

RESVERATROL SUPPLEMENTATION AND MITOCHONDRIAL CAPACITY WITH EXERCISE IN YOUNG ADULTS

by

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(Under the Direction of Kevin McCully)

ABSTRACT

PURPOSE: To determine the effects of a 28 day resveratrol supplementation period in combination with a submaximal exercise protocol on skeletal muscle mitochondrial capacity.

METHODS: Sixteen healthy young adults were randomly assigned in a double blind fashion into two groups. One group received supplementation of 500 mg of resveratrol and 10 mg of piperine to take daily, while the other group received two placebo pills daily. Supplementation lasted 4 weeks and included 3 sessions per week of low intensity forearm endurance training.

Changes in skeletal muscle mitochondrial capacity in the forearm muscle were measured using near-infrared spectroscopy (NIRS). **RESULTS:** A significant increase ($p < 0.05$) in

mitochondrial capacity occurred at post-testing in the resveratrol group (~40%). The placebo group did not have a significant increase in mitochondrial capacity after 4 weeks of forearm

endurance training. **CONCLUSION:** Resveratrol significantly increased mitochondrial capacity in a submaximal endurance training protocol, where the placebo group had no effect.

INDEX WORDS: resveratrol, mitochondrial capacity, NIRS, exercise training

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CHAPTER 1

INTRODUCTION

Nutraceuticals are becoming a prevailing alternative health care method in the United States. These dietary supplements contain concentrated forms of bioactive substances that are originally found in food. Through the use of nutraceuticals it is possible to obtain much higher dosages for a specific substance than the amount obtainable in food. Extracting and isolating particular compounds in food, allows for their properties to be enhanced without consuming other substances found in the food. These compounds are generally sought out to have potential health benefits. Instead of consuming large amounts of specific foods to attain these health benefits, nutraceuticals allow an effective way for important bioactive substances to be obtained in biologically important concentrations, high enough to enhance health (31).

The nutraceutical, resveratrol, has been increasing in popularity in recent years. Resveratrol is a polyphenol antioxidant found in grapes, red wine, peanuts, cranberries, and many other species of plants. This compound has recently become of interest because of its ability to possibly mimic effects of calorie restriction (46, 48). A number of studies done in mice have found that resveratrol supplementation is linked to increasing longevity and overall health (5, 39). In particular, resveratrol has demonstrated increases in mitochondrial biogenesis through elevating AMPK and SIRT1 and activating PGC-1 α (28, 32, 37, 48). A small number of human studies have been done looking at mitochondrial capacity with resveratrol supplementation and most are inconclusive. Other nutraceuticals of interest that have reported impact on mitochondrial biogenesis are Coenzyme Q10, L-carnitine, and alpha-lipoic acid (35).

Mitochondria within skeletal muscle play an important part in the health of an individual. Mitochondrial impairments are associated with a number of neuromuscular diseases, metabolic diseases, and also play a role in aging. Finding ways to increase mitochondrial biogenesis and maintain healthy, functional mitochondria is significant to treating these diseases and maintaining overall health (47).

It is well known that aerobic exercise training enhances skeletal muscle mitochondrial oxidative capacity. Exercise initiates cellular signals that activate kinases, such as AMPK, increasing gene transcription. This enhances mRNA causing increases in mitochondrial proteins and mitochondrial DNA expression. These adaptations lead to mitochondrial biogenesis in the skeletal muscle activated by exercise(23). Therefore, exercise serves as a great method to enhance mitochondria and maintain a healthy quality of life for persons suffering from mitochondrial dysfunction.

Near- infrared spectroscopy (NIRS) is a non-invasive technique that has been used for evaluation of skeletal muscle mitochondrial capacity through measuring muscle oxygenation and oxidative energy metabolism (20, 21). This method involves measuring the recovery rate of muscle oxygen consumption after exercise in skeletal muscle. Studies have also shown that this method is reproducible (44). Furthermore, recent studies have demonstrated that NIRS-measured skeletal muscle mitochondrial capacity results are consistent with more established techniques (6, 13). NIRS has been successfully used to measure changes in skeletal muscle mitochondrial capacity induced by endurance exercise training and detraining (41). This demonstrates that NIRS is an accurate and appropriate way to measure to determine changes in mitochondrial capacity and an appropriate way to measure mitochondrial capacity.

Statement of Problem

Extensive research with resveratrol supplementation has been studied in animal models. Research looking at effects of resveratrol on mitochondrial capacity in humans is minimal and inconclusive. Supplementing with resveratrol may lead to an increase in mitochondrial function and therefore, health benefits.

Significance of Study

The purpose of this study is to determine the effects of resveratrol supplementation by noninvasively measuring skeletal muscle mitochondrial capacity with and without performing submaximal exercise training in young adults. The study will measure mitochondrial capacity of human participants at baseline, comparing baseline testing to after 28 days of resveratrol supplementation. In addition, the study will measure mitochondrial capacity after four weeks of forearm training in young adults, comparing a group supplemented with resveratrol and a placebo control group. This study will provide further information on whether resveratrol, alone or in combination with submaximal exercise, has effects on mitochondrial capacity in young adults.

Specific Aims

Specific Aim 1: Measure mitochondrial capacity of young adults at baseline and after 28 days of submaximal exercise training.

Specific Aim 2: Measure the influence of 28 days of resveratrol supplementation on mitochondrial capacity in young adults.

Specific Aim 3: Measure the influence of 28 days of resveratrol supplementation in combination with submaximal exercise training on mitochondrial capacity in young adults.

Hypotheses

- I. Mitochondrial capacity will be enhanced 20-40% after 28 days of submaximal exercise training.
- II. Mitochondrial capacity will be enhanced from baseline in young adults after 28 days of resveratrol supplementation.
- III. Young adults receiving 28 days of resveratrol supplementation in combination with submaximal exercise training will have a greater magnitude of effect on mitochondrial capacity than those receiving a placebo.

CHAPTER 2

REVIEW OF LITERATURE

Resveratrol

Research on supplementation with resveratrol has suggested that this polyphenol may have significant health benefits. Resveratrol has been shown to have many biological effects, such as improving insulin resistance, increasing blood flow, and reducing oxidative stress and inflammation.

Many animal studies have shown improvements in insulin sensitivity with resveratrol supplementation and even lowered body weight (28). Human studies have shown that type II diabetics experience improved insulin sensitivity with resveratrol supplementation (29). Liu et al. demonstrated in his meta-analysis that resveratrol intervention significantly reduced fasting glucose and insulin concentrations, decreased Hb A_{1c} and also decreased HOMA-IR values in patients with Type 2 diabetes mellitus. However, his analysis included studies involving non-diabetic patients as well who did not experience significant changes in glucose control or insulin sensitivity with resveratrol supplementation (40, 55).

Resveratrol also upregulates endothelial nitric oxide (50), which increases nitric oxide (NO) mediated vasodilation. This vasodilation leads to increases in blood flow. Flow-mediated dilation (FMD) is a common technique used to measure responsiveness to NO. Wong et al. found that acute intake of resveratrol resulted in increased FMD in overweight, obese adults. Dosages of 30, 90, and 270 mg of resveratrol were consumed, resulting in the highest increase in FMD at the highest dosage of resveratrol given (54). Later, Wong also studied the effects of

chronic resveratrol supplementation over six weeks. Chronic supplementation resulted in a 23% increase in FMD compared to placebo (53). Another study completed by Kennedy et al. measured responsiveness of cerebral blood flow to resveratrol supplementation. Healthy young adults were supplemented with 250 mg and 500 mg of resveratrol which resulted in increases in cerebral blood flow during task performance. Increases were dose dependent, with higher dosing resulting in greater blood flow (25).

