UNIVERSITY OF WISCONSIN-LA CROSSE

Graduate Studies

THE EFFECTS OF SHORT-TERM L-CITRULLINE SUPPLEMENTATION ON MICROVASCULAR FUNCTION, SKELETAL MUSCLE OXYGEN AND LIPOLYSIS IN PREDIABETIC OLDER ADULTS: AN EXPLORATORY STUDY

A Manuscript Style Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Science in Clinical Exercise Physiology

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THE EFFECTS OF SHORT-TERM L-CITRULLINE SUPPLEMENTATION ON MICROVASCULAR FUNCTION, SKELETAL MUSCLE OXYGEN AND LIPOLYSIS IN PREDIABETIC OLDER ADULTS: AN EXPLORATORY STUDY

By Christopher R. Martinson

We recommend acceptance of this thesis in partial fulfillment of the candidate's requirements for the degree of Master of Science in Clinical Exercise Physiology

The candidate has completed the oral defense of the thesis.

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ABSTRACT

Martinson, C.R. Effects of L-Citrulline on microvascular function, skeletal muscle oxygen, and lipolysis in healthy and prediabetic older adults: an exploratory study. MS in Clinical Exercise Physiology, December 2019, 62pp., (S, Jaime)

Background: Prediabetes may increase the risk of endothelial dysfunction and reduce skeletal muscle oxygenation. Increased lipolysis during exercise may reduce fat mass and improve insulin sensitivity. L-citrulline improves endothelial function, although there are no data on lipolysis during exercise in humans. The purpose of this study was to measure the effects of a 2-week L-citrulline supplementation on microvascular function, skeletal muscle oxygenation and exercise lipolysis. **Methods**: 16 healthy and prediabetic older adults (age=68.8±9.8 yrs) participated in this study. Each intervention involved a two-week supply of L-citrulline or placebo in a double blind, crossover design. Each participant completed an occlusion and submaximal handgrip protocol, and a perceptually regulated exercise test (PRET) before and after each intervention. **Results**: There were no significant responses in microvascular function, skeletal muscle oxygenation, or lipolysis rates during submaximal exercise to L-citrulline between normal and prediabetic adults. **Conclusion**: As an exploratory study, there were few significant effects of L-citrulline on efficiency of skeletal muscle oxygenation or rates of fat oxidation during exercise in this population. However, the variables were trending in the direction of our hypotheses. A larger sample size may be needed.

ACKNOWLEDGEMENTS

I would like to express the deepest appreciate to my thesis chairperson, Dr. Salvador Jaime, for putting up with my procrastination and lackluster writing abilities. Without Cha this thesis would not have been completed. Cha's assistance in data collection was invaluable during times with multiple subjects and when I had class.

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The testing wouldn't have been completed without the assistance of four undergraduate students from the University of Wisconsin – La Crosse. I am thankful for the help of Taylor Falck, Jordan Gygax, Holly Schwecke, and Mariah Teske. I am also appreciative of our summer intern from the University of Wisconsin – Eau Claire, Elizabeth Schwab, who filled many of the maltodextrin pills.

Finally, I would like to thank all the participants in this study for completing all the testing procedures multiple times. Although the testing was lengthy and unpleasant the participants continually showed up in a timely fashion for all testing sessions.

TABLE OF CONTENTS

PAGE

LIST OF TABLES

TABLE PAGE

LIST OF FIGURES

FIGURE PAGE

LIST OF APPENDICES

APPENDICES PAGE

INTRODUCTION

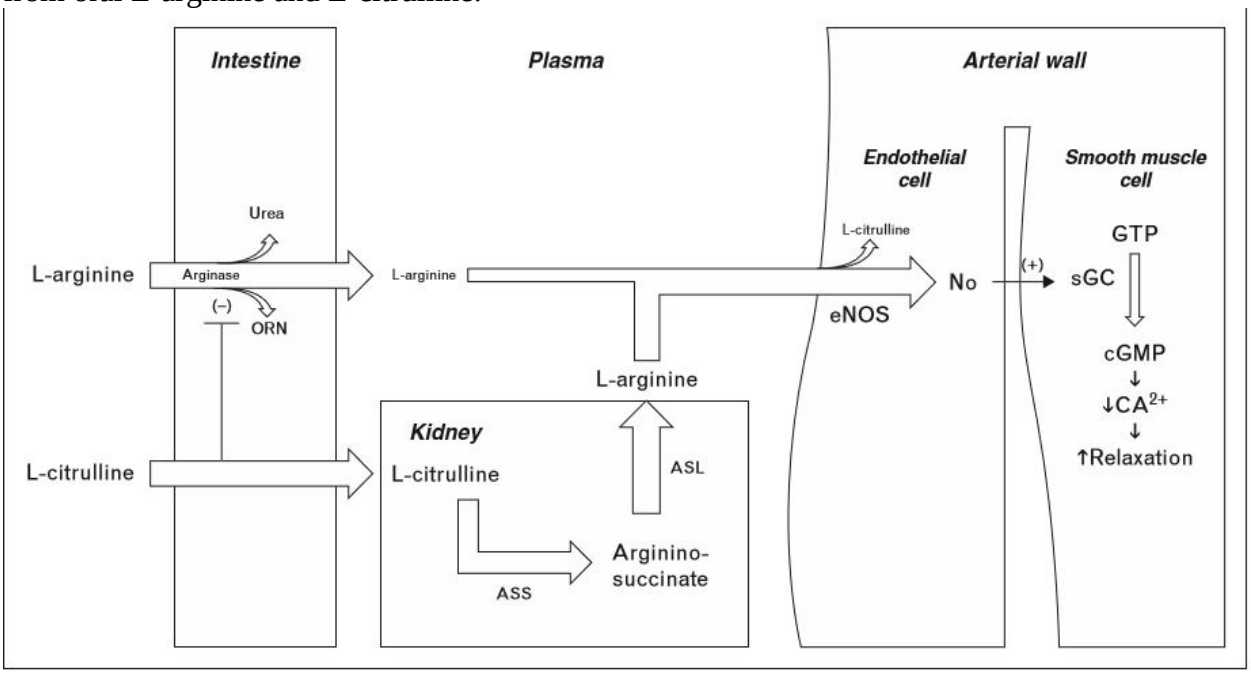
In today's society, prediabetes is becoming more prevalent. It is estimated that almost 81 million or over one-third of U.S. adults have prediabetes (Mozaffarian et al., 2015). This is projected to increase to over 91 million U.S. adults by 2025 (Rowley & Bezold, 2012). Prediabetes is a condition in which blood glucose levels are higher than normal creating a hyperglycemic environment within the circulatory system. Chronic hyperglycemia causes numerous physiological changes, including microvascular (arterioles and capillaries) changes and endothelial dysfunction. Endothelial dysfunction consists of changes in the endothelium, including impaired vasodilation, enhanced generation of reactive oxygen intermediates, inflammatory activation, and impaired angiogenesis and barrier function (Bakker et al., 2009). As blood glucose increases, there is a decrease in endothelium dependent vasodilation in both the macro- and microvasculature (Caballero et al., 1999). Hyperglycemia effects the vasodilatory ability of the microvasculature by increasing the release of endothelin-1 and decreasing the release of nitric oxide (NO) (Bakker et al., 2009). NO is a powerful vasodilator that is released in response to sheer stress along the endothelial wall (Gnasso et al., 2001).

Aging is another factor than can lead to endothelial dysfunction. It has been demonstrated that microvascular function is lower in an elder population compared to a

younger population with a 5-minute occlusion protocol (Rosenbury et al., 2018). Artery intimae have more substances present that can damage vessels including adhesion molecules, matrix metalloproteinases, and proinflammatory cytokines as they age (Ferrari, Radaelli, & Centola, 2003). It has been demonstrated that there is reduced bioavailability of endothelium-derived NO (Loo et al., 2000) and an increase in the bioactivity of the constricting factor endothelin-1 (Donato et al., 2009).

Therefore, to increase NO production, there are non-pharmaceutical options that are of interest. L-citrulline is a non-essential amino acid, which can be found in watermelon (Figueroa, Wong, Jaime, & Gonzales, 2017). L-citrulline is a precursor for the endogenous synthesis of L-arginine and consequentially NO bioavailability. It has been demonstrated that oral L-citrulline increases the amount of L-arginine in both intestinal tissue (Wijnands et al., 2012) and vascular systems (Schwedhelm et al., 2008; Kuhn, 2002). Unlike L-arginine, L-citrulline can pass through the intestines without being catabolized making it to the kidneys where it can be efficiently recycled into L-arginine (Figure 1). As such, L-citrulline can be considered a more effective oral supplement (Figueroa, Wong, Jaime, & Gonzales, 2017).

Figure 1. Figueroa, Wong, Jaime, & Gonzales, J. U. (2017); Pathway of NO production from oral L-arginine and L-citrulline.



L-citrulline has been shown to improve cardiovascular markers in multiple studies. Acutely, 3-grams of L-citrulline has demonstrated a 10-fold increase in endogenous NO synthesis (Kim et al. 2015). Increased NO synthesis has a vasodilatory effect on the arterioles and capillaries increasing blood flow and oxygen to skeletal muscle (Dipla et al., 2017a). One-way to measure the increased NO production is observing the oxygenation of skeletal muscle. This can be performed non-invasively through near-infrared spectroscopy (NIRS).

L-citrulline has also been studied in relation to fat oxidation and muscle mass, however mostly in animal models. L-citrulline has demonstrated the ability to improve insulin sensitivity, increase lean mass, and decrease fat mass (Moinard et al., 2015, Joffin et al., 2014a, Joffin et al., 2014b). L-citrulline supplementation has demonstrated the ability to significantly decrease adipocyte diameter and increase fatty acid β-oxidation from visceral adipose tissue in rats of various ages (Joffin et al., 2014b). L-citrulline

supplementation increased lean body mass by 9 percent and decreased fat mass 13 percent in healthy rats (Moinard et al., 2015). L-citrulline demonstrated an increase in the oxidation of lipoproteins in those rats. L-Citrulline also showed an increase in protein synthesis of old malnourished rats (Osowska et al., 2016).

