Effects of L-citrulline supplementation on nitric oxide and antioxidant markers after high-intensity interval exercise in young men: a randomized controlled trial

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Abstract

L-citrulline (L-Cit) is a nonessential amino acid that stimulates nitric oxide (NO) production and improves exercise performance by reducing muscle damage indices; however, the direct benefits of L-Cit on antioxidant markers are unclear. The aim of this study was to examine antioxidant responses to high-intensity interval exercise following acute L-Cit supplementation. Nine young men (21 ± 1 years) participated in a double-blind crossover study in which they received 12 g of L-Cit and placebo (PL) an hour prior to high-intensity interval exercise on two occasions, separated by a seven-day washout period. Blood samples were obtained before (PRE), immediately after (IP), 10 (10P), and 30 min after exercise (30P) from the cubital vein using standard procedures. Serum concentrations of superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT), and NO metabolites (NOx) were measured. The exercise protocol significantly elevated SOD (p = 0.01) and GPx (p = 0.048) from PRE to 10P in the L-Cit group with greater changes than the PL group. CAT concentrations increased IP (p = 0.014) and remained elevated at 10P (p = 0.03) and 30P (p = 0.015) in both the L-Cit and PL conditions. NOx concentrations increased IP (p = 0.05) in the L-Cit group with greater changes than PL group in PRE to IP, PRE to 10P, and PRE to 30P (p < 0.05). Our data indicate that L-Cit supplementation (single 12 g dose pre-exercise) induces improvements in antioxidant markers following a session of high-intensity interval exercise in young men.

**Keywords:** L-citrulline, Nitric oxide, Arginine, Defense system
Introduction

Physiological stress, such as exercise, leads to oxidative stress that is associated with reactive oxygen species (ROS) and free radicals\(^1\). Oxidative stress occurs as the cellular generation of pro-oxidants exceeds the physiological capacity to eliminate them, which involves the endogenous antioxidant mechanism as well as exogenous antioxidants obtained via diet\(^2\). Evidence suggests that nutritional supplements containing antioxidants including glutathione, vitamins E, A, and C, proteins, and taurine are safe for human consumption (rare side effects) and can have a positive influence on exercise performance and the reduction of exercise-related oxidative stress\(^3; 4; 5; 6; 7; 8; 9; 10; 11\). Thus, the assessment of dietary compounds with antioxidant properties is a vital line of research for the development of different nutritional interventions to help diminish ROS production and oxidative stress after exercise.

L-citrulline is a non-protein and non-essential amino acid with antioxidant properties\(^12\) that is well-known as the precursor of l-arginine in the urea cycle\(^13\). Dietary L-citrulline enters the kidneys, vascular endothelial cells, and other tissues which leads to a rise in plasma and tissue l-arginine levels. L-arginine is the substrate for endothelial production of nitric oxide (NO), a strong vasodilator\(^13; 14; 15\). Animal research and tentative human evidence also show L-citrulline as an anabolic pharmaconutrient that has a beneficial impact on protein synthesis; it is thought that its contribution is mediated by the mechanistic target of rapamycin (mTOR) pathway\(^13; 14; 16; 17\). Recently, Gonzalez et al.\(^10\) indicated an increase in athletic performance and recovery with a decrease in muscle damage following L-citrulline supplementation. Moreover, Stanelle et al.\(^13\) reported that L-citrulline supplementation might lead to a slight improvement in the cycling performance of trained athletes. It was also shown that, due to exercise-induced muscle damage, L-citrulline supplementation can decrease serum creatine kinase concentrations along with increasing perceived recovery\(^13; 18\). According to prior research, L-citrulline supplementation reduces concentrations of lactate and muscle pain 24 hours after exercise\(^13; 16\), which might be related to its antioxidant effects. Furthermore, it has been previously reported L-citrulline supplementation efficiently increases NO metabolites\(^19; 20\). However, limited studies have found whether acute ingestion of L-citrulline alters the response of antioxidant markers after a single bout of exercise\(^21\). Therefore, the purpose of this study was to evaluate the effect of 12 g L-citrulline supplementation on antioxidant markers after acute high-intensity interval exercise in
young men. We hypothesized that acute L-citrulline supplementation would enhance our primary outcome of blood antioxidant markers and secondary outcome of NO metabolites after a session of high-intensity interval exercise.