Macedo et al. indicated resveratrol's role in inflammation after exercise, finding a decrease in the inflammatory cytokine TNF- α compared to placebo after an exercise fitness test in military firefighters (30). High-fat, high-carbohydrate meals also induce inflammatory and oxidative stress. Ghanim et al. found that ingestion of a nutraceutical containing 100mg of resveratrol and 75mg of total polyphenols from grape extract 10 minutes before ingesting a high-fat, high carbohydrate meal resulted in attenuation of inflammatory and oxidative stress responses (15). The implication that resveratrol could reduce the responses to inflammation and oxidative stress is noteworthy in that many diseases are associated with chronic inflammation induced by oxidative stress. Treatment with resveratrol could potentially alleviate these inflammatory effects.

Resveratrol and Mitochondrial Capacity

In the past decade, studies have focused on resveratrol's effects on mitochondrial biogenesis. Lagouge et al. 2006 found that mice supplemented with resveratrol for 15 weeks experienced increases in aerobic capacity of the gastrocnemius muscle as evidenced by increases in mitochondrial size and mtDNA content. Increases in mitochondrial enzymes, succinate dehydrogenase and citrate synthase, also occurred. Expression of PGC-1 α , an indicator of

mitochondrial activity, was measured as well, and increased by resveratrol treatment (28). These findings were completed concurrent to Bauer et al. 2006 who yielded very similar results. After 6 months of treatment of resveratrol, mice exhibited an increase in AMPK and PGC-1 α activity, and increase in mitochondrial number (5).

More recently, research has concentrated on resveratrol's effects in combination with exercise. In a model of aging, mice placed on a 12-week exercise training protocol with resveratrol supplementation exhibited increases in running endurance capacity, oxygen consumption, and mitochondrial function compared to those with exercise alone. Markers of mitochondrial function were expression levels of PGC-1 α and cytochrome c oxidase (34). Dolinsky et al. also found increases in exercise performance by the addition of resveratrol to a 12 week endurance training program in rats. Exercise performance was enhanced by 21% when resveratrol was added to the diet. Increases in cardiac AMPK, PGC-1 α , and citrate synthase activity were observed indicating increases in mitochondrial function in the heart (12). Menzies et al. conducted a study to evaluate the role of SIRT1 in exercise and resveratrol induced skeletal muscle mitochondrial biogenesis. Resveratrol in combination with exercise demonstrated a SIRT-1 synergistic effect on mitochondrial biogenesis. This was accompanied by increases in PGC-1 α , AMPK, cytochrome c oxidase activity, and mitochondrial mass (32).

There seems to be strong evidence to support increases in mitochondrial biogenesis with supplementation of resveratrol in animal models. However, studies in human subjects are minimal and more contradicting. Timmers et al. supplemented healthy, obese men with 150 mg of resveratrol for 30 days. Resveratrol increased SIRT1 and PGC-1 α muscle protein levels, along with activating AMPK, and increasing citrate synthase activity. Mitochondrial function was also assessed by measuring phosphocreatine (PCr) recovery rate after exercise. Mean PCr

recovery was unchanged by resveratrol treatment compared to placebo, indicating mitochondrial *in vivo* function did not improve. The author suggests that the reason for these contradictory findings is the length of treatment of resveratrol. He proposes that with only 30 days treatment, resveratrol mostly affects mitochondrial efficiency, not abundance and this is why increases are only seen in mitochondrial gene expression. With longer or higher dosing, it is likely to see changes in PCr recovery as well (46). Goh et al. designed a study to look at the effects of 12 weeks of resveratrol supplementation on skeletal muscle SIRT1 expression in 10 patients with Type 2 diabetes mellitus. After resveratrol supplementation of 3 g per day for 12 weeks, SIRT1 expression in skeletal muscle was significantly increased compared to the placebo group. AMPK and GLUT4 also increased non-significantly. This data suggests resveratrol's possible exercise-mimetic potential (17). Scribbans et al. examined the effect of 4 weeks of high-intensity interval training (HIIT) 3 days per week and daily resveratrol supplementation of 150 mg. Skeletal muscle gene expression was blunted with resveratrol supplementation. PGC-1 α , SIRT1, and SOD2 were significantly lower compared to placebo, indicating resveratrol supplementation may attenuate mitochondrial adaptations to exercise training (42). Another study indicated no effect of resveratrol to enhance physiological adaptations to exercise training. Gliemann et al. recruited healthy sedentary aged men to undergo an 8 week high intensity exercise training protocol. His findings indicated a greater increase in maximal oxygen uptake in the placebo group compared to the group supplemented with 250mg of resveratrol daily during the exercise intervention. SIRT1 protein expression remained unchanged in both the resveratrol group and placebo group (16).

Pharmacokinetics of Resveratrol

A concern in human studies is the low bioavailability of resveratrol. Many human studies that have assessed potential changes in mitochondrial status supplement with less than 250 mg of resveratrol per day. Maximum plasma resveratrol concentrations (C_{\max}) with this dosage is around 50 ng/mL (1). This is much lower than the *in vitro* concentrations shown to have pharmacological benefits (>1000 ng/mL) (10). This information suggests that a higher dose, and better bioavailability, is potentially needed to produce beneficial pharmacological effects.

Resveratrol's time to reach C_{\max} can range anywhere from 30 minutes to 2 hours depending on whether it is ingested with a meal or ingested during a fasted state. Vaz-da-Silva et al. examined the effects of a single 400 mg oral dose of resveratrol on whether the presence of food affects resveratrol's pharmacokinetics (49). Their findings indicated that the rate of absorption of resveratrol following administration was delayed by the presence of food. On average, C_{\max} levels were reached about 2 hours after administration when resveratrol was ingested with a high fat meal compared to only 30 minutes when consumed alone in a fasted state. However, total absorption of resveratrol was not different between fed and fasted states, as reflected by the area under the plasma concentration versus time curve (AUC), concluding resveratrol can be administered without regard to meals.

Studies have also looked at the pharmacokinetics of resveratrol using repetitive dosing to determine if this helps to increase serum levels of resveratrol. Almeida et al. studied the effect of multiple doses of resveratrol administered at 4 hour intervals for 48 hours (13 doses total). His results indicated that supplementing with 150 mg of resveratrol 6 times/day resulted in increases of resveratrol's half-life, C_{\max} , and AUC compared to a single dose. The author also noted that

serum levels of resveratrol tended to be higher in the morning and decrease along the day (1). Nunes et al. also looked at the effect of repetitive dosing of resveratrol administered in 200 mg doses at 8 hour intervals for 3 days. Her results were similar to Almeida, indicating increases in half life, C_{\max} and AUC compared to a single dose of resveratrol (36). These findings are applicable to intervention studies supplementing with resveratrol over an extended period of time.

