THE EFFECTS OF L-CITRULLINE SUPPLEMENTATION ON PHYSICAL PERFORMANCE

by

Peter Lawrence Stordahl

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ABSTRACT

INTRODUCTION. Recent studies have investigated l-citrulline (CIT) as a possible ergogenic aid. A small number of studies have explored the performance question, with varying methodologies and results. The purpose of this study was to examine CIT influence on cycling time to exhaustion, cardiovascular function, and muscle activity. METHODS. Thirteen healthy subjects volunteered for this study. The first visit was to obtain a maximal power output, where 80% and 50% of maximal power were calculated for the following two visits. Subjects were then randomly assigned into either treatment or placebo groups for the second visit and the opposite treatment for the third visit. Subjects were instructed to drink their treatment 1 hour prior to coming into the laboratory. The treatment drink contained 10g of CIT while the placebo (PBO) was formulated to look and taste like the CIT drink. The second and third visits to the laboratory consisted of EMG from the rectus femoris, vastus medialis, and medial gastrocnemius of the right leg, along with HR monitor and BP. Subjects completed a 40-minute interval ride, consisting of 8 5-minute intervals of 3 minutes at 50% maximal power and 2-minutes at 80% maximal power. After the interval ride, subjects received a second dose of either treatment, 5g of CIT or PBO and were allowed 1-hour rest before the ramped time to exhaustion (TTE) test. RESULTS. There was no significant difference in TTE by treatment (CIT, 20.79 ± 4.48 and PBO, 20.86 ± 3.99). There was no significant main effect of treatment on percent of maximum heart rate ($p = 0.084$), mean arterial pressure ($p = 0.714$), or muscle activity of the rectus femoris ($p = 0.300$), vastus medialis ($p = 0.641$), or medial gastrocnemius ($p = 0.133$) during the TTE test. CONCLUSIONS. There were no differences in cycling TTE between treatments. Further research should investigate the metabolism of CIT under different physiological conditions.

CHAPTER ONE

INTRODUCTION

Development of the Problem

In 2013, Forbes Magazine reported that the supplement industry made $32 billion dollars in revenue and projected that number to exceed $60 billion by the year 2021 (Lariviere, 2013). Many athletes and gym goers purchase supplements online; Bodybuilding.com is a popular source for a wide variety of supplements. A recent article on Bodybuilding.com, listed citrulline malate (CM) as one of the top eight supplements needed for the those pursuing increases in strength and other exercise performance standards (Krissy Kendall, 2016). Citrulline malate is composed of L-citrulline (CIT), a non-essential amino acid (Caruso, Rossi, Gahn, & Caruso, 2017) and malate, an amino acid intermediate in the citric acid cycle (Bendahan et al., 2002). Citrulline malate is a common ingredient in pre-workout supplements and can also be purchased as a solitary supplement. Citrulline can also be found in natural sources, such as watermelon, pumpkin and squash. The increase in CIT popularity is not supported by the current literature on CIT supplementation.

History of Supplement Use Physical ability and competition have played an important role in human culture. Even in the early days of competition, humans sought out means of improving performance. The Olympic Games began in 776 BC ("History," 2017) and there are reports of ancient Greeks ingesting mushrooms in an effort to

improve their performance (Magkos & Kavouras, 2005). This trend of searching for means of improving performance continues today. The history of CIT and CM use is not well documented; however, CM has been used in clinical settings to treat general fatigue for decades. In the 1980s Creff (1982), studied the use of Stimol, which is the pharmaceutical name for CM, and its effectiveness. When CIT and CM were adopted by the fitness community is unclear, but due to its physiological effects on systems in the body that lead to a decrease in fatigue, it is logical that this adoption took place.

Citrulline. As stated above, CIT ($C_6H_{13}N_3O_3$) is a non-essential amino acid that has been gaining popularity in the supplement industry and physiology research community. This is largely due to its role as a precursor for l-arginine (ARG) which is a critical amino acid in the production of the vasodilator, nitric oxide (NO). Increased NO production could benefit athletes by augmenting blood flow to and from active tissues. Thereby, enabling greater nutrient and oxygen delivery while simultaneously removing metabolic wastes produced from the skeletal muscles in use.

Approximately 60 % of the arginine produced in the body is made in the kidneys (Wu & Morris, 1998). Citrulline is formed in the enterocytes of the small intestine from glutamate before being transported to the kidneys for conversion to ARG (Windmueller & Spaeth, 1981). In the kidney, CIT is extracted from the blood and converted into arginine through two enzymatic reactions catalyzed by argininosuccinate synthase (ASS) and argininosuccinate lyase (ASL), which are found in the proximal convoluted tubules (Wu & Morris, 1998). Arginine can then be transformed to NO via an enzymatic reaction

using nitric oxide synthase (NOS) isoenzymes. This reaction produces NO and recycles the amino acid back to CIT.

Citrulline Studies Past and Future Only a handful of studies have been published examining CIT as a possible ergogenic aid(P. T. Cutrufello, Gadomski, & Zavorsky, 2015; Hickner et al., 2006; McKinley-Barnard, Andre, Morita, & Willoughby, 2015; Takashi, Masahiko, Yoshinori, & Ayako, 2016; Tarazona-Díaz, Alacid, Carrasco, Martínez, & Aguayo, 2013) . Unfortunately, with a limited amount of research and varying methodologies between the studies it is still unclear if CIT supplementation is effective at improving various metrics of physical performance (e.g. cycling time to exhaustion), and if so, under what circumstances.

With so many gaps in the research it would be impossible for one study to answer all the remaining questions pertaining to CIT supplementation. However, the use of electromyography (EMG) could offer new insight into the state of the muscle tissue during supplementation. If CIT supplementation does augment blood flow patterns, it could lead to a reduction in fatigue of the skeletal muscles being activated. The fatigue of these tissues could be monitored using accepted and validated EMG techniques. The following study was conducted in hopes of furthering the discussion on CIT supplementation.

Purpose

The purpose of this study is to examine the effects of l-citrulline supplementation on cycling time to exhaustion, cardiovascular function, rating of perceived exertion, and muscle activity.

Hypothesis

It is hypothesized that supplementation with l-citrulline will change subjects time to exhaustion compared to supplementation with a placebo, experience changes in muscle activity throughout the interval ride and Ramped Time to Exhaustion test and have lower heart rates and blood pressures. Also, the l-citrulline condition will lower the subjects rating of perceived exertion at equal workloads.

- H_O: $TTE_{CIT} = TTE_{PBO}$ H_a: $TTE_{CIT} \neq TTE_{PBO}$
- H_O: $HR_{CIT} = HR_{PBO}$ H_a: $HR_{CIT} < HR_{PBO}$
- H_O: $MAP_{CIT} = MAP_{PBO}$ H_a: $MAP_{CIT} < MAP_{PBO}$
- H_O: $RPE_{CIT} = RPE_{PBO}$ H_a: $RPE_{CIT} < RPE_{PBO}$
- H_O: $MA_{CIT} = MA_{PBO}$ H_a: $MA_{CIT} \neq MA_{PBO}$

Dependent Variables

TTE- Time to Exhaustion, HR- Heart Rate, MAP- Mean Arterial Pressure, RPE- rating of perceived exertion and MA- muscle activity

Limitations

The results of this study are only applicable to recreationally active, healthy individuals, and not trained cyclists. Due to a limited budget, we were not able to examine the effects of l-citrulline supplementation on amino acid levels in the blood or in the active muscle tissue. Also, we were not able to monitor nitric oxide or associated metabolites. Therefore, any changes in performance can only be hypothesized to come from l-citrulline's mechanism of action. The study was also limited by the subjects' abilities to follow dietary guidelines and dosing schedules.

Delimitations

Research was conducted in the Movement Science Laboratory and Biomechanics Laboratory at Montana State University. All subjects performed cycling protocols on the same cycle ergometer with the same seat height and cycling cadence to control workloads between trials. Subjects were given at least a five-day washout period between experimental trials to ensure no residual fatigue would influence the second experimental trial. Subjects were required to record all food and fluids consumed in the 48 hours before the test days and during the test days. Subjects were informed to copy their intake of food and fluids for the days leading up to the second experimental trial. All subjects had to provide a urine sample before the experimental trials to ensure adequate hydration.

CHAPTER TWO

REVIEW OF RELATED LITERATURE

Introduction

L-Citrulline (CIT; $C_6H_{13}N_3O_3$) is a non-essential amino acid that derives its name from watermelon (Citrullus) where it was originally isolated (Caruso et al., 2017). Citrulline can be found in other nutritional sources such as melons, squash, and pumpkins. Citrulline is also biosynthesized in the body from two related amino acids: L-arginine (ARG) and L-glutamine (Caruso et al., 2017). Citrulline is a component of three metabolic pathways, the conversion of ammonia to urea in the liver, the de novo synthesis from glutamine to ARG, and nitric oxide synthesis indirectly via ARG (Rabier & Kamoun, 1995).

Citrulline has gained popularity recently as an ingredient in many pre-workout drinks which are touted as performance promotors. The two most common forms of CIT in pre-workout beverages are CIT on its own and CIT in combination with malate to create citrulline malate (CM). Malate is an amino acid intermediate in the citric acid cycle (CAC; (Bendahan et al., 2002). Citrulline is included in pre-workout formulations because CIT is a precursor to ARG which can be converted into a vasodilator, nitric oxide (NO). Increased blood flow to active muscle tissue could raise performance by augmenting metabolic waste removal from the muscle.

Metabolism of Citrulline

Citrulline is most commonly metabolized in the liver, small intestine, and kidney. In the liver, CIT is an intermediate in the urea cycle, which rids the body of ammonia by converting it into urea (Rabier & Kamoun, 1995). Citrulline is synthesized in the mitochondria by hepatocytes: it is then transported out of the mitochondrial matrix into the cytosol where it is immediately converted into arginine and urea (Rabier & Kamoun, 1995). It is important to note that under normal physiological conditions, CIT is neither taken up or exported by the liver (Windmueller & Spaeth, 1981) (Rabier & Kamoun, 1995). The liver will only release CIT when levels of ornithine and ammonia are too high for normal physiological function (Windmueller & Spaeth, 1981).

The small intestine and kidneys work in concert to transform glutamate into arginine (Rabier & Kamoun, 1995). Around 60 % of the arginine produced in the body is made in the kidneys (Wu & Morris, 1998). Citrulline is formed in the enterocytes of the small intestine from glutamate before being transported to the kidneys for conversion to arginine (Windmueller & Spaeth, 1981). In the kidney, CIT is extracted from the blood and converted into arginine through two enzymatic reactions catalyzed by argininosuccinate synthase (ASS) and argininosuccinate lyase (ASL), which are found in the proximal convoluted tubules (Wu & Morris, 1998). Once synthesized, arginine can play a role in numerous pathways throughout the body, but it is arginine's role as a precursor for NO that is of greatest interest here.

Nitric oxide synthase (NOS) isoenzymes have become the most well-known group of enzymes for the metabolism of arginine within the past few years (Wu &

Morris, 1998). Arginine is transported through the blood an taken up by cells primarily through sodium-independent transporters (Wu & Morris, 1998). Once inside the cell, ARG can be catabolized in numerous pathways, including the conversion to NO via NOS isoenzymes.

Nitric oxide diffuses to a layer of smooth muscle that surrounds the blood vessel. This sets off a chain reaction that leads to relaxation of the muscle and therefore, an increase in diameter of the blood vessel, a process known as vasodilation (Tiidus, Tupling, & Houston, 2012). The role of nitro-compounds as vasodilators has been established in scientific literature for decades (Kukovetz, Holzmann, Wurm, & Pöch, 1979). Perfusion of blood through muscle is essential during exercise because blood delivers nutrients required for activity and removes waste products created during bouts of exercise.

Muscle Fatigue and Blood Flow

Skeletal muscle fatigue is not the result of a single system failure but a phenomenon with numerous variables that need to be considered. Due to its complexity, many definitions have been given for fatigue such as the reversible decline of muscle performance due to intensive use, (Allen, Lamb, & Westerblad, 2008). Also, a decrease in force production (Enoka, Rankin, Joyner, & Stuart, 1988). The latter definition being widely accepted in research.

Oxygen must be present in the mitochondria of muscle cells for oxidative phosphorylation to occur. The delivery of oxygen to the muscle fiber depends on blood

flow and the ability of oxygen to diffuse from the capillaries across the interstitial space and into the muscle fiber (Allen et al., 2008). The increased metabolic demands of the muscle during exercise creates a scenario where the delivery of oxygen to the muscle becomes crucial for sustained performance. Therefore, the supply of oxygen from the capillaries to the muscle fibers provides one limit to performance (Allen et al., 2008). The use of CIT or CM could increase blood flow and perfusion of the active muscle thereby increasing oxygen delivery and improving performance.

Metabolism of Malate

The citric acid cycle (CAC) is also known as the tricarboxylic acid cycle (TCA) or Kreb's Cycle and is one part of a larger metabolic process known as oxidative phosphorylation. Oxidative phosphorylation is defined as the formation of cellular energy from the transfer of electrons to coenzymes to oxygen. (Tiidus et al., 2012). These processes occur in the mitochondria of cells. The main function of the CAC is the oxidation, or removal of electrons, from a fuel source, so that those electrons can be transported to the second stage of oxidative phosphorylation, known as the electron transport chain (ETC). Malate plays a key role in the CAC. Malate undergoes an oxidation reaction to form oxaloacetate, which is needed to start the CAC (Tiidus et al., 2012). Supplementation of CM could lead to increased amounts of malate present in the mitochondria. Having an abundance of all the intermediaries for the CAC is important because an intermediate may be used in other pathways in the cell, and a decrease in concentrations will slow the rate of the CAC (Tiidus et al., 2012). This decrease may be a cause for fatigue during exercise. A review of the literature has only found one article

that mentions CM use increasing cellular energy production (Jackson & Stoppani, 2004). However, this article is not found in academic literature, rather a fitness magazine. Therefore, more research in this area is needed to elucidate the role of CM on cellular energy.

