



Article

Physiological, Performance and Perceptual Effects of Acute Intake of an L-Arginine and L-Citrulline Beverage Prior to a Repeated Bout of Intensive Running Among University Soccer Players

Siphamandla Nyawose ^{1,*} , Rowena Naidoo ¹, Nenad Naumovski ^{2,3,4} and Andrew J. McKune ^{1,3,5,6} ¹ Discipline of Biokinetics, Exercise, and Leisure Sciences, School of Health Sciences, University of KwaZulu Natal, Durban 4000, South Africa; naidoor3@ukzn.ac.za (R.N.); andrew.mckune@canberra.edu.au (A.J.M.)² Discipline of Nutrition and Dietetics, Faculty of Health, University of Canberra, Canberra, ACT 2601, Australia; nenad.naumovski@canberra.edu.au³ Functional Foods and Nutrition Research (FFNR) Laboratory, University of Canberra, Bruce, ACT 2617, Australia⁴ Department of Nutrition and Dietetics, Horokopio University, 17671 Athens, Greece⁵ Discipline of Sport and Exercise Science, Faculty of Health, University of Canberra, Canberra, ACT 2601, Australia⁶ Research Institute for Sport and Exercise, University of Canberra, Canberra, ACT 2601, Australia

* Correspondence: nyawoses2@ukzn.ac.za

Abstract: The purpose of this study was to determine whether a combined L-arginine and L-citrulline beverage can enhance total nitric oxide (NO_x), as well as physiological (cardiopulmonary metrics) and perceptual (rate of perceived exertion) responses to a repeated bout of high-intensity exercise among university soccer players. Thirty male soccer players were included in a randomized double-blind, placebo-controlled, parallel design. Participants performed two bouts of high-intensity running, spaced two hours apart. Forty minutes before the second bout only, participants consumed a 500 mL beverage containing 6 g L-arginine and 6 g L-citrulline (n = 15) or placebo (n = 15). Blood NO_x concentration was measured immediately before and after both bouts. There was no significant increase in NO_x or significant interaction effects for physiological, performance, or perceptual variables between the L-arginine/L-citrulline and placebo groups. The peak volume of oxygen uptake of the L-arginine/L-citrulline group was significantly higher in the second compared with the first exercise bout (54.92 ± 4.81 vs. 50.54 ± 9.22 mL/kg/min; $p = 0.01$). In the second bout of exercise, time to exhaustion in the L-arginine/L-citrulline group increased by 8.5% (~60 s) compared to the first. In conclusion, these results suggest that a single dose of L-arginine/L-citrulline beverage did not increase NO_x yet seemed to impact aerobic metabolism in university soccer players.

Keywords: nitric oxide; exercise performance; cardiopulmonary exercise test



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1. Introduction

Maintaining sufficient vascular endothelial cell nitric oxide (NO) production is crucial in overall metabolic health and significantly impacts exercise performance [1]. Inadequate NO production can elevate vascular tone, altering systemic vascular resistance and renal perfusion and increasing the risk of hypertension and compromising cardiovascular health [2]. Increased vascular resistance can decrease blood flow, restricting oxygen and essential nutrient delivery to muscles during exercise [3]. Impaired oxygen delivery to skeletal muscles can reduce exercise performance by altering bioenergetics, leading to a shift towards less efficient energy production, increased metabolic stress, and prolonged clearance of metabolic by-products, delaying recovery both during and after exercise [4].

NO is a signaling molecule involved in several physiological functions, including vasodilation, glucose uptake, calcium handling, and muscle contractility [5]. Blood NO

in vivo has a short lifespan, as it is quickly converted to the stable, reliable metabolites nitrate (NO_3^-) and nitrite (NO_2^-) [6]. NO synthesis is produced by the oxidation of L-arginine and the action of nitric oxide synthase (NOS) enzymes. The NOS has three isoforms: neural (nNOS or NOS-1); cytokine-inducible (iNOS or NOS-2); and endothelial NOS (eNOS or NOS-3) [7]. L-arginine, a semi-essential amino acid, is metabolized to NO and L-citrulline. This complex oxygen-dependent process requires reduced nicotinamide adenine dinucleotide phosphate (NADPH) as a co-substrate, as well as flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD), tetrahydrobiopterin, and heme group as co-factors [8]. About 60% of orally administered L-arginine is extracted in the small intestine through sodium-dependent cationic amino acid transporters, catalyzed by arginase, and further metabolized in the liver [9,10]. Arginase is highly expressed in the small intestine and the liver, which may limit NO production via the L-arginine substrate [11]. L-citrulline, a non-proteinogenic amino acid with the highest concentrations recorded in watermelon, is associated with improving L-arginine bioavailability, bypassing hepatic metabolism [12]. Following its absorption in the small intestine, L-citrulline helps recycle L-arg in the kidneys via the urea cycle with the enzymes argininosuccinate synthase and argininosuccinate lyase [13]. This metabolism maintains L-arginine bioavailability and NO synthesis.

