Influence of exercise training with resveratrol supplementation on skeletal muscle mitochondrial capacity

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Abstract

Background: Physical inactivity reduces, and exercise training increases, mitochondrial capacity. In rodents, exercise training effects can be augmented by large doses of resveratrol supplementation but whether this can occur in humans with a smaller dose is unclear.

Objective: This study sought to determine the effects of resveratrol supplementation in combination with exercise training on skeletal muscle mitochondrial capacity.

Design: Sixteen healthy young adults were randomly assigned in a double blind fashion to consume either placebo or 500 mg of resveratrol plus 10 mg of piperine, a bioenhancer to increase bioavailibilty and bioefficacy of resveratrol. Participants ingested the pills daily for 4 weeks and completed 3 sessions per week of submaximal endurance training of the wrist flexor muscles of the non-dominant arm. The contralateral arm served as an untrained control. Skeletal muscle mitochondrial capacity was measured using near-infrared spectroscopy (NIRS).

Results: Changes in mitochondrial capacity from baseline to post testing indicated significant differences between the resveratrol+piperine trained arm and the placebo trained arm (p=0.02), with the resveratrol+piperine group increasing about 40% from baseline (Δk= 0.58), while the placebo group increased about 10% from baseline (Δk= 0.13). Neither the placebo nor the resveratrol+piperine group exhibited changes in mitochondrial capacity in the untrained arm.

Conclusions: Low intensity exercise training can increase forearm skeletal muscle mitochondrial capacity when combined with resveratrol and piperine supplementation.

Key words: resveratrol, skeletal muscle, mitochondrial capacity, NIRS, exercise training, submaximal endurance training

Introduction

Dietary supplements that target health and performance are a large global commercial market, with billions of dollars spent per year (Hardy 2000). One type of dietary supplement is polyphenols, which are the most abundant antioxidants in the diet and have recently gained attention due to their potential beneficial effects on metabolic health and aging (Scalbert et al. 2005; Schrauwen and Timmers 2014). A popular supplement known as resveratrol has been the focus of much research due to its reported anti-inflammatory, anti-oxidant, and anti-tumorigenic properties (Smoliga et al. 2011).

Resveratrol is a polyphenol antioxidant found in grapes, red wine, peanuts, cranberries, and other plant species. Resveratrol has become of particular interest in the past decade in part because of its potential ability to stimulate the expression of the SIRT1-AMPK-PGC1α pathway in skeletal muscle, leading to enhanced mitochondrial capacity (Lagouge et al. 2006; Timmers et al. 2011; Ungvari et al. 2011; Menzies et al. 2013; Goh et al. 2014; Kulkarni and Canto 2014). Exercise training also activates this pathway and results in enhanced mitochondrial capacity (Winder et al. 2000; Jager et al. 2007; Hood 2009).

Several experiments in rodents have reported an augmented effect on skeletal muscle mitochondrial capacity of exercise training when it was combined with resveratrol supplementation (Murase et al. 2009; Dolinsky et al. 2012; Menzies et al. 2013). In humans, there have been few studies assessing resveratrol's effect on mitochondrial capacity and the research to date has produced contradictory findings. Some studies show upregulation of mitochondria and the pathways that lead to increased mitochondrial capacity (Timmers et al. 2011; Goh et al. 2014), while others show minimal(Voduc et al. 2014) or attenuated training adaptations (Gliemann et al. 2013; Olesen et al. 2014; Scribbans et al. 2014; Voduc et al. 2014) .

These inconsistent findings in human studies could be due to multiple factors including the health of the participants, variations in the muscles trained, the training stimulus, and the dose and bioavailability of the resveratrol.

A concern in human studies is the low bioavailability of resveratrol (Amri et al. 2012). Maximum plasma resveratrol concentrations (C_{max}) typically are around 75 ng/mL or less with a single dose of 500mg of resveratrol (Boocock et al. 2007). This is much lower than the *in vitro* concentrations shown to have pharmacological benefits (>1000 ng/mL), which has been achieved in plasma of rodents treated with large doses of resveratrol (de la Lastra and Villegas 2005). This suggests that a higher dose, or enhanced bioavailability, is potentially needed to produce beneficial effects in humans. Piperine has been used in combination with neutraceuticals such as coenzyme Q10, beta-carotene, and resveratrol to enhance their bioavailability and bioefficacy (Badmaev et al. 1999; Badmaev et al. 2000; Johnson et al. 2011; Wightman et al. 2014). Therefore, it seems plausible that co-supplementing resveratrol with piperine could lead to enhanced bioavailability and bioefficacy of resveratrol and improve the ability to produce physiological benefits in humans yet minimize the potential risks associated with high doses (Mennen et al. 2005).