Citrulline Studies

Supplementation of CIT has been overlooked until recently, but it is an established intermediary in the urea cycle (Tarazona-Díaz et al., 2013). A handful of studies have examined the effectiveness of CIT on various markers of athletic performance with differing results. One study took seven male volunteers that were physically active men but not competing in any specific sport (Tarazona-Díaz et al.),. The subjects completed a cycling protocol on three different days with five days rest between the tests. For each test, the men were randomly assigned either a watermelon juice drink containing 1.17g of CIT, an enriched watermelon drink containing the 1.17g of CIT plus another 4.83g of CIT, and a placebo drink made to look and taste like the other treatments. The results of this study showed no differences in cycling performance, heart rate, blood lactate levels, and perceived exertion. Although neither of the drinks containing CIT made significant improvement in performance compared to the placebo, they did demonstrate a significant difference on muscle soreness twenty-four hours post-test. This result might have been be due to the increased amino acid content in the watermelon drinks (Tarazona-Díaz et al., 2013).

In 2016 (Takashi et al.) tested CIT supplementation on 22 trained male subjects. The subjects received either 2.4g of CIT or placebo each day for one week, and on the eighth

day all subjects ingested their supplement or placebo 1 hour before completing a 4km cycling time trial. During the time trial, the subjects power output to VO_2 ratio was recorded along with plasma nitrite and nitrate levels, amino acid profiles, and visual analog scale scores to evaluate fatigue. Those authors reported supplementation of CIT lead to greater concentrations of CIT and ARG in subjects when compared to the subjects on the placebo. Plasma CIT concentration (nmol/ml) on placebo, 40.0 ± 1.4 and on CIT, 475 ± 37. Plasma ARG (nmol/ml) on placebo 110 ± 4 and on CIT 192 ± 9. However, no significant differences in nitrite and nitrate values were observed between the test and placebo groups. When analyzing the power output to VO_2 ratio, the test group values tended to be higher but were not significantly different than the placebo group. Interestingly, the supplementation of citrulline significantly decreased the subjects feeling of muscle fatigue and improved the cycling time trial completion time (569 ± 14 seconds) by 1.5% compared to the placebo (578 ± 15 seconds). These positive results further support the claim that CIT could be used as an ergogenic aid. This study was unique in that the subjects ingested small amounts of CIT over eight days before testing compared to ingestion of a larger acute dose before testing. The positive results of this study should lead future researchers to consider applying a similar dosing design to further investigate dosing strategies.

Another recent study by (P. T. Cutrufello et al., 2015) investigated supplementation of a high dose of CIT (6g) compared to a low dose (1g) and a placebo on eleven men and eleven women college athletes. The study was a double-blind, randomized, crossover, that consisted of one trial per condition. The trials were held one week apart to give

adequate time for the subjects to recover from the exercise. To begin working out the timing variable with supplementation of CIT, ten of the subjects began exercising one hour after ingestion and the other twelve started two hours after ingestion. High intensity exercise performance was tested using a weightlifting protocol. The subjects first worked up to a one repetition maximum on a chest press machine, then 80% of the one repetition maximum was calculated and this weight was to be used during the three tests. The tests consisted of the same warm up and then five sets of maximum repetitions with thirty seconds rest between the sets. The total number of sets was used to measure performance. The study revealed no significant differences between any of the conditions and was consistent across both genders and both timing variables (P. T. Cutrufello et al., 2015).

Citrulline has even been shown to decrease athletic performance. (Hickner et al., 2006) studied the effects of two different doses and timing procedures on performance in a graded exercise test. There were 17 total participants in the study, both men and women, who were randomly assigned to either the placebo, ingesting 9g of CIT over a 24-hour period, or ingesting 3g of CIT three hours before the test. All participants completed each of the test conditions, and the results showed a negative relationship between CIT ingestion and performance. Both CIT conditions corresponded to a reduction in time-to-exhaustion during the exercise test compared to the placebo group (CIT, 888.2 ± 17.7 and placebo, 895.4 ± 17.9). These findings were the first of their kind when CIT was given to healthy subjects. With a limited number of studies consisting of varying protocols and differing results, a clear relationship between CIT supplementation and high intensity exercise performance cannot be made.

Citrulline Malate Studies

There are numerous gaps in the literature on CM and high intensity exercise performance. However, there are a potentially limitless number of future studies, such as studies regarding dosage, timing, exercise type, and exercise duration. Two recent studies, one by (Perez-Guisado & Jakeman, 2010)and the other by (Glenn et al., 2017), both examined CM supplementation and resistance training performance. Future studies examining the effects of CM supplementation can build off these studies.

The first study, (Perez-Guisado & Jakeman, 2010), studied the response to an acute dose of 8g of CM on barbell bench press performance compared to a placebo. The study was a randomized, double-blind, crossover design with 41 male participants who all trained with weights for over three hours each week. Each subject had three sessions, the first session was to familiarize them with the study's process and complete a one-repetition maximum lift on the bench press. The second and third were identical test sessions. For the second and third sessions, every participant received a randomly assigned drink. The CM drink and placebo were both 200ml in volume and were formulated to have similar taste, smell, consistency, and appearance. One hour after ingestion the subjects performed a total of eight sets of maximum repetitions with 80% of their previously established one repetition maximum. The results revealed a significant increase in number of repetitions on six of the 8 recorded sets from the placebo trial and the CM trial: Set 3 (placebo 7.44 ± 1.58, CIT 8.22 ± 1.56), Set 4 (placebo 6.00 ± 1.61, CIT 7.05 ± 1.73), Set 1' (placebo 9.24 ± 2.08, CIT 10.32 ± 1.75), Set 2' (placebo 6.90 ± 1.95, CIT 8.37 ± 1.76), Set 3' (placebo 5.12 ± 1.78, CIT 6.88 ± 1.71), and Set 4' (3.59 ±

1.41, CIT 5.49 ± 1.53) data expressed as mean ± standard error. This study had the largest number of participants out of all the CM and high intensity exercise studies. It also monitored muscle soreness and found significant decreases in soreness in the CM trial. This is a factor that was not examined in the following study.

The other study, (Glenn et al., 2017), investigated the effects of the same 8g dose of CM on weightlifting performance but used female lifters rather than male. (Glenn et al., 2017) only had 15 participants compared to the 41 used by (Perez-Guisado & Jakeman, 2010), and did not examine muscle soreness as did the 2010 study. However, the protocols between the studies were generally similar. Glenn et al. (2017) had the participants report to the facility the first time to become familiar with the study design and to find a one-repetition maximum in both the bench press and leg press. On the testing days, subjects randomly received either the CM or placebo drink one hour before testing. Then the subjects completed six sets of maximum repetitions with 80% of their maximum in the bench press and the leg press. During this time the subjects were monitored for heart rates and their rating of perceived exertion. This study also showed significant increases in total repetitions in both upper body (CIT 34.1 ± 5.7, placebo 32.9 ±6.0) and lower body movements (CIT 66.7 ± 30.5, placebo 55.13 ± 20.64), along with significantly lower ratings of perceived exertion during the bench press for CIT compared to placebo (CIT 7.9 ± 0.3, placebo 8.6 ± 0.2). There was no change in heart rate between the groups (Glenn et al., 2017).

Glenn has also studied CM supplementation in older adults. In 2016 he published a study, *Acute Citrulline-Malate Supplementation Improves Maximal Strength and*

Anaerobic Power in Female, Masters Athletes Tennis Players, Glenn, Gray, Jensen, Stone, and Vincenzo (2016). Seventeen women with an average age of 51 years participated in the study. All participants were considered low risk by the American College of Sports Medicine, played tennis at least two times a week, competed in events, and had not ingested any CM or other vasodilator supplements in the past year. The subjects received doses of 8g of CM along with 12g dextrose or 12g of dextrose for the placebo, one hour before testing. During the test, subjects were required to perform grip strength tests, vertical jumps, and a standard 30 second Wingate test against 7.5% of their bodyweight. Minimal time was given between each of the test exercises. To assess isometric grip strength, participants used their dominant hand to squeeze a dynamometer for 5 seconds, three times, with one minute between attempts. The vertical jumps were performed with one minute of rest between attempts until the subject had two consecutive failed attempts. The subjects then moved onto the Wingate test where they were given 5 minutes to warm up before the test commenced.

Analysis of the data revealed that ingestion of CM significantly improved maximal ($p = 0.042$) and average ($p = 0.045$) grip strength compared to the placebo. However, no differences were seen comparing the vertical power data gathered during the vertical jump tests. Using the Wingate test data, anaerobic capacity, relative peak power, explosive power and sustained power were all calculated. While no differences were seen in anaerobic capacity and sustained power, there were significantly higher peak and explosive power values when the subjects ingested CM over the placebo ($p <$

.001 for both variables). Leading the researchers to conclude that ingestion of CM might improve performance.

In 2016, (Cunniffe et al.) tested CM supplementation using cycling performance as the metric. A key question this study addressed was CM effects on acid base balance during high intensity exercise. The acid base balance was measured by analyzing pH, lactate, bicarbonate, and base-excess. Ten well trained males consumed 12g of CM in beverage form or a placebo 1 hour before completing two separate cycling trials. Each trial consisted of 10, 15 second maximal sprints with 30 seconds recovery intervals between. Following the interval sprints the subjects rested for 5 minutes and then completed a time to exhaustion ride at 100% of their power output. Blood analysis revealed that the 12g dose of CM caused an increase in CIT plasma concentrations (343±41 μm) at peak compared to (39±12μm) for initial values. Unlike other CIT and CM studies this group did not monitor plasma arginine concentrations or NO markers. The analysis of markers that effect acid base balance revealed no difference between the CM and placebo conditions. The time to exhaustion test data showed no difference between CM and placebo with only 50% of the subjects increasing their time to exhaustion on the CM condition. These results show that 12g of CM was not an effective supplement for increasing high intensity exercise performance in well trained males.

Electromyography

The proposed ability of CIT to alter blood flow patterns during exercise could reduce or delay the onset of fatigue in exercising muscle. Fatigue is defined here as the relative decline in force over the course of the activity (Enoka et al., 1988).

Electromyography (EMG) is an analytical tool commonly used in kinesiology and physiology research. Surface electromyography (sEMG) measures the electrical activity in superficial skeletal muscles. Skeletal muscles are excited by action potentials that are propagated along alpha motor neurons. The electrodes used in sEMG detect these electrical currents and transmit the data into waves that can be analyzed to determine the qualities of the muscle.

Type II muscle fibers have a characteristic high frequency wave while Type I fibers have low frequency EMG waves. Since Type II fibers are more susceptible to fatigue, a frequency shift in EMG waves from high to low can be an indication of the onset of fatigue (Komi & Tesch, 1979). Further analysis of the EMG wave signal can provide insight into the recruitment of motor units during exercise. Increased motor unit activation could indicate fatigue because the central nervous system will not activate additional motor units to perform a task until the original motor unit(s) can no longer produce enough force to perform the activity.

Conclusion

Supplementation with CIT has gained popularity amongst athletes in recent years. It has also been the subject of a growing number of research studies. The current state of the discussion on CIT supplementation's effectiveness remains unclear. This is largely due to the varying methodologies used while studying CIT. With such large discrepancies in the subjects being used, the tasks being performed, and the doses given, any conclusions need to be made with caution. Future work in this area could benefit from the use of EMG. EMG can offer new insights into the state of the muscle during

exercise. Particularly, valuable information on the muscle recruitment patterns and the muscle fibers being used during exercise could be obtained. Thus, furthering the discussion on CIT supplementation as an ergogenic aid.

CHAPTER THREE

THE EFFECTS OF L-CITRULLINE SUPPLEMENTATION ON PHYSICAL PERFORMANCE

Contribution of Author and Co-Authors

Manuscript in Chapter 3**.**

Author: Peter Lawrence Stordahl

Contributions: Primary investigator and developer of study methodology, managed data collection, processed and analyzed data, developed conclusions, and authored manuscript.
Co-Authors: John G. Seifert

Contributions: Dr. Seifert assisted with the development of study methodology, helped process and analyze data, reviewed manuscript, and aided in the refinement of conclusions.
Co-Authors: Mary Miles

Contributions: Dr. Miles reviewed the study design and manuscript and recommended changes.
Co-Authors: Dawn Tarabochia

Contributions: Dr.Tarabochia reviewed the study design and manuscript and recommended changes.

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Peter L. Stordahl, John G. Seifert, Mary Miles, Dawn Tarabochia

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ABSTRACT

INTRODUCTION. Recent studies have investigated l-citrulline (CIT) as a possible ergogenic aid. A small number of studies have explored the performance question, with varying methodologies and results. The purpose of this study was to examine CIT influence on cycling time to exhaustion, cardiovascular function, and muscle activity. METHODS. Thirteen healthy subjects volunteered for this study. The first visit was to obtain a maximal power output, where 80% and 50% of maximal power were calculated for the following two visits. Subjects were then randomly assigned into either treatment or placebo groups for the second visit and the opposite treatment for the third visit. Subjects were instructed to drink their treatment 1 hour prior to coming into the laboratory. The treatment drink contained 10g of CIT while the placebo (PBO) was formulated to look and taste like the CIT drink. The second and third visits to the laboratory consisted of EMG from the rectus femoris, vastus medialis, and medial gastrocnemius of the right leg, along with HR monitor and BP. Subjects completed a 40-minute interval ride, consisting of 8 5-minute intervals of 3 minutes at 50% maximal power and 2-minutes at 80% maximal power. After the interval ride, subjects received a second dose of either treatment, 5g of CIT or PBO and were allowed 1-hour rest before the ramped time to exhaustion (TTE) test. RESULTS. There was no significant difference in TTE by treatment (CIT, 20.79 ± 4.48 and PBO, 20.86 ± 3.99). There was no significant main effect of treatment on percent of maximum heart rate ($p = 0.084$), mean arterial pressure ($p = 0.714$), or muscle activity of the rectus femoris ($p = 0.300$), vastus medialis ($p = 0.641$), or medial gastrocnemius ($p = 0.133$) during the TTE test. CONCLUSIONS. There were no differences in cycling TTE between treatments. Further research should investigate the metabolism of CIT under different physiological conditions.