It is recommended that individuals with prediabetes lose weight without decreasing muscle mass. With the increase of fat oxidation that L-citrulline has demonstrated in rat models, this could be a secondary benefit. During aerobic exercise the body generates adenosine triphosphate (ATP) from two primary methods, glycolysis and β-oxidation. Beta-oxidation is the degradation of a fatty acid chain to produce ATP and other products that are later converted into ATP. A non-invasive way to measure fat oxidation is with the respiratory exchange ratio (RER) during exercise (Jeukendrup & Wallis, 2005). RER is the ratio of produced carbon dioxide (CO_2) to consumed oxygen (O_2). Stoichiometry shows that fat has a ratio of 16 to 23 or about 0.70 whereas carbohydrates have a ratio of 6 to 6 or 1.0 (Jeukendrup & Wallis, 2005). When intensity increases enough the RER will go above 1.0 due to an increased production of CO_2 from the bicarbonate buffering system (Jeukendrup & Wallis, 2005).

The purpose of this study is to measure the effects of short-term L-citrulline supplementation on the microvasculature, skeletal muscle oxygenation, and lipolysis in prediabetic, older adults. The results of this study could demonstrate the effectiveness of a supplement treating certain pathophysiological effects of prediabetes.

METHODS

Subjects

Subjects for this study were 20 apparently healthy prediabetic (n=9) and nondiabetic (n=11) individuals. During the study, four subjects failed to comply with protocol, leaving 16 apparently healthy prediabetic (n=8) and nondiabetic (n=8) individuals. Prediabetes during this study was measured as having a fasting blood glucose of 100 mg/dL to 125 mg/dL or an HbA1c ranging from 5.7-6.5. Subjects were between the ages of 45 and 95 without any irregular arrhythmia, a pacemaker, or being unable to complete testing. Prior to participation in this study, each subject provided written informed consent. This study was approved by the Institutional Review Board at the University of Wisconsin La Crosse.

Experimental Design

This experiment was a double blind, within-subjects group randomized control trial. Each subject completed an initial screening, which included informed consent and protocol familiarization. Following familiarization, each subject attended four testing sessions separated by two weeks. After the first and third testing sessions, each participant received a randomized placebo or supplement.

Supplement

Subjects are provided a pill bottle with a 14-day supply of either L-Citrulline or the placebo (maltodextrin). The pill bottle contains 112, 750mg L-Citrulline or

maltodextrin pills. Subjects are required to ingest four pills in the morning and four pills night every day for two weeks.

Procedures

Prior to arrival for testing, each participant was informed to be fasting, and avoid caffeine and medications for 12 hours. Upon arrival for testing, demographic information including subject number, date of birth, age, height, weight, blood pressure, right forearm skinfold, waist and hip circumference, and HbA1c was collected. Waist circumference was measured at the midpoint between the lowest rib and the superior portion of the iliac crest and hip circumference was measured at the widest point of the hips. Body composition was measured through bioelectric impedance analysis using a RJL Quantum IV (RJL Systems, Clinton Township, MI) (Fakhrawi et al. 2009). Maximal voluntary contraction (MVC) was obtained prior to testing using a Vernier LabQuest 2 (Vernier, Beaverton, OR).

The first test performed was a forearm occlusion protocol. Briefly, this protocol requires a NIRS device (Moxy, Fortiori Design LLC, Hutchinson, Minnesota) to be placed on the flexor digitorum muscle immediately distal to the occluding cuff to measure skeletal muscle oxygenation (SmO_2). In the supine position, the protocol begins with one-minute non-occlusion to determine baseline values followed by cuff inflation to a pressure of 250mmHg of pressure to occlude the target muscles (Dipla et al., 2017). This pressure was maintained for five minutes, then once released, SmO_2 was measured for three minutes. Following this protocol, area under-the curve is calculated by removing a rectangular area (time above baseline * baseline SmO_2) from the sum of trapezoidal areas after exceeding baseline SmO_2.

For the second test, the NIRS device remained on right flexor digitorum and the participant remained in a supine position. The second test was a three-minute isometric handgrip protocol. Participants applied 35 percent of their MVC for four-second bouts followed by three-second bouts of rest. This seven second set was continuously performed for the entirety of the 3-minute period (Dipla et al., 2017).

The final test was a sub-maximal graded exercise test on a Lode Excalibur sport cycle ergometer (Lode, Groningen, Netherlands). This test utilized open-circuit indirect calorimetry (TrueOne2400, PARVO Medics, Sandy, UT) and was calibrated in accordance with the manufacturer's guidelines prior to every test. The participants completed a 12-minute perceptually regulated sub-maximal exercise test (PRET) with self-selected intensities at 9, 11, 13, and 15 on the Borg perceived exertion scale (Faulkner et al., 2007). The participant completed each intensity for 3 minutes where rating of perceived exertion (RPE), heart rate (HR), respiratory exchange ratio (RER), and power output (PO) were recorded.

Statistical Analysis

As this project was part of a larger study, we utilized previous research (Figueroa et al., 2010) to conduct a power calculation done *a priori* to determine a population of 24 subjects, with an effect size of 0.30 and power of 80% to observe a significant difference (α=0.05) between placebo and L-citrulline treatments for the primary variable unrelated to this thesis manuscript. Normality was confirmed using the Shapiro-Wilk test for all measurements. An independent t-test was used to measure potential differences between groups at baseline. A two-way repeated measure analysis of variance (RMANOVA) was used to determine differences within and between interventions in muscle oxidation

during the occlusion and submaximal handgrip exercise and fuel utilization during the PRET. The alpha level was set at $P<0.05$ to determine statistical significance. Data will be analyzed using SPSS version 25.0 (SPSS Inc., Chicago, IL) and Microsoft Excel 2017 (Microsoft, Redmond, WA).

RESULTS

All data are presented as means ± SD. Sixteen subjects complied with the procedures and completed the study. Subject characteristics are presented below in Table 1. No significant difference was found between the non-diabetic and prediabetic subjects for any of the descriptive statistics.

Table 1. Descriptive Characteristics of Subjects (N=16)

	Non-diabetic (n=8)	Prediabetic (n=8)
Age (yrs)	71.9 ± 7.43	71.0 ± 7.35
Height (cm)	170.2 ± 8.00	167.5 ± 8.78
Weight (kg)	73.7 ± 11.45	77.4 ± 16.45
Body Fat (%)	33.3 ± 5.75	38.4 ± 8.51
VO_2Max (ml/kg/min)	25.0 ± 9.79	23.7 ± 9.50
MVC (kg)	21.3 ± 7.74	21.5 ± 7.35

Values represent mean ± standard deviation.

Subjects skeletal muscle oxygenation (SmO_2) during the occlusion protocol are summarized in Table 2. Trends of SmO_2 at baseline and following each supplement are demonstrated for non-diabetics and prediabetic in Figure 1 and Figure 2, respectively.

Table 2. Skeletal Muscle Oxygenation (SmO_2) during Occlusion Protocol (N=16)

	Non-diabetic (n=8)	Prediabetic (n=8)
Initial Resting SmO_2	57.4 ± 5.07	60.9 ± 7.73
Initial Minimum SmO_2	12.9 ± 10.27	20.0 ±15.33
Initial Maximum SmO_2	75.1 ± 11.45	70.7 ± 10.90
Placebo Resting SmO_2	56.4 ± 8.94	58.6 ± 3.80
Placebo Minimum SmO_2	9.5 ± 8.59	17.3 ± 14.23
Placebo Maximum SmO_2	71.0 ± 12.27	66.7 ± 11.51
L-Citrulline Resting SmO_2	57.0 ± 5.90	61.3 ± 9.66
L-Citrulline Minimum SmO_2	8.0 ± 10.51	21.2 ± 18.36
L-Citrulline Maximum SmO_2	75.0 ± 7.38	70.1 ± 13.87

Values represent mean ± standard deviation.

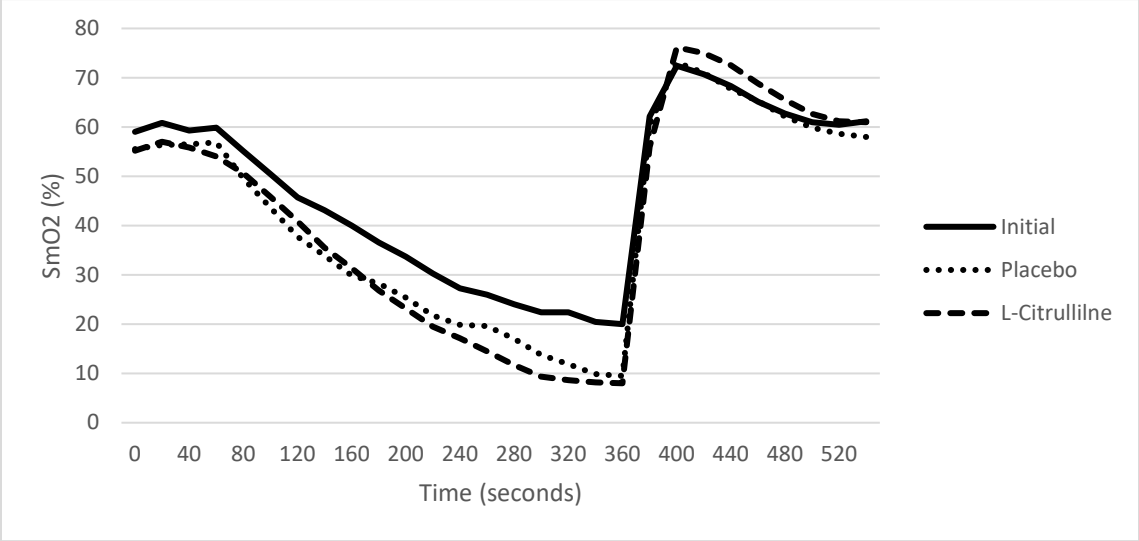


Figure 2. Skeletal muscle oxygenation curves during arterial occlusion and reperfusion from mean data in non-diabetic subjects at rest, following the placebo, and following L-Citrulline.

