (FEV1), was performed using a spirometer (Koko; PDS
Instrumentation, Louisville, CO). Each patient completed the spirometry at least twice at
each visit to ensure the values were consistent with their first attempt. Bioelectrical
impedance (BIA) (Prism BIA 101A, RJL Systems, Clinton Twp, MI) was performed to
determine body composition. Fat-free mass (FFM), percent body fat (%BF) and total

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body water (TBW) were calculated from the resistance and reactance using the equations supplied by the manufacturer.

**Venous blood sampling and urine collection.** Whole blood was collected from an antecubital vein into pre-chilled evacuated tubes containing either heparin (for lactate analysis) or EDTA (for the determination of CoQ_{10}) and centrifuged at 2500 rpm for 10 min. The plasma was stored at -80°C. Each patient provided a spot urine sample, of which approximately 10 mL was quickly frozen and stored at -80°C for subsequent analysis of creatine, creatinine, 8-hydroxy-2'-deoxyguanosine (8-OHdG), and 8-isoprostane (8-IsoP).

**Biochemical analyses.**

**Lactate.** Plasma lactate concentrations were determined using the YSI 2300 STAT Plus lactate analyzer (YSI, Yellow Springs, Ohio). The inter- and intra-assay coefficients of variation for lactate were 2.1% and 1.7%, respectively.

**CoQ_{10}.** Plasma CoQ_{10} concentrations were determined with high performance liquid chromatography (HPLC) using an electrochemical detector. Plasma (0.5 mL) was aliquoted into a 10 mL vacutainer containing 1 mL of 1-propanol and 0.5 mL of coenzyme Q_{9} (CoQ_{9}), mixed for 5 min and then centrifuged at 300 g for 5 min. The sample was filtered using a 0.22 µm syringe filter and transferred to a chromatography bottle for direct analysis by HPLC. The CoQ_{9} was added to the mixture to act as an internal standard as the levels of CoQ_{9} are negligible in human blood. The resultant sample was injected onto a reversed-phase stainless steel column (150 X 3 mm) RP-C18 with 3 µm packing with an electrochemical detector (ESA, Bedford, Mass) connected to a guard cell with a single electrode (Model 5020; E = +350 mV) and a coulometric analytical cell with dual electrodes (Model 5011; E1 = -400 mV, E2 = +300 mV). A mobile phase of mixed and degassed methanol, 1-propanol and ethanol (70:20:10) containing 50 mM of lithium acetate as conductivity salt was used at a flow rate of 0.5 mL/min and the total run time was less than 15 min. CoQ_{10} was measured first by reducing ubiquinone (E = -400 mV) and then by oxidizing the resultant ubiquinol (E = +300 mV). Both CoQ_{10} and CoQ_{10}H_{2} were detected at the last electrode at the highest sensitivity. The correlation coefficient of the standard curve was 0.997. The coefficient of variation was determined to be <2%.

**Creatine and creatinine.** The concentrations of creatine, creatinine and the ratio of creatine:creatinine in urine were determined using HPLC. Urine (1 mL) was aliquoted into microcentrifuge tubes and centrifuged at 10 000 rpm for 10 min. The urine supernatant was diluted to a 1 in 10 dilution using ddH_{2}O (0.1 mL supernatant to 0.9 mL ddH_{2}O). The diluted urine supernatant was kept at 10°C using a refrigerated autosampler. The sample was injected onto a 250 X 4.6 mm C18 Phenomenex 10µ Hydro-RP 80 column using a Hewlett Packard LC1100 series HPLC (Agilent, Mississauga, ON) with the UV detector set at λ = 210 nm. The Hewlett Packard LC1100 data analysis program generated the calibration curve and analyzed the resultant data. The mobile phase was...
potassium dihydrogen phosphate (20 mM) adjusted to pH 5.0 using potassium hydroxide using a flow rate of 1.0 mL/min. The coefficient of variation was 3.1%.

**8-IsoP.** Urinary concentrations of 8-IsoP were determined using a commercial enzyme-linked immunosorbent assay (ELISA) (MediCorp Inc, Montreal, Quebec) following the manufacturer’s instructions. The correlation coefficient of the standard curve was 0.988. The coefficient of variation was 10.5%. The 8-IsoP values are expressed relative to creatinine (g).

**8-OHdG.** Urine concentrations of 8-OHdG were determined using HPLC as previously described (Bogdanov et al 1999). The 8-OHdG values are expressed relative to creatinine (g).