L-arginine is taken in doses ranging from 3 to 6 g daily, while L-citrulline is often consumed in doses of 6 to 8 g daily [14]. For optimal physiological effects, both supplements are recommended to be taken 30 to 60 min before exercise to allow full absorption in the body [15,16]. Research suggests that combined intake of L-arginine and L-citrulline can enhance absorption and more effectively elevate circulating L-arginine concentrations and NO levels than either L-arginine -Arg or L-citrulline alone [14,17]. Pre-exercise supplementation with L-arginine/L-citrulline has been shown to improve exercise performance by increasing blood flow and oxygen delivery to working muscles, as well as delaying the onset of fatigue markers.

L-citrulline supplementation over several days reportedly improved fatigue tolerance during high-intensity exercise, increased endurance performance, and reduced perceived feelings of exhaustion [18]. It was reported that oral supplementation of combined L-arginine and L-citrulline may increase the circulating L-arginine bioavailability and plasma NO_2^- concentration effectively more effectively than either standalone L-arginine or L-citrulline supplementation [17,19]. Some research has indicated that the ingestion of combined L-arginine and L-citrulline over several days led to improvements in exercise performance markers such as improved time-trial performance in endurance runners, improved swim trial during high-intensity interval swimming protocol, and alleviated exercised-induced central fatigue in taekwondo athletes [15,19,20]. The current trend in studying L-arginine and L-citrulline supplementation entails studying their effects over several days [15].

As an alternative to the endogenous pathway, dietary NO_3^- supplementation provides an exogenous pathway for improving circulating NO, independent of eNOS [21]. Unlike the L-arginine pathway, which relies on the availability of eNOS, this pathway thrives even under physiological concentration levels of L-arginine [22]. Research by Alvares et al. (2012) reported that, under normal circumstances in a healthy individual, L-arginine concentration is sufficient to saturate available eNOS. Thus, supplementation following the L-arginine pathway may not increase circulating NO [23]. Therefore, supplementation via the L-arginine pathway has reported less favorable outcomes in exercise performance, particularly in trained athletes [16,24]. In this context, the current literature has only determined the efficacy of supplementing the L-arginine pathway under an eNOS physiologically saturated condition. Therefore, a gap in the literature still exists that seeks to determine the efficacy of L-arginine and L-citrulline supplementation on NOx and exercise performance markers following a bout of exercise performance.

In light of findings from the previous studies that utilized high-intensity exercise, supplementing with L-arginine and L-citrulline has the potential to enhance repeated bouts

of exercise performance [15,25,26]. The efficacy of the supplementation may be supported by the ‘L-Arginine paradox’, a phenomenon that explains that supplementing with L-arginine should increase NO production, despite that intracellular L-arginine concentration far exceeding the Michaelis constant of the NOS [27]. However, some literature suggests that supplementing with an NOS-dependent pathway precursor should be effective in situations where NO biosynthesis is depleted [23]. This is based on the notion that when NOS reaches saturation its substrate, L-arginine, supplementation may not increase NO production. Given the conflicting findings regarding the efficacy of L-arginine and L-citrulline supplementation under different physiological conditions, the current study’s aim was to determine whether a combined L-arginine and L-citrulline beverage can enhance NO bioavailability and improve performance in repeated high-intensity exercise. We hypothesized that a high-intensity exercise bout would deplete NO bioavailability and that a single dose of L-arginine and L-citrulline consumed before the second bout of exercise would increase NO bioavailability and as well as improve physiological, performance, and rating of perceived exertion responses, compared to placebo, in trained university soccer players. This study addresses the gap in the literature by focusing on the effects of this supplementation strategy on NO levels and exercise performance markers during repeated bouts of high-intensity exercise, where NO bioavailability is presumed to be depleted.

2. Materials and Methods

2.1. Study Selection

Thirty male university soccer players (Table 1), classified as Tier Three level athlete participants based on the recommended classification system, who were competing in the local regional league and university football participated in the study [28]. These students participate in organized intercollegiate soccer competitions within the university sports system. The University of KwaZulu-Natal’s Biomedical Research Ethics Committee approved the study (BREC/00001656/2020), and all participants provided written consent. The trial is registered with the South African National Clinical Trials Register (DOH-27-022022-5186). All participants completed the adult pre-exercise screening tool to identify individuals with a higher risk of an adverse event from exercise [29].