The purpose of this study was to evaluate the influence of co-ingesting 500 mg of resveratrol with 10 mg of piperine on changes in muscle mitochondrial capacity with submaximal endurance training in healthy subjects compared to a placebo group. It was hypothesized that 4 weeks of supplementation of resveratrol+piperine in combination with submaximal endurance training would produce a greater increase in muscle mitochondrial capacity than exercise training or resveratrol+piperine alone.

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 also excluded from the study if there exercise regimen involved excessive use of the forearm muscle, such as rowing, rock climbing, or Crossfit. 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 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 4 weeks. 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 similar to previous endurance training programs (Ryan et al. 2013a). During baseline testing, participants performed a maximal voluntary isometric contraction (MVIC) to determine the appropriate weight for exercise training and testing. MVIC was determined using a JAMAR

® handgrip dynamometer (Sammons Preston Rolyan, Bolingbrook, IL). Participants were given the hand dynamometer and instructed to perform three MVIC's on each hand. Participants were instructed to rest 20 seconds between each contraction and the highest value of the three contractions was recorded. 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 (Boocock et al. 2007). 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 containing10 mg of piperine (n=8); or two identically appearing 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 was not a member of the investigational team and 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.

Mitochondrial Capacity Procedures and Measurements

Mitochondrial capacity measurements took 45 minutes and were made at weeks 0, 2, 3, and 4. Mitochondrial capacity, measured using near infrared spectroscopy (NIRS) (Ryan et al. 2012; Ryan et al. 2013a), yields results highly correlated to gold standard measures of mitochondrial function including magnetic resonance spectroscopy (Ryan et al. 2013b) and biochemical analysis of muscle biopsies (Ryan et al. 2014). 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 (O_2Hb) and deoxygenated (HHb) hemoglobin/myoglobin were corrected for changes in blood volume as previously described (Ryan et al. 2012).

Mitochondrial capacity was measured using a short bout of voluntary exercise to increase metabolic rate, and the rate of recovery of metabolic rate after the exercise was measured (McCully et al. 2009; Ryan et al. 2013a). The testing protocol consisted of a 5 – 10 second bout of voluntary exercise followed by a series of 17-20 short duration arterial occlusions (5-10 seconds) (**Figure 1A**). The exercise/occlusion protocol was performed twice. The rate of recovery of oxygen consumption measured by the difference signal ($Hb_{difference}= O_2Hb – HHB$) 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 (**Figure 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

The differences between baseline testing and time points 2, 3 and 4 were first calculated and a two-way repeated measures ANOVA was used to compare the effect of group

(resveratrol+piperine vs. placebo) and time (difference between weeks compared to baseline) for mitochondrial capacity data in the trained and untrained arms. Examination of individual data indicated considerable variability in initial values of mitochondrial capacity among study participants. ANCOVA was therefore used to control for individual variation in baseline mitochondrial capacity that plausibly could influence responsiveness to the intervention. Because of equipment problems, missing data occurred at week 3 for one individual in the placebo trained arm group and a second individual in the placebo untrained arm. These missing values were replaced with the last value carried forward. Fishers 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 ± 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**. Compliance was monitored via daily text messages and determined using a post-screening questionnaire which was administered after completion of the study (Wei et al. 2011). Based on the questionnaire, there was a 99.1% adherence to supplementation and no participant missed more than 2 out of 28 days of capsules. All training 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 was not significant in either the trained arms (p=0.55) or the untrained arms (p=0.43). Baseline strength

197 measurements were not different between the resveratrol group and the placebo group, and did
198 not change during the course of the intervention.