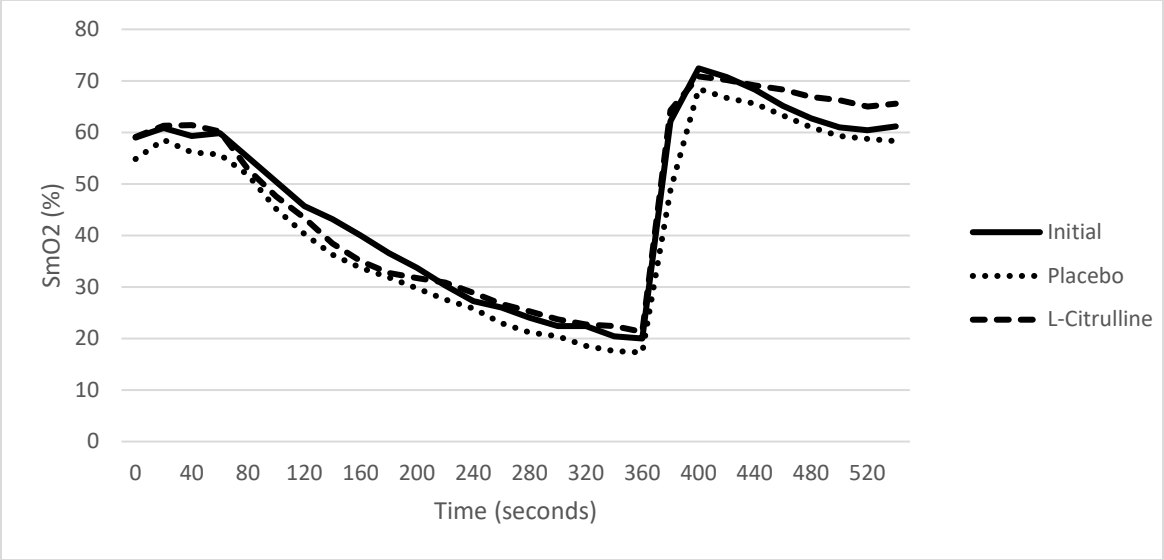


Figure 3. Skeletal muscle oxygenation curves during arterial occlusion and reperfusion from mean data in pre-diabetic subjects at rest, following the placebo, and following L-Citrulline.

There was no statistically significant interactions or main effects on resting SmO_2, minimum SmO_2, or maximum SmO_2 during the occlusion protocol ($p>0.05$) or reactive hyperemia. Subjects SmO_2 during each minute of the handgrip protocol are summarized in Table 3. Trends of SmO_2 at baseline and following each supplement are demonstrated for non-diabetics and prediabetic in Figure 3 and Figure 4, respectively.

Table 3. Skeletal Muscle Oxygenation (SmO_2) during Hand Grip Protocol (N=16)

	Non-diabetic (n=8)	Prediabetic (n=8)
Initial SmO_2 1st Minute	53.9 ± 10.41	58.6 ± 9.41
Initial SmO_2 2nd Minute	52.5 ± 10.85	57.7 ± 9.41
Initial SmO_2 3rd Minute	54.9 ± 11.09	59.4 ± 7.37
Placebo SmO_2 1st Minute	52.8 ± 7.69	56.6 ± 10.47
Placebo SmO_2 2nd Minute	51.0 ± 9.48	56.0 ± 9.76
Placebo SmO_2 3rd Minute	53.1 ± 9.04	56.8 ± 9.70
L-Citrulline SmO_2 1st Minute	51.3 ± 11.35	55.3 ± 3.60
L-Citrulline SmO_2 2nd Minute	50.9 ± 14.44	51.3 ± 3.69
L-Citrulline SmO_2 3rd Minute	52.1 ± 12.98	52.5 ± 4.26

Values represent mean ± standard deviation.

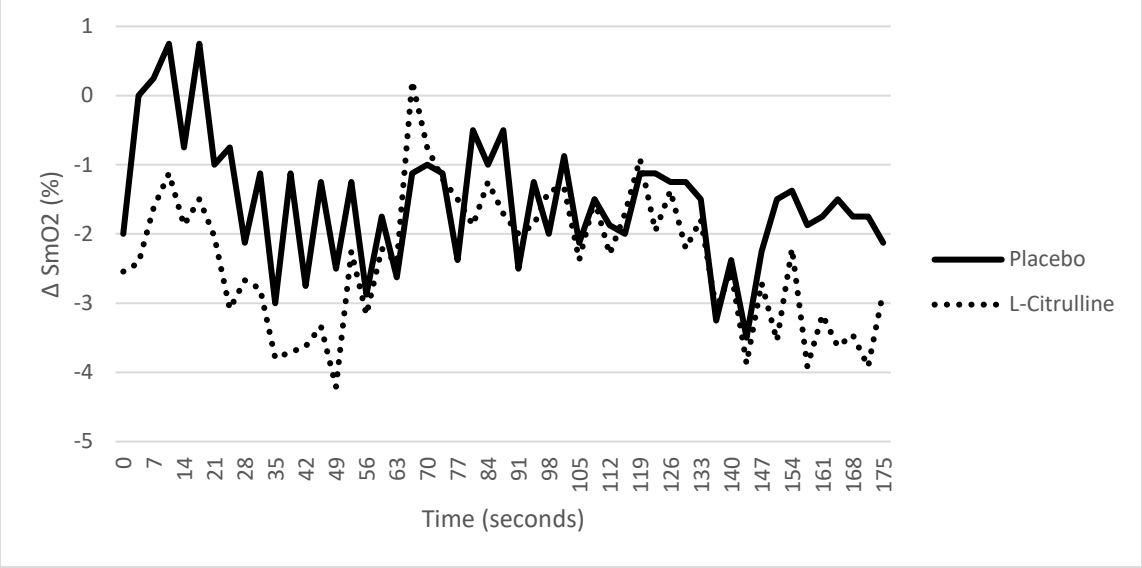


Figure 4. Changes in tissue oxygenation during the hand grip protocol for non-diabetic individuals.

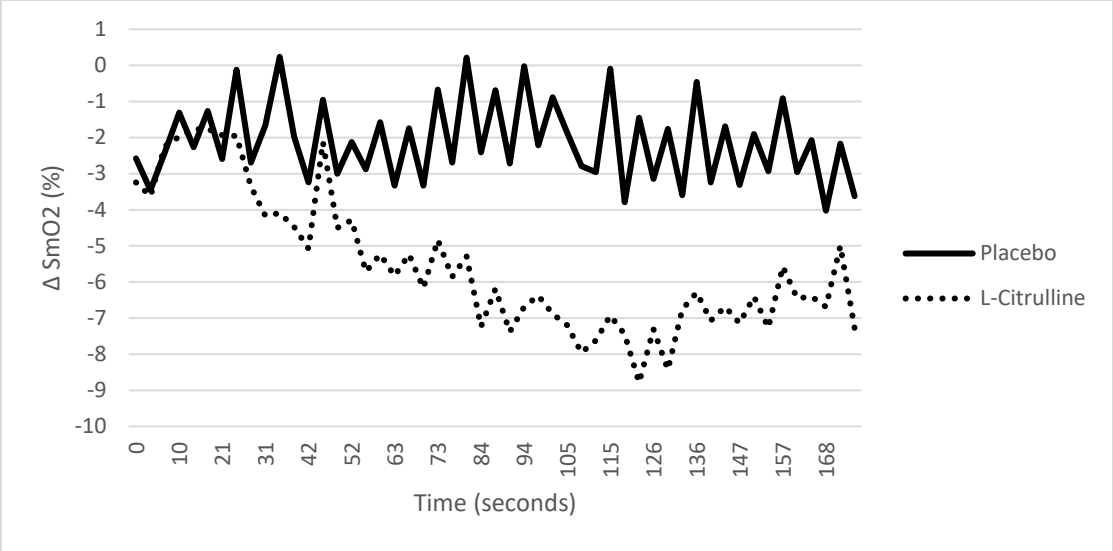


Figure 5. Changes in tissue oxygenation during the hand grip protocol for prediabetic individuals.

There was no statistically significant interaction or main effect on the first minute, the second minute, and the third minute of the handgrip protocol (p>0.05).

Subjects RPE, HR, PO, and RER during the final minute of each stage of the PRET are summarized in Table 4. Changes in RPE for each supplement over time are presented in Figure 5 for non-diabetic subjects and Figure 6 for prediabetic subjects. Changes in HR for each supplement over time are presented in Figure 7 for non-diabetic subjects and Figure 8 for prediabetic subjects. Changes in PO for each supplement over time are presented in Figure 9 for non-diabetic subjects and Figure 10 for prediabetic subjects. Changes in RER for each supplement over time are presented in Figure 11 for non-diabetic subjects and Figure 12 for prediabetic subjects.