Experimental Methods

Participants
Nine trained young men (see participant’s characteristics in table 1) from the University of Guilan were recruited for this study. The participants were familiar with resistance and endurance exercises and trained 5 times per week (≥90 min per session) for at least 6 months prior to the present study. Inclusion criteria were; 1) healthy and free of any injury 2) no history of surgery and musculoskeletal disorders for two years prior to the study 3) no history of ergogenic aids, supplements, and drug/medication use for at least 6 months before the study. All participants were made aware of the research procedures and gave written consent ahead of data collection. This study was authorized by the ethical review board and the university research council (IR.GUMS. RES1397/428), registered at the Iranian Registry of Clinical Trials (IRCT20210323050758N1) and conducted in the Exercise Physiology Laboratory of the University of Guilan in accordance with the Declaration of Helsinki II.

Study design
This study was performed using a random sampling, double-blind, cross-over, and placebo-controlled design. Overall, participants visited the laboratory three times. Anthropometrics (height, body mass, and body fat percentage) were assessed during an initial familiarization visit for participants, which included a review of study procedures and exercise techniques. In the second visit (a week following the first visit), the participants were randomly allocated into either an acute L-citrulline (12g) or placebo (PL) supplementation trials that were subsequently completed. After the second visit, there was a one-week washout period before the third visit, in which participants performed the other trial (cross-over approach, Figure 1). Blood samples were obtained from the brachial vein at baseline (PRE, 15 min before exercise), immediately after exercise (IP), at 10 (10P) and 30 minutes (30P) post-exercise to assess serum concentrations of the antioxidant markers including superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT), and nitric oxide (NO). The participants were asked to refrain from any exercise
for at least 48 hours, and to avoid caffeinated beverages at least 12 hours prior to each visit. The study schematics are illustrated in Figure 1.

Procedures

Randomization and blinding
Randomization was carried out by utilizing a random number table; for this, an independent coordinator, not otherwise involved in the study, created the allocation sequence assigning participants to the L-citrulline or PL trials. The independent coordinator also provided and witnessed supplement consumption. Both the participants and research team members were blinded to the treatment allocation until the database was unlocked and data analysis was completed.

Anthropometric measures
Height was measured using a wall-mounted stadiometer with an accuracy of 0.1 cm (Seca 222, Terre Haute, IN). Body mass was measured using a scale (Camry, FB9003, Japan) with a precision of 0.1 kg. Body fat percentage was acquired from the skinfold thickness of 3 different points (i.e., pectoral, quadriceps, and abdominal) on the right side of the body using a caliper (Lafayette caliper, model 01128, USA). All measurements were calculated based on Jackson Pollock’s body fat percentage equations\(^{(22)}\).

L-citrulline and placebo supplementations
One hour before exercise, the participants consumed either 12g of L-citrulline powder dissolved in 200 ml of water (L-citrulline 1200 mg, NOW® Sports, IL, USA) or PL (12g of maltodextrin) with the same appearance, taste, smell, and color). The selected dose was based on previous studies that showed increases in plasma L-citrulline and NO metabolites levels without the negative effects of saturation\(^{(21; 23)}\). We used a one-week washout period between acute L-citrulline and PL supplementations, which was based on a previous study\(^{(14)}\).\)

Acute high-intensity interval exercise
Before initiation of the exercise protocol, the participants performed a 10-min warm-up, which consisted of 5 min cycling (with the minimal workload, 50-60% of maximum heart rate) and 5 min dynamic stretching (total body) exercise. Thereafter, participants performed 12 consecutive rounds of the 2-hand kettlebell swing exercise including (30 seconds of exercise and 30 seconds
of rest) using a 16-kg kettlebell (Wirecutter, NY, USA); a weight that has been shown to induce significant metabolic and cardiorespiratory stress and seems to be standardized in prior kettlebell exercise research\(^{(24)}\). The swing exercises were performed at a rate of 1-second eccentric/ 1-second concentric phase (15 swings per round) and were controlled by using a metronome\(^{(22)}\) . The participants were verbally encouraged, and feedback was given on their performance by an experienced trainer.