Table 1. Participants’ anthropometric characteristics (mean $\pm$ SD).

Participants	n = 30	L-Arg/L-Cit	PLA	p-Value
Age (y)	22.467 $\pm$ 2.67	22.69 $\pm$ 2.75	22.20 $\pm$ 2.90	0.65
Height (cm)	171.7 $\pm$ 6.23	171.7 $\pm$ 4.170	171.8 $\pm$ 7.95	0.10
Weight (kg)	66.69 $\pm$ 6.39	65.35 $\pm$ 3.55	65.01 $\pm$ 6.48	0.26
BMI (kg/m ²)	22.60 $\pm$ 1.54	22.94 $\pm$ 0.96	22.01 $\pm$ 1.37	0.05

PLA = placebo, L-Arg = L-arginine, L-Cit = L-citrulline, BMI = body mass index.

The purposive sampling method was employed to select participants for the study. The inclusion criteria required male university soccer players aged at least 18 years; actively competing in local or intercollegiate competitions; and classified as Tier Three athletes. Participants had to be in good health, as well as having no history of cardiovascular or metabolic disorders. Participants were excluded from the study if they were at increased risk of injury-related exercise; if they were recently injured and could not run on a treadmill; or if they were taking medication or supplements that interact with L-arginine, L-citrulline, or NO production. Specific medications focused on were antihypertensive medication (diuretics, beta-blockers, and angiotensin-converting enzyme inhibitors) and vasodilators (Sildenafil, Tadalafil, and Vardenafil). The researchers did not record the additional medications that the participants may have been taking such as corticosteroids or nonsteroidal anti-inflammatory medications.

Two participants were excluded from the analysis because they did not complete the second exercise bout following a power outage (power cut or load shedding) at the University. Unfortunately, this was a weekly occurrence at the time of data collection.

2.2. Study Design

This study used a randomized, double-blind, placebo-controlled, parallel design. Participants were randomly assigned to groups receiving a single treatment containing 6 g of L-arginine and 6 g of L-citrulline (L-arginine/L-citrulline) or a placebo. The supplementation followed similar protocols that have examined the effect of combined L-arginine and L-citrulline supplementation on NO bioavailability and exercise performance [15,17].

2.3. Study Protocol

Participants reported to the laboratory on two separate occasions. At the first clinic, participants were informed of the purpose of the study and the methods used, and they provided written consent. Participants were requested to refrain from consuming foods rich in L-arginine and L-citrulline a day before the second laboratory visit. These were presented in broad categories such as nuts and seeds, meat products, legumes, seaweed, and watermelon. Participants were requested to have their last meal before 21.00 h the night before the laboratory visit, and participants were reminded to refrain from consuming L-arginine and L-citrulline foods. Additionally, a day before the second clinic, participants were reminded to avoid consuming foods containing L-arginine and L-citrulline.

Participants were randomly assigned to the placebo or L-arginine/L-citrulline group at the second clinic. All compounds used in the preparation of supplements were purchased from commercially viable sources (Bulk Nutrients, 7 Crabtree Road, Grove, TAS 7109, Australia). The production and allocation of the beverage formulation (powder) to placebo or L-arginine/L-citrulline was performed by a researcher (NN) who had no contact with the participants throughout the study. All powders were prepared at the Functional Foods and Nutrition Research (FFNR) Laboratory at the University of Canberra (Canberra, Australia). Each powder (L-arginine/L-citrulline treatment or placebo) was assigned a selected number using the random number generation function in Microsoft Excel 2016 (v16, Microsoft, Pennant Hills, NSW, Australia), before randomly assigning each category of the beverages (placebo or L-arginine/L-citrulline treatment) to participants for consumption using the online application Research Randomiser (<https://www.randomizer.org/>). Powders in sealed zip bags with allocated numbers were mailed to researchers at the University of KwaZulu-Natal via regular post. On the day of clinic two, a research assistant at the University of KwaZulu-Natal, who was not part of the testing team, mixed the powders with tap water in a 500 mL dark-colored bottle and assigned the beverages to the participants. At the end of the study, the bag numbers of the powders used were sent to NN, who then disclosed the treatment (L-arginine/L-citrulline or placebo) administered to each participant.