Introduction

L-citrulline (CIT), $C_6H_{13}N_3O_3$, is a non-essential amino acid that has been gaining popularity in the supplement industry and physiology research community. Researchers have hypothesized that one benefit of CIT ingestion is its role as a precursor to the amino acid, L-arginine (ARG) (P. T. Cutrufello et al., 2015; Hickner et al., 2006; McKinley-Barnard et al., 2015; Takashi et al., 2016; Tarazona-Díaz et al., 2013) . Arginine plays a role in numerous metabolic pathways throughout the body. The key pathway of interest for this study is ARG conversion into nitric oxide (NO) via nitric oxide synthase (NOS) isoenzymes (Wu & Morris, 1998). Nitric oxide is a brief, yet potent vasodilator, that can alter blood flow patterns throughout the body.

Increased blood flow can improve performance in physical activities because the active muscle tissue will have a greater delivery of key nutrients, such as glucose and oxygen. The increase in blood perfusion throughout the muscle could also boost the removal of metabolic waste from the tissue. The combination of these two mechanisms could lead to an overall increase in performance by raising the tissues ability to recover (Bescos, Sureda, Tur, & Pons, 2012). However, there appears to be a trend in the research investigating NO supplementation which shows that NO products are only effective in untrained individuals (Bescos et al., 2012). For this reason, the following study excluded trained cyclists from the subject pool.

One consequence of CIT's proposed ability to increase blood flow could be a reduction of muscular fatigue and improved athletic performance. Muscular fatigue in this study is defined as a relative decrease in force production over the course of the test

(Enoka et al., 1988). Surface electromyography (EMG) will be implemented in the study to monitor muscle activity. The EMG data will provide leading insights CIT supplementation's effects on active muscle tissue.

Supplementation with CIT has gained popularity in recent years amongst athletes and fitness enthusiasts. However, the current literature does not show consistent positive results in CIT supplementation studies. These discrepancies could partially be attributed to the wide range of methodologies being implemented in the studies and inconsistent subject selections. To our knowledge, no CIT study has investigated CIT's effect on muscle activity. Therefore, the purpose of this study is to examine the effects of CIT supplementation on cycling time to exhaustion, cardiovascular function, rating of perceived exertion, and muscle activity.

Methodology

Informed Consent Approval of the study was obtained from the University Institutional Review Board (IRB) before the experiment commenced. Subjects were provided with an Informed Consent document prior to participation. The Informed Consent explained the risks associated with the study and it was made known to all subjects that they could withdraw from the experiment at any time. Written informed consent was obtained by all subjects.

Subjects Thirteen healthy subjects ages 19-41 years, who were non-competitive athletes, were recruited from the Rocky Mountain Region to participate in the study (Table 1). Subjects completed the National Academy of Sports Medicine's Physical

Activity Readiness Questionnaire (PAR-Q). Subjects who reported as smokers or had certain diseases (i.e. diabetes) were excluded in the study, as these conditions affect nitric oxide production (Glenn et al., 2017). All female subjects completed their testing outside of the follicular phase to control for hormonal influences (Glenn et al., 2017).. The variations in cardiovascular function throughout the menstrual cycle have not been extensively researched (Moran, Leathard, & Coley, 2000). However, one important hormone that needs consideration in this study is estrogen. Estrogen has been shown to increase NO production (Chambliss & Shaul, 2002). Consequently, all female participants were tested during the follicular phase of the menstrual cycle when estrogen levels were lower compared to ovulatory or luteal phases.

Table 1. Subject Demographics (mean ±SD).

Sex	Number	Age (Years)	Height (Meters)	Weight (Kilograms)	Maximal Power (Watts)
Female	2	24.5 ± 6.6	1.65 ± 0.1	62.3 ± 10.9	130 ± 11.3
Male	11	26.6 ± 7.8	1.79 ± 0.1	84.4 ± 1.6	192.7 ± 27.7

Citrulline and Placebo Treatments The CIT used in this study was purchased from NutraBio Labs Inc. (Middlesex, NJ, USA). Ten grams of unflavored CIT was weighed on (name of scale) and added to 250 milliliters of water along with 0.5 grams of zero calorie Kool-Aide and mixed until the solution was uniform. To formulate the placebo, 0.75

grams of zero calorie Kool-Aide was added to 250 milliliters of water and mixed so the appearance and taste matched the treatment condition.

Design The study was a randomized double-blind placebo crossover design.

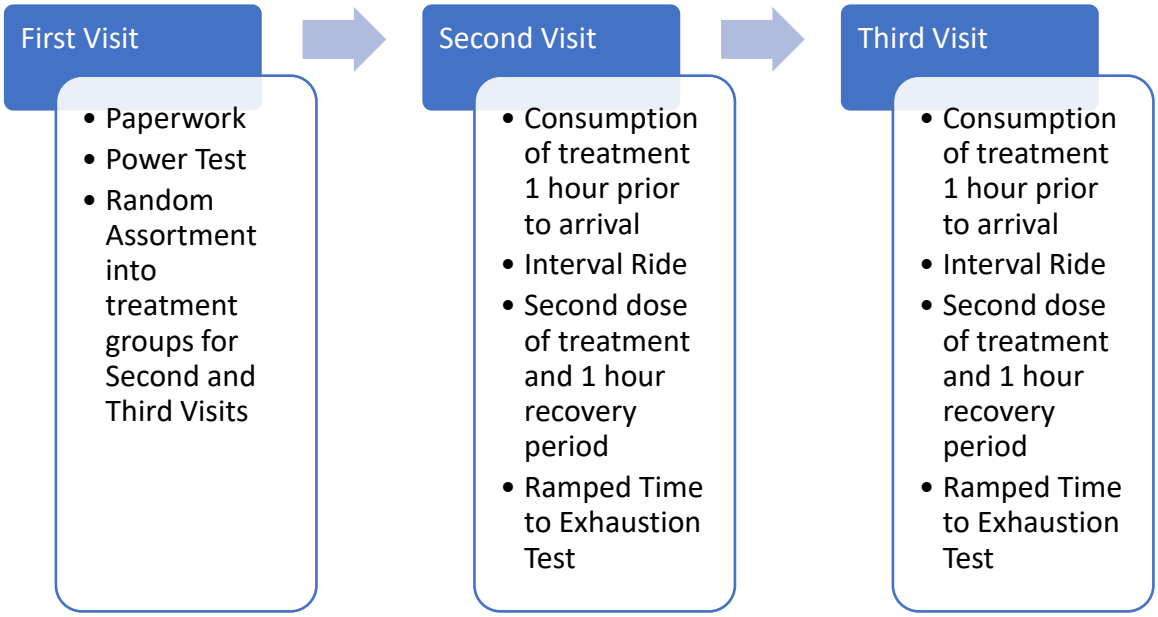
Procedures Each subject reported to the laboratory three times. Each visit was separated by at least one week to allow for a washout period as well as time for the subjects to recover. Subjects reported to the laboratory at the same time of day for all three visits to control for diurnal rhythms.

First Visit The first visit was for the subjects to receive an overview of the protocol, fill out required paperwork, and to perform a power test to determine maximal power. The ramped power test consisted of 5-minute stages, with the resistance increasing by 0.5kg every stage. Subjects continued to ride until they could no longer maintain their selected cadence. The last completed stage was used to determine maximal power.

All rides were performed on a Monark 828E Ergometer (Vansbro, Sweden), with the bike and seat height recorded to ensure consistency between rides. Subjects were then fitted with a Monark Heart Rate Monitor (Vansbro, Sweden) and familiarized with the Borg Scale (1-10) of exertion. The revolutions per minute (RPM), heart rate (HR), rating of perceived exertion (RPE), and blood pressure (BP) were collected at the end of each stage. After completion of the power test, the subjects' maximal power, 50% and 80%maximal power were calculated for use during the interval rides. Lastly, the subjects

were randomly sorted into the supplement or placebo group for their second rides (see Figure 1).

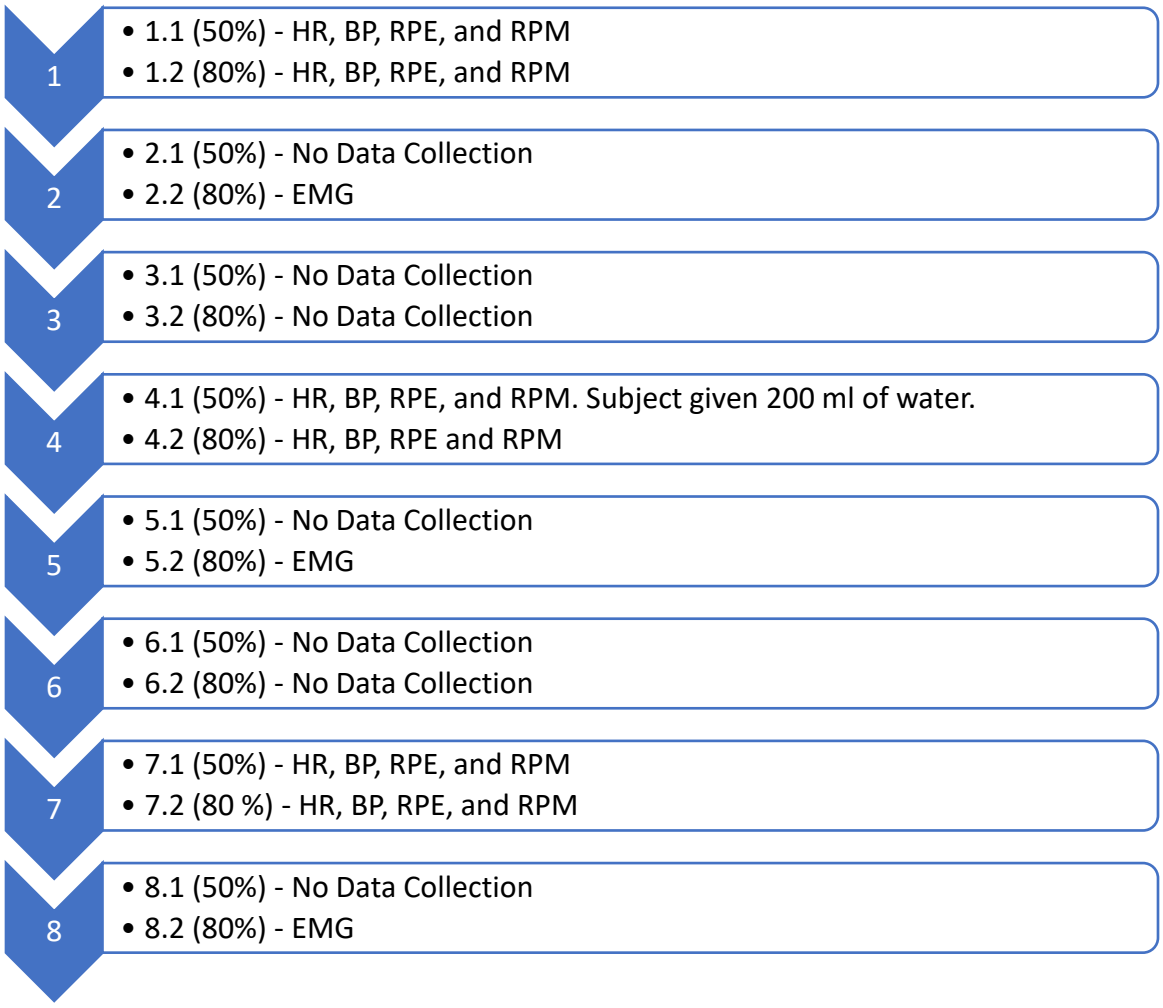
Figure 1. Study Design Overview.



Second and Third Visits Subjects were asked to consume a premade drink containing their first dose (10g) of CIT and second dose (5g) of CIT or a placebo drink that had been flavored to match the CIT solution, one hour before reporting to the laboratory for their ride. Dosage was determined from the literature (Moinard et al., 2008). Subjects were instructed to only drink the first dose (500 ml of solution) before arrival and bring the remaining second dose (250 ml of solution) to consumer later. Upon arrival, subjects provided a urine sample to be tested for specific gravity to verify adequate hydration using an ATAGO Manual Master Refractometer (Japan). The subjects' urine had to be < 1.020 in order to ensure euhydration status (Paul T.

Cutrufello, Dixon, & Zavorsky, 2016). If subjects did not pass the initial hydration test, they were given water until the hydration level was acceptable. Three surface electromyography (EMG) sensors were then fixed to: *rectus femoris* (RF), *vastus medialis* (VM), and *medial head of gastrocnemius* (MG) on the subject's right leg. Each subject was also fitted with a Monark Heart Rate Monitor. Subjects rode for a 5-minute warm up at 0.5kg resistance. After the warm-up subjects began the first of eight 5-minute intervals. Subjects rode at 50% of their maximal power output for the first three minutes of the interval followed by two minutes at 80% (see Figure 2). Electromyography data was collected for 15 seconds during the final 30 seconds of the 1st, 4th, and 8th interval. Heart rate, RPE, BP, and RPM were recorded at the end of the 3-minute stages and 2-minute stages of intervals, 2, 5, and 7. Subjects received 200 mL of water after completion of the 4th interval. After completion of the intervals, subjects had a 5-minute cool down riding at a selected cadence against 0.5 kg of resistance. Immediately upon completion of the cool down subjects received their second dose (5g) of either the treatment or placebo and were given a one-hour recovery period. During the recovery period, subjects received another 200 mL of water.