**Statistics.** Statistical analyses were performed using either a three-way (group X treatment X time) or two-way (group X treatment) repeated measures ANOVA. Given the a priori hypotheses that the combination (COMB) therapy would lower oxidative stress due to the fact that each of the three components have antioxidant properties, we used a one-tailed test for the oxidative stress markers. When a significant result was found, a Tukey HSD post hoc test was run. All analyses were performed using Statistica version 5 software (StatSoft Inc, Tulsa, OK). P<0.05 was considered statistically significant. All data are given as mean ± SD.

**Results**

**CoQ10 and creatine:creatinine.**

As expected, the plasma CoQ10 and urinary creatine:creatinine ratios were higher on the COMB compared with the placebo phase of the trial. The plasma CoQ10 concentration was 172% higher after COMB (1.94 ± 0.89 µg/mL) compared with placebo (0.71 ± 0.24 µg/mL) (P<0.05; n=14). The creatine:creatinine ratio was 600% higher after COMB (2.45 ± 2.03) compared with placebo (0.35 ± 0.20) (P<0.05).

**Plasma lactate.**

A treatment X time interaction was found for plasma lactate (P<0.05, one-tailed), where plasma lactate concentrations were lower during the COMB therapy phase with no effect observed during the placebo phase (Figure 4).

**Body composition.**

A three-way interaction (group X treatment X time) was observed for FFM, TBW and %BF (P<0.05) (Figure 2), with an increase in FFM and TBW, and decrease in %BF significant only for the MELAS group (Figure 5).

**Pulmonary function.**

There were no effects of treatment, group or time observed for FVC or FEV1 (P>0.05) (Table 4).
**Strength measures.**

There were no effects of treatment, group or time for peak handgrip strength; although, there was a nonsignificant trend for peak handgrip strength to be lower at the end of each phase regardless of treatment (P=0.054). There were also no treatment, group or time effects for handgrip fatigue (expressed as either peak or area fatigue), ankle dorsiflexion fatigue (peak or area fatigue) or for peak knee extension strength. However, a two-way interaction (treatment X time) was observed for peak ankle dorsiflexion strength, with a decline in ankle dorsiflexion peak strength apparent following the placebo but not the COMB phase (P<0.05; Figure 6).

**Urinary 8-OHdG and 8-IsoP.**

There was no treatment or group effect for urinary 8-OHdG; however, a nonsignificant trend for lower 8-OHdG/creatinine following treatment with COMB compared with placebo was found (P=0.065; Figure 7A). A treatment effect was observed for 8-IsoP, such that lower urinary 8-IsoP/creatinine content was observed following the COMB phase compared with the placebo phase (P<0.05; Figure 7B).

**Discussion**

Treatment with a combined therapy of CrM, CoQ₁₀ and lipoic acid (COMB) resulted in lower resting lactate concentrations, prevention of a reduction in peak ankle dorsiflexion strength and a lowering of oxidative stress as reflected by a significant reduction in urinary 8-IsoP excretion and a directional trend in 8-OHdG excretion in all groups. In addition, positive changes in body composition (increased FFM and TBW, and decreased %BF) were observed for patients in the MELAS group. The COMB therapy had no effect on pulmonary function, peak handgrip or knee extension strength, or handgrip or ankle dorsiflexion percent or area fatigue.

An elevated plasma lactate concentration is a common clinical feature in mitochondrial disease (Mahoney et al 2002, Schmeidel et al 2003, Tarnopolsky et al 1997). Mitochondrial disease results from mutations that lead to a defect in oxidative phosphorylation, which results in an increased reliance on nonaerobic energy sources (Mahoney et al 2002, Tarnopolsky and Raha 2005). Either the phosphocreatine (PCr) system, adenylate kinase/AMP deaminase, or glycolysis/glycogenolysis can be used to supply ATP; however, as increased reliance on glycolysis/glycogenolysis results in elevated lactate (Tarnopolsky et al 1997), CrM was included in the COMB therapy used in the present study. The increase in the urinary creatine:creatinine and the lower plasma lactate concentrations following the COMB phase in the patients, indirectly suggest that the CrM component of the COMB therapy may have provided an alternative anaerobic energy source for muscle contraction.

Low levels of total creatine (Tarnopolsky and Parise 1999) and PCr (Kornblum et al 2005) have been observed in muscle from patients with mitochondrial disease, further supporting a potential benefit from CrM supplementation in such patients. A recent study by Kornblum et al (2005) examined the effect of CrM supplementation on intramuscular PCr in patients with CPEO or KSS. Contrary to results previously observed in healthy subjects (Burke et al 2003, Harris et al 1992), CrM supplementation did not result in
increased intramuscular PCr concentrations as measured by phosphorous-31 magnetic resonance spectroscopy ($^{31}$P-MRS) despite significant increases in plasma concentrations of creatine (Kornblum et al. 2005). A limitation of the current study is that creatine and/or PCr content were not measured in brain or skeletal muscle in the patients. However, Burke et al. (2003) showed that when CrM was combined with lipoic acid in healthy volunteers, muscle PCr and total creatine concentrations were significantly higher than when supplemented with CrM alone. The latter results provided the rationale for the inclusion of lipoic acid in our COMB therapy. Therefore, lipoic acid may have enhanced CrM uptake in the patients in the current study, leading to the observed lower resting plasma lactate concentrations.