Piperine as a Bioenhancer

A bioenhancer is an agent that can be used to enhance the bioavailability of nutraceuticals and drugs(2). Piperine is a common bioenhancer and is the compound found in black pepper that contributes to the pungency of the spice. Piperine has been used in combination with nutraceuticals such as coenzyme Q10, beta-carotene, and resveratrol to enhance their bioavailability. Addition of 5 mg of piperine to coenzyme Q10 increased human plasma levels of coenzyme Q10 by 30% (4). When supplementing with beta-carotene and 5 mg of piperine, plasma levels of beta-carotene increased by 50% (3).

Previous research has found that resveratrol can increase cerebral blood flow. Wightman et al. was able to show an even higher increase in cerebral blood flow when co-administrating 250 mg of resveratrol with 20 mg of piperine. The combination of the two significantly augmented cerebral blood flow during task performance compared to resveratrol alone (51).

Co-supplementing resveratrol with piperine may lead to enhanced bioavailability and therefore produce significant pharmacological benefits in humans.

Near-Infrared Spectroscopy

Mitochondrial capacity can be measured by taking muscle tissue samples and measuring the concentrations and activity levels of mitochondrial enzymes such as AMPK, PGC-1 α , and citrate synthase (23). However, more recent technology has been developed to measure mitochondrial capacity non-invasively. Near-infrared spectroscopy (NIRS) is a non-invasive technique used to measure the recovery of muscle oxygen consumption after exercise and is used as an index of skeletal muscle oxidative capacity (6, 7, 41, 44). This technique has been used to measure adaptations to exercise training regimens to see changes in mitochondrial capacity. Ryan et al. found a 64% improvement in mitochondrial capacity after 4 weeks of endurance exercise training (41). This is in agreement with studies that have used in vitro measurements with muscle biopsies (18, 22). NIRS has proven to be a reliable technique to detect changes in mitochondrial capacity.

CHAPTER 3

RESVERATROL SUPPLEMENTATION AND MITOCHONDRIAL CAPACITY WITH
EXERCISE IN YOUNG ADULTS

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Abstract

PURPOSE: To determine the effects of a 28 day resveratrol supplementation period in combination with a submaximal exercise protocol on skeletal muscle mitochondrial capacity.

METHODS: Sixteen healthy young adults were randomly assigned in a double blind fashion into two groups. One group received supplementation of 500 mg of resveratrol and 10 mg of piperine to take daily, while the other group received two placebo pills daily. Supplementation lasted 4 weeks and included 3 sessions per week of low intensity forearm endurance training.

Changes in skeletal muscle mitochondrial capacity in the forearm muscle were measured using near-infrared spectroscopy (NIRS). **RESULTS:** A significant increase ($p < 0.05$) in

mitochondrial capacity occurred at post-testing in the resveratrol group (~40%). The placebo group did not have a significant increase in mitochondrial capacity after 4 weeks of forearm

endurance training. **CONCLUSION:** Resveratrol significantly increased mitochondrial capacity in a submaximal endurance training protocol, where the placebo group had no effect.

INDEX WORDS: resveratrol, mitochondrial capacity, NIRS, exercise training

Introduction

Dietary supplements that target health and performance are a large global commercial market, with billions of dollars spent per year (12). One type of dietary supplement is polyphenols, which are the most abundant antioxidants in the diet and have recently been brought to attention due to their potential beneficial effects on metabolic health and aging (25, 26). A popular supplement known as resveratrol has been the focus of much research due to its anti-inflammatory, anti-oxidant, and anti-tumorigenic properties (28).

Resveratrol is a polyphenol antioxidant found in grapes, red wine, peanuts, cranberries, and many other species of plants. Resveratrol has become of particular interest in the past decade because of its ability to stimulate the expression of the SIRT1-AMPK-PGC1 α pathway in skeletal muscle, leading to enhanced mitochondrial biogenesis (11, 17, 18, 21, 30, 31). Exercise also activates this pathway, producing training adaptations signaling skeletal muscle mitochondrial biogenesis (14, 15, 35). Recent research on resveratrol supplementation has focused on its effects on skeletal muscle mitochondrial capacity in combination with exercise. Most research has been in rodent models, showing ingestion of daily resveratrol in combination with exercise training augment responses to training, increasing mitochondrial biogenesis (9, 21, 22). In humans, there have been few studies assessing resveratrol's effect on mitochondrial capacity and the research has produced contradictory findings. Some studies show upregulation of mitochondria and the pathways that lead to mitochondrial biogenesis with resveratrol supplementation (11, 30), while others suggest minimal effects (10, 27). These inconsistent findings in human studies could be due to factors such as the health of the participants, training stimulus or dosage amounts.

A concern in human studies is the low bioavailability of resveratrol (2). Maximum plasma resveratrol concentrations (C_{max}) typically are around 75 ng/mL or less with a single dose of 500mg of resveratrol (6). This is much lower than the in vitro concentrations shown to have pharmacological benefits (>1000 ng/mL) (8). This suggests that a higher dose, or enhanced bioavailability, is potentially needed to produce beneficial pharmacological effects. Piperine, the compound found in black pepper, is a common bioenhancer. Piperine has been used in combination with nutraceuticals such as coenzyme Q10, beta-carotene, and resveratrol to enhance their bioavailability (3, 4, 16, 34). Therefore, co-supplementing resveratrol with piperine may lead to enhanced bioavailability of resveratrol and improve the ability to detect physiological benefits in humans.

The purpose of this study was to evaluate the influence of co-ingesting 500 mg of resveratrol with 10 mg of piperine on mitochondrial capacity before and after a submaximal endurance training protocol in healthy subjects compared to a placebo group. It was hypothesized that 4 weeks of supplementation of resveratrol and piperine in combination with submaximal endurance training would produce a greater increase in mitochondrial capacity than those who exercise trained and consumed placebos.

Methods

Participants

A total of 16 healthy young adults (9 males, 7 females) volunteered for the study. Participants were excluded if they were currently taking medications other than oral contraceptives or any vitamin supplements. Participants were instructed to maintain exercise and dietary habits and abstain from vigorous forearm activity during the study. Participants were

instructed to consume their study pills each morning upon waking. All experimental procedures performed on human participants were approved by the Human Subjects Institutional Review Board at the University of Georgia. Verbal and written explanations of the experimental protocol and associated risks were provided to all participants prior to obtaining written informed consent.

Exercise Training Procedures

This was a longitudinal training study in which participants performed 30 minutes of supervised forearm wrist flexor exercises of the non-dominant arm 3 times per week over a 4 week period. The wrist flexor muscles were chosen as the muscle of interest because they are relatively detrained, non-weight bearing muscles compared to muscles of the lower limb. The dominant arm was not trained and used as the untrained control arm for each subject.

The exercise training regimen used in this study was a submaximal endurance training protocol, based on previous maximal endurance training programs (24). During baseline testing, participants performed a maximal voluntary isometric contraction (MVIC) to determine the appropriate weight for exercise training and testing. Participants trained with dumbbell weights adjusted to 12-15% of their MVIC and performed the exercise on a flat surface, such as the arm of a chair, with the elbow at 90 degrees of flexion. Training was performed during the morning hours of the day before 11am so that the time of training coincided with the time resveratrol reaches peak plasma concentrations after ingestion of pills (6). Participants began training with a contraction frequency of 1 contraction every 3.5 seconds (514 contractions per session) during week one. Starting with week two the frequency was increased to 1 contraction every 2.5

seconds (720 contractions per session) and remained at this frequency for the remaining 2 weeks of the study.