**Blood sampling and analysis**

Blood samples (8 ml) were obtained from the brachial vein in a seated position, then evacuated in test venoject tubes. The blood was clotted at room temperature for 30 min and centrifuged at 1500\(\times\)g for 10 min. To further analyze, the serum layer was removed and frozen in various aliquots at -70\(^{\circ}\)C. The serum concentrations of SOD, GPx, CAT, and NO metabolites (NO\(_x\)) (circulating levels of nitrite + nitrate) were measured by existing commercial enzyme-linked immunosorbent assays (ELIZA) kits (Zellbio GmbH Veltliner, Ulm, Germany). The intra- and inter-assay coefficient of variation for all blood measurements were <7\%.

**Statistical analysis**

All participants who completed the study were included in the data analysis. Statistical analysis was conducted using SPSS 24 for Windows (SPSS, Inc, Chicago, IL, USA). Estimation of an appropriate sample size was conducted using the G*Power analysis software\(^{(25)}\). Our rationale for sample size was based on a previous study evaluating changes in blood NO\(_x\) responses to high-intensity exercise after acute L-citrulline supplementation in 9 young men\(^{(18;26)}\). This study revealed an effect size of 0.4 for the increase in NO\(_x\) after high-intensity exercise. A total sample size of 9 participants was determined with an effect size of 0.4, and 80\% power at the predetermined level of \(\alpha=0.05\). It was relevant to have sufficient power to detect changes in NO\(_x\) as an enhanced NO bioavailability leads to increases in blood flow, which in turn eliminates metabolites such as H\(^+\) and free radicals from muscle tissue and may improve antioxidant production\(^{(12)}\). Data were expressed as mean ± standard deviation (SD). The normality of data was examined and confirmed using the Shapiro-Wilk test. For analysis of SOD, CAT, GPx, and NO data, repeated measures ANOVA (2 \(\times\) 4, group \(\times\) time) was used (SPSS 21.0). Bonferroni post hoc test was used to change the different measurement steps after a significant F value. In each variable, for one percent of the difference, the difference between the basic time and all
subsequent time points was calculated. An independent t-test was used between groups and the standard trapezoidal method to the area under the curve (AUC) was assessed for dependent variables. Significance levels were considered at p ≤ 0.05.

Results
Between December 6 and 23 of 2018, we screened 12 trained young men. Of these, 9 qualified for baseline evaluation and were subsequently randomized to either the L-citrulline or PL trials. After randomization, no participants dropped out of the study, and no harm or unintended effects were reported. Data are presented for the 9 participants that successfully completed our study protocol (Figure 2). At baseline concentrations, no significant differences were observed in the dependent variables between the two conditions (i.e., SOD, GPx, CAT, and NO) (p > 0.05).

There was no significant interaction for group × time for SOD (F = 1.02, p = 0.39). Only the L-Citrulline showed a significant increase in SOD from PRE to 10P (p = 0.01). The AUC analysis for SOD did not show a significant difference (p = 0.13) between the conditions. Due to the % change in SOD, the L-Citrulline showed more changes than PL group in PRE to 10P [(p = 0.042), ~29%, 15.3 u/ml, 95% CI (-0.284, 30.882)] (Figure 3).

No significant interaction was noted for group × time for GPx (F = 0.24, p = 0.86). The L-Citrulline group showed a slight [3%, 95% CI (-22.449, 28.989)] but significant increases in GPx at 10P compared to PRE (p = 0.048). The AUC analysis for GPx showed no noticeable difference (p = 0.58) between groups. Based on % change in GPx, the L-Citrulline group indicated higher changes than PL group in PRE to 10P (p = 0.05) ((Figure 4)).