An alternative or additive explanation for the lower lactate concentrations could be that the COMB therapy improved mitochondrial ATP production. CoQ$_{10}$ is an electron acceptor in the ETC that transfers electrons from complexes I and II to complex III (Mahoney et al. 2002, Marriage et al. 2003, Tarnopolsky and Beal 2001). The goal of CoQ$_{10}$ supplementation is to bypass defects in the ETC to maximize ATP production (Mahoney et al. 2002). One study using cultured lymphocytes from patients with mitochondrial cytopathy observed an increase in mitochondrial ATP production with a combination therapy that included CoQ$_{10}$ (Marriage et al. 2004). Using control lymphocytes, the authors attributed approximately 49% of this increase to CoQ$_{10}$ (Marriage et al. 2004). In contrast, the results from human studies are not conclusive for some reports have reported a beneficial effect of CoQ$_{10}$ (alone or in combination with other cofactors) in decreasing resting plasma lactate concentrations in patients with mitochondrial disease (Artuch et al. 1998, Berbel-Garcia et al. 2004), while others have not (Marriage et al. 2004, Matthews et al. 1993, Tarnopolsky et al. 1997). Unlike previous reports, the patients in the present study were also given lipoic acid. Lipoic acid is found naturally within the mitochondria and is an essential cofactor for pyruvate dehydrogenase and α-ketoglutarate dehydrogenase (Tarnopolsky and Beal 2001). Lipoic acid is thought to act as a potent antioxidant (Smith et al. 2004, Tarnopolsky and Beal 2003) and has been shown to decrease a marker of oxidative stress in healthy volunteers (Marangon et al. 1999). Increased ROS scavenging by lipoic acid could slow the ‘vicious cycle’ that is thought to occur in mitochondrial disease (Mahoney et al. 2002). This ‘vicious cycle’ is a positive feedback loop where increased ROS generation leads to mutations in mtDNA, which can exacerbate the defect in oxidative phosphorylation, which leads to the generation of more ROS (Mahoney et al. 2002). Thus, in combination with lipoic acid, CoQ$_{10}$ may have had the ability to increase ATP production, resulting in decreased utilization of alternative energy sources and a decrease in resting plasma lactate concentrations.

The COMB therapy attenuated the decrease in peak ankle dorsiflexion strength that was observed following the placebo phase. It was hypothesized that the CrM component in the COMB therapy would lead to improved strength values compared with placebo as CrM has been shown to improve strength in patients with mitochondrial disease (Tarnopolsky and Martin 1999, Tarnopolsky et al. 1997), Duchenne muscular dystrophy (Tarnopolsky et al. 2004), as well as in older healthy volunteers (Brose et al. 2003). Given that we did not directly measure creatine or PCr content in the muscle in the
present study, we cannot conclusively state that the CrM component of the COMB therapy resulted in the attenuation of peak ankle dorsiflexion strength. Other studies have shown improved strength in patients with mitochondrial disease with CoQ10 supplementation. For example, both Bresolin et al (1990) and Chen et al (1997) found that CoQ10 increased the Medical Research Council index score, an index of global muscle strength.

Previous studies have shown improvements in body composition with CrM supplementation (Brose et al 2003, Tarnopolsky et al 2004). The MELAS group in the present study demonstrated improvements in body composition – increased FFM and TBW, and decreased %BF – following supplementation with the COMB therapy; however, these improvements were not seen in the patients from the CPEO/KSS or Other groups. Patients with MELAS demonstrate a more severe clinical phenotype than do patients with the other forms of mitochondrial disease represented in the other two groups in the present study (eg, CPEO, KSS, LHON, etc). For example, as another marker of disease severity, plasma lactate concentrations were highest in patients with MELAS (3.52 mmol/L) versus 1.78 mmol/L for the Other group and 1.96 mmol/L for the CPEO/KSS group (P=NS). Consequently, patients with MELAS may have greater room for improvement with all of the variables measured in the present study, including body composition.