Supplementation Procedures

Participants were randomly assigned in a double blind fashion to receive one pill containing 500 mg of resveratrol and one pill containing 10 mg of piperine (n=8); or two placebo pills containing flour (n=8). Participants were asked to consume the pills upon waking each morning. Participants were randomized in a double-blind fashion and given pills by a researcher who did not participate in either the training or testing of participants. Randomization was done using randomizer.org. The resveratrol supplement contained 99% Pure Trans Resveratrol (MegaResveratrol, Danbury, USA) in size “0” vegetable capsules and the piperine supplement (BioPerine®) was provided by Sabinsa and contained 95% Piperine prepared in to size “3” vegetable capsules. All pills were prepared by the lead researcher and coded by a third party researcher who did not participate in either the training or testing of participants. No member of the investigative team was aware of the contents of the capsules until all training and mitochondrial capacity measurements were completed and analyzed. Compliance was monitored via daily text messages and determined using a post-screening questionnaire which was administered after completion of the study (33).

Mitochondrial Capacity Procedures and Measurements

Participants reported to the lab for an additional 45 minutes for baseline testing of mitochondrial capacity measurements and at weeks 2 and 3 during the study and again following completion of the 4 week training and supplementation period. Mitochondrial capacity was

measured using near infrared spectroscopy (NIRS) (23, 24). The NIRS protocol was performed on both the non-dominant (training arm) and dominant arm (non-training arm). The participant was placed supine on a padded table with the test arm extended 90 degrees from the body. The NIRS probe was placed over the superficial wrist flexor muscles (flexor carpi radialis, palmaris longus, and flexor carpi ulnaris) approximately 2-3 cm distal to the medial epicondyle of the humerus. A blood pressure cuff (Hokanson SC5, Bellevue, WA) was placed above the elbow joint and was attached to a rapid cuff-inflation system (Hokanson E20 cuff inflator) powered by a 30-gallon commercial air compressor (Husky VT6315, Kenosha, WI).

NIRS signals were obtained using a continuous wave NIRS device (Oxymon MK III; Artinis Medical Systems, The Netherlands). Adipose tissue thickness (ATT) was measured at the site of the NIRS probe using B-mode ultrasound (LOGIQe; GE HealthCare, USA). The NIRS probe had two source detector separation distances that were set based on the amount of adipose tissue thickness (ATT) on top of the muscle of interest. The transmitter and receiver on the NIRS probe were set to a distance at least twice the ATT depth in order to assure NIRS penetration depth was adequate to reach the muscle. NIRS data were collected at 10 Hz. NIRS signals that represent oxygenated (O₂Hb) and deoxygenated (HHb) hemoglobin/myoglobin were corrected for changes in blood volume as previously described (23).

Mitochondrial capacity was measured using a short bout of exercise to increase metabolic rate, and measuring the rate of recovery of metabolic rate after the exercise (20, 24). The testing protocol consisted of a short bout of exercise (5-10 seconds) followed by a series of 17-20 short duration arterial occlusions (5-10 seconds) (Fig. 3.1A). The exercise/occlusion protocol was performed twice. The rate of recovery of oxygen consumption measured by the difference signal was calculated as the slope of the change during arterial occlusion using linear regression. The

repeated measures were fit to a monoexponential curve and a mitochondrial rate constant (k) was determined for each fit (Fig. 3.1B). Prior to mitochondrial capacity tests, a five minute ischemic calibration was used to normalize NIRS signal, completely deoxygenating the muscle tissue under the probe. The cuff was then released to obtain a peak hyperemic response which was used to indicate 100% oxygenation. This calibration was also used to monitor depletion of oxygen saturation during the short bout of exercise in the recovery protocol. Oxygen depletion was kept between 30-50% of the 100% hyperemic response to ischemia to reduce the likelihood that rate of oxygen utilization during the ischemic cuff periods were limited by oxygen delivery.

Statistical Analysis

A two-way repeated measures ANOVA was used to compare the effect of group (resveratrol+piperine vs. placebo) and time (baseline, weeks 2, 3 and 4) for mitochondrial capacity data in the trained and untrained arms. A two-way repeated measures ANCOVA was then used to account for inter-individual variability in baseline mitochondrial rate constants (Fig. 3.3a, b). Missing data occurred at 1 time point in the placebo trained arm and the untrained arm. Missing data was replaced with the last value carried forward. Fischers LSD was used for the post-hoc pair-wise comparisons. A two-way repeated measures ANOVA was applied to strength measurement data with a within subjects factor of time (pre- and post-) and a between subjects factor of group. All statistical analysis were performed using SPSS 19.0 (IBM®, Armonk, NY). Statistical significance was accepted at $p < 0.05$ and all data are presented as means \pm SD.

Results

All participants completed the supplementation, exercise training, and testing without any adverse events. The physical characteristics of the participants in this study are shown in Table 1. There was a 99.1% adherence to supplementation and no participant missed more than 2 out of 28 days of capsules. All testing sessions were completed by all participants (12/12) resulting in 100% adherence to exercise training.

The statistical analysis of the strength measurements revealed that the interaction (group*time) was not significant in either the trained arms ($p=0.55$) or the untrained arms ($p=0.43$). Strength was not different between the resveratrol group and the placebo group, and did not change during the course of the intervention.

A two-way ANOVA model was first used for the analysis of the change in mitochondrial rate constants over time. The analysis identified a trend towards a significant group \times time interaction effect in the trained arm ($p=0.067$). Examination of individual baseline data indicated substantial heterogeneity among subjects within each group. Therefore, we performed a two-way ANCOVA, which revealed that inter-individual variability in baseline mitochondrial capacity significantly affected the resveratrol and placebo groups ($p=0.05$). By using baseline mitochondrial capacity values as a covariate in the ANCOVA model, the analysis identified a significant group \times time interaction effect in the trained arm ($p=0.02$), indicating differences in the NIRS rate constants between the resveratrol and placebo group. Pairwise comparisons indicated significant differences in the NIRS rate constants at the final time point of post-testing in the resveratrol group only ($p=0.01$) (Fig. 3.2a), increasing about 40% from baseline (Fig.3.4). There were no significant differences among time points in the placebo group ($p=0.44$). When comparing the resveratrol trained arm to the placebo trained arm at post intervention, differences

in mitochondrial rate constants between the two groups were approaching significance ($p=0.08$). A two-way ANCOVA did not identify a significant interaction effect in the untrained arms between the 2 variables (group \times time) ($p=0.65$) (Fig. 3.2b), meaning that the untrained arms NIRS rate constants did not differ significantly between groups or over time.

Discussion

The primary finding in this study was that 4 weeks of resveratrol supplementation combined with a submaximal endurance training stimulus significantly increased mitochondrial oxidative capacity, compared to placebo. Previous studies have shown administration of resveratrol enhances AMPK and SIRT1, leading to activation of PGC-1 α (18, 21, 30, 31) which is the primary pathway associated with endurance exercise adaptations leading to mitochondrial biogenesis (14, 15, 35). Therefore, resveratrol along with exercise, could enhance this pathway to a greater extent than exercise alone. This is supported by studies in mice and rats that have shown resveratrol enhances physiological adaptations to exercise training (9, 13, 21), which are consistent to the findings in this study demonstrating that mitochondrial capacity was increased to a greater extent in the resveratrol group than the placebo group.