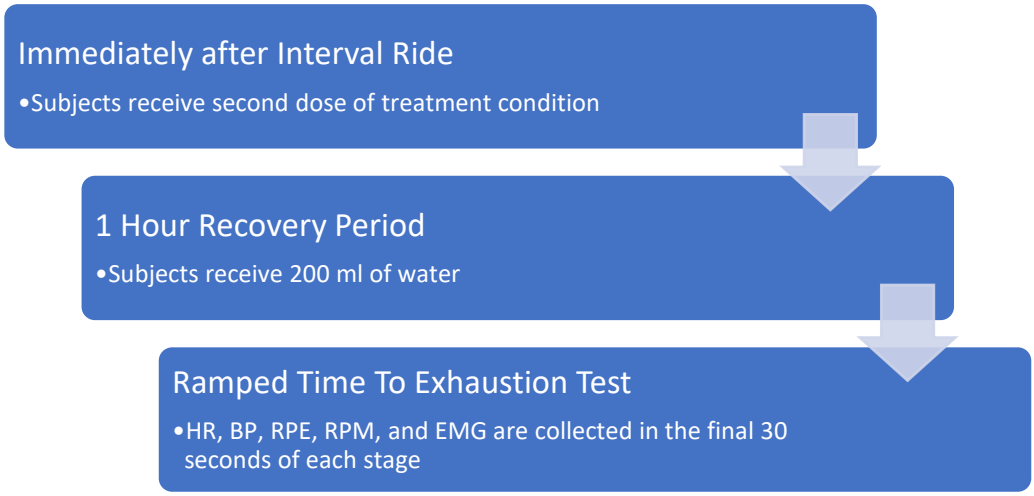
Figure 2. Study Design. Interval Ride. Shown are the phases of each interval during the interval ride along with the data collected at each phase and when water was given.



Following the one-hour recovery period, subjects performed a ramped time to exhaustion test (see Figure 3). Each stage of this test lasted five minutes. In the initial stage the resistance was set to 1kg and 0.5kg was added for each successive stage. Subjects cycled until they could no longer maintain 90% of their selected cadence for the ride. Electromyography data was collected for 15 seconds during the final 30 seconds of each stage. Heart rate, RPE, BP, and RPM were recorded at the end of each stage. After an approximately one-week washout period, subjects returned to the lab to repeat the

interval ride and ramped time to exhaustion test, this time using the opposite treatment (placebo or supplement) of the first visit. Standardized verbal encouragement was given to subjects during TTE test.

Figure 3. Study Design. Post Interval Ride and Ramped Time to Exhaustion Test.



Instrumentation and Measurements Heart rates were taken using Monark Heart Rate Monitors and BP was assessed using a Sprague Rapapport Stethoscope and an Aneroid Sphygmomanometer (Fullerton, CA, USA). Data was collected at the end of the 3-minute stages and 2-minute stages of intervals, 2, 5, and 7.

Surface electromyography electrodes, 4 -bar bipolar Ag/AgCl electrodes (5x1mm electrodes, 5mm interelectrode spacing, CMMR >80dB at 60Hz, 16 bit A/D conversion, Gain=300, 2 mΩ input impedance, Trigno IM, Delsys INC, Natick, MA, USA were placed over the belly of the RF, VM, and MG on the right leg of the subject. The RF was chosen due to its ability to create hip flexion as well as knee extension. The VM was chosen because of its ability to extend the knee and the MG was selected because of its capacity to create plantarflexion at the ankle. These actions are central to the task of

cycling. These muscle selections are also consistent with EMG cycling literature (Jorge & Hull, 1986; Raasch, Zajac, Ma, & Levine, 1997; Ryan & Gregor, 1992)

Before placement of the electrode took place, the subjects' skin was shaved and then cleaned with an alcohol wipe. These steps were taken to reduce the skin-electrode impedance and obtain a signal with less electrical interference (Hermens, Freriks, Disselhorst-Klug, & Rau, 2000). The subject was placed in a standing start position so anatomical landmarks could be palpated to help determine proper location for each sensor (Hermens et al., 2000). The sensors were placed halfway between the most distal motor endplate zone and distal tendon and in line with the pentation angle of the fibers, this was in accordance with SENIAM recommendations (Hermens et al., 2000). The electrodes were then fixed to the skin using athletic tape.

Food logs were distributed to all participants during their first visit to the Movement Science Laboratory (MSL) at Montana State University. Subjects were instructed on how to fill out the log for the 72 hours prior to their testing and were informed to not consume any watermelon due to its high concentration of citrulline. Food and beverages were broken down by content and number of servings, and each subject was instructed to ring in their food log before each subsequent ride to clarify any questions in the log. They were then given copies of the food log and asked to replicate the intake before the next trial.

Data Processing Relative peak heart rate (HR_{peak}) was expressed as a percent of maximum heart rate (HR_{max}=220-Age). Relative average heart rate (HR_{mean}) was measured across the intervals and stages during the Ramped Time to Exhaustion test as a

percent of maximum heart rate (HR_{max}=220-Age). Mean arterial pressure (MAP) was calculated using the following equation:

$$MAP = \frac{(2\ x\ diastolic\ blood\ pressure) + systolic\ blood\ pressure}{3}$$

MAP was used to determine the profusion pressure of the blood through the muscle. Rating of Perceived Exertion data was compared across intervals and for each stage of the Ramped Time to Exhaustion test to determine if any significant changes were found between treatments.

Electromyographic data was recorded in Delsys EMGWorks® Acquisition software and collected at 1926 Hz. Data was converted from digital to analog via a 16-bit A/D board (National Instruments USB-6225, Austin, Texas, USA). Electromyographic data was analyzed in MATLAB (Mathworks, Natick, MA, USA) and filtered using a zero-phase low pass 4^{th} order Butterworth filter with a low end cutoff of 30 Hz and a high end cutoff of 300 Hz, which was selected to be consistent with the literature (Ryan & Gregor, 1992). Filtered and cropped signals were normalized using a mean task method. The mean value for each signal is divided by the range for that signal creating a percent of muscle activation so comparisons between measurements and subjects could be made (Burden, 2010).

The food logs were visually inspected for gross differences in types and amounts of food consumed.

Statistical Analyses Data was analyzed with SPSS statistical software. The TTE test data was analyzed using a paired samples t-test. The HR, MAP, and EMG data were analyzed using a 2x5 ANOVA with repeated measures because there are two treatments and five time points where data was collected. A Kruskal Wallace non-parametric test was used to analyze RPE data. For the interval ride data, HR and MAP were analyzed using a 2x6 ANOVA with repeated measures because there are two treatments and six time points where data was collected. The EMG data was analyzed using a 2x3 ANOVA with repeated measures because there are two treatments and three time points where data was collected. Upon a significant interaction, an LSD Post Hoc test was used to differentiate means. Treatment (CIT and PBO) were the independent variables. Alpha level of significance was set at $p \leq 0.05$.

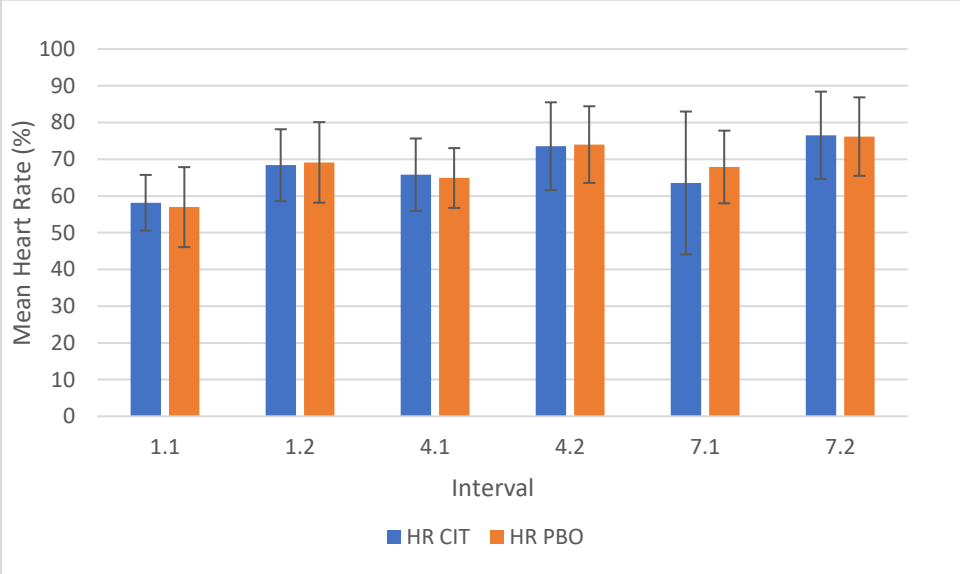
Results

Interval Ride-Heart Rate There was no significant interaction between treatment and time for percent of maximal heart rate during the 50% of maximal power intervals (*p = 0.304*) or 80% interval (*p = 0.716*). The HR data is shown below in Table 2 and Figure 4. There was no significant main effect of treatment for the heart rate throughout the interval ride at 50% of maximum power (*p = 0.668*) and 80% of maximum power (*p = 0.803*). There was a trivial main treatment effect size found (*d = 0.05*). There were significant main effects of time on percent of maximal heart rate over time for the 50% interval (*p = 0.001*) and 80% interval (*p = 0.000*).

Table 2. Percent of Maximum Heart Rate by Treatment During the Interval Ride (mean ±SD).

	Interval 1.1	Interval 1.2	Interval 4.1	Interval 4.2	Interval 7.1	Interval 7.2
CIT	58.14 ± 7.59	68.37 ± 9.78	65.78 ± 9.87	73.55 ± 11.95	63.53 ± 19.46	76.51 ± 11.90
PBO	56.96 ± 10.89	69.14 ± 10.97	64.89 ± 8.14	73.99 ± 10.44	67.89 ± 9.89	76.16 ± 10.68

Figure 4. Percent of Maximum Heart Rate by Treatment During the Interval Ride (mean ±SD).



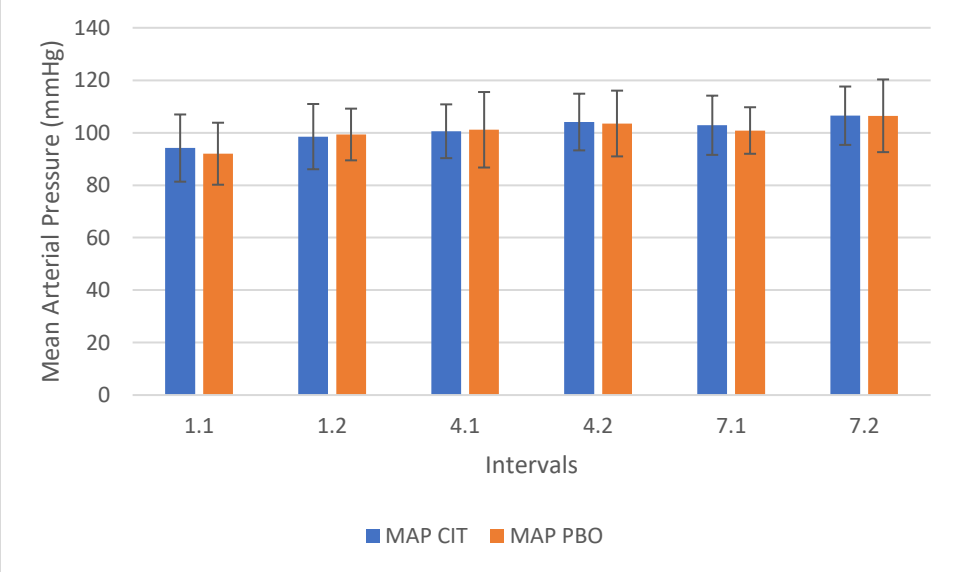
Interval Ride-Mean Arterial Pressure There was no significant interaction between treatment and time for MAP during the 50% interval ($p = 0.658$) or 80% interval ($p = 0.885$). The MAP data is shown below in Table 3 and Figure 5. There was no significant main effect of treatment for the MAP throughout the interval ride at 50% interval ($p = 0.677$) and 80% interval ($p = 0.978$). There was a trivial main treatment

effect size observed (*d = 0.05*). There was a significant main effect of time on MAP over time for the 50% interval (*p = 0.000*) and 80% interval (*p = 0.000*).

Table 3. Mean Arterial Pressure by Treatment During the Interval Ride (mean ±SD).

	Interval 1.1	Interval 1.2	Interval 4.1	Interval 4.2	Interval 7.1	Interval 7.2
CIT	94.15 ± 12.81	98.51 ± 12.44	100.56 ± 10.25	104.08 ± 10.80	102.85 ± 11.29	106.49 ± 11.12
PBO	92 ± 11.81	99.33 ± 9.85	101.13 ± 14.39	103.51 ± 12.54	100.85 ± 8.87	106.46 ± 13.86

Figure 5. Mean Arterial Blood Pressure by Treatment During the Interval Ride (mean ±SD).



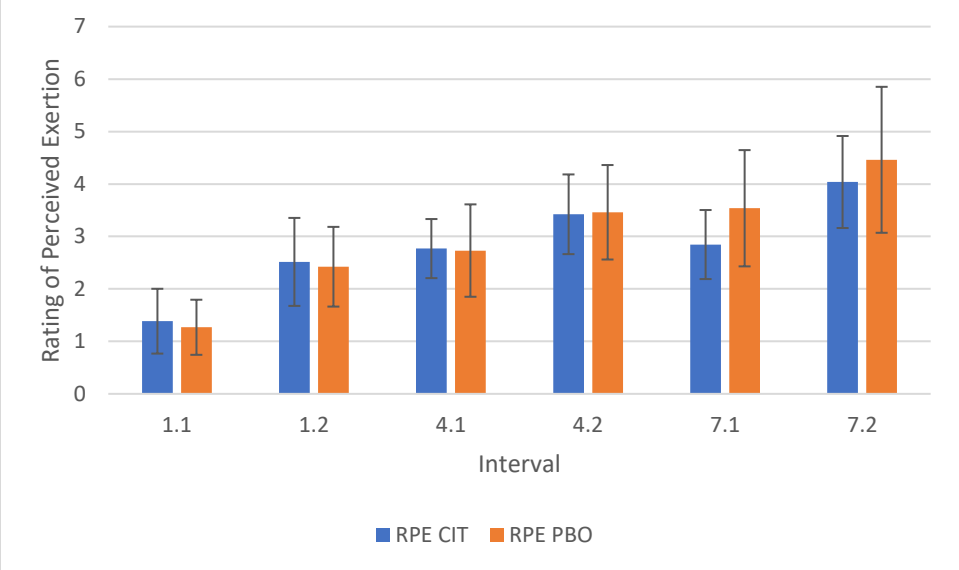
Interval Ride-Rating of Perceived Exertion There was no significant difference in the RPE for the 50% intervals (Interval 1.1, *p = 0.671*, Interval 4.1, *p = 0.956*, and Interval 7.1, *p = 0.090*) or the 80% intervals (Interval 1.2, *p = 0.627*, Interval 4.2, *p =*

0.058, and Interval 7.2, *p = 0.352*). There was a small main effect of treatment observed (*d = 0.18*). The RPE data during the interval ride is shown below in Table 4 and Figure 6.