Table 4. Perceptually Regulated Exercise Test Measurements (N=16)

	Non-diabetic (n=8)	Prediabetic (n=8)
Stage 1 RPE		
Initial	10.4 ± 1.55	9.8 ± 1.04
Placebo	9.9 ± 0.92	9.7 ± .74
L-Citrulline	10.4 ± 1.00	9.0 ± .74
Stage 1 HR		
Initial	86.1 ± 13.23	78.3 ± 10.85
Placebo	87.2 ± 10.67	82.8 ± 14.63
L-Citrulline	87.0 ± 13.52	76.0 ± 7.63
Stage 1 Watts		
Initial	28.6 ± 23.26	25.6 ± 12.94
Placebo	25.9 ± 19.59	25.4 ± 14.63
L-Citrulline	27.3 ± 23.55	28.5 ± 19.58
Stage 1 RER		
Initial	0.77 ± 0.075	0.76 ± 0.034
Placebo	0.75 ± 0.048	0.78 ± 0.055
L-Citrulline	0.74 ± 0.034	0.77 ± 0.028
Stage 2 RPE		
Initial	11.5 ± 1.07	11.5 ± 0.76
Placebo	11.0 ± 0.73	11.5 ± 0.60
L-Citrulline	11.5 ± 1.43	11.1 ± 0.53
Stage 2 HR		
Initial	97.6 ± 10.60	82.8 ± 13.35
Placebo	95.5 ± 12.98	89.3 ± 22.88
L-Citrulline	99.7 ± 19.96	82.1 ± 10.71
Stage 2 Watts		
Initial	37.5 ± 25.09	39.8 ± 24.08
Placebo	38.9 ± 28.15	41.1 ± 30.5
L-Citrulline	42.0 ± 37.32	49.5 ± 30.42
Stage 2 RER		
Initial	0.83 ± 0.073	0.81 ± 0.072
Placebo	0.82 ± 0.066	0.85 ± 0.078
L-Citrulline	0.85 ± 0.050	0.80 ± 0.052[ac]
Stage 3 RPE		
Initial	13.4 ± 0.52	13.1 ± 0.64
Placebo	13.1 ± 0.35	13.5 ± 0.73
L-Citrulline	13.8 ± 0.38	13.0 ± 0.46

Stage 3 HR		
Initial	112.3 ± 14.83	92.8 ± 20.36
Placebo	108.1 ± 19.48	97.6 ± 29.34
L-Citrulline	114.1 ± 21.52	96.0 ± 16.66
Stage 3 Watts		
Initial	59.4 ± 46.40	56.6 ± 36.89
Placebo	58.6 ± 41.83	54.4 ± 38.86
L-Citrulline	62.7 ± 47.03	71.9 ± 40.02
Stage 3 RER		
Initial	0.88 ± 0.087	0.87 ± 0.073
Placebo	0.85 ± 0.079	0.89 ± 0.102
L-Citrulline	0.92 ± 0.081	0.88 ± 0.075[ac]
Stage 4 RPE		
Initial	14.6 ± 1.18	15.0 ± 1.07
Placebo	14.7 ± 0.89	15.4 ± 0.38
L-Citrulline	14.8 ± 0.42	15.1 ± 0.37
Stage 4 HR		
Initial	120.5 ± 19.93	105.5 ± 23.82
Placebo	119.7 ± 24.77	104.6 ± 9.38
L-Citrulline	120.4 ± 25.49	110.1 ± 19.32
Stage 4 Watts		
Initial	80.9 ± 49.42	76.9 ± 46.67
Placebo	87.0 ± 48.77	70.2 ± 48.19
L-Citrulline	80.1 ± 48.55	90.1 ± 50.00
Stage 4 RER		
Initial	0.93 ± 0.113	0.93 ± 0.061
Placebo	0.90 ± 0.083	0.90 ± 0.076
L-Citrulline	0.95 ± 0.082	0.97 ± 0.104[abc]

Values represent mean ± standard deviation.
[a]Significantly different pill x blood glucose classification interaction ($p < 0.05$).
[b]Significantly different time x pill interaction ($p < 0.05$).
[c]Significantly different three-way interaction ($p < 0.05$).

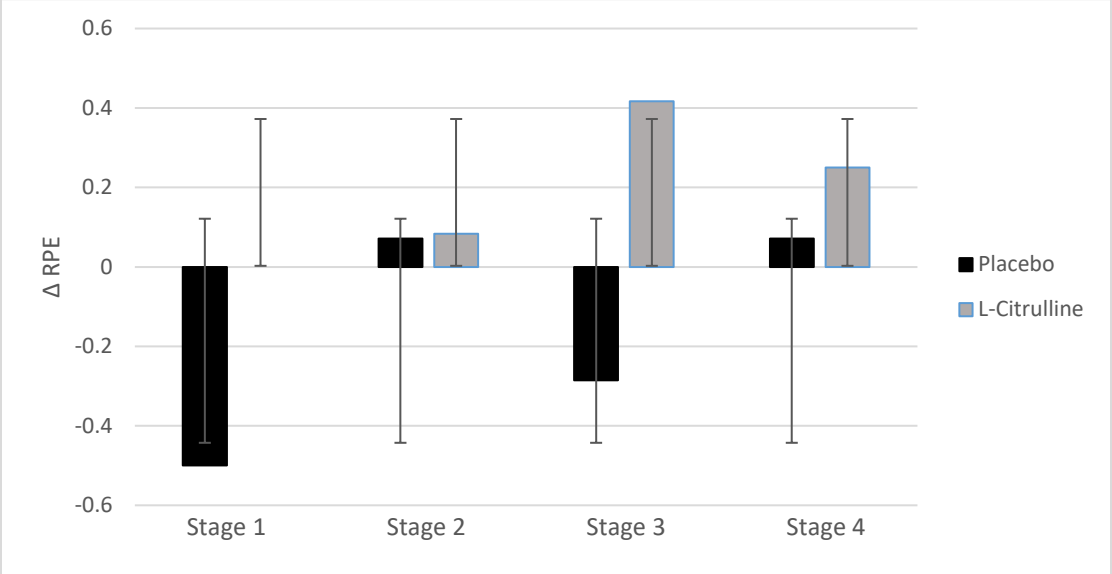


Figure 6. Changes in RPE over time in non-diabetic subjects for both the placebo and L-Citrulline at each stage.

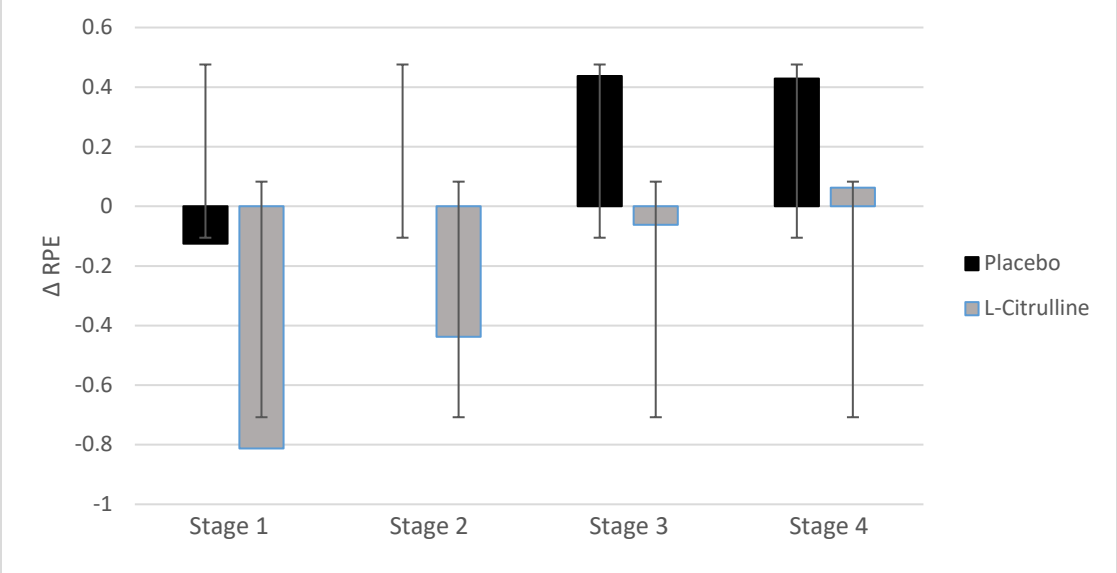


Figure 7. Changes in RPE over time in prediabetic subjects for both the placebo and L-Citrulline at each stage.

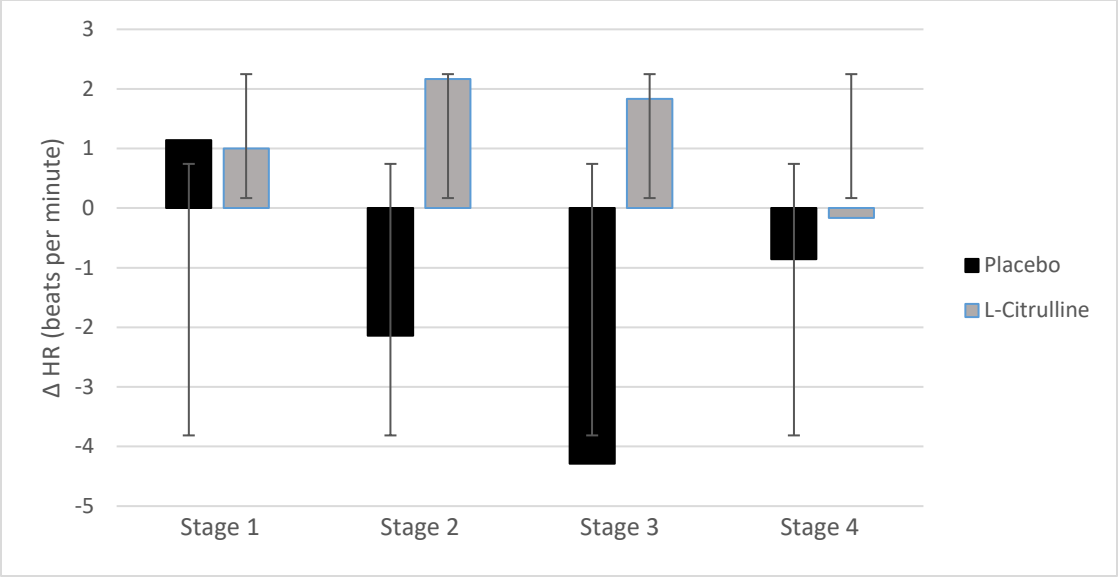


Figure 8. Changes in HR over time in non-diabetic subjects for both the placebo and L-Citrulline at each stage.