However, recent studies in humans have mixed results. Studies done in obese and diabetic populations have been consistent with rodent models, indicating increases in skeletal muscle SIRT1 and AMPK protein levels with resveratrol supplementation (11, 30). However, studies combining exercise and resveratrol supplementation in healthy adults have reported attenuated training induced adaptations with supplementation of resveratrol. A study examining the effects of 4 weeks of high-intensity interval training (HIIT) found that skeletal muscle gene expression was unchanged with resveratrol supplementation. PGC-1 α and SIRT1 gene

expression were lower compared to placebo, indicating resveratrol supplementation may attenuate mitochondrial adaptations to exercise training (27). Other studies have reported resveratrol has no effect on physiological adaptations to training, such as whole body maximal oxygen uptake (10, 19, 32). A potential difference between these studies and the current study may be due to the intensity of the exercise training regimens and the dosage amounts. For example, both Scribbans et al. (27) and Gliemann et al. (10) performed HIIT protocols 3 times per week. Having an intense training stimulus could potentially mask effects resveratrol might have on physiological outcomes. By using a submaximal exercise training stimulus, this allowed for a synergistic effect to be detected. Based on our data, it seems possible that resveratrol could enhance adaptations to lower intensity training stimuli.

An additional consideration is the amount of resveratrol necessary to produce physiological changes. Scribbans et al. (27) and Gliemann et al. (10) supplemented with 250mg/day or less of resveratrol. Resveratrol is rapidly metabolized and results in the formation of various resveratrol metabolites and conjugates. These conjugates could act differently than the parent compound, possibly inducing different physiological effects, however further research needs to be done to better understand the biological activity of resveratrol and its conjugates (5, 7). Increasing the dosage of resveratrol and increasing its bioavailability may be significant in inducing beneficial adaptations and increases in mitochondrial capacity.

In the current study, significant enhancement of mitochondrial capacity did not occur until the last measurement in the resveratrol group. This suggests that supplementation of resveratrol may need a preloading period to increase concentrations in the blood high enough to see effects (1, 7). Another concern is the bioavailability of resveratrol. In this study, resveratrol was combined with piperine to enhance the bioavailability of resveratrol. Combining resveratrol

with piperine has been shown to increase the absorption and maximum serum levels of resveratrol concentration by ~1500% in mice given 100 mg/kg resveratrol and 10 mg/kg piperine (16). Along these lines, it has also been reported that the bio-efficacy of resveratrol is also enhanced when co-supplementing with piperine in healthy human subjects (34). Therefore, combining piperine with resveratrol could have been a contributing factor to the significant changes seen in this study.

Another interesting outcome in this study is that the submaximal exercise stimulus was not sufficient enough to induce any changes in mitochondrial capacity within the placebo group, only 3 /8 individuals indicating increases in mitochondrial rate constants from baseline (Fig.3.3b). When comparing this response to the change seen in the resveratrol group, 7/8 individuals indicating increases in mitochondrial rate constants from baseline (Fig. 3.3a), this could lead to the assumption that the increase in mitochondrial capacity in the resveratrol group was solely due to supplementation. However, no effect of resveratrol supplementation was observed in the untrained arm of the resveratrol group, suggesting that the combination of exercise and resveratrol is needed for eliciting mitochondrial adaptations. This suggests that small changes at the molecular level with this submaximal stimulus could have occurred, but the signals were not enough to elicit training adaptations at the physiological level in the placebo trained arm. This conclusion is supported by Timmers et al. who detected increases in AMPK, PGC-1 α , and citrate synthase- all biomarkers of mitochondrial biogenesis- but did not find a change in mitochondrial function in vivo when measuring phosphocreatine recovery rate after exercise (30). Therefore, our findings indicating no changes in the resveratrol untrained arm lead to the conclusion that effects of resveratrol may depend on the energy state of the muscle, and

small changes at the molecular level produced by low intensity exercise may be enough to see these effects.

Some of the limitations of this study were that serum levels of resveratrol in the blood were not assessed, muscle tissue samples were not taken to determine activation of signaling pathways, and the effect of piperine on mitochondrial capacity was not assessed independent of co-supplementation with resveratrol. Measuring resveratrol concentrations in the blood could have led to better understanding of the bioavailability and dosage amounts needed to induce physiological effects, and how repeated dosing at the amount given over 28 days effects C_{\max} values. Diet was also not controlled or accounted for in this study, which may be a confounding variable. Muscle tissue samples could have provided more insight in to mechanistic pathways resveratrol activates and made this study easier to compare to others indicating activation of proteins such as SIRT1 and AMPK. Finally, future studies may determine the independent and combined effects of piperine and resveratrol on mitochondrial adaptations to training (29).

Conclusions

This is the first human study to show that resveratrol supplementation could enhance mitochondrial capacity when taken in combination with a low intensity, submaximal exercise training stimulus. The synergistic effect between low intensity exercise and resveratrol supplementation could benefit older populations as well as diseased populations who are unable to exercise at high intensities. Using piperine to increase bioavailability and bioefficacy may be a key component when supplementing with resveratrol, although further studies are needed to determine whether piperine definitively augments resveratrol-mediated improvements in mitochondrial training adaptations. More studies are needed to determine the biochemical

pathways in which resveratrol increases mitochondrial capacity and the optimal dosage that these physiological adaptations occur.

Figure Legends

Figure 3.1: **(A)** NIRS oxygenated hemoglobin/myoglobin signal during a mitochondrial capacity recovery protocol, consisting of ~5-10 seconds of voluntary exercise followed by a series of short duration arterial occlusions. **(B)** Results from the NIRS recovery test measuring the rate of recovery of metabolic rate after ~5-10 seconds of voluntary exercise.

Figure 3.2: **(A)** Mitochondrial rate constant (k) for the recovery of muscle oxidative capacity in the resveratrol (closed diamonds) and placebo (open diamonds) trained arms. **(B)** Mitochondrial rate constant (k) for the recovery of muscle oxidative capacity in the resveratrol (closed circles) and placebo (open circles) untrained arms. * $p < 0.05$ compared to time points 2 and 3.

Figure 3.3: **(A)** Individual subjects pre- and post-intervention mitochondrial rate constants (k) for the recovery of muscle oxidative capacity in the resveratrol training arm. **(B)** Individual subjects pre- and post-intervention mitochondrial rate constants (k) for the recovery of muscle oxidative capacity in the placebo trained arm.

Figure 3.4: Percent changes in mitochondrial rate constant from baseline measurements to post-testing in untrained and trained arms of the resveratrol and placebo group.