199 Average mitochondrial rate constants between groups are displayed in **Figures 2A and**
200 **2B**. Examination of individual baseline data indicated substantial heterogeneity among subjects
201 within each group (**Figure 3A, 3B**). When using baseline mitochondrial capacity values as a
202 covariate in the ANCOVA model, the analysis identified a significant group × time interaction
203 effect in the trained arm (p=0.002). In the resveratrol+piperine group only, pairwise
204 comparisons indicated significant differences in the change in mitochondrial rate constants from
205 baseline to post testing compared to changes at weeks 2 (Δk= 0.03 vs Δk= 0.58, p=0.001) and
206 weeks 3 (Δk= 0.13 vs Δk= 0.58, p = 0.005) (**Figure 4**). There were no significant differences
207 among time points in the placebo group. When comparing the resveratrol+piperine trained arm
208 to the placebo trained arm, the change from baseline to post testing indicated significant
209 differences between the two groups (p=0.02), the resveratrol+piperine group increasing about
210 40% from baseline (Δk= 0.58), where the placebo group increased about 10% from baseline
211 (Δk= 0.13) . A two-way ANCOVA did not identify a significant interaction effect in the
212 untrained arms between the 2 variables (group × time) (p=0.66), meaning that the untrained arms
213 NIRS rate constants did not differ significantly between groups or over time.
214
215 **Discussion**

216 The primary finding in this study was that 4 weeks of resveratrol and piperine
217 supplementation combined with a submaximal endurance training stimulus significantly
218 increased mitochondrial oxidative capacity, compared to exercise training with a placebo.
219 Previous studies have shown administration of resveratrol enhances AMPK and SIRT1, leading

to activation of PGC-1α (Lagouge et al. 2006; Timmers et al. 2011; Ungvari et al. 2011; Menzies et al. 2013) which is the primary pathway associated with endurance exercise adaptations leading to mitochondrial biogenesis (Winder et al. 2000; Jager et al. 2007; Hood 2009). Therefore, resveratrol along with exercise training, 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 (Dolinsky et al. 2012; Hart et al. 2013; Menzies et al. 2013), which are consistent with the findings in this study demonstrating that mitochondrial capacity was increased after 4-weeks of arm training in the resveratrol+piperine group, but not the placebo group.

Recent studies investigating the interactive effects of training and resveratrol supplementation in humans have produced mixed results. Studies performed in obese and diabetic populations have been consistent with rodent models, indicating increases in skeletal muscle SIRT1 and AMPK protein levels with resveratrol supplementation (Timmers et al. 2011; Goh et al. 2014). 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) of the legs found that mitochondrial biogenesis-related gene expression in skeletal muscle was unchanged from baseline with resveratrol supplementation. Specifically, PGC-1α and SIRT1 gene expression were increased in the placebo group, and unchanged in the resveratrol group after the training period, indicating resveratrol supplementation may attenuate mitochondrial adaptations to leg exercise training (Scribbans et al. 2014). Another study examining the effects of 8 weeks of a daily dosage of 250 mg of resveratrol in combination with high intensity exercise training also found no effects of resveratrol on SIRT1, AMPK, and PGC-1α. However, exercise

training alone did increase these mitochondrial markers (Olesen et al. 2014). Other studies have reported resveratrol has no effect on physiological adaptations to training, such as whole body maximal oxygen uptake (Gliemann et al. 2013; Voduc et al. 2014; Macedo et al. 2015). A potential difference between these studies and the current study may be due to the muscle group trained, the intensity of the exercise training regimens or the dosage amounts. For example, Scribbans et al. (Scribbans et al. 2014) ,Gliemann et al. (Gliemann et al. 2013) and Olesen et al. (Olesen et al. 2014) performed leg HIIT protocols 3 times per week. Based on our data, it seems possible that resveratrol+piperine could enhance adaptations to lower intensity training stimuli in relatively untrained muscle, controlling for baseline differences in mitochondrial capacity. Future studies are needed to systematically examine whether resveratrol differentially modifies adaptations to low *vs.* high intensity exercise training, or arm *vs.* leg training.