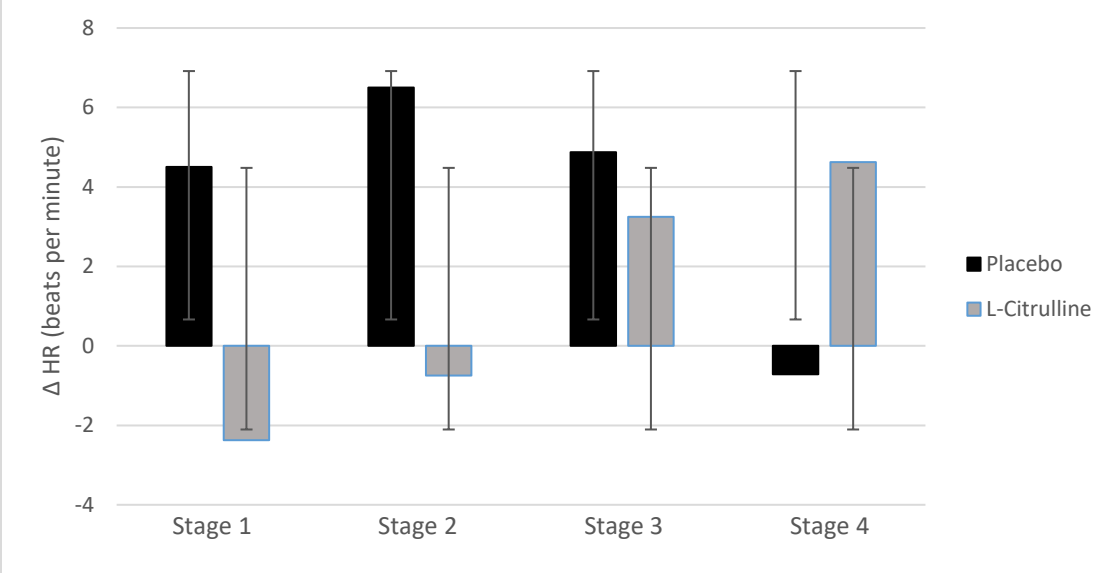


Figure 9. Changes in HR over time in prediabetic subjects for both the placebo and L-Citrulline at each stage.

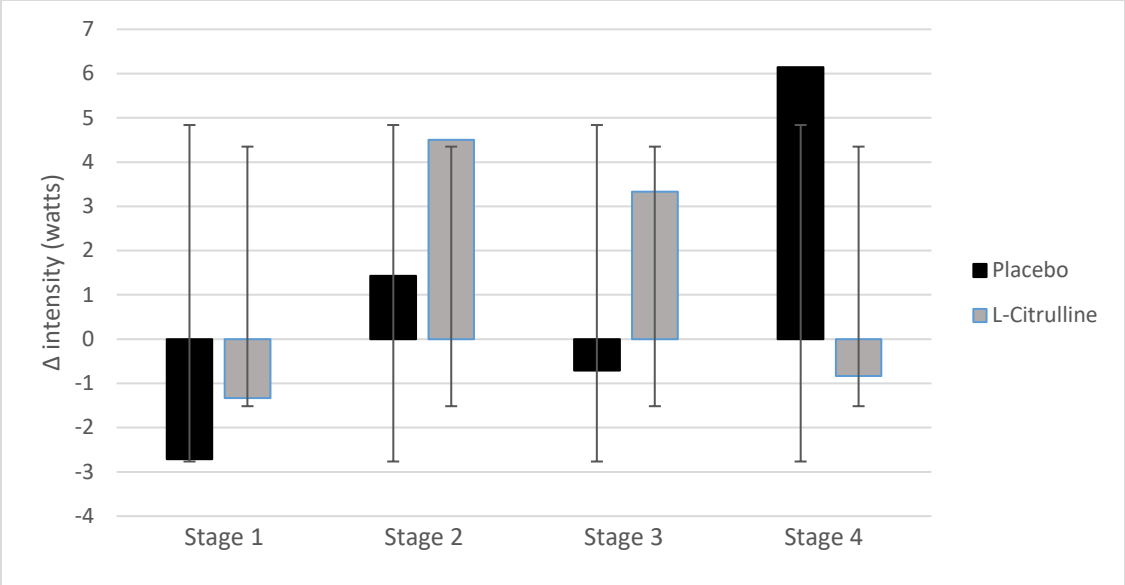


Figure 10. Changes in watts over time in non-diabetic subjects for both the placebo and L-Citrulline at each stage.

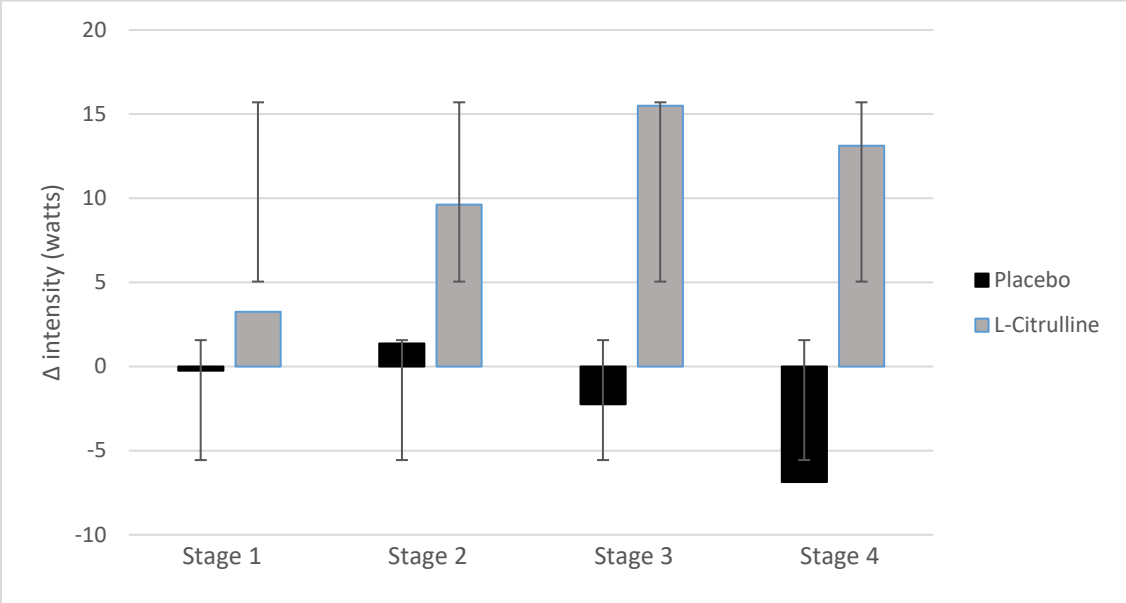


Figure 11. Changes in watts over time in prediabetic subjects for both the placebo and L-Citrulline at each stage.

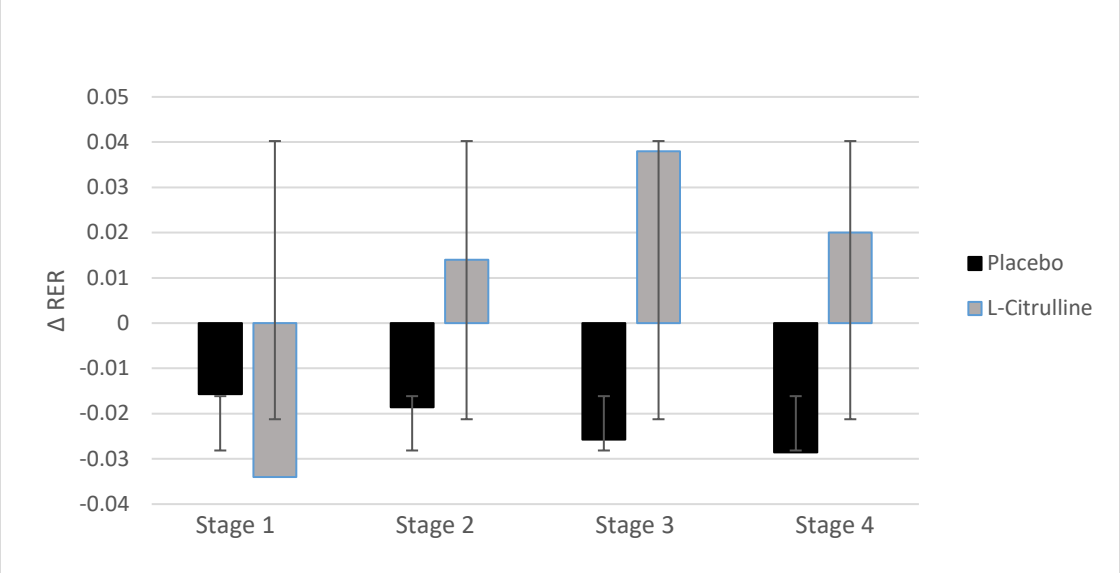


Figure 12. Changes in RER over time in non-diabetic subjects for both the placebo and L-Citrulline at each stage.

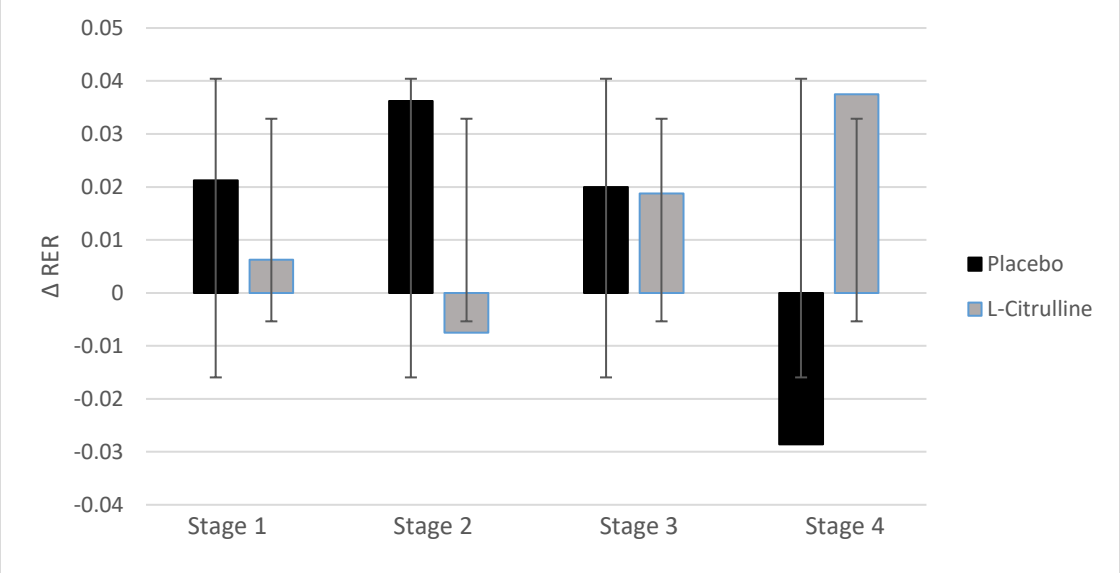


Figure 13. Changes in RER over time in prediabetic subjects for both the placebo and L-Citrulline at each stage.