Table 3.1. Participant characteristics (N=16)

	Placebo (n=8)		Resveratrol (n=8)	
	Males (n=5)	Females (n=3)	Males (n=4)	Females (n=4)
Age (y)	21.0±2.4	19.7±0.6	20.0±0.8	19.0±0.8
Height (cm)	176.9±5.1	166.0±16.5	175.9±7.0	162.9±7.9
Body mass (kg)	75.4±13.1	58.1±18.0	78.3±17.0	59.1±3.5
ATT (mm)				
Nondominant Arm	3.6±1.1	5.5±1.4	4.8±1.6	6.1±1.8
Dominant Arm	3.4±1.1	6.0±2.4	4.4±1.4	5.7±1.1

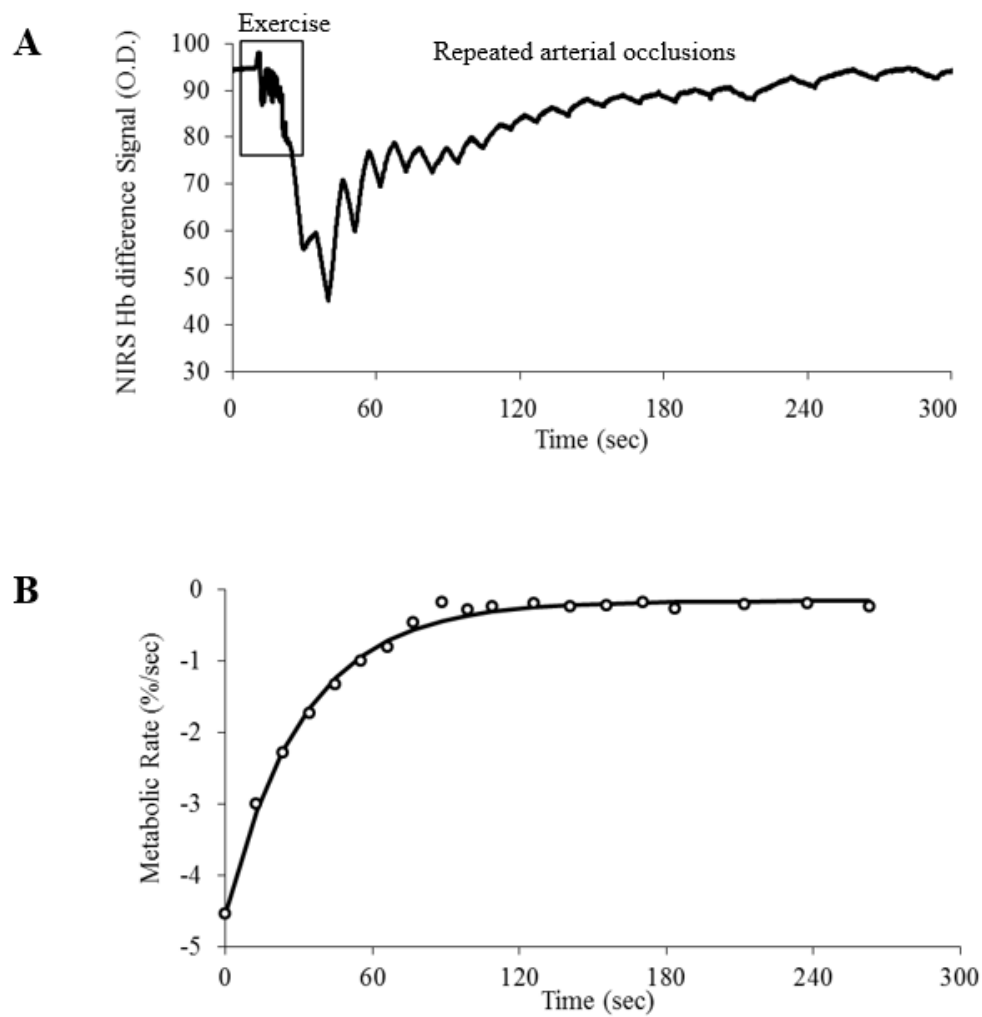
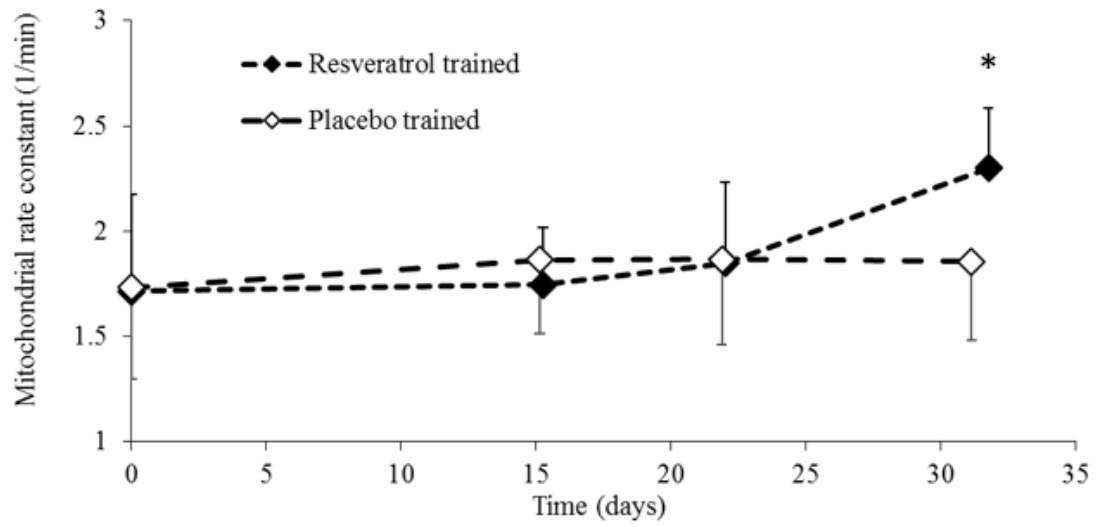
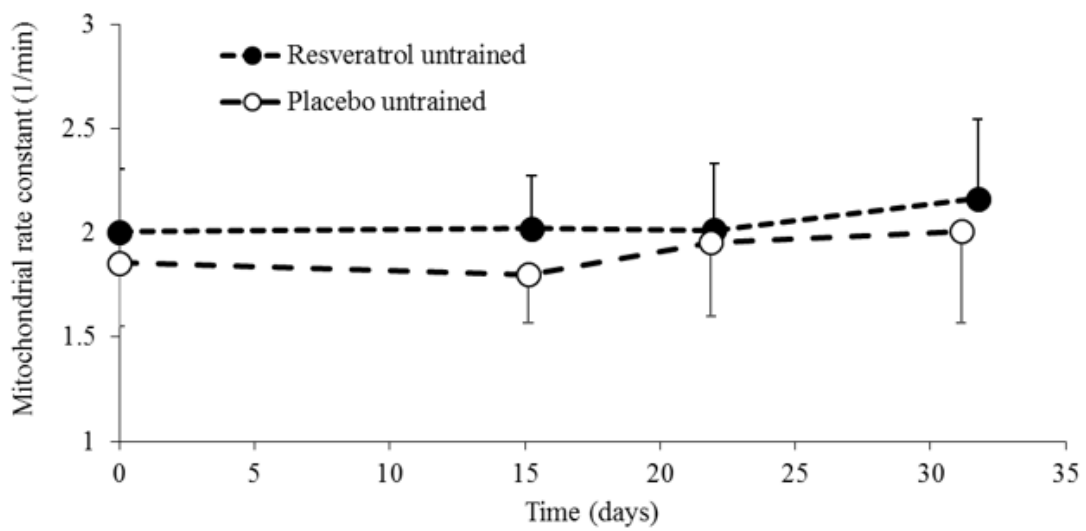