Standard anthropometric height and weight measurements were taken using BW-1110H NAGATA (Lane 404, Chung Cheng S. Road, Yung Kang City, Taiwan). Participants performed two bouts of treadmill running maximal exercise tests, separated by at least two hours of rest. The two-bout exercise protocol was adapted from the protocol developed to assess physiological and hormonal responses and performance markers for the diagnosis of overtraining [30]. The current study employed the first bout to induce fatigue and potentially lower NOx concentration. The rationale relating to the depletion of NOx was based on research showing that endothelial wall shear stress caused by high-intensity exercise can lead to excessive reactive oxygen species production, which can inhibit NO production [31]. Subsequently, the second bout was employed to investigate the potential of supplementation with the L-arginine/L-citrulline beverage in elevating circulating NOx concentration and improving recovery from the first exercise bout, leading to enhanced physiological and performance indicators as well as a lower rate of perceived exertion (RPE) compared to a placebo.

One hour before each exercise bout, participants received a standardized pre-exercise meal comprising four Weet-Bix™ biscuits, 400 mL full cream milk, and four drizzles of honey (2315 kJ, 73% carbohydrate, 19% protein, 8% fat). Supplementation was not administered before or during the first exercise bout. Heart rate, blood pressure, and

blood samples were taken immediately before and after each exercise bout. Following the pre-exercise meal for the second exercise bout protocol, participants ingested the 500 mL L-arginine/L-citrulline treatment or placebo 40 min before the exercise test. Figure 1 represents the CONSORT study flow diagram [32].