There was no statistically significant interactions or main effects on RPE at any stage during the PRET (p>0.05). There was no statistically significant interactions or main effects on HR at any stage during the PRET (p>0.05). There was no statistically significant interactions or main effects on intensity at any stage during the PRET (p>0.05). There was no statistically significant interactions or main effects on RER at stage 1 during the PRET (p>0.05). Statistical significance was found for RER at Stage 2 for a three-way interaction (p=0.020) and pill X blood glucose classification interaction (p=0.020). Statistical significance was found for RER at Stage 3 for a three-way interaction (p=0.010) and pill X blood glucose classification interaction (p=0.010). Statistical significance was found for RER at Stage 4 for a time X pill interaction (p=0.007), a pill X blood glucose classification interaction (p=0.035), a three-way interaction (p=0.035), and a main effect for the pill (p=0.007).

DISCUSSION

This exploratory study investigates the effects of L-citrulline on prediabetic older adults. Using an occlusion protocol, SmO_2 characteristics were observed to have the expected changes, however they were non-significant. SmO_2 changes were also noted during the handgrip protocol with a visible difference between the second and third minute for prediabetics on L-citrulline. During the PRET, there is an increased intensity and decreased RPE while RER stays consistent in prediabetics on L-citrulline.

Since no current studies look at a prediabetic population, this exploratory study could precede other studies and assist in finding clinical application of L-citrulline on individuals with prediabetes. Prediabetics elevated glucose level create a hyperglycemic environment which could lead to endothelial dysfunction. In addition to potential endothelial dysfunction, many prediabetics are not medicated and 90% of prediabetics do not know they are prediabetic (CDC, 2017). These factors make prediabetes a significant risk factor for developing cardiovascular diseases later in life.

With initial, placebo, and L-citrulline supplementation result from the occlusion protocol, it is demonstrated that nondiabetic individuals have a non-significant lower minimum SmO2 and higher maximum SmO2 values than prediabetic individuals. These trends, while not significant, are also seen in previous research as a blunted SmO_2 response to occlusion in healthy older adults (Rosenbury et al., 2018), women with gestational diabetes (Dipla et al., 2017a), and hypertensive patients (Dipla et al., 2017b). These values offer insights of muscles ability to extract and utilize oxygen and the

microvasculature's response to stimuli. The lower minimum values demonstrated by non-diabetic individuals possibly show a greater ability to utilize oxygen by improving oxidative capacity and mitochondrial function. The maximum values potentially demonstrate a decreased microvasculature response in prediabetics. Those with prediabetes might have this delayed response due to a reduced release of vasodilators or an inability of the terminal arterioles to properly dilate (Dipla et al., 2017a).

During the hand-grip protocol, L-citrulline displays a decreased SmO_2 during the second and third minute in prediabetic individuals which could be due to improved oxygen utilization in the muscle and increased oxygen delivered to the muscle. The muscle contraction of the forearm during the hand grip protocol causes microvascular occlusion. The increased microvascular response observed during this protocol is likely due to improvements in NO synthesis from the L-citrulline supplement (Kim et al. 2015). Increased NO leads to increased vasodilation which allows for a greater reduction in oxygen saturation during exercise. This improved oxygen utilization will help to increase exercise tolerance. Previous research has demonstrated a greater decrease in SmO_2 in control groups because they were able to maintain the same intensity during a handgrip protocol (Dipla et al., 2017a). In another study of 18 healthy men, citrulline/malate demonstrated a reduction in the sensation of fatigue (Bendahan et al., 2002). Improved exercise tolerance will allow prediabetics to exercise longer or at a higher intensity. This will increase the benefits gained from exercise including increased cardiac output, increased muscle mass, improved body composition, increased strength, and improved overall cardiovascular health (Colberg et al., 2016).

During the PRET, prediabetic participants on L-citrulline demonstrated an increase in intensity and a decrease in perceived effort while RER only slightly shifted. This could be explained by a shift in primary fuel source during exercise. If this shift is present, long-term supplementation could potentially lead to increased lipolysis. This result would be similar to the animal studies that demonstrated increased lean mass, and decreased fat mass (Joffin et al., 2014a) along with decreased adipocyte diameter and increased visceral adipose tissue β-oxidation (Joffin et al., 2014b). Both groups on L-citrulline also see an overall increase in intensity. Like with L-citrulline, arginine supplementation has seen to decrease fat mass in both rat and pig studies (McKnight et al., 2010). One study with three weeks of arginine supplementation in humans demonstrated a larger decrease in fat mass and waist circumference compared to placebo. (Lucotti et al., 2006). Decreasing fat mass is beneficial to prediabetics in many ways including decreasing the chance of cardiovascular disease and progressing to diabetes (Colberg et al., 2016).

These later improvements might be seen due to enhanced muscle power output with an improved oxidative energy capacity (Bendahan et al., 2002) and improvements in skeletal muscle metabolism and contractile efficiency. (Bailey et al., 2015). Bailey (2015) noted an improvement in healthy adults after 7 days of supplements which could improve oxidative metabolism and exercise performance while showing an improved O_2 availability or distribution at the microvascular level. When given citrulline/malate, healthy adults had a 34% increase in oxidative ATP production during and saw a 20% improvement in phosphocreatine recovery following exercise (Bendahan et al., 2002.)

Several aspects limited the effectiveness of this study including supplementation period, population, and testing procedures. Having a two-week supplementation period only demonstrates acute effects. Increased duration of supplementation needs to be researched further to determine the presence and extent of chronic adaptations. Locating participants that qualified and were willing to complete all aspects of the testing proved difficult. This study ended with 16 participants completing the study and complying with supplementation out of 20 participants found and 24 participants desired. While conducting the PRET, some participants seemed to report RPE values associated with the stage instead of actual RPE. A set intensity or ramp protocol might be more beneficial in looking at L-citrulline's effects on fat oxidation.

Benefits from conducting similar studies focusing on one variable with a larger sample size could be applied to a clinical population. Further testing on the effects of L-citrulline on oxygen utilization in the muscle has the potential for practical application in a prediabetic population.

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APPENDIX A

INFORMED CONSENT FORM

1. **INFORMED CONSENT FOR "The effects of short-term L-citrulline supplementation on arterial and muscle-oxygen function in prediabetic adults"**

Principal Investigator: **Salvador Jaime, PhD**
UW-La Crosse
142 Mitchell Hall
La Crosse, WI 54601
(608)785-6518

2. I, _____, give my informed consent to participate in this study designed to determine the effectiveness of L-citrulline as a natural supplement to attenuate vascular dysfunction in prediabetic individuals. I have been informed that the study is under the overall direction of Salvador Jaime, Ph.D. who is an Assistant Professor in the Department of Exercise and Sport Science at the University of Wisconsin-La Crosse. I consent to the presentation, publication and other release of summary data from the study which is not individually identifiable.

3. I have been informed that my participation in this study will require 5 total visits to the human performance laboratory (Mitchell 225). Each visit to the lab requires fasting for at least 12 hours (food, alcohol, and caffeine), no strenuous exercise for at least 48 hours, and no morning vasoactive medication (I will bring to the lab for consumption following the tests). The subsequent measures will be taken before and after each treatment (L-citrulline and placebo) and are as follows:

    a. The first visit will involve the following:
        1) You will be given further detail regarding the nature of the study and then complete the written informed consent. Following the agreement, we will take your height and weight.
        2) Next, we will take a small amount of blood, from a finger-stick blood draw or venous blood draw from the antecubital vein, to evaluate HbA1c and plasma nitrate levels.
        3) Next, you will be asked to lie supine on an athletic training table for 10 minutes of silent rest. During this time, we will place four electrodes, two on your right hand and two on your right foot. A small and imperceptible electrical current will be sent through your body to measure body composition (amount of fat and fat free mass).
        4) Following 10 minutes of rest, blood pressure cuffs will be placed around your upper left thigh and arm. We will also use a pencil-like pressure sensor (tonometer) to measure the pulses in your neck area (carotid artery).
        5) Following the resting measures, we will place your hand in a bucket of ice water (4°C) for 3 minutes (cold pressor test). During the last minute, we will take your blood pressures of your arm and leg.
        6) After the cold pressor test, we will begin the muscle oxygenation test. The near-infrared spectroscopy device (Moxy) will be placed around your lower right thigh. This device will use light to measure the amount of oxygen in the muscle. In order to accurately depict its readings, we must completely devoid the muscle of oxygen. This is known as the deoxygenation protocol.
        7) The deoxygenation protocol will involve a blood pressure cuff wrapped around your upper right thigh. This cuff will be inflated to at least 50 mmHg above your normal systolic blood pressure. It will remain inflated for 5 minutes. Following instantaneous deflation, you will remain in the supine position for 3 minutes. The entire deoxygenation protocol will last 8 minutes.
        8) Lastly, you will undergo a submaximal exercise test on a cycle ergometer. This will require you to cycle at a self-selected resistance (keeping the revolutions per minute constant) for four 3-minute stages at a perceived exertion of 9, 11, 13, and 15 on the Borg RPE scale.
        9) **<u>This visit will last approximately 90 minutes.</u>**

b. The rest of the visits (4) will consist of everything in the first visit except for point 1. **Each of these visits will take approximately 75 minutes.**

c. Supplementation:
   1) After your second and fourth visit, you will receive an unlabeled bottle of capsules [L-citrulline (6g/day) or placebo (maltodextrin)].
   2) You will consume 8 capsules per day for 14 days. 4 capsules before breakfast, 4 capsules before bed.
   3) Following the 14-day period, your post-treatment visit will take place within 48-72 hours.
   4) Between your third and fourth visit, you will have 14 days with no capsule consumption. This is known as the wash-out period.