Table 4. Rating of Perceived Exertion by Treatment During the Interval Ride (mean ±SD).

	Interval 1.1	Interval 1.2	Interval 4.1	Interval 4.2	Interval 7.1	Interval 7.2
CIT	1.38 ± 0.62	2.51 ± 0.84	2.77 ± 0.56	3.42 ± 0.76	2.85 ± 0.66	4.04 ± 0.88
PBO	1.27 ± 0.52	2.42 ± 0.76	2.73 ± 0.88	3.46 ± 0.90	3.54 ± 1.11	4.46 ± 1.39

Figure 6. Rating of Perceived Exertion by Treatment During the Interval Ride (mean ±SD).



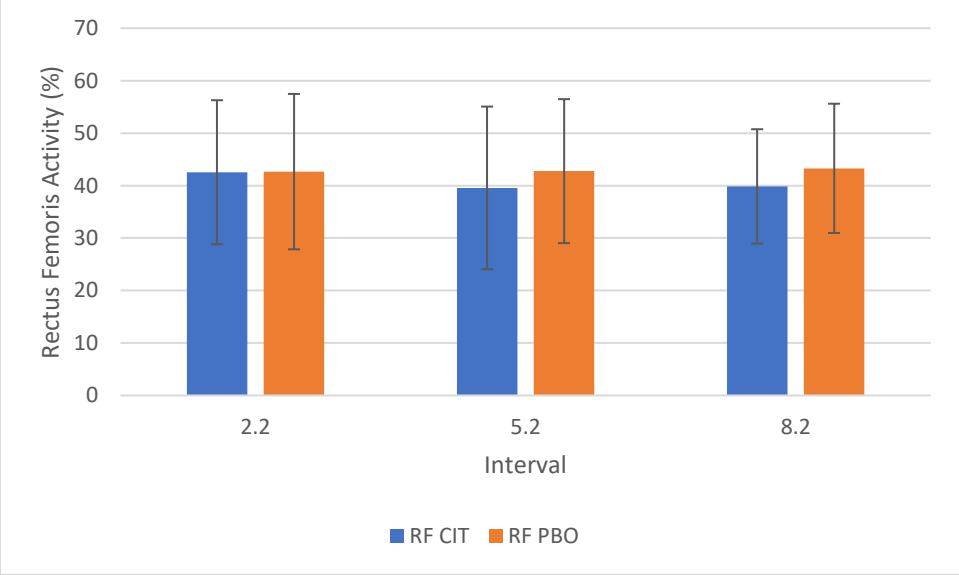
Interval Ride-Electromyography There was no significant interaction between treatment and time on percent of RF activity during the interval ride (*p = 0.565*). The RF muscle activity data is shown below in Table 5 and Figure 7. There was no significant main effect of treatment on RF activity (*p = 0.212*). There was a small main effect of

treatment found (*d = 0.171*). There was no significant main effect of time on RF activity (*p = 0.624*).

Table 5. Percent Rectus Femoris Activity by Treatment During the Interval Ride (mean ±SD).

	Interval 2.2	Interval 5.2	Interval 8.2
CIT	42.55 ± 13.72	39.56 ± 15.50	39.83 ± 10.91
PBO	42.66 ± 14.81	42.76 ± 13.72	43.29 ± 12.32

Figure 7. Percent Rectus Femoris Activity During the Interval Ride (mean ±SD).



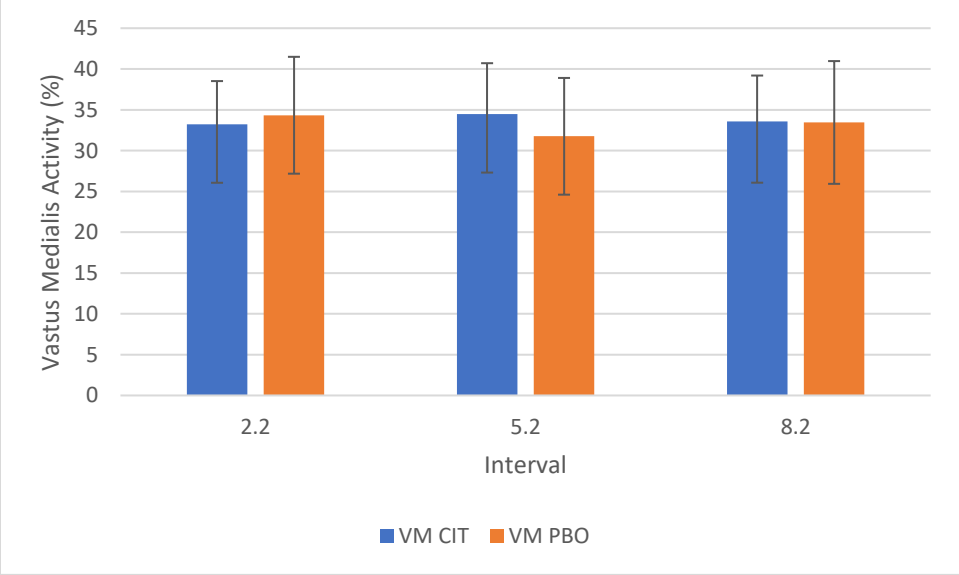
There was no significant interaction between treatment and time on percent of VM activity (*p = 0.221*). The VM muscle activity data is shown below in Table 6 and Figure 8. There was no significant main effect of treatment on VM activity during the interval ride (*p = 0.943*). There was a trivial main treatment effect found (*d = 0.023*).

There was no significant main effect of time on VM activity over time during the interval ride (*p = 0.879*).

Table 6. Percent Vastus Medialis Activity by Treatment During the Interval Ride (mean ±SD).

	Interval 2.2	Interval 5.2	Interval 8.2
CIT	33.22 ± 5.30	34.46 ± 6.25	33.59 ± 5.61
PBO	34.33 ± 7.16	31.75 ± 7.15	33.45 ± 7.52

Figure 8. Percent of Vastus Medialis Activity During the Interval Ride (mean ±SD).

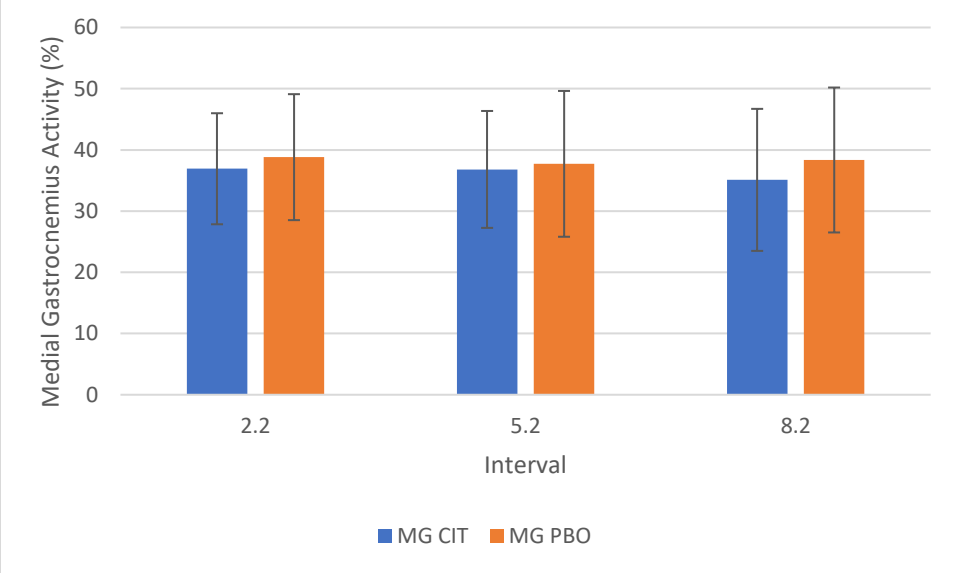


There was no significant interaction between treatment and time on percent MG activity (*p = 0.901*). The MG muscle activity data is shown below in Table 7 and Figure 9. There was no significant main effect of treatment on percent MG activity (*p = 0.218*). There was a small main treatment effect size found (*d = 0.181*). There was no significant main effect of time on percent MG activity (*p = 0.336*).

Table 7. Percent Medial Gastrocnemius Activity by Treatment During the Interval Ride (mean ±SD).

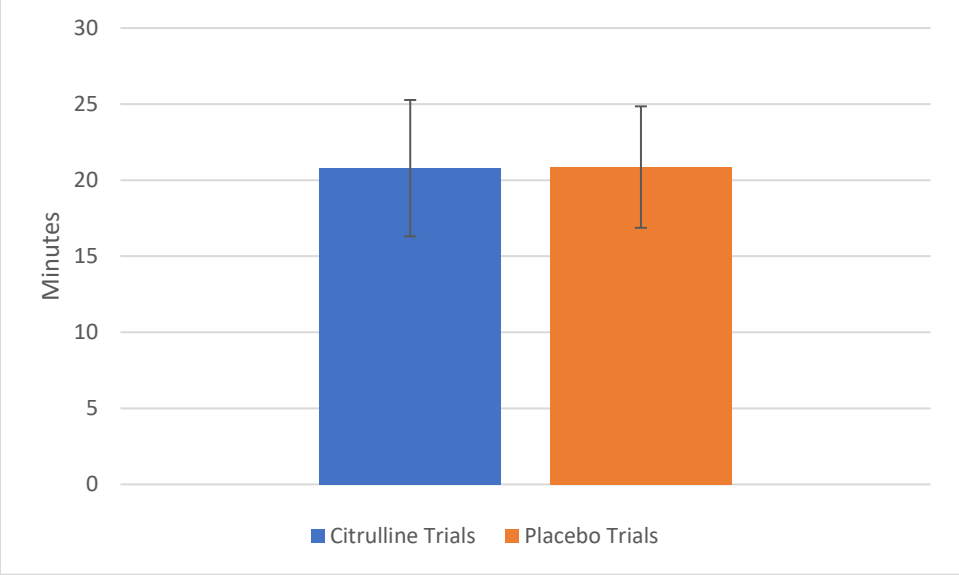
	Interval 2.2	Interval 5.2	Interval 8.2
CIT	36.92 ± 9.07	36.81 ± 9.55	35.10 ± 11.60
PBO	38.81 ± 10.29	37.72 ± 11.91	38.35 ± 11.84

Figure 9. Percent of Medial Gastrocnemius Activity During the Interval Ride (mean ±SD).



Ramped Time to Exhaustion Test-Time to Exhaustion There was no significant difference in the time to exhaustion test between the CIT and PBO groups ($p = 0.913$). The PBO TTE was (20.86 ± 3.99 min) and the CIT TTE was (20.79 ± 4.48 min). The mean time to exhaustion times by treatment group are shown in Figure 1.

Figure 10. Time to Exhaustion Times by Treatment (mean ±SD).

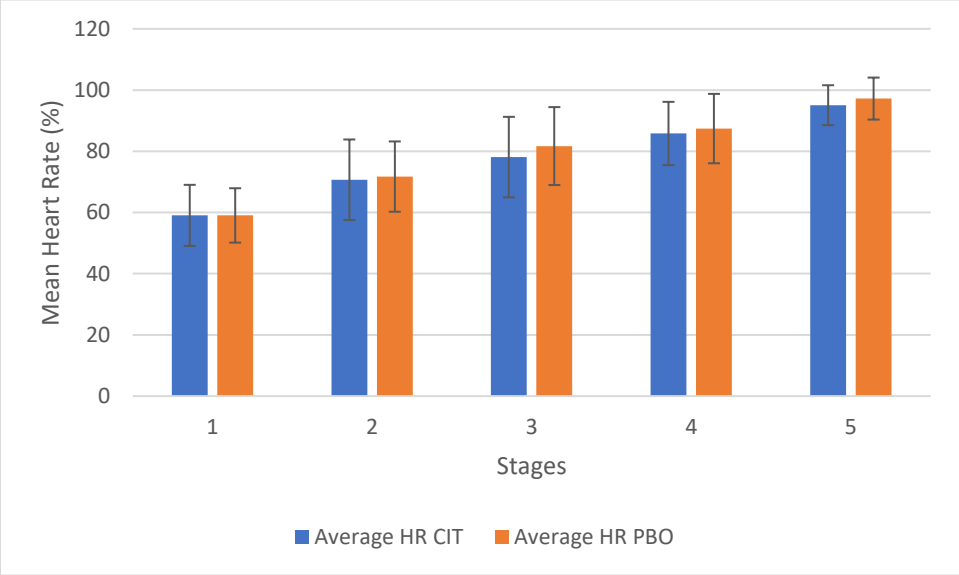


Ramped Time to Exhaustion Test-Heart Rate There was no significant interaction between treatment and time for percent of maximum heart rate during the TTE test (*p = 0.366*). The HR data is shown below in Table 8 and Figure 11. There was no significant main effect of treatment for the percent of maximum heart rate during the TTE test (*p = 0.084*). There was a small main treatment effect size (*d = .279*). The data is shown in Table 2. There was however a significant main effect of time for the percent of maximum heart rate during the TTE test (*p = 0.000*).

Table 8. Percent of Maximal Heart Rate by Treatment During Ramped Time to Exhaustion Test (mean ±SD).

	Stage 1	Stage 2	Stage 3	Stage 4	Stage 5
CIT	59.05 ± 9.98	70.69 ± 13.17	78.09 ± 13.15	85.81 ± 10.33	95.06 ± 6.52
PBO	59.03 ± 8.88	71.72 ± 11.49	81.70 ± 12.73	87.41 ± 11.33	97.22 ± 6.86

Figure 11. Percent of Maximum Heart Rate by Treatment During the Ramped Time to Exhaustion Test (mean ±SD).