Figure 3.1

A**B****Figure 3.2**

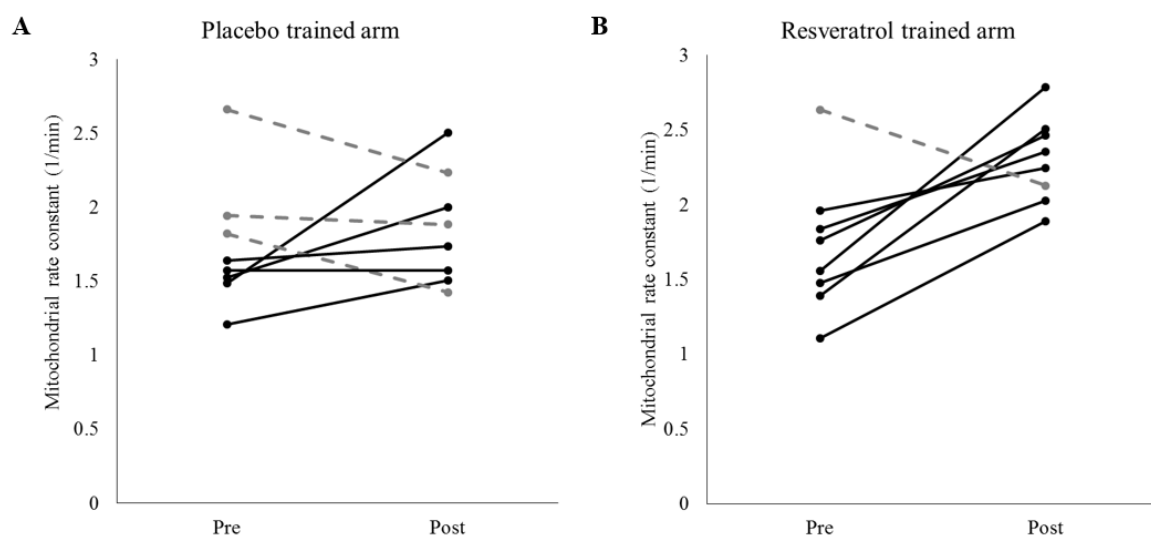


Figure 3.3

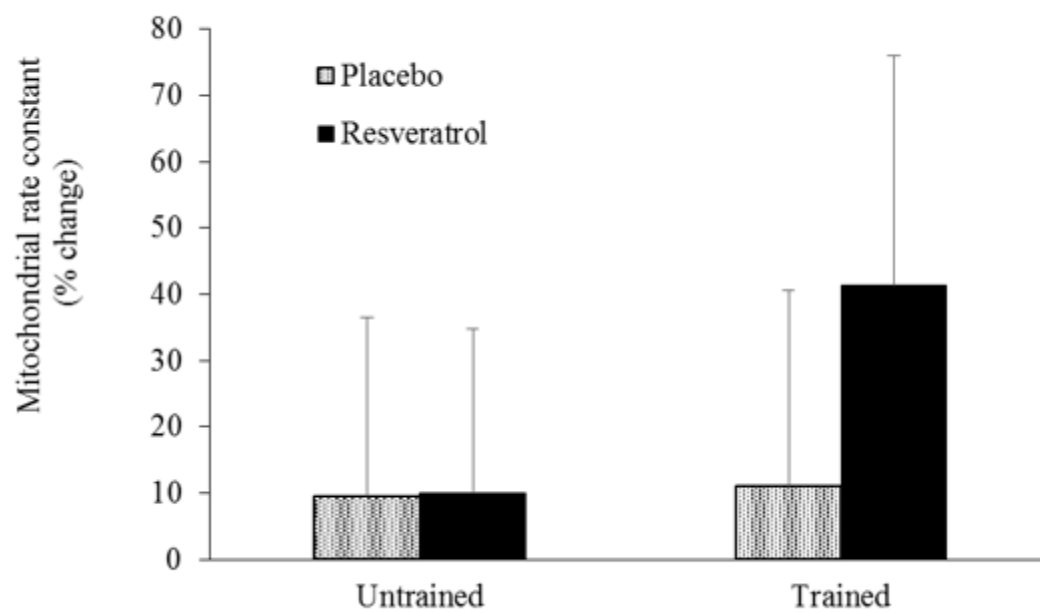


Figure 3.4

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CHAPTER 4

SUMMARY AND CONCLUSION

Significance of Study

The major finding in this study is that resveratrol supplementation with the addition of piperine combined with a submaximal exercise stimulus enhanced mitochondrial capacity to a greater extent than the placebo group. This study is impactful in that it reports possible benefits from resveratrol supplementation in humans where most research on resveratrol supplementation has been in rodent models. This study is also novel in its use of a submaximal exercise training stimulus with resveratrol supplementation. Previous studies looking at physiological adaptations in healthy humans have used intense exercise regimens and have found no further beneficial adaptations to exercise training with supplementation of resveratrol.

This study was also different in that a bioenhancing agent was used to increase absorption of resveratrol in the blood. This was used because the bioavailability of resveratrol is very low and is rapidly metabolized by humans. The addition of piperine or other bioenhancers may be necessary to see physiological outcomes with this nutraceutical.

Supplementation of Resveratrol

In this study, a dosage of 500 mg of resveratrol was used, as well as 10 mg of piperine to increase the bioavailability of resveratrol. This dosage seemed to be well tolerated with no adverse events being reported. Most studies that have shown beneficial effects of resveratrol on mitochondrial biogenesis have been done in animals. It is difficult to compare this dosage to

animal studies because of differences in size and metabolic rate between humans and rodents. This dosage was based off of previous studies supplementing healthy adults. The dosage given was higher than previous studies in healthy adults and combined with piperine to try and acquire higher concentrations in the blood, increasing bioavailability and bioefficacy. This dosage appeared to work in our favor, indicating a greater magnitude of change in mitochondrial capacity in the resveratrol trained arm. This dose seemed to be successful for a healthy population, however, patient populations may need higher dosage amounts in order to elicit beneficial effects of resveratrol.

This study did not take in to account amount supplemented per individual based on body weight. This might have been a limitation when supplementing a bigger male versus a tiny female. We also did not do a diet recall and ask for specific details as to what each participant ingested the pills with each morning, which may possibly alter bioavailability (49).

Submaximal Exercise Training Program

A key method to this study was the submaximal exercise training protocol. The submaximal wrist flexor training program in this study was designed based off of previous studies using 4 weeks of wrist flexor exercise to elicit a maximal response to training (41, 43). All training sessions were performed under the supervision of the lead investigator to control for adherence. This resulted in 100% adherence to exercise training, each participant attending all 12 training sessions over the course of the study. We felt a submaximal exercise stimulus was a key factor to be able to see changes in mitochondrial capacity, where a high maximal exercise stimulus may have overpowered and masked the effects of resveratrol on mitochondrial capacity.

This stimulus was successful in that we were able to get an effect of the supplement on mitochondrial capacity compared to the placebo.