4. This study will involve submaximal cycling exercise. As such, it is possible that subjects will experience fatigue and muscle soreness. If at any time you should feel overly fatigued or sore due to your participation, please alert the investigators and you may discontinue participation. Due to the nature of the cold pressor and deoxygenation protocols, you may feel sensations of pain. This will subside as soon as the stimulus is removed with no lasting effects.

5. You will receive information regarding your current cardiovascular health and physical fitness levels (i.e. maximal oxygen consumption and ventilatory threshold). You will also receive $50 in monetary compensation following the completion of the study.

6. I have been informed that the investigator will answer questions regarding the procedures throughout the course of the study.

7. I have been informed that I am free to decline to participate or to withdraw from the study at any time without penalty.

8. Concerns about any aspects of this study may be referred to Dr. Salvador Jaime at (608)785-6518. Questions about the protection of human subjects may be addressed to the Chair of the UW-L Institutional Review Board (608)785-6892.

_____ _____
Signature of Participant Date

_____
Printed Name of Participant

_____ _____
Signature of Witness Date

APPENDIX B

DATA COLLECTION FORMS

The effects of L-citrulline on vascular and skeletal muscle function in prediabetic older adults

Subject Number: _____ **Visit #:_____** **Date: _____**

Have you been fasting for 12 hours?	Yes	No	(Circle One)
Did you take your medications today?	Yes	No	(Circle One)
Have you consumed caffeine in the past 12 hours?	Yes	No	(Circle One)

Baseline Measurements

DOB:	Age (years):	Height (in):	Weight (lbs):
_____	_____	_____	_____
Waist Circ (cm):	Hip Circ (cm):	Marker (cm):	HbA1C:
_____	_____	_____	_____

Hand Grip Test

MVC 1:	MVC 2:	MVC 3:	35% MVC:
_____	_____	_____	_____

BIA

Resistance:	Reactance:	Impedance:	Phase Angle:
_____	_____	_____	_____

Resting PWA/PWV

SC to carotid(mm):	SC to cuff (mm):	Femoral to cuff (mm):	Resting BP (mmHg):
_____	_____	_____	_____

CPT BP: _____

Skinfold (mm): _____

MOXY	Time
Power On	
Start 9 min timer (CLICK)	
At 1 min start occlusion	
At 6 min end occlusion	
At 9 min (CLICK):	
Grip test start (CLICK)	
At 3 min (CLICK)	

Minute	Goal RPE	Actual RPE	Heart Rate	RER	Resistance (W)
1:00	9				25 W
2:00	9				
3:00	9				*Increase*
4:00	11				
5:00	11				
6:00	11				*Increase*
7:00	13				
8:00	13				
9:00	13				*Increase*
10:00	15				
11:00	15				
12:00	15				Ending Watts:

APPENDIX C

BORG RPE SCALE

Rating	Perceived Exertion
6	No exertion
7	Extremely light
8	
9	Very light
10	
11	Light
12	
13	Somewhat hard
14	
15	Hard
16	
17	Very hard
18	
19	Extremely hard
20	Maximal exertion

APPENDIX D

REVIEW OF LITERATURE

Background Information

Throughout history there have been breakthroughs in life, medicine, and aseptic technique. With each of these changes to lifestyle there has been an increase in longevity. However, with this increased longevity there is also an increased risk for cardiovascular disease (CVD) and diabetes. Ortman et al. (2014) reported that 13.7 percent of the population is beyond the age of 65. This is followed up by the prediction of 20.3 percent of the population in this category by the year 2030. According to Mozaffarian et al. (2015) we see that 32.6 percent or roughly 80 million U.S. adults have hypertension. Also shown in this study were death rates from CVD including that deaths related to CVD have decreased by 15.5 percent, but it still accounts for 31.3 percent of all deaths in the United States. Another important aspect of CVD is cost of operation and procedures which cost over 320 billion dollars in 2011.

Along with CVD, diabetes is becoming more prevalent in the modern world. Mozaffarian (2015) shows that 1 in 10 U.S. adults have diabetes mellitus, over 90 percent being type 2 diabetes. Diabetes affected 382 million people as of 2013 and is projected to affect over 590 million people by the year 2035 (Guariguata et al., 2014). Another study estimated a worldwide prevalence of diabetes at 2.8 percent in 2000 and projects that 4.4% of people will suffer diabetes in the year 2030 (Wild et al., 2004). The growing amount of diabetes will cause a strain on healthcare. According the American Diabetes Association we see that the estimate cost of diabetes is 327 billion dollars. This study also estimates that those with diabetes cost approximately 2.3 times more than if they didn't have diabetes. Before an individual becomes diabetic, they become prediabetic. It is estimate that almost 81 million or over one-third of adults have prediabetes (Mozaffarian

et al., 2015). Ligthart et al. (2016) found that from the age of 45 individuals that were diagnosed with prediabetes had a 74 percent lifetime risk of progressing to diabetes. This is projected to increase to over 91 million U.S. adults by 2025. In the state of Mississippi, the amount of prediabetic adults are projected to increase from roughly 757,000 to 782,000 by 2025 (Rowley & Bezold, 2012).

These results illustrate the severity of CVD and diabetes and highlight the importance of finding ways to prevent this affliction. Any improvement in preventing CVD could go a long way towards decreasing death, cost of healthcare, and improving quality of life in an aging or diabetic population.

Effects of Aging

Given the aforementioned rates of deaths caused by CVD, the cardiovascular system remains of major clinical importance in the aging population. Rosenbury et al. (2018) demonstrated that microvascular function was lower in an elder population when compared to a younger population with a 5-minute occlusion protocol. Aging arteries are elongated, have an enlarged lumen, and a thickened wall (Ferrari, Radaelli, & Centola, 2003). Artery intimae have more substances present that can damage vessels including adhesion molecules, matrix metalloproteinases, and proinflammatory cytokines as they age (Ferrari, Radaelli, & Centola, 2003). Further evidence of this can be displays in studies by Loo et al. (2000) demonstrating reduced bioavailability of endothelium-derived nitric oxide (NO) and Donato et al. (2009) whom reported an increase in the bioactivity of the constricting factor endothelin-1. Another study by van der Heijden-Spek et al. (2000) shows that aortic distensibility decreases with age and no difference was noted in muscular

brachial arteries. It is also shown that both arterial stiffness and wave reflections are increased as people age regardless of the presence of atherosclerosis (Mitchell et al., 2004). These studies all help to illustrate the idea that as the body ages the cardiovascular system progressively deteriorates. While changes to the larger vessels are important, there can also be dysfunction at the microvascular level. The most prevalent impairment is endothelial dysfunction, which is improper function of the endothelium due to an imbalance between vasoconstrictor and vasodilator substances (Kalani, 2008). Endothelial dysfunction consists of changes in the endothelium, including impaired vasodilation, increased endothelial products in the plasma, inflammatory activation, and angiogenesis and barrier function (Bakker et al., 2009 Another key impairment from endothelial dysfunction is the compromise of a specific endothelial activation stage that favors atherogenesis (Anderson et al., 1999). Arterioles, capillaries, and venules make up the microvascular system where the oxygen is delivered to the tissue.

A valid non-invasive technique to measure skeletal muscle oxygenation and subsequently microvascular function is using non-infrared spectroscopy (NIRS). NIRS works by measuring the relative change in oxygenated and deoxygenated hemoglobin (Jones et al., 2016). These measurements allow the oxygen saturation of skeletal muscle as well as blood flow to be determined in a dynamic environment. Some limitations that the NIRS device has are the unknown contribution of myoglobin, skin perfusion and pigmentation during exercise, and can be impacted by adipose tissue over the targeted muscle. NIRS has been shown to be a valid and reliable measurement tool compared to phosphorus magnetic resonance spectroscopy (Ryan et al., 2013) as well as between NIRS devices (Crum et al., 2017; McManus et al., 2018). Jones (2016) stated that NIRS has good

agreement with the clinical gold standard techniques. NIRS has been used to continuously monitor skeletal muscle oxygenation and therefore microvascular function (Dipla et al., 2017).

Effects of Diabetes

Diabetes is measured as having a fasting blood glucose of over 125 mg/dL, a hemoglobin A1C (HbA1C) of over 6.5%, or a plasma glucose of over 200 mg/dL and has many problems associated with it. HbA1C is the percentage of glycated hemoglobin in the blood. HbA1C gives a measure of blood glucose over the lifetime of a blood cell (about 90 days), making it a better predictor of average blood glucose. Some of the more common problem known with diabetes include a higher risk for CVD, nephropathy, retinopathy, depression, neuropathy, and complication from neuropathy. There are numerous studies that have observed the link between elevated glucose levels and CVD risk factors and mortality shown in a meta-analysis by the Emerging Risk Factors Collaboration (2010). Along with CVD risk factors, some studies have shown that arterial stiffness is increased in those with diabetes (Cameron et al., 2003), while other studies have shown no correlation between the two (Gomez-Sanchez et al., 2017).

Diabetes impairs the ability of skeletal muscle to metabolize both glucose and fatty acids due to dysregulation of oxidation. Mitochondria were also measured to be smaller in individuals with diabetes compared to a healthy population. These factors show impaired bioenergetic capacities of mitochondria from diabetes and obesity (Kelley et al., 2002). Changes from diabetes play a large part in the cardiovascular system and the capacity of skeletal muscle to properly preform. Diabetes, as well as prediabetes can

increase the risk of endothelial dysfunction by effecting how endothelin-1 works. As blood glucose increases endothelium-dependent vasodilation the skins microvasculature decreases (Caballero et al., 1999).