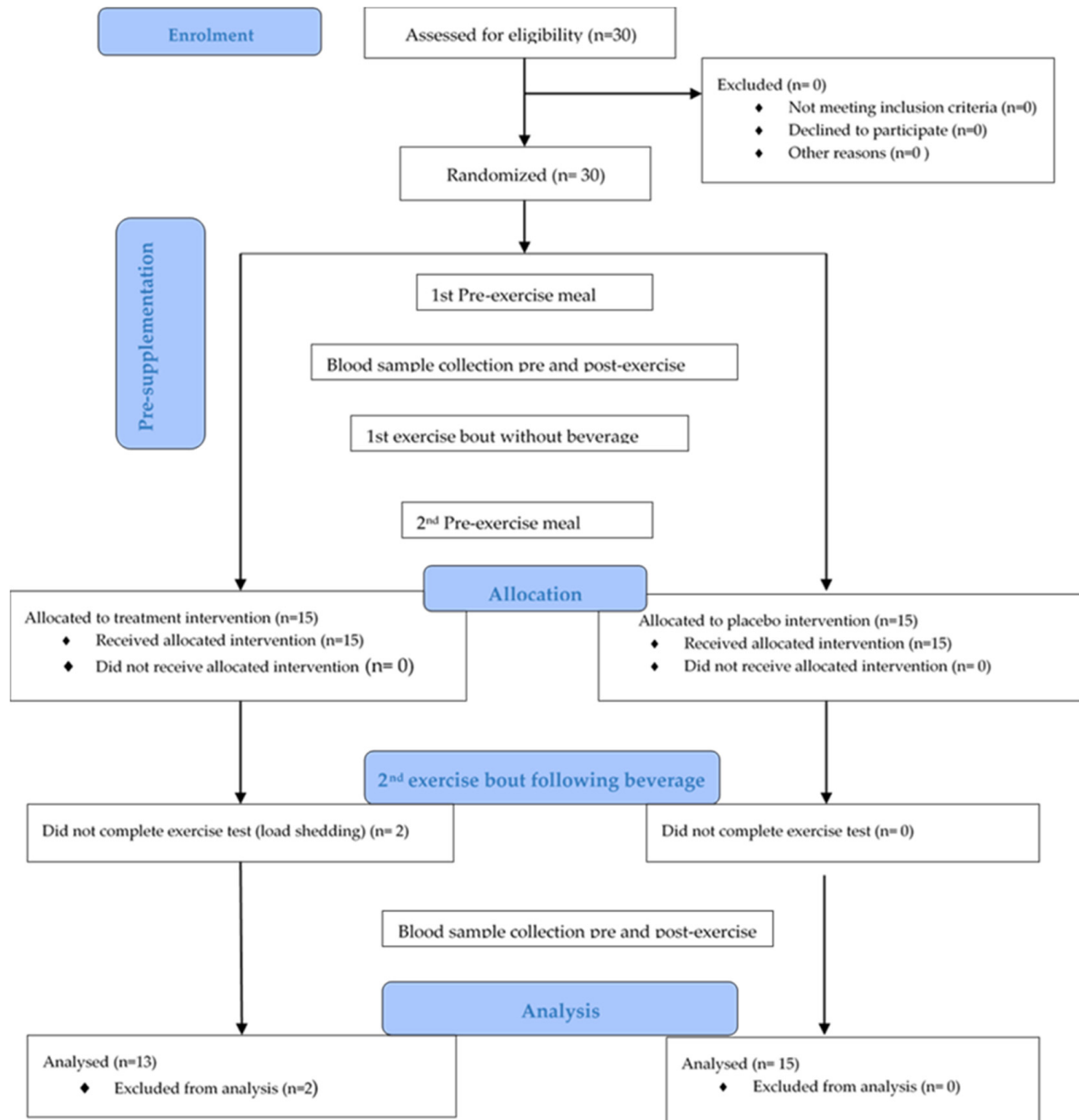


Figure 1. CONSORT study flow.

2.4. Exercise Bouts

The incremental exercise test, adapted from semi-professional soccer protocols, was administered using a treadmill ergometer (Medisoft model 870A, Hagen, Germany) [33]. Participants wore a 7450 V2 series reusable mask, and the oxygen and carbon dioxide concentrations of exhaled air and minute ventilation were continuously measured using gas and flow sensors, respectively, integrated into the Ergocard CPX pro (PAE De sorinnes, Sorinnes Route de la Voie Cuivree, 1 Dinant, B-5503 Belgium). This setup enabled continuous gas analysis averaging 30 s. The volume of oxygen uptake ($\dot{V}O_2$), carbon dioxide output ($\dot{V}CO_2$), minute ventilation ($\dot{V}E$), respiratory exchange ratio (RER), anaerobic threshold (AT), time to exhaustion (TTE), and peak oxygen uptake ($\dot{V}O_{2\text{ peak}}$) were all recorded.

during the tests. Heart rate was continually measured during the exercise test using the Suunto smart heart rate monitor chest strap (Suunto Oy, Tammiston Kauppatie 7, 01510 Vantaa, Finland). The research team ran a standard seven-minute dynamic warm-up for all participants, followed by five minutes of rest. The trial started with participants standing on a treadmill for three minutes to stabilize the ventilation rate and observe resting gas analysis measurements. Once the RER was below 0.82, the test began.

The first stage started at a 10 km/h running speed for five minutes. Thereafter, speed increased by 1 km/h every two minutes. At 16 km/h, the gradient increased by 0.5% and continued increasing every two minutes by 0.5% until volitional exhaustion. The rating of perceived exertion (Borg 6–20 scale) was used to record participants' subjective feelings of exertion [34]. Afterwards, there was a five-minute walk at a speed of 2.74 km/h. Before and after each exercise test, about 20 µL of blood was drawn to determine blood lactate concentration using the Lactate Scout 4 (Bautzner, Str. 67, 04347, Leipzig, Germany). Blood pressure was assessed before and after the exercise test and five minutes into the recovery period, and heart rate was recorded using the Omron m7 Intelli IT blood pressure monitor (HEM-7322T-E).

Once participants' RER was below 0.82, resting $\dot{V}O_2$ was recorded. The $\dot{V}O_2$ peak was determined as the highest average value before volitional exhaustion. The AT by ventilatory gas analysis was determined as a noticeable deviation from the linear relationship between $\dot{V}O_2$ and $\dot{V}CO_2$. This was verified by an analysis of panels 5, 6, and 9, using the original Wasserman nine-panel plot [35]. Time to exhaustion was determined as the total time in seconds from the first stage of the test up to volitional exhaustion. To ensure data accuracy, the Ergocard CPX was calibrated according to the manufacturer guidelines and a consistent temperature of 21 °C was maintained in the Human Performance Laboratory during both exercise bouts.

2.5. Blood Sample Collection and Handling

A qualified team of nurses and phlebotomy technicians collected venous blood samples through venipuncture from the median cubital vein of the cubital fossa before and after running the exercise test. Approximately 5 mL of blood was sampled into an EDTA tube and centrifuged at $1000 \times g$ within 15 min. Plasma was removed, aliquoted, and stored at ≤ -20 °C until further analysis. Plasma samples can be stored at this temperature for up to a month.

The time between freezing and thawing for testing was five days, and laboratory tests were completed in one day. Thawed plasma samples were filtered with the 10,000 molecular weight cut-off filters (OMEGA NANOSEP 10K). Filtered samples (100 µL) were diluted with 100 µL of reaction diluent.