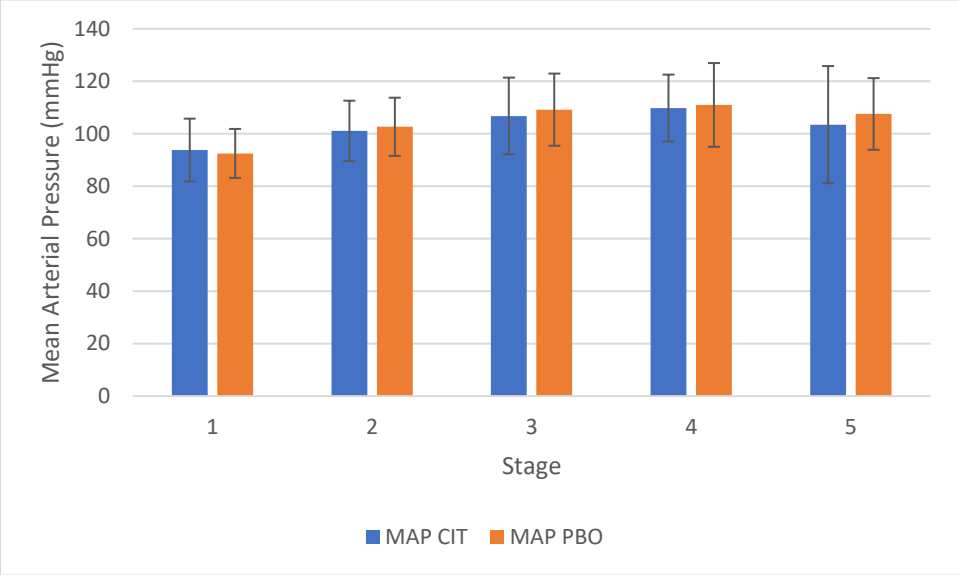


Ramped Time to Exhaustion Test-Mean Arterial Pressure There was no significant interaction between treatment and time for mean arterial pressure during the TTE test (*p = 0.824*). The MAP data is shown below in Table 9 and Figure 12. There was no significant main effect of treatment on the mean arterial pressures during the time to exhaustion test *(p = 0.714*) along with a small main effect size of treatment (*d = 0.107*). There was a significant main effect of time on mean arterial pressure *(p = 0.000*).

Table 9. Mean Arterial Pressure by Treatment During the Ramped Time to Exhaustion Test (mean ±SD).

	Stage 1	Stage 2	Stage 3	Stage 4	Stage 5
CIT	97.97 ± 11.98	101.08 ± 11.54	106.79 ± 14.62	109.8 ± 12.75	103.5 ± 22.31
PBO	92.51 ± 9.33	102.67 ± 11.09	109.19 ± 13.76	111 ± 16.97	107.55± 13.67

Figure 12. Mean Arterial Pressure by Treatment During the Ramped Time to Exhaustion Test (mean ±SD).

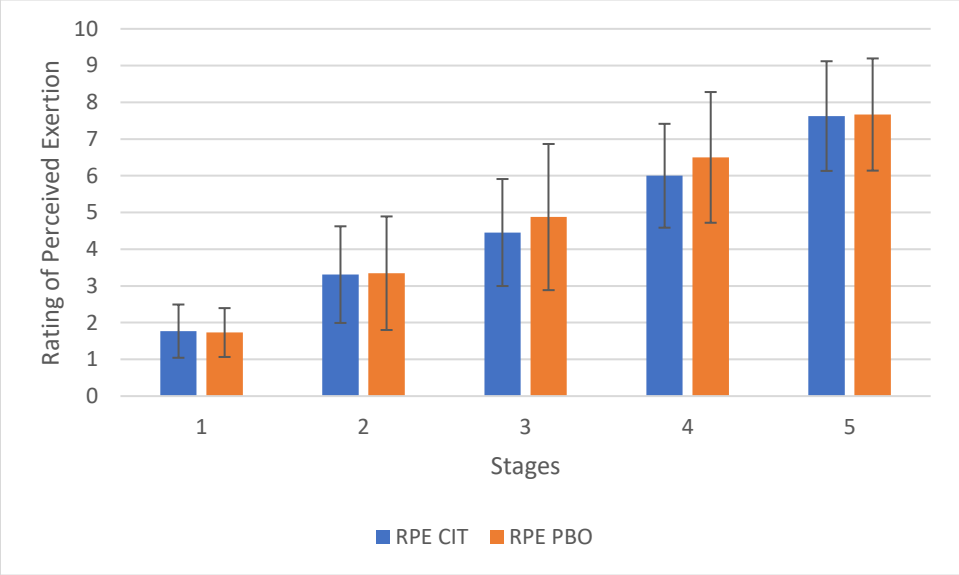


Ramped Time to Exhaustion Test-Rating of Perceived Exertion There was no significant difference in the ratings of perceived exertion for any of the stages of the time to exhaustion test (stage 1, $p = 0.955$, stage 2, $p = 0.979$, stage 3, $p = 0.755$, stage 4, $p = 0.522$, and stage 5, $p = 1.000$). The RPE data is shown below in Table 10 and Figure 13.

Table 10. Rating of Perceived Exertion by Treatment During the Ramped Time to Exhaustion Test (mean ±SD).

	Stage 1	Stage 2	Stage 3	Stage 4	Stage 5
CIT	1.77 ± 0.72	3.31 ± 1.31	4.45 ± 1.46	6 ± 1.41	7.62 ± 1.49
PBO	1.73 ± 0.66	3.35 ± 1.55	4.87 ± 1.99	6.5 ± 1.78	7.67 ± 1.53

Figure 13. Rating of Perceived Exertion by Treatment During the Ramped Time to Exhaustion Test (mean ±SD).

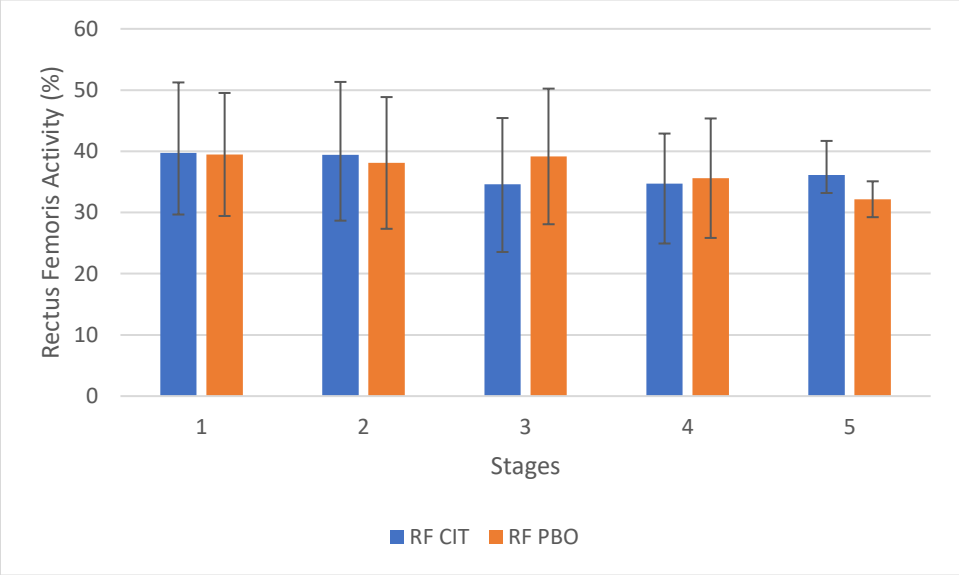


Ramped Time to Exhaustion Test-Electromyography There was no significant interaction between treatment and time for percent of activity for the RF during the TTE test (*p = 0.754*). The RF muscle activity data is shown below in Table 11 and Figure 14. There was no significant main effect of treatment on RF muscle activity throughout the TTE test *(p = 0.300*). A small main treatment effect size was found (*d = 0.185*). There was no significant main effect of time in percent of activity for the RF (*p = 0.701*).

Table 11. Percent Rectus Femoris Activity by Treatment During the Ramped Time to Exhaustion Test (mean ±SD).

	Stage 1	Stage 2	Stage 3	Stage 4	Stage 5
CIT	39.72 ± 11.53	39.43 ± 11.90	34.63 ± 10.80	34.69 ± 8.20	36.11 ± 5.58
PBO	39.48 ± 10.05	38.09 ± 10.76	39.15 ± 11.08	35.60 ± 9.76	32.16 ± 2.93

Figure 14. Percent Rectus Femoris Muscle Activity by Treatment During the Ramped Time to Exhaustion Test (mean ±SD).

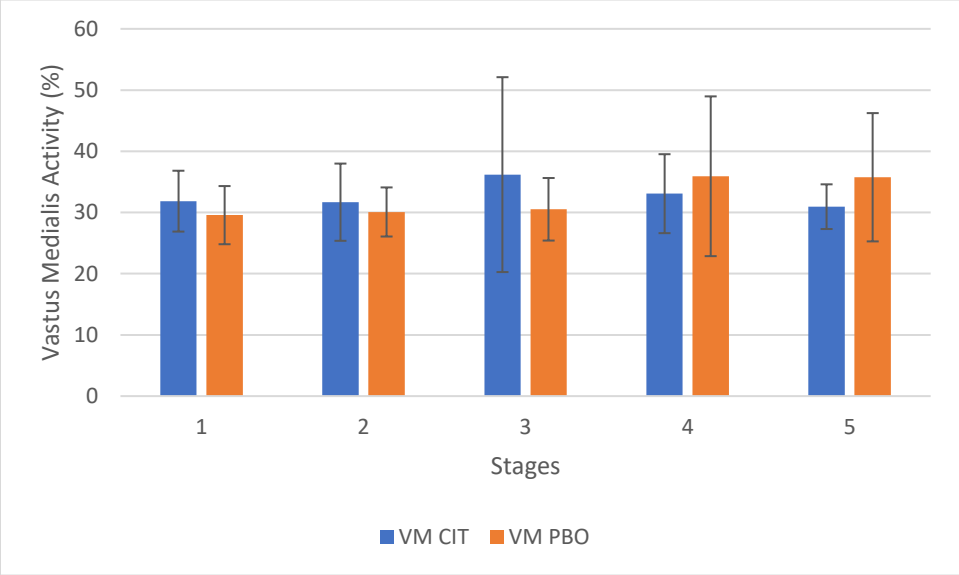


There was no significant interaction between treatment and time for the percent of activity of the VM *(p = 0.648*). The VM muscle activity data is shown below in Table 12 and Figure 15. There was no significant main effect of treatment on VM muscle activity throughout the TTE test (*p = 0.641*). A trivial main treatment effect size was found (*d = 0.098*). There was a significant main effect of time on percent of activity of the VM (*p = 0.616*).

Table 12. Percent Vastus Medialis Activity by Treatment During the Ramped Time to Exhaustion Test (mean ±SD).

	Stage 1	Stage 2	Stage 3	Stage 4	Stage 5
CIT	31.85 ± 4.97	31.68 ± 6.31	36.18 ± 15.92	33.07 ± 6.45	30.95 ± 3.65
PBO	29.56 ± 4.75	30.08 ± 4.01	30.52 ± 5.11	35.92 ± 13.05	35.75 ± 10.48

Figure 15. Percent Vastus Medialis Muscle Activity by Treatment During the Ramped Time to Exhaustion Test (mean ±SD).

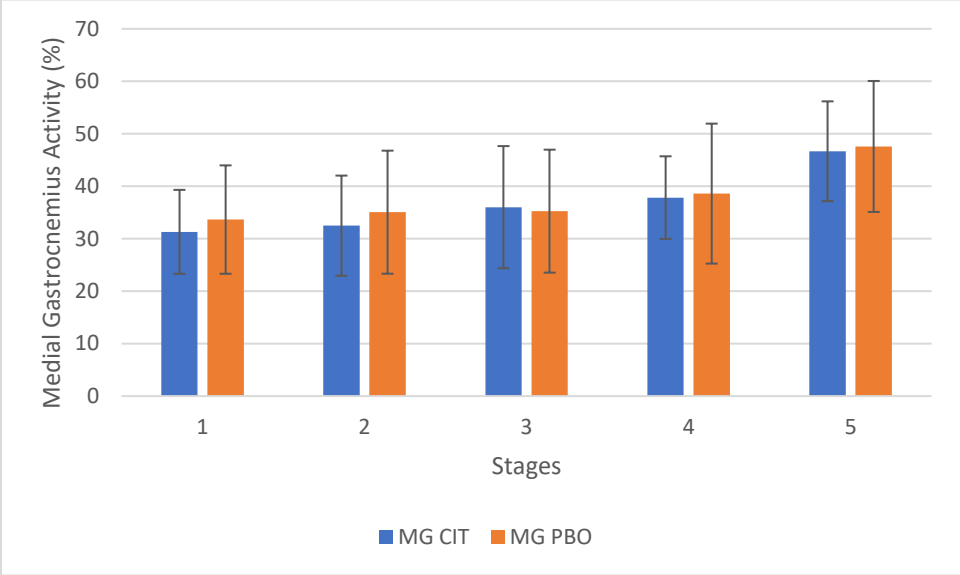


There was no significant interaction between treatment and time on percent activity of the MG during the TTE test (*p = 0.309*). The MG muscle activity data is shown below in Table 13 and Figure 16. There was no significant main effect of treatment on MG muscle activity between the two treatments throughout the TTE test (*p = 0.133*). There was a small main treatment effect found (*d = 0.182*). There was a significant main effect of time on percent of muscle activity of the MG (*p = 0.007*).

Table 13. Percent Medial Gastrocnemius Activity by Treatment During the Ramped Time to Exhaustion Test (mean ±SD).

	Stage 1	Stage 2	Stage 3	Stage 4	Stage 5
CIT	31.31 ± 8.00	32.48 ± 9.55	36.01 ± 11.64	37.84 ± 7.87	46.67 ± 9.51
PBO	33.65 ±10.33	35.06 ± 11.73	35.25 ± 11.72	38.60 ± 13.34	47.58 ± 12.48

Figure 16. Percent Medial Gastrocnemius Muscle Activity by Treatment During the Ramped Time to Exhaustion Test (mean ±SD).