During resting oxidative phosphorylation, there is electron leak from the ETC in the mitochondria, which leads to the formation of ROS (Mahoney et al 2002). High levels of ROS and oxidative stress have been implicated in the pathophysiology of mitochondrial disease. For example, higher levels of oxidative stress have been reported in patients with mitochondrial disease compared with controls (Migliore et al 2004, Yen et al 2004) and in patients with higher degrees of heteroplasmy for a mtDNA mutation (Canter et al 2005). In addition, antioxidant supplementation has been shown to decrease levels of oxidative stress in patients with mitochondrial disease (Migliore et al 2004). All three compounds in the COMB therapy have been shown to have properties that would decrease oxidative stress. Creatine has been shown to have direct antioxidant properties in cell-free systems (Lawler et al 2002) and to provide cytoprotection to mammalian cells incubated with a variety of oxidative agents (Sestili et al 2006). CoQ10 is thought to act as an antioxidant in lipid and mitochondrial membranes (Geromel et al 2002, Tarnopolsky and Beal 2001) and may also decrease electron leak from the ETC via its role in bypassing defects in oxidative phosphorylation (Geromel et al 2002). Lastly, lower levels of urinary isoprostanates were found in healthy volunteers following supplementation with lipoic acid (Marangoni et al 1999). We observed lower 8-IsoP concentrations following treatment with the COMB therapy compared with the placebo phase; however, only a trend for lower levels of 8-OHdG was observed. Isoprostanes are prostaglandin-like compounds formed by the peroxidation of arachadonic acid (Milne et al 2005, Montine et al 2004, Montuschi et al 2004). Isoprostanes are chemically stable; are formed in vivo; and are a peroxidation-specific product that is detectable at steady-state levels in a variety of human tissues and fluids (Montuschi et al 2004). All of which contribute to 8-IsoP being considered the most reliable marker to assess oxidative stress in vivo (Montine et al 2004, Montuschi et al 2004). It is unknown why the COMB therapy resulted in
significantly lower 8-IsoP concentrations and only a trend for lower 8-OHdG concentrations. 8-OHdG is formed by the hydroxylation of guanosine residues (Rodriguez and Tarnopolsky 2003) and is often used as a biomarker of ROS damage to DNA (Rodriguez and Tarnopolsky 2003, Yen et al 2004). Because 8-OHdG is a biomarker for oxidative damage to all DNA, not only mtDNA, it is possible that the presence of the nuclear DNA may have masked or diluted a beneficial effect of the combined therapy for decreasing oxidative damage to mtDNA.

Few randomized, controlled trials have examined the effect of nutraceutical compounds in patients with mitochondrial disease. Of those that have conducted rigorous examinations, they have only examined the effect of a single compound, such as CrM (Klopstock et al 2000, Kornblum et al 2003, Tarnopolsky et al 1997) or CoQ10 (Chen et al 1997). Other studies that have examined the effect of a combined therapy (Artuch et al 1998, Marriage et al 2004, Matthews et al 1993, Panetta et al 2004, Peterson 1995, Tanaka et al 1997) did not use the same rigorous study design as was used in the present study. As a result, direct comparison with these studies is extremely difficult, particularly when combined with the fact that different compounds, combinations, outcome measures, etc were examined in different mitochondrial disease populations. For example, in an open trial, Marriage et al (2004) gave 12 patients CoQ10, carnitine, vitamin B complex, vitamin C and vitamin K1. In another open trial, Matthews et al (1993) examined the effect of CoQ10, vitamin K3, vitamin C and a multivitamin tablet in 16 patients. Comparing the present study with these two trials alone, only CoQ10 was present in all of the treatments studied. The dose of CoQ10 varied among the three studies: 240 mg/day (present study) versus 300 mg/day (Matthews et al 1993) versus 5 mg/kg/day (Marriage et al 2004). We measured body composition changes and markers of oxidative stress, while the other two studies did not; however, Marriage et al (2004) measured changes in ATP synthesis in patient lymphocytes, whereas this was not measured by Matthews et al (1993) or the present study. The differences shown in study design, compounds used, dosages of similar compounds and outcome measures, strongly suggest that comparison of results among studies examining nutritionally based therapies in patients with mitochondrial disease may not always be the most accurate method when judging the therapeutic efficacy of any strategy.