An additional consideration is the amount of resveratrol necessary to produce physiological changes. Scribbans et al. (Scribbans et al. 2014),Gliemann et al. (Gliemann et al. 2013), and Oleson et al. (Olesen et al. 2014) supplemented with 250mg/day or less of resveratrol. Increasing the dosage of resveratrol given in this study to 500mg instead of the 250mg given in the previous studies may have been important in inducing beneficial adaptations and increasing mitochondrial capacity. 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 significantly increase blood concentrations to produce physiological effects (Almeida et al. 2009; Brown et al. 2010). Another concern is the bioavailability and bioefficacy of resveratrol. In this study, resveratrol was combined with piperine in hopes to enhance the bioavailability and bioefficacy of resveratrol. Resveratrol is rapidly metabolized and results in the formation of various resveratrol metabolites and

266 conjugates. These conjugates could act differently than the parent compound, possibly inducing
267 different physiological effects, however further research needs to be done to better understand
268 the biological activity of resveratrol and its conjugates (Baur and Sinclair 2006; Brown et al.
269 2010). The use of piperine in the current study could have been a key component due to its
270 reported ability to inhibit glucuronidation of polyphenol antioxidants (Shoba et al. 1998;
271 Lambert et al. 2004; Srinivasan 2007), therefore possibly preventing extensive metabolism of
272 resveratrol and the formation of resveratrol conjugates, allowing a greater amount of
273 untransformed resveratrol to be absorbed. Combining resveratrol with piperine has been shown
274 to increase the absorption and maximum serum levels of resveratrol concentration by ~1500% in
275 mice given 100 mg/kg resveratrol and 10 mg/kg piperine (Johnson et al. 2011). Along these
276 lines, it has also been reported that the bioefficacy of resveratrol is also enhanced when co-
277 supplementing with piperine in healthy human subjects. This study also confirmed that
278 resveratrol metabolites and conjugate serum levels were lower with the addition of piperine,
279 indicating piperine's inhibition of resveratrol glucuronidation (Wightman et al. 2014). Therefore,
280 combining piperine with resveratrol could have been a contributing factor to the changes
281 observed in this study.

282 This study is also different than previous studies in our use of a low intensity exercise
283 stimulus instead of a high-intensity exercise stimulus combined with resveratrol
284 supplementation. The low intensity stimulus was chosen because previous literature has only
285 assessed resveratrol supplementation in combination with high intensity exercise training and
286 was chosen to mimic an intensity that is feasible for all populations, including patient
287 populations who may be unable to exercise at high intensities. It is interesting that the
288 submaximal exercise stimulus in this study was not sufficient to increase mitochondrial capacity

289 within the placebo group, only 3 of 8 individuals within the placebo group exhibited training-
290 induced increases in mitochondrial rate constants from baseline. Conversely, in the
291 resveratrol+piperine group, supplementation and training increased mitochondrial rate constants
292 in 7 of 8 participants. Moreover, no changes in mitochondrial capacity were observed in the
293 untrained arm of the resveratrol+piperine group. Taken together, these findings indicate that
294 combination of exercise and resveratrol is needed for eliciting muscle mitochondrial adaptations
295 to low-intensity training programs such as that used in the present study. We speculate that
296 small changes at the molecular level with this submaximal stimulus could have occurred, but the
297 signals were not enough to elicit training adaptations at the physiological level in the placebo
298 trained arm. This conclusion is supported by Timmers et al. who detected resveratrol-induced
299 increases in muscle AMPK, PGC-1α, and citrate synthase expression- all biomarkers of
300 mitochondrial biogenesis- but did not find a change in mitochondrial function *in vivo* when
301 measuring phosphocreatine recovery rate after acute exercise (Timmers et al. 2011).
302 Some of the limitations of this study were that plasma levels of resveratrol in the blood
303 were not assessed, muscle tissue samples were not taken to determine activation of mitochondrial
304 biogenesis- related signaling pathways, and the effect of piperine on mitochondrial capacity was
305 not assessed independent of co-supplementation with resveratrol. Measuring resveratrol
306 concentrations in the blood could have led to a better understanding of the bioavailability and
307 dosage amounts needed to induce physiological effects, and how repeated dosing at the amount
308 given over 28 days effects C_{max} values. Measuring circulating resveratrol could have provided
309 insight as to whether piperine did indeed raise resveratrol levels in the blood, and would have
310 served as another indicator of participant compliance. Diet was also not controlled or accounted
311 for in this study, which may be a confounding variable. However, dietary intake of resveratrol is

estimated to be small relative to the doses administered in this study. Muscle tissue samples could have provided more insight in to the effects of training and resveratrol+piperine on mitochondrial capacity, as well as the mechanistic pathways resveratrol activates. Muscle biopsy analysis would have also 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 (Srinivasan 2007).