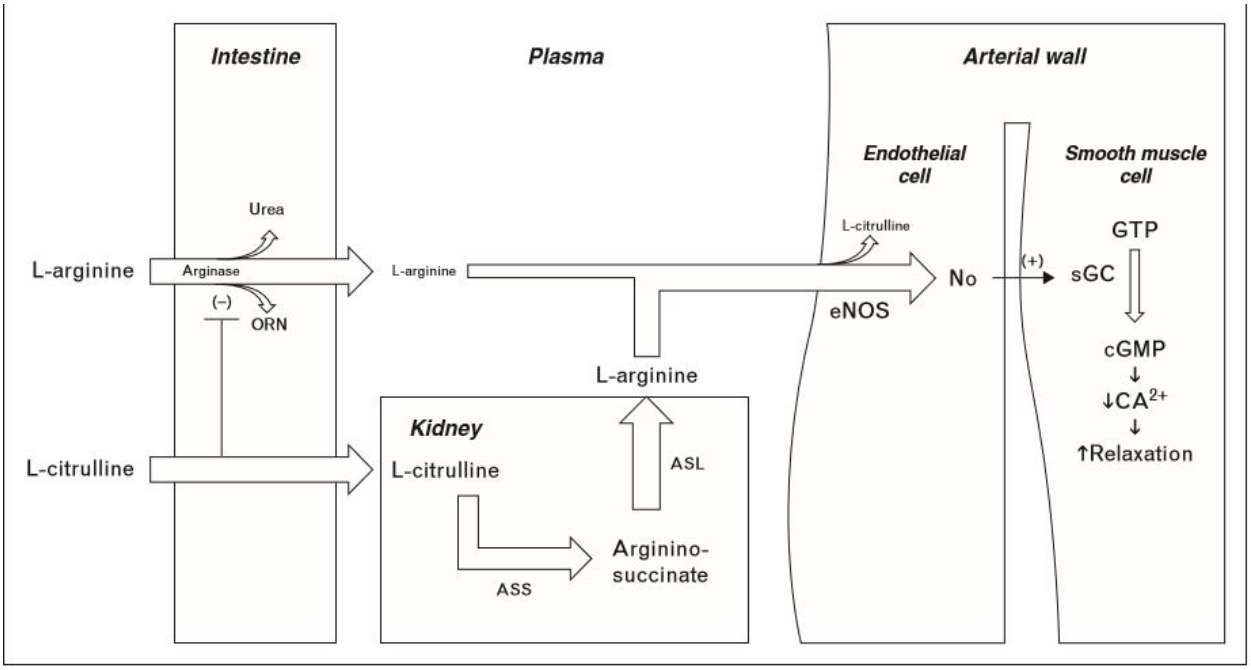
Prediabetes is measured as having a fasting blood glucose of 100 mg/dL to 125 mg/dL, an HbA1c ranging from 5.7-6.5%, or a plasma glucose between 140 mg/dL and 199 mg/dL. Even though prediabetes is more common that diabetes and still has health consequences it is not studied as much as diabetes. The minimal amount of research on L-citrulline on a prediabetic population makes it an ideal research population. Hyperglycemia effects the vasodilatory ability of the microvasculature by increasing the release of endothelin-1 and decreasing the release of NO (Bakker et al., 2009)

L-citrulline

L-citrulline is a non-essential amino acid which can be found in watermelon. Until recently, L-citrulline was viewed as unimportant because it isn't used for protein synthesis (Breuillard, Cynober, & Moinard, 2015). Within the plasma, the L-arginine-NO pathway works by having eNOS (endothelial nitric oxide synthase) transform L-arginine into L-citrulline and NO (Le Roux et al., 2017). Knowledge of this pathway lead to an increase in studies looking the effects of L-arginine on NO production (Lerman et al., 1998; Hambrecht et al., 2000; Siani et al., 2000). Now, because L-citrulline is a precursor of L-arginine and nitric oxide (NO) bioavailability in the body it is starting to be studied more. More recently, it has been demonstrated that oral L-citrulline increases the amount of L-arginine in both tissue (Wijnands et al., 2012) and in the vascular system (Schwedhelm et al., 20008; Kuhn, 2002). Unlike L-arginine, L-citrulline can pass through

the intestines without being catabolized making it to the kidneys where it can be efficiently recycled into L-arginine (Figure 2). L-citrulline is viewed as a more effective oral supplementation because it is able to bypass liver uptake and degradation (Figueroa, Wong, Jaime, and Gonzales, 2017). After L-citrulline bypasses the liver and intestines it reaches the kidneys where it is then converted to L-arginine and NO.

Figure 2.



L-citrulline has been shown to improve cardiovascular markers in multiple studies. Use of acute L-citrulline has demonstrated a 10-fold increase in NO synthesis with only 3 grams (Kim et al. 2015). With L-citrulline working by affecting NO bioavailability it is important to look at how NO functions within the vessel. L-citrulline decreased resting diastolic blood pressure and blood flow responses to exercise were improved in men. L-citrulline supplementation also increased femoral blood flow and vascular conductance during lower-limb exercise in men (Gonzales et al., 2017).

NO functions as a potent vasodilator to control vascular tone (Vanhoutte, Zhao, Xu, & Leung, 2016). This article discusses how endothelial dysfunction is caused when the ability to produce NO is blunted and reduced NO-mediated responses start the atherosclerotic process. When this article mentions sources for NO, nitrates, nitrites, and the substrate L-arginine are listed. Dipla et al. (2017) explained how endothelial dysfunction has been associated with a higher risk for cardiovascular disease in patients with diabetes. This showed that the hyperglycemic environment promotes micro- and macrovascular dysfunction. Diabetes likely reflects a reduced release of vasodilators from the vascular endothelium, which leads to an inability of small vessels to dilate in response to stimuli and/or capillary needs. L-citrulline's increase in NO also has a vasodilatory effect on the arterioles and capillaries increasing blood flow and oxygen to skeletal muscle. Le Roux (2017) demonstrated a larger response in total hemoglobin and hemoglobin oxygen saturation following occlusion for those taking L-citrulline supplementation. This suggest an increase in vasodilation following tissue ischemia, potentially due to an increased availability of NO. L-citrulline supplementation has been shown to decrease VO2 mean response time, improve severe-intensity exercise tolerance, and increased the total amount of work completed during exercise (Bailey et al., 2015).

L-citrulline has also been studied in relation to muscle mass. Breuillard (2015) discusses how muscle protein synthesis was restored in rats with L-Citrulline supplementation. It was also noted that the muscle protein synthesis rates were preserved in female rats during food restricted periods. Other areas that are benefited by L-citrulline are reducing fatty infiltration in the muscle and decreasing overall fat-mass (Joffin et al., 2014). L-citrulline has also demonstrated enhanced muscle power output with an

improved oxidative energy capacity (Bendahan et al., 2002). This could be due to improvement in skeletal muscle metabolism and contractile efficiency. (Bailey et al., 2015).

Lipolysis

Obesity is a risk factor for CVD, diabetes, and can cause earlier mortality. Mozaffarian et al. (2015) discovered that obesity predisposes individuals to the most risk factors and is among the leading causes of death and disability in the United States. Decreasing fat mass and maintaining or increasing lean mass can help the body better store glucose and decrease blood sugar. Numerous research studies have been conducted on the vasoactive effects of L-citrulline as an effective way to increase NO bioavailability. Recent studies have discovered some additional functions of L-citrulline including improving insulin sensitivity, increasing lean mass, and decreasing fat mass (Moinard et al., 2015, Joffin et al., 2014a, Joffin et al., 2014b). An early study by Osowska et al. (2016) showed an increase in protein synthesis of old malnourished rats. Jourdan et al. (2015) reports an anabolic muscle protein synthesis action in adults who are on a low-protein diet while taking L-citrulline. This is noteworthy because this is reversed from the normal impaired protein metabolism measured in an elderly population (Felgines et al., 1999). L-citrulline supplementation has also demonstrated the ability to significantly decrease adipocyte diameter and increase fatty acid β-oxidation from visceral adipose tissue in rats of various ages (Joffin et al., 2014b). While these studies have only been demonstrated in rats, there is reason to believe the same mechanism can be seen in humans.

L-citrulline has demonstrated a potential shift in muscle energy metabolism in rats. In a study of healthy rats, Moinard et al. (2013) shows that L-citrulline supplementation increased lean body mass by 9 percent and decreased fat mass 13 percent. L-citrulline had shown an increase in oxidation of lipoproteins in those rats. Several studies have looked the catabolism of carbohydrates and fats during exercise to find the appropriate exercise intensity for maximal fat oxidation. Maintaining peak fat oxidation would be an additional benefit of L-citrulline, helping to maintain a healthy body mass. The goal in this study is to determine if the effects of L-citrulline change the intensity or duration of oxidizing fat as the primary fuel source. This idea come from a study showing 66 percent of variation in between oxidation of fat and aerobic training is unaccounted for. It is believed that a large portion of this relationship could be explained by diet (Jeukendrup & Wallis, 2005.). Fat oxidation can be measured by the respiratory exchange ratio (RER) during exercise (Daniel, C.G. 2009). RER is the ratio of produced carbon dioxide (CO_2) to consumed oxygen (O_2). Stoichiometry shows that fat has a ratio of 16:23 or about 0.70 whereas carbohydrates have a ratio of 6:6 or 1.0. When intensity increases enough the RER will go above 1.0 due to an increased production of CO_2 from the bicarbonate buffering system. This study by Daniel (2009) shows that maximal fat oxidation occurred at 54.2 percent of maximal oxygen consumption (VO_{2max}).

Conclusion

The purpose of this study is to look at the effects of short-term L-citrulline supplementation on microvasculature, skeletal muscle oxygenation, and lipolysis in prediabetic older adults. The hypothesis is that there will be an increased response of

oxygen saturation following the release of occlusion and an increase in lipolysis during exercise while taking L-citrulline supplementation.

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