Our results suggest that a combination therapy with CrM, CoQ10 and lipoic acid targeting three consequences of mitochondrial dysfunction leads to improvements in resting plasma lactate concentrations, body composition, ankle dorsiflexion strength and oxidative stress compared with placebo. However, because one patient group showed greater benefits than did other groups with this therapy (MELAS > CPEO/KSS = Other), one therapeutic strategy may not be universally applicable to all mitochondrial diseases. Future research using larger sample sizes in relatively homogeneous groups will be required to determine which combinations (different compounds with different concentrations) show the greatest improvements for specific mitochondrial diseases using as rigorous a study design as was used in the present study.
Figure 4. Plasma lactate concentrations before and after each treatment phase for each of the three groups (n=15). *P<0.05, one-tailed. COMB Combination therapy; CPEO Chronic progressive external ophthalmoplegia; KSS Kearns-Sayre syndrome; MELAS Mitochondrial encephalopathy, lactic acidosis and stroke-like episodes.

Figure 5. Measures of body composition as determined by bioelectric impedance (n=15). (A) Fat-free mass (FFM), (B) total body water (TBW) and (C) percent body fat (%BF) before and after each treatment phase for each of the three groups. *P<0.05; **P<0.05, one-tailed. COMB Combination therapy; CPEO Chronic progressive external ophthalmoplegia; KSS Kearns-Sayre syndrome; MELAS Mitochondrial encephalopathy, lactic acidosis and stroke-like episodes

Figure 6. Ankle dorsiflexion strength values before and after each treatment phase. There was a significant interaction between treatment x time (P<0.05, n=16). *P<0.05. COMB Combination therapy.

Figure 7. (A) Urinary 8-hydroxy-2'-deoxyguanosine (8-OHdG) concentrations and (B) urinary 8-isoprostane (8-IsoP) concentrations following the placebo or combination (COMBO) treatment phases. *P<0.05, one-tailed (n=14)
A

![Chart A](chart_a.png)

B

![Chart B](chart_b.png)

C

![Chart C](chart_c.png)

36
**Dorsiflexion Torque (Nm)**

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*Comparative plot showing changes in dorsiflexion torque before (Pre) and after (Post) intervention for Placebo and COMB groups.*
Table 3. Patient characteristics

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CPEO Chronic progressive external ophthalmoplegia; KSS Kearns-Sayre syndrome; LHON Leber hereditary optic neuropathy; MELAS Mitochondrial encephalopathy, lactic acidosis and stroke-like episodes; MNGIE Mitochondrial neurogastrointestinal encephalopathy (absent thymidine phosphorylase activity, high thymidine levels).
Table 4. Pulmonary function (n=11)

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FEV₁ Forced expiratory volume in 1 s; FVC Forced vital capacity
References


DISCUSSION

The present study demonstrated that a combination therapy (COMB) comprising creatine monohydrate, CoQ₁₀ and lipoic acid targeting three consequences of mitochondrial dysfunction – decreased ATP production; increased reliance on alternative anaerobic energy sources; and increased production of ROS – resulted in improvements in a variety of surrogate markers. Treatment with the COMB therapy attenuated a decline in peak ankle dorsiflexion strength, improved resting plasma lactate concentrations and lowered oxidative stress as shown by a significant decline in urinary 8-isoprostane concentrations as well as a directional trend toward a decrease in urinary 8-hydroxy-2'-deoxyguanosine (8-OHdG) concentrations in all groups of patients with mitochondrial disorders. Patients with MELAS (mitochondrial encephalopathy, lactic acidosis and stroke-like episodes) also showed a significant increase in fat-free mass and total body water, and a significant decrease in percent body fat. Although, no improvements were observed in forced vital capacity or forced expiratory volume in 1 s, handgrip or ankle dorsiflexion peak or area fatigue, or handgrip or knee extension peak strength following COMB supplementation.

A plethora of previous research has shown that supplementation with creatine monohydrate, alone or in combination with resistance training, results in increases in muscle strength and/or positive changes in body composition in young, healthy individuals (Becque et al 1999, Bemben et al 2001, Maganaris and Maughn 1998, Tarnopolsky and MacLennan 2000, Volek et al 1997), in healthy older adults (Chrusch et al 2001, Brose et al 2003), as well as in patients with neuromuscular disease, such Duchenne muscular dystrophy (Tarnopolsky et al 2004) and mitochondrial disorders (Tarnopolsky et al 1997, Tarnopolsky and Martin 1999). Based on these data, it was originally hypothesized that the creatine component of the COMB therapy would result in improvements in strength for the patients in the present trial. An attenuation in a decline in peak ankle dorsiflexion strength was observed following supplementation with the COMB therapy. It would be attractive to attribute this improvement in ankle dorsiflexion strength solely on the creatine component of the COMB therapy based on our a priori hypothesis; however, a recent study by Kornblum et al (2006) demonstrated that creatine monohydrate supplementation did not result in increased intramuscular PCr concentrations as measured by phosphorous-31 magnetic resonance spectroscopy in patients with CPEO (chronic progressive external ophthalmoplegia) or KSS (Kearns-Sayre syndrome). These results suggest it is possible that a different component of the COMB therapy may be responsible for the improvement in peak ankle dorsiflexion strength. Other studies have shown improved strength in patients with mitochondrial disease with CoQ₁₀ supplementation. Both Bresolin et al (1990) and Chen et al (1997) found that CoQ₁₀ increased the Medical Research Council index score, an index of global muscle strength.