2.6. Estimation of Total Nitric Oxide Concentration

NOx was measured using the nitrate reduction assay of the NOx and NO_3^-/NO_2^- parameter assay kit (#KGE001, R&D Systems, Minneapolis, MN, USA). As per the assay datasheet, all samples registered levels below the lowest standard of 3.13 µmol/L NO_2^- . In EDTA plasma, NOx levels varied between 10 and 92 µmol/L. The minimum detectable dose of this assay ranged from 0.009–0.78 µmol/L. This assay has been previously employed by other researchers [36–39]. Optical density was measured using a SPECTROstar Nano microplate reader (BMG Labtech, Ortenburg, Germany) at 540 nm. A standard curve was constructed by using GraphPad Prism 8.0.2.263 (225 Franklin Street, Boston, MA 02110, USA) to determine NOx concentration in µmol/L from the optical density.

2.7. Materials and Reagents

The 10,000 molecular weight cut-off filters (OMEGA NANOSEP 10K) were purchased from Lichro Chemical and Laboratory Supplies (8 Baumann Rd, Queensburgh, 4147, South Africa). The NOx and NO_3^-/NO_2^- parameter assay kit was purchased through Whitehead Scientific (Unit 9, Van Biljon Industrial Park, Cape Town, 7530, South Africa). Using

commercially available ingredients, the L-arginine/L-citrulline and PLA powders were prepared at the FFNR Laboratory at the University of Canberra (Canberra, Australia).

2.8. Statistical Analysis

Differences in cardiopulmonary variables between the two exercise bouts within the same group were analyzed using the paired *t*-tests. Unpaired *t*-tests were utilised to compare outcomes between L-arginine/L-citrulline and placebo conditions to determine the effects of the supplementation on NO_x concentrations. Mixed ANOVA was conducted to evaluate the interaction effects between treatment and time on NO_x levels across the two exercise bouts (within-subjects factor) and treatment conditions (between-subject factor). ANCOVA was conducted to compare rating perceptual scores while controlling for baseline fitness level as a covariate. Statistical analyses were conducted using SPSS v29.0.0.0 (IBM, Armonk, NY, USA). The level of significance was set at $p < 0.05$.

3. Results

The results below show the findings when comparing measurements to a repeated bout of high-intensity exercise protocol without supplementation in the first bout and with supplementation with L-arginine/L-citrulline or placebo in the second bout.

3.1. Total Nitric Oxide

The L-arginine/L-citrulline treatment administered in this study was well tolerated by participants, with no adverse side effects reported. The analysis excluded two participants from the L-arginine/L-citrulline group who did not complete the study protocol. Plasma NO_x concentrations of the two exercise bouts, pre and post the exercise incremental running to exhaustion test, are shown in Figure 2. There was no significant effect of time on NO_x levels across the four time points, $F(1.873, 50.6573) = 0.755$, ($p > 0.05$). Additionally, the interaction between the time and treatment was not significant, ($p > 0.05$). There were no significant differences between the L-arginine/L-citrulline and placebo groups in the first exercise bout, pre-exercise (24.31 ± 6.24 vs. 20.83 ± 6.46 $\mu\text{mol/L}$), post-exercise (25.02 ± 7.37 vs. 25.90 ± 18.17 $\mu\text{mol/L}$), $p > 0.05$; and the second exercise bout, pre-exercise (24.41 ± 6.48 vs. 24.77 ± 13.72 $\mu\text{mol/L}$), post-exercise (25.29 ± 5.96 vs. 22.58 ± 4.74 $\mu\text{mol/L}$), $p > 0.05$. However, following the second exercise bout, plasma NO_x concentration increased by 3.6% for the L-arginine/L-citrulline group, whereas it decreased by 8.8% with placebo. However, this was not significant ($p > 0.05$).