No significant interaction was seen for group × time for CAT (F = 0.24, p = 0.86). Both the L-Citrulline and PL conditions showed a significant increase in CAT at IP (p = 0.014) and did not show any significant difference (p = 0.69) between the conditions. Due to the % change in CAT, the PL revealed more changes than the L-Citrulline group in PRE to IP [(p = 0.023), ~33%, 0.84 u/ml, 95% CI (-0.107, 1.787)] and PRE to 10P [(p = 0.036), ~45%, 1.1 u/ml, 95% CI (-0.005, 2.205)]. In contrast, the L-Citrulline showed more changes than PL group in IP to 30P (p = 0.041) and 10P to 30P (p = 0.039) ((Figure 5)).

There was no noticeable interaction for group × time for NO (F = 1.8, p = 0.17). The L-Citrulline group showed a small [~4%, 0.44 umol/l, 95% CI (-0.553 to 1.433)] but significant increase in NO at IP compared to PRE (p = 0.05). The AUC analysis for NO showed a significant difference (p = 0.041) between the conditions. Based on the % change in NO, the L-Citrulline group
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revealed higher changes than PL group in PRE to IP [(p = 0.013), ~4.5%, 0.48 umol/l, 95% CI (-0.625, 1.584)], PRE to 10P [(p = 0.04), ~4%, 0.44 umol/l, 95% CI (-0.454, 1.334)] and PRE to 30P [(p = 0.042), ~4%, 0.41 umol/l, 95% CI (-0.288, 1.108)] (Figure 6).

Discussion

The major findings of our study are that L-citrulline supplementation induced significant changes to antioxidant markers in response to an acute bout of high-intensity interval exercise in young men. Our results were in line with our hypothesis that acute L-citrulline supplementation (12g) an hour prior to high-intensity interval exercise would significantly increase antioxidant markers compared to the PL condition. To the best of our knowledge, this is the first study to investigate the effects of L-citrulline supplementation on antioxidant markers following acute high-intensity interval exercise in humans.

The effects of L-citrulline supplementation on the antioxidant and NO response to exercise

Higher concentrations of L-arginine are transported into the cell during physical activity to sustain adequate amounts of this amino acid and allow for its optimum coupling with inducible NO synthase (iNOS). As a result of the ability to restrict NO activity in the absence of sufficient L-arginine, iNOS will primarily use O₂ to form O₂⁻, possibly resulting in oxidative stress. Although the fact of the antioxidant properties of L-citrulline was first discovered in leaves of drought-tolerant wild watermelon plants(27), our current knowledge of the role of L-citrulline and iNOS in the regulation of skeletal muscle antioxidant defense mechanisms is insufficient, and further studies are needed(28). The primary aim of this research was to assess whether the L-citrulline supplementation would improve the antioxidant markers and NOx concentrations in response to high-intensity interval exercise in young men. In the current study, the findings showed increased serum concentrations of SOD, GPx, and CAT after exercise in the L-citrulline trial. In line with our results, da Silva et al.(2014)(29) indicated that taurine supplementation increases SOD, GPx, and CAT concentrations following eccentric exercise (29). In contrast, Lee et al. (2020)(30) showed no changes in SOD and GPx concentrations following four weeks of high-intensity exercise combined with aspirin consumption. It seems that disparities in exercise and trial duration (acute vs longitudinal), as well as supplementation type, are probable reasons for these inconsistencies (30). Antioxidant defensive systems are in control of scavenging ROS in
order to avoid oxidative stress. The main antioxidant molecules in the antioxidant defense are SOD, GPx, and CAT. SOD converts superoxide anions to less harmful hydrogen peroxide, which CAT and GPx then further eliminate. Anti-oxidative enzyme production is a response to ROS activities that commonly occurs in aerobic exercise\(^{31; 32}\). L-citrulline increases blood flow as well as enhances NO bioavailability, which in turn eliminates metabolites such as H\(^+\) and free radicals from muscle tissue and eventually improves anti-oxidative production\(^{12}\). Indeed, our findings suggest that L-citrulline supplementation before exercise can be an effective antioxidant agent that enhances SOD, GPx, and CAT through its antioxidant properties. In fact, L-citrulline has been shown to have a protective effect against ROS and oxidative stress, which can be attributed to its antioxidant capacity\(^{12}\). However, this area is relatively unexplored and further studies are needed to clarify the beneficial effects of L-citrulline supplementation on antioxidant enzyme activity following physical exercise.