As discussed above, previous studies have shown improvements in body composition with CrM supplementation (Becque et al 1999, Bemben et al 2001, Brose et al 2003, Chrusch et al 2001, Tarnopolsky et al 2004b); however, only the patients in the MELAS group demonstrated improvements in body composition following supplementation with the COMB therapy. Patients with MELAS demonstrate a more severe clinical phenotype than do patients with CPEO, KSS, or LHON (Leber hereditary
optic neuropathy) (mitochondrial disorders represented in the other two groups in the present trial). Therefore, patients with MELAS may have greater room for improvement with all of the variables measured in the present study, including body composition.

The mitochondrial dysfunction observed in mitochondrial disorders results in an increased reliance on non-aerobic energy sources (Kornblum et al 2005, Tarnopolsky and Parise 1999), which can result in an elevated plasma lactate concentration – a common clinical feature in mitochondrial disease (Schmeidel et al 2003). Creatine was included in the COMB therapy as it can be used as an alternative energy source that does not result in increased lactate concentration. The increase in the urinary creatine:creatinine and the lower plasma lactate concentrations following the COMB phase in the patients, indirectly suggest that the creatine component of the COMB therapy may have provided an alternative anaerobic energy source for muscle contraction.

High levels of ROS and oxidative stress have been implicated in the pathophysiology of mitochondrial disease - higher levels of oxidative stress have been reported in patients with mitochondrial disease compared with controls (Migliore et al 2004, Yen et al 2004) and in patients with higher degrees of heteroplasmy for a mtDNA mutation (Canter et al 2005). All three compounds in the COMB therapy have been shown to have properties that would decrease oxidative stress. Isoprostanes are prostaglandin-like compounds formed by the peroxidation of arachidonic acid (Milne et al 2005, Montine et al 2004, Montuschi et al 2004) and are considered by some to be the most reliable marker to assess oxidative stress in vivo (Montine et al 2004, Montuschi et al 2004). Lower 8-isoprostane concentrations were observed following treatment with the COMB therapy compared with the placebo phase; however, only a trend for lower levels of 8-OHdG was observed. As 8-OHdG is a biomarker for oxidative damage to all DNA, not only mtDNA, it is possible that the presence of the nuclear DNA may have masked or diluted a beneficial effect of the combined therapy for decreasing oxidative damage to mtDNA.

The present study was the first randomized, double-blind, placebo-controlled, crossover trial examining the effects of a targeted combination therapy combining creatine monohydrate, CoQ10 and lipoic acid in patients with mitochondrial disorders. Despite the fact that one patient group showed greater benefits than did other groups with this therapy (MELAS > CPEO; KSS = Other), our results strongly suggest that this combination of nutraceutical cofactors is beneficial for patients with a variety of mitochondrial disorders.
FUTURE DIRECTIONS

Although the present trial demonstrated that a nutraceutical cocktail combining creatine, CoQ₁₀ and lipoic acid led to a variety of improvements in all patient groups, patients with MELAS demonstrated greater benefits with this therapy did patients in the other groups. This suggests that one therapeutic strategy may not be universally applicable to all mitochondrial diseases. Future studies should:

1. Evaluate the effect of short-term and long-term creatine monohydrate supplementation on muscle total creatine and PCr in a variety of mitochondrial diseases using muscle biopsies.

2. Evaluate the effect of the combination therapy used in the present trial on clinical outcome and quality of life to determine if the biochemical improvements observed in the present trial translate into long-term functional improvements for patients with mitochondrial disease.

3. Narrow the field of potential nutraceutical cofactors to those that showing greatest potential for patients with mitochondrial disease for future clinical trials with in vitro assays using cells from patients with different mitochondrial diseases.

4. Determine which combination(s) of nutraceutical cofactors demonstrate the greatest improvements biochemically and clinically in patients with specific mitochondrial diseases.
REFERENCES


30. Folkes K, Simonsen R. Two successful double-blind trials with coenzyme Q\textsubscript{10} (vitamin Q\textsubscript{10}) on muscular dystrophies and neurogenic atrophies. *Biochim Biophys Acta* 1995;1271:281-6.