Conclusions

This is the first study to show that the combination of resveratrol+piperine supplementation and low-intensity, submaximal exercise training enhances mitochondrial capacity in humans. Using piperine as a bioenhancer 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 also needed to determine the cellular mechanisms by which resveratrol increases mitochondrial capacity and the optimal dosage of resveratrol required to safely elicit these physiological adaptations.

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335 analyzed data. All authors contributed to and reviewed the final publication. None of the

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Table 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

Note: Data are presented as means ± SD. All measures were made at the start of the study. ATT, adipose tissue thickness.

Figure Legends

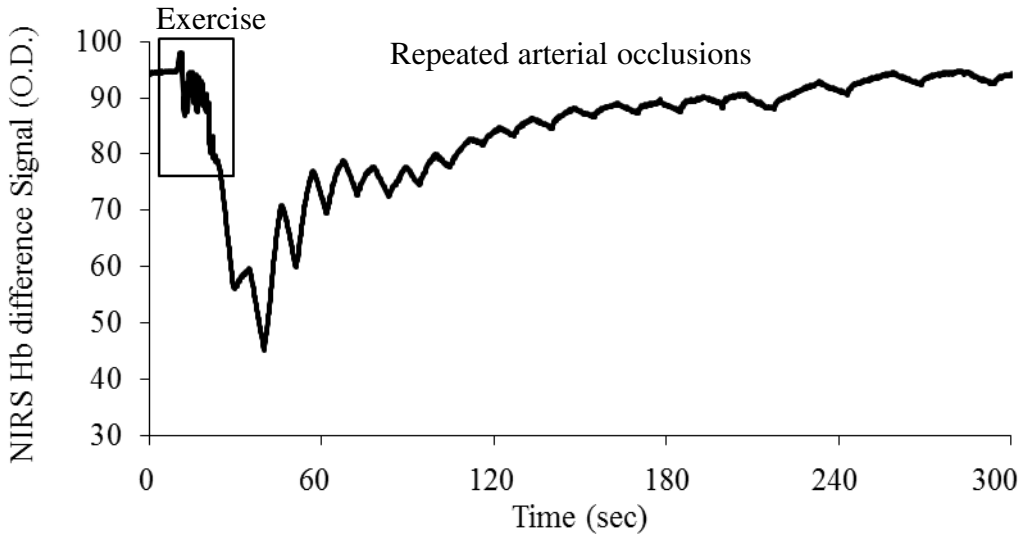
Figure 1: (A) NIRS oxygenated hemoglobin/myoglobin signal during a mitochondrial capacity recovery protocol, consisting of ~5-10 seconds of voluntary exercise followed by a series of short duration arterial occlusions. (**B)** Results from the NIRS recovery test measuring the rate of recovery of metabolic rate after ~5-10 seconds of voluntary exercise.

Figure 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.

Figure 3: (A) Individual subjects pre- and post-intervention mitochondrial rate constants (k) for the recovery of muscle oxidative capacity in the placebo trained arm. **(B)** Individual subjects pre- and post-intervention mitochondrial rate constants (k) for the recovery of muscle oxidative capacity in the resveratrol training arm.

Figure 4: ANCOVA change scores of mitochondrial rate constants from baseline at weeks 2, 3, and 4 in the placebo (open boxes) and resveratrol (dark boxes) trained arms. * Significantly different from changes at weeks 2 and 3 ($p < 0.05$). † Significantly different from placebo change at week 4 ($p < 0.05$).