Our results indicate that NOx concentrations increased significantly in the L-citrulline group after exercise. These findings are consistent with the hypothesis of the study as well as previous researchers who have reported significant improvements in NOx concentrations following L-citrulline supplementation \(^{33; 34}\). Also, our findings are in line with the results of studies by McKinley et al. (2015)\(^{35}\) and Fu et al. (2013)\(^{36}\). However, in another study by Hickner et al. (2015)\(^{37}\), L-citrulline supplementation following a graded exercise test did not cause any significant increase in NOx concentrations. The discrepancy in the type of exercise protocol and supplemental dose are probably reasons for this difference because participants consumed 3-g L-citrulline over 3 hours or 9-g L-citrulline over 24 hours prior to treadmill testing to exhaustion\(^{37}\). It has been shown that L-citrulline supplementation leads to an increase in L-arginine concentrations both at rest and during exercise in humans\(^{16; 37}\). L-arginine is the main substrate for NO synthesis as a blood flow modulator\(^{34}\). Indeed, it has been reported that L-citrulline supplementation not only may lead to an indirect increase in NO synthesis but also blood NO concentrations which rises the blood flow of active muscles\(^{34}\). Accordingly, L-citrulline supplementation is not only considered a precursor to L-arginine but is also a viable alternative to increasing plasma L-arginine concentrations for greater NO generation\(^{33}\), which is consistent with the findings of the present study. Additionally, L-citrulline may play an important role in preventing the oxidation of low-density lipoprotein, which leads to an improved endothelial dysfunction \(^{38; 39}\).
The effect of high-intensity interval exercise on antioxidant enzymes and NOx

It is clear that during high-intensity training, free radicals and ROS are continually produced by cells as part of metabolic activity, while an antioxidant defense system can neutralize both components\(^{40, 41}\). SOD and GPx are both vital for vascular tone and normal risk to cells. Throughout the exercise, SOD and GPx act in the cell as a progressive resistance to ROS generation and oxidative stress as a primary antioxidant defense mechanism against superoxide radicals\(^{40}\).

In this study, SOD concentrations did not show any significant change in the PL trial. It seems that high-intensity interval exercise has no effect on SOD and GPx concentrations. Our results support previous research that found high-intensity exercise has no effect on SOD and GPx concentrations on its own\(^{42, 43}\). According to the studies by Saritas et al. (2011)\(^{42}\) and Miazaki et al. (2001)\(^{43}\), SOD concentrations in humans did not improve after exhausting exercise. In contrast to our findings, Parker et al. (2018)\(^{44}\) showed a significant decrease in SOD concentrations response to a high-intensity interval exercise protocol in healthy adults. This seems to be due to the disparity between protocol and population, in which 8 untrained men and women completed the high-intensity interval exercise protocol, which consisted of five 4-min bouts, and SOD concentrations were assessed 3 hours after the exercise\(^{44}\). In our study, the redox state was evaluated up to 30 minutes following exercise, which was based on previous research that showed significant differences in antioxidant markers (such as SOD and GPx) at this post-exercise timepoint\(^{26}\). However, we may not have captured optimum plasma oxidative stress and antioxidant function as total antioxidant capacity has been reported to peak around 2 hours post-exercise\(^{45}\). In addition, due to the participants’ fitness levels, it appears that the variation in outcomes is most likely because SOD activity tends to assess the impact of exercise on redox hemostasis, and trained men with higher basal SOD activity have a lower risk of exercise-induced oxidative stress than untrained-men with lower basal SOD activity. Similarly, in our study, there were no major differences in GPx concentrations in the PL condition.Wiecek et al. (2018)\(^{26}\) found significant differences in GPx concentrations after high-intensity exercise, which is contrast to our findings. In the current study, it can be observed that there was a significant increase in CAT and NOx concentrations in the PL condition. In line with our study, Rowinski et al. (2013)\(^{46}\) reported that performing physical activities has a positive effect on antioxidant enzyme (i.e., CAT) activity in older adults. Similarly, other researchers have reported
increased serum CAT concentrations after exercise\textsuperscript{(47; 48; 49)} which supports our findings. On the other hand, Djordjevic et al. (2012)\textsuperscript{(50)} showed a significant decrease in CAT and NOx concentrations after acute exercise in young athletes. This disparity is likely due to a different type of exercise protocol and various characteristics of participants (age, fitness level, and health). It seems that athletes have a significantly higher basal concentrations of CAT and NOx which provide for proficient elimination of excess exercise-produced H\textsubscript{2}O\textsubscript{2} and consequently decline their concentrations after exercise in this population\textsuperscript{(50)}.