Food Logs. All subjects were consistent with their dietary intake. There were no apparent differences in the types or amounts of food consumed.

Discussion

The purpose of this study was to examine the effects of CIT supplementation on cycling time to exhaustion, cardiovascular function and muscle activity. One of the strongest theories of CIT mechanism of advancing physical performance is in CIT role as a precursor to ARG which can be transformed into NO. We hypothesized that increasing the amount of NO would lead to increased vasodilation would allow for greater nutrient delivery and waste removal from the active muscle tissue. Thereby, leading to increased cycling performance by improving recovery. This study tested this hypothesis under relative and absolute working conditions.

Interval Ride This study employed the use of an interval ride before the main TTE test. The interval work allowed us to investigate CIT supplementation on an exercise protocol that was the same relative workload for all subjects. There were no significant interactions for treatment and time on any of the measures along with no main effect of treatment on any of the measures. However, there were significant main effects of time for HR and MAP. Both HR and MAP values increased over the course of the interval ride. The rise in HR under consistent intensities could be explained by cardiac drift due to hyperthermia or a decrease in blood plasma volume. This increase in HR could have contributed to the rise in MAP throughout the interval ride because HR is a component of the formula for calculating BP.

The subjects' RPE showed no significant difference between treatments for any interval ride. Also, there were no observable differences in muscle activity between the treatment groups or over time. Although these findings do not speak to any improvement in cycling performance, they do show a high level of reliability for the interval ride protocol. This was critical to our study's design and effort to normalize the subjects' physical condition before testing them in the ramped TTE test.

Ramped Time to Exhaustion Test The use of the ramped TTE test tested CIT supplementation under absolute workload conditions. There was no significant difference in TTE between CIT and PBO conditions. The only statistically significant results were the main effect of time on HR, MAP, and muscle activity of MG. There is a connection to between heart rate and blood pressure as blood pressure is determined by the following formula:

$$Blood\ pressure = Heart\ Rate \times Stroke\ Volume\ \times Systemic\ Vascular\ Resistance$$

Therefore, the change in percent of maximal heart rate could have contributed to the change in MAP. Although statistically significant values were found, they did not result in an increase in TTE performance. The trend of increasing MG activity correlates to the increase in intensity of the ramped TTE test.

Literature Comparisons This study was unique in its dosing and timing protocol compared to previous CIT studies. The 10g of CIT given one hour before the interval ride began. To our knowledge this is the largest dose of CIT given before an exercise test. It was chosen due to findings from a dosing study done by Moinard et al. (2008). This study found that 10g of CIT caused the largest increase in ARG plasma levels. Moinard et al. (2008), concluded that saturation of CIT to ARG conversion in the kidney occurs around this 10g dose and that 10g of CIT is the most appropriate dose for clinical practice. Lastly, they found that CIT levels peak around 45 minutes to an hour post ingestion and taper off for the next 3 to 5 hours. It is this tapering of plasma amino acid levels that led us to give a second dose of CIT, immediately after the interval ride concluded and one hour before the TTE test began, in an effort to keep plasma CIT and ARG elevated. The other CIT studies by (P. T. Cutrufello et al., 2015; Hickner et al., 2006; Takashi et al., 2016; Tarazona-Díaz et al., 2013) all picked different dosing and timing protocols for their studies. Due to the limited number of CIT studies and the inconsistency of critical methodology, dosing and timing, it is difficult for researchers to answer these foundational questions surrounding CIT supplementation.

Another important methodological difference in this study from the studies before is the use of a preliminary exercise bout before the main exercise test started. The interval ride allowed us to see how CIT supplementation effected subjects under relative conditions, but it also acted to normalize the subjects' physical state prior to the ramped TTE test. This study implemented a 40-minute interval ride and a 1-hour rest period before the TTE test to normalize the physical readiness of the subjects prior to the TTE test. To our knowledge, no other CIT used this protocol. Some similarities do exist between the current CIT studies. P. T. Cutrufello et al. (2015) used a treadmill graded TTE test with their subjects and found no significant change in TTE. This finding is consistent with the current study's results. Hickner et al. (2006) also used a treadmill graded TTE test with their subjects and interestingly found that CIT supplementation decreased the TTE compared to the placebo group. To our knowledge the only positive result for performance from CIT supplementation was found by Takashi et al. (2016). Their study found that CIT supplementation reduced the time to complete a 4km cycling time trial. Their study utilized a weeklong CIT dosing protocol. Subjects received 2.4g/day for 7 days and one final dose of 2.4g one hour prior to their time trial on day 8. The reduction in time trial performance was 1.5% (Placebo: 578 ± 15 s, L-citrulline: 569 ± 14 s). The authors postulated that the mechanism of the improved performance on CIT was due to a significantly larger power output at a given oxygen consumption (VO_2). With such a limited amount of literature any conclusions on CIT supplementations effectiveness need to be made with caution.

This study did not test the hypothesized mechanisms of action for CIT. As previously stated, the theoretical mechanisms for CIT's ergogenic capacity were via the conversion of CIT to ARG at the kidney, followed by the uptake of ARG by endothelial cells and conversion of ARG to NO. This increase in NO production was thought to possibly benefit muscle activity by increasing the delivery of nutrients and oxygen to the tissue and more importantly, removal of metabolic waste products. Since metabolites of NO, blood flow changes, and plasma levels of CIT and ARG were not assessed, surface electromyography was employed to monitor muscle activity. It was hypothesized that if CIT supplementation led to the aforementioned physiological changes, a change in muscle activity could be seen when comparing CIT and placebo trials. This study found no significant difference in percent of muscle activity for the RF, VM, and MG for any stage of the TTE test or any interval during the interval ride when comparing the CIT and placebo groups. To our knowledge this study is the first to implement electromyography to monitor muscle activity while testing CIT. Due to this unique approach, no comparisons to prior studies can be made. These findings are not surprising when viewed alongside the other results of this study. Specifically, the TTE results which showed only a 4.2 second difference in mean TTE between groups. Although the results are not statistically significant, they do speak to the high reliability of the results.

Due to this study's absence of mechanistic insight, the possible causes for the obtained results can only be speculated. The dosing study by Moinard et al. (2008) shed light on one metabolic pathway for citrulline. That study showed that the absorption of CIT into the bloodstream does not appear to be the limiting factor in metabolism. Rather

it appears that saturation occurs in the kidneys. Specifically, the proximal convoluted tubules are the sight of conversion for CIT to ARG. However, a large question remains in what occurs to the newly generated ARG molecules once they enter circulation. It might be premature to assume that a significant amount of the ARG would be converted to NO. One possible metabolic pathway for ARG is the stimulation of the pancreas's beta cells to produce insulin. An increase in insulin could result in an increase in protein synthesis through the stimulation of the mammalian target of rapamycin (mTOR) pathway. One study has looked at the possible benefit of CIT supplementation on recovery. Tarazona-Díaz et al. (2013) found that ingestion of CIT resulted in a decrease in muscle soreness after exercise.

The study of CIT as a possible ergogenic aid is a relatively new branch of supplementation research. There have only been a few studies on CIT and all of them have large differences in methodologies and corresponding results. With respect to dosage and timing of CIT, Takashi et al. (2016) gave 2.4 g of CIT for one week and another 2.4 g 1 hour before testing. P. T. Cutrufello et al. (2015) fed 6 g and 1 g CIT 1 or 2 hours prior to testing and Hickner et al. (2006) gave either 3 g 3 hours before testing or 9 g over the 24 hours prior to testing. These studies also all used different subjects. Takashi et al. (2016) used 22 trained male subjects, P. T. Cutrufello et al. (2015) tested 11 male and 11 female collegiate athletes, and Hickner et al. (2006) tested 17 healthy adults. Another discrepancy between the studies is the performance metric. Takashi et al. (2016) utilized a 4-km cycling time trial, P. T. Cutrufello et al. (2015) tested subjects on total repetitions performed, time to exhaustion, VO_2 max, and anaerobic threshold, and

Hickner et al. (2006) had subjects perform a graded treadmill time to exhaustion test. The variables of dosage, timing, subjects, and exercise selection must all be considered when testing a supplement. One review of NO supplements, (Bescos et al., 2012), found that training status of the subjects seems to be an important variable when designing supplement studies that impact NO levels. These authors stated highly trained subjects received no positive effect on physical performance. This is a direct contrast to the findings of Takashi et al. (2016). Since there is a limited amount of literature on CIT supplementation, insights into proper CIT use could be gained by looking at other NO boosting supplements.

The utility of NO supplements rests in their ability to create vasodilation. It is hypothesized that increased vasodilation will lead to better performance through the delivery of nutrients and removal of wastes from active muscle tissue. Beetroot juice is a common supplement used to increase NO levels and thereby alter blood flow patterns through the same vasodilation mechanism that CIT supplementation utilizes. There are numerous studies on beetroot juice supplementation, and some have found improvements in TTE performance (Wylie et al., 2013). Another recent study, (de Castro, de Assis Manoel, Figueiredo, Figueiredo, & Machado, 2019), found that beetroot supplementation improved markers of aerobic exercise performance. Although CIT and beetroot supplementation protocols cannot be identical, due to differing biochemical pathways, researchers of CIT supplementation could look to the studies of beetroot juice to gain insight into the types of exercise metrics could be improved due to increases in NO and vasodilation.

Further research should investigate the metabolism of CIT under different physiological conditions. This information could then be used to better understand where and when CIT could be used as a performance enhancer. The results of this study showed that although it is possible for CIT to alter cardiovascular function, these changes may not be large enough to elicit a change in performance. Additional research needs to be performed looking into the dose response time for CIT supplementation, metabolism of CIT under physical stress, and use of CIT as a post-workout supplement to aid in muscle repair.

Conclusion This study found that ingestion of CIT did not result in improved cycling performance during a ramped TTE test or changes in muscle activity. Statistically significant changes in cardiovascular function were found during the TTE test. However, these alterations did not create an improved performance during the test. Additional research on the metabolism of CIT under different physiological conditions is needed to further the discussion of CIT as a possible ergogenic aid. At this point there is not enough information to fully support or deny CIT supplementation's utility.

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APPENDICIES

APPENDIX A

INSTITUTIONAL REVIEW BOARD APPLICATION

MONTANA
STATE UNIVERSITY

INSTITUTIONAL REVIEW BOARD
For the Protection of Human Subjects
FWA 00000165

960 Technology Blvd. Room 127
c/o Microbiology & Immunology
Montana State University
Bozeman, MT 59718
Telephone: 406-994-6783
FAX: 406-994-4303
E-mail: cherylj@montana.edu

Chair: Mark Quinn
406-994-4707
mquinn@montana.edu
Administrator:
Cheryl Johnson
406-994-4706
cherylj@montana.edu

MEMORANDUM

TO: Peter Stordahl and John Seifert

FROM: Mark Quinn Mark Quinn cfj
Chair, Institutional Review Board for the Protection of Human Subjects

DATE: February 20, 2019

SUBJECT: *""The Effects of L-Citrulline Supplementation on Physical Performance""*
[PS111918]

This is to acknowledge receipt of the request dated February 20, 2019 for minor modifications to the above protocol. The request for the following modifications is approved:

- Subjects will now be compensated with a $30 gift card;
- Revised consent form to indicate compensation.

Full Committee Review ☐

Expedited Review ☐

MONTANA STATE UNIVERSITY

Institutional Review Board Application for Review

(revised 06/01/15)

****

THIS AREA IS FOR INSTITUTIONAL REVIEW BOARD USE ONLY. DO NOT WRITE IN THIS AREA

Application Number: Approval Date:

Disapproved: IRB Chair's Signature:

****

Date:

I. Investigators and Associates (list all investigators involved; application will be filed under name of first

person listed)

NAME: **Peter Stordahl** TITLE: **Graduate Student**

DEPT: HHD PHONE #: **406-600-5030**

COMPLETE ADDRESS: **406 Nelson Story Tower Bozeman, MT 59715**

E-MAIL ADDRESS: **peterstordahl@montana.edu**

DATE TRAINING COMPLETED: **on record** *[Required training: CITI Training; see website for link]*

SIGNATURE (PI or ADVISOR): _____

NAME: **John Seifert** TITLE: **Professor**

DEPT: **HHD** PHONE #: **994-7154**

COMPLETE ADDRESS:

E-MAIL ADDRESS: **john.seifert@montana.edu**

DATE TRAINING COMPLETED: *[Required training: CITI Training; see website for link]*

(repeat for additional investigators if needed; or delete extra if not necessary)

Do you as PI, any family member or any of the involved researchers or their family members have consulting agreements, management responsibilities or substantial equity (greater than $10,000 in value or greater than 5% total equity) in the sponsor, subcontractor or in the technology, or serve on the Board of the Sponsor? _____ YES __XX___ NO

If you answered Yes, you will need to contact Kellie Peterson, Legal Counsel-JD at 406-994-3480.

II. Title of Proposal: *[please try to keep title on front page; use smaller font and delete excess lines if necessary]*

The effects of l-citrulline supplementation on physical performance

III. Beginning Date for Use of Human Subjects:

November 2018

IV. Type of Grant and/or Project (if applicable)

Research Grant:

Contract:

Training Grant:

Classroom Experiments/Projects:

Thesis Project: **XX**

Other (Specify):

V. Name of Funding Agency to which Proposal is Being Submitted (if applicable):

VI. Signatures

Submitted by Investigator

Typed Name: **Peter Stordahl**

Signature:

Date: **9/30/18**

Faculty sponsor (for student)

Typed Name: **Dr. John Seifert**

Signature:

Date: **9/30/18**

VII. Summary of Activity. Provide answers to each section and add space as needed. Do not refer to an accompanying grant or contract proposal.

A. RATIONALE AND PURPOSE OF RESEARCH. (What question is being asked?)

The purpose of this study is to test the effectiveness of l-citrulline supplementation versus a placebo on physical performance. L-citrulline is a non-essential amino acid that is converted into the amino acid l-arginine in the kidneys. L-arginine can then act as a precursor for the vasodilator, nitric oxide. Nitric oxide is a potent vasodilator. The increased diameter of blood vessels allows for a larger amount of blood to be delivered to exercising muscle. The greatest exercise performance benefit of the increased blood flow is the removal of metabolic wastes from the muscle tissue.