However, no changes in mitochondrial capacity training adaptations were seen at all in the placebo group. Specifically, the submaximal training protocol consisted of 3 sessions per week with the frequency of the contractions only increasing after week 1. Small training adaptations were expected to occur. The frequencies were uniform across participants and based off of previous data performed in our lab. This may have been a factor as to why changes in mitochondrial capacity were not seen in the placebo trained arm. Frequencies were not adjusted specifically to individual's ability to perform the exercise or endurance capacity in the forearm which may have contributed to the lack of adaptation in the placebo trained arm. Individualizing the protocol for each subject may help to elicit a training response. Taking in to account a subject's baseline mitochondrial capacity measures may be one way to better elicit a submaximal training program to see training adaptations occur.

Antioxidant Vitamins and Polyphenols: A Difference?

Vitamin antioxidants, such as vitamins C and E, have been thoroughly researched and are known for their ability to scavenge free radicals and protect the cell from oxidative stress and excessive production of reactive oxygen species (ROS). Diseased populations as well as older aging populations experience high rates of ROS production and oxidative stress (8), which may be slightly alleviated with antioxidant vitamin supplementation (33). High muscle activity also increases concentrations of free radicals and ROS production in the muscle (9). This has led to the belief that supplementation of vitamin antioxidants may be beneficial to persons who participate in high amounts of exercise, especially targeting athletes who train at very high

exercise intensities. It was believed that decreasing exercised-induced ROS would help in muscle fatigue and inflammation after training, benefiting the athlete (11). However, recent research has found that vitamin antioxidants may not be beneficial for the athlete trying to obtain training adaptations. ROS produced in the muscle during exercise is a signaling mechanism involved in physiological adaptations to training (45). Because vitamin antioxidants blunt the ROS response to training, recent research has shown that this leads to attenuation of training adaptations (19, 38). Therefore, using antioxidants in combination with a training stimulus to reduce ROS production in the muscle and improve fatigue and recovery, may not be beneficial in the long run, reducing the ability for the muscle to adapt to the exercise stimulus.

Polyphenols are a class of antioxidants that have been brought to the attention of researchers for their potential beneficial effects on health. Because of polyphenols antioxidant properties, it may be thought that polyphenols, like vitamin antioxidants, directly scavenge ROS as well, attenuating exercise training adaptations. However, polyphenol antioxidants seem to act through various cell signaling pathways, not necessarily directly inhibiting exercise-induced ROS (52). A study comparing the effects of a polyphenol mixture (oligomerized lychee fruit extract) and a vitamin C and E mixture found a significant increase in submaximal endurance performance from baseline in the polyphenol group only. Maximal oxygen uptake remained consistent with baseline measurements after intervention in both the polyphenol group and the placebo group, however significantly decreased in the vitamin C and E group. These findings indicate that ingestion of the vitamin C and E mixture slightly reduced exercise performance compared to the polyphenol and placebo groups. This data suggests that polyphenol antioxidants may not inhibit ROS induced muscle adaptations to exercise, allowing for performance

enhancements to occur, where vitamin antioxidants have an inhibitory effect on performance outcome measures (24).

To add to the complexity of polyphenols oxidative mechanisms, polyphenols are also shown to have pro-oxidant effects, signaling cell apoptosis and preventing tumor growth. This chemopreventive action is especially unique due to polyphenols ability to differentiate and selectively target cancer cells, sparing normal cells (26). However, polyphenols biological effects extend beyond simply modulation of oxidative stress. Resveratrol is a key example, showing calorie-restrictive like effects and possible exercise mimetic capability through its activation of the SIRT1, AMPK, PGC-1 α pathway (17, 27). All in all, polyphenols seem to work through various signaling pathways and induce a vast array of physiological effects, differentiating them from vitamin antioxidants. The different workings of polyphenols oxidative effects compared to vitamin antioxidants mechanisms of action may be slightly different and therefore, should be regarded separately when being studied. Future research should focus on understanding the molecular actions that underlie the various biological effects of polyphenols to better evaluate their potential use as possible preventative therapies for disease risk.

Evaluation of NIRS Testing

Mitochondrial capacity measurements were assessed using NIRS technology. Using this method had many benefits. The device is easy to use and non-invasive. Set up time for the device is quick and analysis of the data takes minutes. However, there were limitations to using this technique as well.

NIRS measures oxygenated and deoxygenated hemoglobin/myoglobin. Because of this, distinguishing if changes in the muscle are due to mitochondrial number or function of the

mitochondria are hard to decipher. The device can only detect changes in the muscles ability to use oxygen. So it is unclear whether changes seen with this device are due to mitochondrial density or efficiency of the mitochondria. Therefore, changes seen in the measurement are referred to as changes in mitochondrial oxidative capacity. Another limitation in the use of the NIRS recovery test in this study is that voluntary forearm exercise was used as the exercise stimulus to increase metabolic rate before the repeated cuff recovery protocol to calculate mitochondria rate constants. Oxygen saturation levels drop with voluntary exercise and there is currently rising evidence in our lab indicating that it is important to activate the muscle in order to increase metabolic rate of the muscle, but levels of oxygen saturation may play a role in outcome measures of the mitochondrial rate constants. Decreases in oxygen saturation are not seen in protocols utilizing electrical stimulation to activate the muscle (14). This may make electrical stimulation a better alternative to control for drops in oxygen saturation seen in voluntary exercise protocols. However, in the current study, oxygen saturation levels were carefully monitored and kept consistent between trials. Depletion of oxygen saturation was kept between 30-50% of the 100% calibration. This consistency between trials within subjects may contribute to the accuracy of the measurement and should not skew results that were obtained. As more research is done on the effect of oxygen saturation levels on this measurement, it may be important to consider electrical stimulation as a mode of increasing metabolic rate before recovery tests are performed to easily control for oxygen saturation levels. The current research involving NIRS methodology to measure muscle oxygenation levels is still young and evolving. There are many publications supporting the use of this method to measure mitochondrial capacity (6, 13, 41, 43, 44), but more research involving the details of the protocol need to be to

be done to assure complete confidence in the method and establish precise measurements with lower variability.

Future of Resveratrol

More research needs to be done with resveratrol supplementation in humans, specifically using bioenhancers in combination with this polyphenol to increase bioavailability in the body. The findings in this study indicate resveratrol may have clinical and therapeutic applications. Resveratrol may have the most benefit in diseased populations and older individuals who do not have the ability to exercise at high intensities. The addition of resveratrol to a low exercise stimulus could enhance training adaptations to that of higher intensity exercise, making it a key dietary supplement to be considered in older adults and various clinical populations. Future studies should strive to more fully understand these interactions.

Using resveratrol in combination with other polyphenols may be a key direction to take the administration of polyphenols in the next decade to enhance beneficial effects from this class of antioxidants. Development of a polyphenol cocktail in combination with a bioenhancing agent may be key in enhancing polyphenols low bioavailability and inducing effective physiological changes.

All together resveratrol has shown implications towards improving health status, including enhancement of training adaptations such as mitochondrial capacity as shown in this study. It is important to understand the molecular pathways in which this polyphenol induces these changes to effectively apply the use of this nutraceutical. Future research should focus on effective dosing levels of resveratrol, enhancing its low bioavailability, and better understanding cellular pathways this polyphenol regulates.

CHAPTER 5

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