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APPENDIX I
Letter of Information/Consent Form

The Potential Benefit of Anti-Oxidant and Creatine Supplementation In Patients with Mitochondrial Disorders

Mark A. Tarnopolsky, M.D., Ph.D.
Jay R. MacDonald, Ph.D.
Heather Naylor, M.Sc.
Department of Medicine, McMaster University

I consent to participate in a study designed by the above individuals examining the effects of creatine, co-enzyme Q₁₀, and lipoic acid on individuals diagnosed with mitochondrial disorders.

OUTLINE

Mitochondrial disorders are a group of serious neuromuscular disorders that results in the body’s cells not being able to provide sufficient energy. There is evidence that three nutritional supplements (creatine, co-enzyme Q₁₀ and lipoic acid, all of which are also produced within the body) may prevent cell damage and increase energy production of the cells.

If I consent to participate in this study, I will be asked to complete the following 60 day protocol on two separate occasions. During one of the trials, I will be ingesting a cocktail, twice daily of 3 grams of creatine, 300 mg of lipoic acid and 120 mg of co-enzyme Q₁₀. During the other trial, I will be ingesting an identical looking placebo. I will not know which substance I am receiving during each trial.

Trial Outline:
Prior to taking the cocktail or placebo, I will report to the neuromuscular laboratory after a 4 hour fast and undergo baseline testing of the following parameters (as described below):

Voluntary Strength as assessed by 1) maximal handgrip strength, 2) foot strength and 3) knee kicking strength.
Respiratory (breathing) strength as assessed by maximal expired volume over 1 sec. (FEV1)
Quality of Life as assessed by the modified Rand 36-Item Health Survey (RAND)
A small sample of blood (30 mL) will also be drawn for analysis. A urine sample will also be requested.
The following day, I will begin consuming the cocktail or placebo for a period of 60 days, taking 1 at 9:00 a.m. and another at 9:00 p.m. On days 20, and 40, I will have completed the RAND and quality of life questionnaires at 12:00 noon.

On day 60, I will report to the laboratory to return my questionnaires and undergo determinations of strength as above.
Following a minimum of a 5 week wash out period, where I will take no drug or placebo, I will undergo a second trial of identical testing while ingesting the alternate substance (the cocktail or placebo).

Explanation of Tests and Potential Risks

1. Cocktail Ingestion
This trial will require me to take a cocktail of 3 grams of creatine, 300 mg of lipoic acid and 120 mg of co-enzyme Q₁₀ twice daily for a period of 60 days. All three of these substances are also made by the body, and therefore, most people find this cocktail free of side effects. It is possible that you may experience some stomach upset early in the trial, although this is not common. Creatine may also cause you to gain a small amount of weight (<1kg) (muscle).

2. Maximal Voluntary Handgrip Strength/Forearm Ischaemic Test
This test will require me to squeeze a measuring device as hard as I can. I will have to do this for a period of one minute with a 1 second rest every nine seconds. During this time, I will have an inflated blood pressure cuff around my arm. I will also have a "near infrared spectroscopy" unit taped to my arm. This unit sits on top of the skin and measures the amount of oxygen in your blood and muscle. A maximal grip contraction may temporarily increase blood pressure (as long as the contraction is maintained). The forearm muscles will feel tired and may burn a bit during the test, however, this will disappear rapidly when the test is over. After the test, there will be a 5 minute recovery period.

3. Maximal Dorsiflexion (Foot) Strength
This test will require me to be strapped into a "boot-like" device. Using my right leg, I will have to try and raise the front of my foot up against the top of the boot as hard as I can. I will have to do this test three times in a row. As with the handgrip test, the risks are minimal. This test will take approximately 5 minutes to complete.

4. Maximal Knee Extensor (Kicking) Strength.
During this test, I will have my lower leg strapped to a metal plate. While in a sitting position, I will have to try and extend my leg as hard as possible. I will have to do this test three times in a row. As with the handgrip test, the risks are minimal. This test will take approximately 5 minutes to complete.

5. Respiratory Strength.
During this test, I will be asked to take a deep breath in and then breathe out as quickly and forcefully as possible into a mouthpiece on three occasions. There are no foreseeable risks from this test. This test will last approximately 3 minutes.

6. Modified Rand 36-Item Health Survey and Quality of Life Questionnaire
The Rand 36-Item Health Survey is a self-report questionnaire used to measure eight health concepts: physical functioning, bodily pain, role limitations due to physical health problems, role limitations due to personal or emotional problems, emotional well-being, social functioning, energy/fatigue, and general health perceptions. In responding to the questionnaires at 12:00 p.m. on days 0, 20, 40, and 60, I will be asked to answer the items with regards to my satisfaction with life over the past 20 days. These questionnaires are expected to take approximately 3 minutes to complete.
7. Urinalysis
Urine collection will occur during each visit to the laboratory.