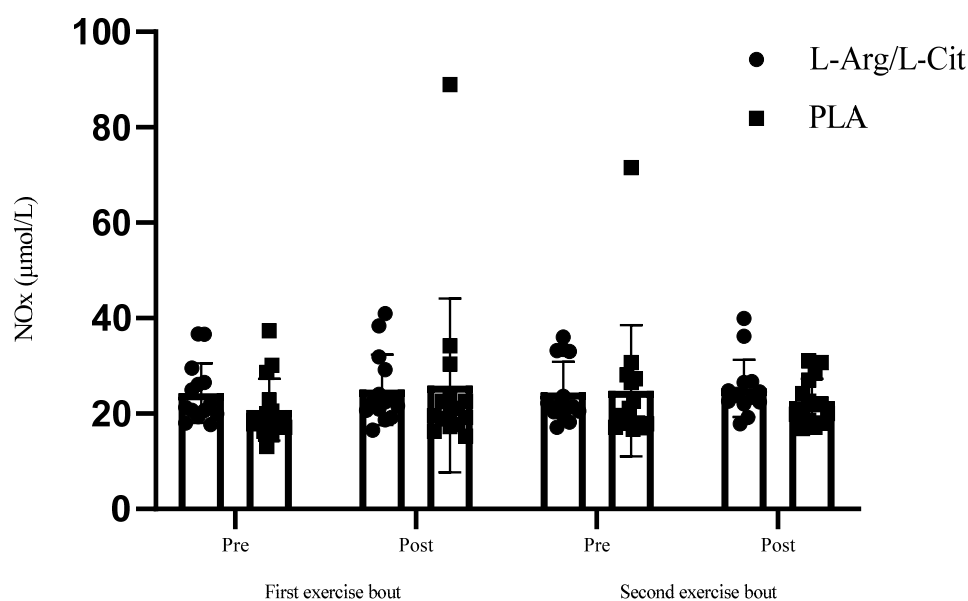


Figure 2. Total nitric oxide concentration pre and post the two bouts of exercise.

3.2. Pulmonary Gas Exchange Variables, Heart Rate and Rate of Perceived Exertion

Pulmonary gas exchange and RPE responses to the two exercise bouts are shown in Table 2. There were no significant differences in $\dot{V}O_2$ uptake at any time between the L-arginine/L-citrulline and placebo groups ($p > 0.05$). The $\dot{V}O_2$ peak for the L-arginine/L-citrulline group was significantly higher in the second bout compared to the first bout (54.92 ± 4.81 vs. 50.54 ± 9.22 mL/kg/min; $p = 0.01$). The $\dot{V}O_2$ peak of the placebo group showed no significant changes in the second bout compared to the first bout (54.27 ± 7.53 vs. 54.40 ± 8.06 mL/kg/min; $p > 0.05$). There were no significant changes in $\dot{V}E$ between the placebo and L-arginine/L-citrulline groups for both exercise bouts, ($p > 0.05$). The AT showed no significant difference between the L-arginine/L-citrulline and placebo groups for both exercise bouts, ($p > 0.05$). In the second bout of exercise, the L-arginine/L-citrulline group showed a significantly higher AT compared to the first bout (31.95 ± 4.86 vs. 28.15 ± 5.41 mL/kg/min; $p = 0.04$). There were no significant changes in TTE between the L-arginine/L-citrulline and placebo groups for both exercise bouts, ($p > 0.05$). For the second bout of exercise, TTE in the L-arginine/L-citrulline group increased by 8.5% compared to the first bout. No changes were observed in the placebo group. The RPE was higher in the placebo group compared to the L-arginine/L-citrulline group for the first exercise bout (11.08 ± 2.2 vs. 13.13 ± 1.78 , $p = 0.01$). There were no significant differences between the placebo and L-arginine/L-citrulline groups in RPE for the second exercise bout. To further explore these findings, ANCOVA was conducted to compare the post-exercise RPE scores while controlling for baseline fitness level as a covariate. The analysis showed that L-arginine/L-citrulline or placebo did not significantly affect RPE scores, ($p > 0.05$). Resting and peak heart rate showed no differences between groups both before and in response to the two exercise bouts ($p > 0.05$). The mean peak heart rate for both exercise bouts indicated that participants in the placebo and L-arginine/L-citrulline reached 93% and 94% of their age-predicted maximum heart rate, respectively. For the second exercise bout, the peak heart rate increased by 2% for the L-arginine/L-citrulline group but remained the same for the placebo group.

Table 2. Pulmonary gas exchange variables, heart rate, and rate of perceived exertion (mean $\pm$ SD).