Current literature suggests that l-citrulline may be effective as an ergogenic aid. The question becomes is l-citrulline more effective than a placebo at enhancing muscle activity and blood flow during physical activity?

B. RESEARCH PROCEDURES INVOLVED. Provide a short description of sequence and methods of procedures that will be performed with human subjects. Include details of painful or uncomfortable procedures, frequency of procedures, time involved, names of psychological tests, questionnaires, restrictions on usual life patterns, and follow up procedures. **If you are planning on posting flyers, posters, etc. anywhere on Campus, you must check with the building managers and/or departments located in MSU buildings and obtain their approval prior to the posting.**

Pre-testing Protocol: Subjects will report to the Movement Science Laboratory three times. The first visit, subjects will fill out the prerequisite paperwork of an informed consent and the National Academy of Sports Medicine's Physical Activity Readiness Questionnaire (PAR-Q) . Subjects will then complete a maximal power test to determine their workloads for subsequent visits. The maximal power test will begin with the subject warming up on a cycle ergometer for 5 minutes at a chosen cadence with 1 kilogram of resistance. Subjects will continue to ride at their chosen cadence, with 0.5 kilograms being added every five minutes, until they can no longer sustain their cadence. The subjects heart rate, and rating of perceived exertion will be recorded at the end of every 5-minute interval.

Treatment Protocol: Subjects will report to the Movement Science Laboratory two more times, one for each treatment. Subjects will ingest either 10 grams of l-citrulline mixed in water and flavored with Kool-Aid or placebo drink consisting of Kool-Aid mixed in water one hour before completing an exercise protocol. The reason for the one-hour time window is based on the literature it takes approximately one hour for ingested citrulline levels to reach maximum values in the blood. Before the subjects begin exercising they will be fitted with surface electromyography sensors and near infrared spectroscopy sensors. The electromyography and near infrared spectroscopy sensors are attached to the skin with tape and are used to non-invasively assess muscle activity and blood flow, respectively. Subjects will begin the exercise protocol by cycling for 5 minutes at a cadence of their choosing with 1 kilogram of resistance on a cycle ergometer. Subjects will then complete eight 5-minute intervals. The intervals alternate between 3 minutes at 50% of their maximal power and 2 minutes at 80% of their maximal power. Subjects' heart rate will be taken at the end of each phase of every interval along with the subjects rating of perceived exertion. Subjects will receive 200 milliliters of water after the fourth interval. After completing the final interval subjects will cool down for 5 minutes by riding at a cadence of their choice against 1 kilogram of resistance.

After completion of the cool down subjects will consume 5 grams of l-citrulline or 5 grams of the placebo and rest for one hour. During this rest period subjects will be given 200 milliliters of water. After the rest period subjects will perform a ramped time to exhaustion cycling test. This protocol begins with the subject cycling for 5 minutes at a cadence of their choosing against 1 kilogram of resistance. 0.5 kilograms of resistance will be added every five minutes until the subject can no longer sustain their cadence. The subjects heart rate and rating of perceived exertion will be recorded at every 5 minute interval.

Rating of perceived exertion will be based off the Borg scale (1-10). Heart

rate will be recorded by a Polar HR Monitor (RC3; Finland).

Electromyography data will be collected during the 1st, 3rd, 5th, and 7th interval along with the final minute of each stage of the ramped time to exhaustion test.

Near infrared spectroscopy data will be collected during the 1st, 3rd, 5th, and 7th interval along with the final minute of each stage of the ramped time to exhaustion test.

Blood Pressure will be recorded before the ride starts, and after the fourth and eighth interval.

Statistical Analyses. Data will be analyzed with StatPac and SPSS statistical software using a 2x3 ANOVA with repeated measures and a 1 way ANOVA for the ramped time to exhaustion test data.

C. DECEPTION - If any deception (withholding of complete information) is required for the validity of this activity, explain why this is necessary and attach debriefing statement.

D. SUBJECTS

1. Approximate number and ages

   How Many Subjects: **up to 15**

   Age Range of Subjects: 1**8-45**

   How Many Normal/Control: **up to 15; this is a crossover design**

   Age Range of Normal/Control: **18-45**

2. Criteria for selection: **Must be healthy with no known metabolic or cardiac symptoms. Must have the ability to cycle for up to one hour.**

3. Criteria for exclusion: **Trained cyclists, inability to follow dietary guidelines, and symptoms of cardiac impairments or metabolic syndrome**

4. Source of Subjects (including patients): **Bozeman area**

5. Who will approach subjects and how? Explain steps taken to avoid coercion.

   Peter Stordahl will approach potential subjects. Recruitment will take place through word of mouth and by contacting known sources such as cycling classes at local gyms. Subjects will be informed that they can discontinue participation at any time.

6. Will subjects receive payments, service without charge, or extra course credit? Yes or **No**
   (If yes, what amount and how? Are there other ways to receive similar benefits?)

7. Location(s) where procedures will be carried out.

   Montana State University's Movement Science Laboratory in Romney Gym

E. RISKS AND BENEFITS (ADVERSE EFFECTS)

1. Describe nature and amount of risk and/or adverse effects (including side effects), substantial stress, discomfort, or invasion of privacy involved.

   Since the subjects will be healthy and in moderate physical condition the risks associated with exercise are low. However, subjects may experience sore muscles and fatigue. There is a very slight risk of sudden death due to the maximal power output test that subjects will perform to determine their workloads for the interval rides. The health risks associated with l-citrulline ingestion are very minimal. The literature states that ingestion of l-citrulline is safe and well tolerated by the digestive system in doses up to 15 grams. L-citrulline ingestion may lead to a decrease in blood pressure and an increase in heart rate as it is a precursor to nitric oxide, a known vasodilator. A drop in blood pressure may also create the potential for syncope. A non-typical blood pressure response will lead to immediate termination of the experiment.

   Subjects will also be informed that they can terminate their participation in the experiment at any time without any questions asked by the researchers.

2. Will this study preclude standard procedures (e.g., medical or psychological care, school attendance, etc.)? If yes, explain.

   None

3. Describe the expected benefits for individual subjects and/or society.

   Subjects will benefit from participation in the study by learning about the potential performance benefits associated with l-citrulline supplementation. Subjects will also receive a free maximal power test to assess their cycling capabilities.

F. ADVERSE EFFECTS

1. How will possible adverse effects be handled?

   By investigator(s):

   Referred by investigator(s) to appropriate care:

   Other (explain): **The university police will be immediately notified for any adverse side effects. Researchers will not administer any medical treatment.**

2. Are facilities/equipment adequate to handle possible adverse effects? **Yes** or No

   (If no, explain.)

3. Describe arrangements for financial responsibility for any possible adverse effects.

   MSU compensation (explain): **None**

   Sponsoring agency insurance:

   Subject is responsible: **Yes**

   Other (explain):

G. CONFIDENTIALITY OF RESEARCH DATA

1. Will data be coded? **Yes** or No

2. Will master code be kept separate from data? **Yes** or No

3. Will any other agency have access to identifiable data? Yes or **No**
   (If yes, explain.)

4. How will documents, data be stored and protected?
   Locked file:
   Computer with restricted password: **Yes**
   Other (explain):

VIII. Checklist to be completed by Investigator(s)

A. Will any group, agency, or organization be involved? Yes or **No**
   (If yes, please confirm that appropriate permissions have been obtained.)

B. Will materials with potential radiation risk be used (e.g. x-rays, radioisotopes)? Yes or **No**

1. Status of annual review by MSU Radiation Sources Committee (RSC). Pending or Approved
   (If approved, attach one copy of approval notice.)

2. Title of application submitted to MSU RSC (if different).

C. Will human blood be utilized in your proposal? Yes or **No**
   (If yes, please answer the following)

1. Will blood be drawn? Yes or **No**

(If yes, who will draw the blood and how is the individual qualified to draw blood? What procedure will be utilized?)

2. Will the blood be tested for HIV? Yes or **No**

3. What disposition will be made of unused blood?

4. Has the MSU Occupational Health Officer been contacted? Yes or **No**

D. Will non-investigational drugs or other substances be used for purposes of the research? Yes or **No**

Name:

Dose:

Source:

How Administered:

Side effects:

E. Will any investigational new drug or other investigational substance be used? Yes or **No**

[*If yes, provide information requested below and one copy of: 1) available toxicity data; 2) reports of animal studies; 3) description of studies done in humans; 4) concise review of the literature prepared by the investigator(s); and 5) the drug protocol.]*

Name:

Dose:

Source:

How Administered:

IND Number:

Phase of Testing:

F. Will an investigational device be used? Yes or **No**

(If yes, provide name, source description of purpose, how used, and status with the U.S. Food and Drug Administration FDA). Include a statement as to whether or not device poses a significant risk. Attach any relevant material.)

G. Will academic records be used? Yes or **No**

H. Will this research involve the use of:

Medical, psychiatric and/or psychological records Yes or **No**

Health insurance records Yes or **No**

Any other records containing information regarding personal health and illness Yes or **No**

If you answered "Yes" to any of the items under "H.", you must complete the **HIPAA worksheet**.

I. Will audio-visual or tape recordings or photographs be made? **Yes** or No

J. Will written consent form(s) be used? (**Yes** or No. If no, explain.) (Please use accepted format from our website. Be sure to indicate that participation is voluntary. Provide a stand-alone copy; do not include the form here.)

APPENDIX B

INFORMED CONSENT

Subject Consent Form For Participation in Human Research At Montana State University

Effects of L-Citrulline Supplementation on Physical Performance

You have been invited to participate in a research study that is investigating the changes in blood flow patterns and muscle activity due to l-citrulline supplementation during cycling.

Pre-testing Protocol

You will report to the Movement Science Laboratory three times. The first visit, you will fill out the prerequisite paperwork of an informed consent and health history questionnaire. You will then complete a maximal power test to determine your workloads for subsequent visits. The maximal power test will begin with you warming up on a cycle ergometer for five minutes at a chosen cadence with 1 kilogram of resistance. You will continue to ride at your chosen cadence, with 0.5 kilograms being added every five minutes, until you can no longer sustain your cadence. Your heart rate, and rating of perceived exertion will be recorded at the end of every 5-minute interval.

Treatment Protocol

You will report to the Movement Science Laboratory two more times, one for each treatment. You will ingest either 10 grams (just under 1 tablespoon) of l-citrulline mixed in water along with flavoring or a placebo drink with water and the same flavoring one hour before completing an exercise protocol. The reason for the one-hour time window is based on the literature it takes approximately one hour for ingested citrulline levels to reach maximum values in the blood. Before you begin exercising you will provide a urine sample to check for adequate hydration. Then you will be fitted with surface electromyography sensors and near infrared spectroscopy sensors. You will begin the exercise protocol by cycling for 5 minutes at a cadence of your choosing with 1 kilogram of resistance on a cycle ergometer. You will then complete eight 5-minute intervals. The intervals alternate between 3 minutes at 50% of your maximal power and 2 minutes at 80% of your maximal power. Your heart rate will be taken at the end of each phase of every interval along with your rating of perceived exertion. You will receive 200 milliliters (just under 7 fluid ounces) of water after the fourth interval. After completing the final interval, you will cool down for 5 minutes by riding at a cadence of your choice against 1 kilogram of resistance.

After completion of the cool down you will consume 5 grams of l-citrulline or 5 grams of the placebo and rest for one hour. During this rest period you will be given 200 milliliters of water. After the rest period you will perform a ramped time to exhaustion cycling test. This protocol begins with you cycling for 5 minutes at a cadence of your choosing against 1 kilogram of resistance. 0.5 kilograms of resistance will be added every five minutes until you can no longer sustain your cadence. Your heart rate and rating of perceived exertion will be recorded at every 5-minute interval.

Benefits

Results from this study will be used to add to the current literature base on l-citrulline supplementation. Results will also be given to you for your education on how l-citrulline supplementation effects physical performance. You will also receive a free maximal power test to assess your cycling capabilities.

Compensation

None

Confidentiality

Your personal information and data will be stored on a laptop computer that is password protected. Data used in analysis will be coded so personal information will not be identifiable on the data sheets.

Risks

The risks associated with exercise are low. However, you may experience sore muscles and fatigue. There is a very slight risk of sudden death due to the maximal power output test that you will perform to determine your workloads for the interval rides. The health risks associated with l-citrulline ingestion are very minimal. The literature states that ingestion of l-citrulline is safe and well tolerated by the digestive system in doses up to 15 grams. L-citrulline ingestion may lead to a decrease in blood pressure and an increase in heart rate as it is a precursor to nitric oxide, a known vasodilator. The drop in blood pressure may result in syncope (fainting). A non-typical blood pressure response would be grounds for immediate termination of the experiment.

Questions

Your decision to participate or not will not jeopardize your relationship with the MSU Movement Science/Human Performance Laboratory. You are free to discontinue participation at any time without negative effect on your relationship with MSU or the researchers. If you have any questions, please ask. If you have any additional questions later, Peter Stordahl at peterstordahl@montana.edu or Dr. John Seifert (406-994-7154 or john.seifert@montana.edu) will be happy to answer them.

Additional questions about the rights of human subjects can be answered by the Chairman of the Institutional Review Board, Dr. Mark Quinn, (406) 994-4707.

Freedom of Consent

I have been given ample opportunity to read this document in its entirety and to ask questions which have been answered to my satisfaction. I hereby consent to become

a participant in this study knowing the health risks involved and that I may withdraw my consent at any time, for any reason. I am covered by a health insurance program. I also understand that project personnel may screen me from this study for any reason deemed appropriate. Such reasons may include abnormal physiological responses to exercise. I declare that I am fit and a capable cyclist.

AUTHORIZATION: I have read the above and understand the discomforts, inconvenience and risk of this study. I, _____ *(name of subject),* agree to participate in this research. I understand that I may later refuse to participate, and that I may withdraw from the study at any time. I have received a copy of this consent form for my own records.

Signed: _____

Witness: _____

Investigator: _____

Date: _____