A



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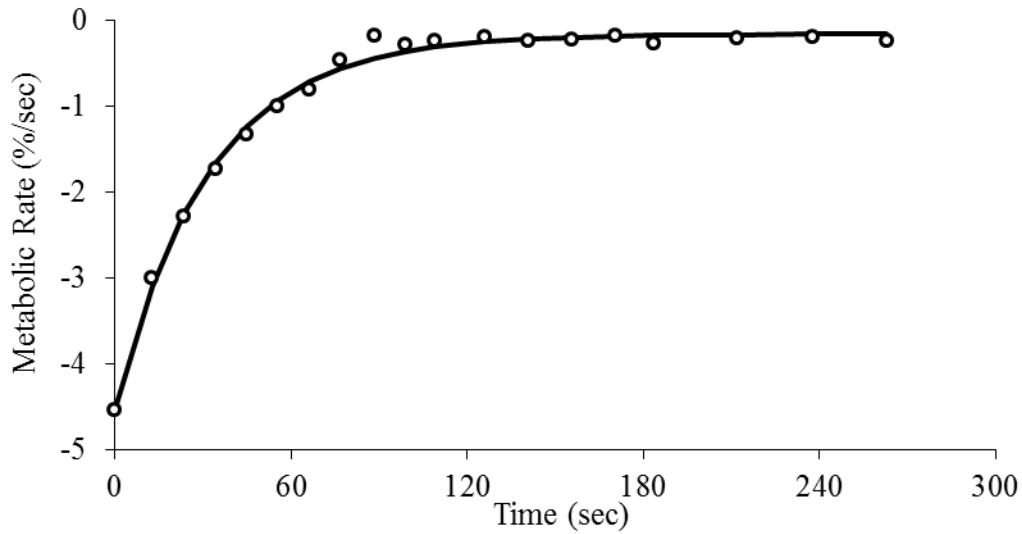

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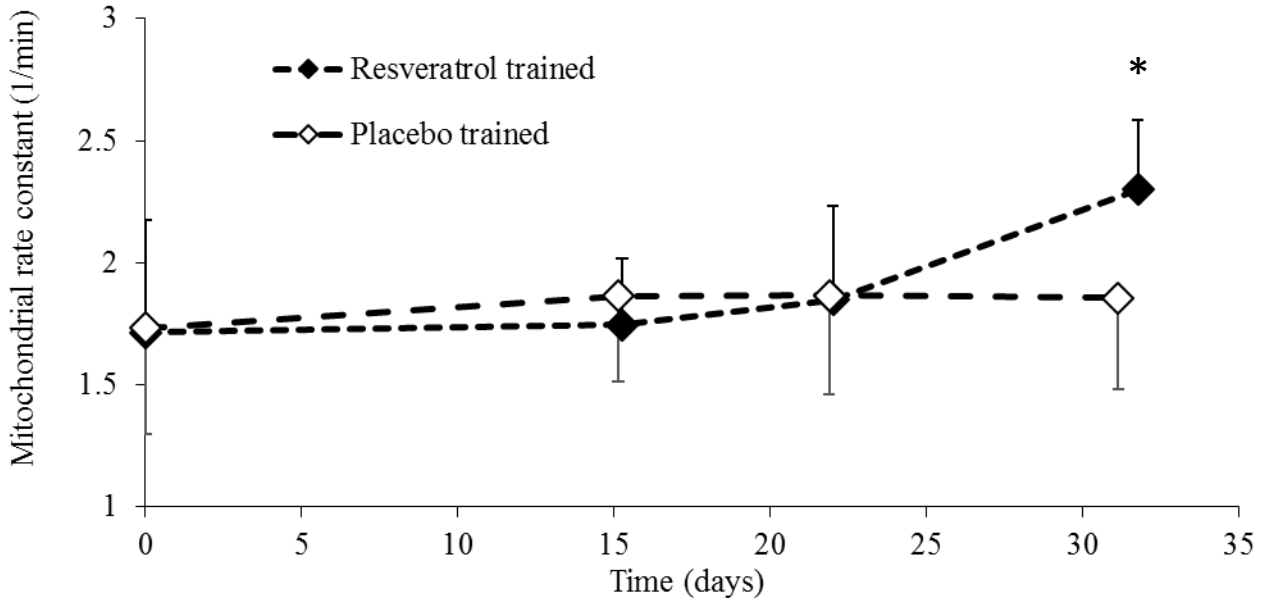

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