**Study strengths and limitations**

A key strength of the present research is its randomized, double-blind, cross-over, and placebo-controlled design. Another strength of this work is that, to our knowledge, few studies have assessed the effect of L-citrulline on antioxidant responses to exercise. Our research is limited by relatively small sample size, although our results reached statistical significance. Additionally, we were unable to measure plasma L-citrulline/L-arginine concentrations due to a limited budget; yet prior studies have shown an increase in plasma L-citrulline/L-arginine concentrations with doses of \(\geq 6\) g\textsuperscript{(21; 23)}. Moreover, our participants were young, trained men, and consequently our findings may not apply to other cohorts with dissimilar age, sex, and training status. Even though the antioxidant changes found in the L-citrulline were statistically significant compared to the PL trial, these remained modest, and consequently further investigations are needed to establish the clinical relevance of our findings. Another limitation of this investigation may be that we did not evaluate pro-oxidant markers (reactive oxygen species), exercise performance, recovery, or muscle fatigue, which would have strengthened our study design. However, the acute effects of L-citrulline supplementation on exercise performance, recovery, and muscle fatigue have been formerly described and highlighted in various review articles\textsuperscript{(10; 15; 51)}, including a couple of meta-analyses\textsuperscript{(16; 52)}. Future research should aim at assessing the effects of acute L-citrulline supplementation on post-exercise oxidation products.

**Conclusion**

In conclusion, L-citrulline supplementation (12 g dose before exercise) can boost antioxidant markers and significantly increase NOx concentrations after a high-intensity interval exercise session in young men.
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Author statement
AW and JM formulated the research question and designed the study. KV performed data collection, analyzed the evidence, interpreted the results, and wrote the paper. Paper revisions were completed by AW and KV. The final manuscript was read and accepted by all authors.
References:


Figure 1. Study design.

Supplementation of placebo or L-citrulline 1 hour prior to

Blood sampling at pre, immediately post, 10 and 30 min post exercise
Figure 2. Participants’ Flow Diagram.
Figure 3. Changes in superoxide dismutase (SOD) levels after exercise. Values are mean ± SD. L-Cit: L-Citrulline. PL: placebo.* denotes significant differences compared to PRE, ** denotes significant differences compared to PL. PRE: before, IP: immediately after, 10P: 10 min after exercise, 30P: and 30 min after exercise.
Figure 4. Changes in glutathione peroxidase (GPx) levels after exercise. Values are mean ± SD. L-Cit: L-Citrulline. PL: placebo. *denotes significant differences compared to PRE, **denotes significant differences compared to PL. PRE: before, IP: immediately after, 10P: 10 min after exercise, 30P: and 30 min after exercise.
Figure 5. Changes in catalase (CAT) levels after exercise. Values are mean ± SD. L-Cit: L-Citrulline. PL: placebo. *denotes significant differences compared to PRE, **denotes significant differences compared to PL. ***denotes significant differences compared to L-Cit PRE: before, IP: immediately after, 10P: 10 min after exercise, 30P: and 30 min after exercise.
Figure 6. Changes in nitrite+nitrate (NO) concentrations after exercise. Values are mean ± SD. L-Cit: L-Citrulline. PL: placebo.*denotes significant differences compared to PRE, **denotes significant differences compared to PL. PRE: before, IP: immediately after, 10P: 10 min after exercise, 30P: and 30 min after exercise.