Blood Collection
Blood collection will occur twice per trial (4 in total). During each instance, a small needle will be placed in a large arm vein and approximately 30 mL (2 tablespoons) of blood will be taken. A small bruise may occur in about 5% of people. This test will take approximately 5 minutes.

I understand that the results of this study will be made available to the scientific community, although neither my name nor any reference to me will be used in compiling or publishing these results.

I understand that I may withdraw from the study at any time without any negative repercussions. I understand that I will receive $50 for my time to complete the study.

I have had the study explained to me by one of the investigators. I am aware that Dr. Tarnopolsky will be available on a 24 hour basis if I have any questions or concerns during the study. I will be given a signed copy of this form to keep in case I have any questions during the study.

Dr. Mark Tarnopolsky: 521-2100 ext. 76593, or pager 2888
Dr. Jay MacDonald: 521-2100 ext. 76452
Ms. Heather Naylor 521-2100 ext. 76371
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### INCLUSION CRITERIA

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<td>Increased lactate (mmol)</td>
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<td>At least 2 of: Migraines (early onset (&lt;40))</td>
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<td>Seizures (encephalopathy)</td>
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<td>Severe exercise intolerance</td>
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### EXCLUSION CRITERIA

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Comments: 

________________________________________________________________________

________________________________________________________________________

________________________________________________________________________

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### RELEVANT HISTORY/PHYSICAL EXAM

- **Approximate date of diagnosis of mitochondrial disorder**

- **Genetic Diagnosis:** __ unknown □

- **Comment on any relevant history or physical findings**

### CONCURRENT DISEASE

- **None □ OR**

List below all disease states

### MEDICATION

- **None □ OR**

List below, next to associated disease condition, all medication

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**VARIABLES and FLOW CHART**

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<th>Date of visit (yy/mm/dd)</th>
<th>Baseline</th>
<th>End of Period 1</th>
<th>End of Washout</th>
<th>End of Period 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mL</td>
<td>mL</td>
<td>mL</td>
<td>mL</td>
</tr>
<tr>
<td>Urinary 8OhdG</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Urinary Isoprostanes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Urinary creatinine</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma CoQ10</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma Lipoic Acid</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma Lactate</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Plasma Pyruvate</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Whole Blood GSSH</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Other</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RAND 36-item Health Survey</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2120 packets CR, 2/10 tabs CoQ10 and 24tabs Lipoic Acid dispensed</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diary dispensed</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RAND 3 dispensed (x2)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of packets and pills returned</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>mL</td>
<td>mL</td>
<td>mL</td>
</tr>
<tr>
<td>Complete diary reviewed</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patient probed for adverse events?</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Report below changes in concomitant meds</td>
<td>None</td>
<td>none</td>
<td>none</td>
<td></td>
</tr>
</tbody>
</table>

**Comments:**

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1. Urine samples to be collected for 24 hours (from 8 am to 8 am) PRIOR to starting the treatment period, and for the last day of each treatment period (i.e., the 24-hour period ending before the first tablet is taken, and the 24-hour period ending the morning after the last tablet is taken).

2. For the first week, 1 tablet in the morning on awakening and one tablet at noon. For the second week, two tablets in the morning and two tablets at noon. If patient has headaches, nausea, or dizziness, decrease the noon dose back to one tablet.

3. To be completed at bedtime every night from the SECOND WEEK of each treatment period.
Did patient complete the study according to protocol?  
Yes ☐  No ☐  
If “No” why not ☐  

- Adverse clinical event (complete Adverse Event form) ☐  
- Unsatisfactory therapeutic response ☐  
- Patient unwilling to continue ☐  
- Other  
- Protocol violation ☐  
- Progression of disease state ☐  
- Intercurrent illness ☐  

Please comment on reasons for termination (use comment form).

PREFERENCE.  

Compare the two 2-week treatment periods.  

<table>
<thead>
<tr>
<th>The best 60 day period was the:</th>
<th>Patient</th>
<th>Physician</th>
</tr>
</thead>
<tbody>
<tr>
<td>1st period</td>
<td>2nd period</td>
<td>1st period</td>
</tr>
<tr>
<td>The difference between periods was:</td>
<td>Mild</td>
<td>Moderate</td>
</tr>
</tbody>
</table>

COMMENTS:  

I declare that I have reviewed for completeness and accuracy all case report forms for this patient; that the information contained on these pages accurately reflects the patient's medical record; and that, to the best of my knowledge, all protocol requirements were followed.

Principal Investigator's signature:  

Date:  

yy / mm / dd