	First Exercise Bout			Second Exercise Bout		
	L-Arg/L-Cit	PLA	<i>p</i> -Value	L-Arg/L-Cit	PLA	<i>p</i> -Value
$\dot{V}O_2$ uptake (mL/kg/min)						
Rest	7.54 ± 2.53	6.53 ± 2.73	0.34	7.46 ± 1.99	7.07 ± 2.72	0.68
Peak	50.54 ± 9.22	54.40 ± 8.06	0.27	54.92 ± 4.81	54.27 ± 7.53	0.80
Peak (L-Arg/L-Cit) 1st vs. 2nd bout	50.54 ± 9.22		0.01	54.92 ± 4.81 *		
Peak (PLA) 1st vs. 2nd bout		54.40 ± 8.06	0.96		54.27 ± 7.53	
$\dot{V}CO_2$ output (L/min)						
Rest	0.48 ± 0.17	0.42 ± 0.15	0.37	0.51 ± 0.19	0.61 ± 0.49	0.50
Peak	3.36 ± 0.76	3.34 ± 0.48	0.94	3.61 ± 0.39	3.31 ± 0.49	0.11
Minute ventilation (L/min)						
Rest	17.59 ± 7.13	15.32 ± 66.01	0.38	18.09 ± 6.35	17.13 ± 6.25	0.70
Peak	113.91 ± 20.52	122.15 ± 16.99	0.27	124.38 ± 14.42	116.23 ± 20.26	0.26
Respiratory exchange ratio						
Rest	0.82 ± 0.18	0.82 ± 0.14	0.67	0.82 ± 0.18	0.82 ± 0.14	0.72
Peak	0.97 ± 0.57	0.95 ± 0.67	0.32	0.97 ± 0.48	0.95 ± 0.72	0.54

Table 2. Cont.

	First Exercise Bout			Second Exercise Bout		
	L-Arg/L-Cit	PLA	p-Value	L-Arg/L-Cit	PLA	p-Value
Anaerobic threshold (mL/kg/min)	28.15 ± 5.41	30.64 ± 5.67	0.26	31.95 ± 4.86	30.41 ± 6.90	0.52
(L-Arg/L-Cit) 1st vs. 2nd bout	28 ± 5.41		0.04	31.95 ± 4.86 *		
PLA 1st vs. 2nd bout		30.64 ± 5.67	0.92		30.41 ± 6.90	
Time to exhaustion (s)	902 ± 157		0.85	976 ± 150	914 ± 164	0.31
(L-Arg/L-Cit) 1st vs. 2nd bout	902 ± 157		0.10	976 ± 150		
PLA 1st vs. 2nd bout		914 ± 167			914 ± 164	0.99
Rate of perceived exertion	11.08 ± 2.2	13.13 ± 1.78 *	0.01	12.23 ± 1.97	13.47 ± 1.63	0.09
Heart rate (b/min)						
At rest	63 ± 9	60 ± 6	0.29	63 ± 8	60 ± 4	0.19
Peak	186 ± 13	184 ± 13	0.66	189 ± 10	184 ± 11	0.31
Age predicted	197 ± 3			198 ± 3		

Significant difference = * $p < 0.05$. PLA = placebo, L-Arg = L-arginine, L-Cit = L-citrulline, $\dot{V}O_2$ = volume of oxygen uptake, $\dot{V}CO_2$ = volume of carbon dioxide output.

3.3. Blood Lactate, Blood Pressure and Recovery Heart Rate

Table 3 presents blood lactate and blood pressure responses measured in a seated position before each exercise bout and test and after five minutes of active recovery, walking on a treadmill at 2.74 km/h. After the five-minute recovery walk, the heart rate was also taken in a seated position. Blood lactate showed no significant changes in the L-arginine/L-citrulline and placebo groups before and after both exercise bouts ($p > 0.05$). Recovery heart rate and systolic, diastolic blood, and mean arterial pressure were not significantly different between the two groups, before or after each exercise bout ($p > 0.05$).

Table 3. Heart rate, blood pressure, and blood lactate responses before and after the two exercise bouts (mean ± SD).

	First Exercise Bout			Second Exercise Bout		
	L-Arg/L-Cit	PLA	p-Value	L-Arg/L-Cit	PLA	p-Value
Blood lactate (mmol/L)						
Pre-Run	1.69 ± 0.60	1.95 ± 0.56	0.13	2.54 ± 1.50	2.44 ± 0.83	0.78
Post-Run	7.38 ± 2.04	7.66 ± 3.12	0.54	8.06 ± 3.47	8.47 ± 3.88	0.89
Heart rate (b/min)						
Recovery	86 ± 9	85 ± 10	0.80	84 ± 14	88 ± 8	0.39
Systolic BP (mmHg)						
Pre-Run	118.5 ± 8.69	122.33 ± 8.47	0.19	123.36 ± 10.22	119.93 ± 9.55	0.68
Post-Run	118.35 ± 11.22	123.33 ± 13.72	0.29	121.57 ± 8.86	122.4 ± 10.33	0.83
Diastolic BP (mmHg)						
Pre-Run	71.64 ± 11.41	77.33 ± 7.78	0.10	76.21 ± 10.26	78.93 ± 8.83	0.49
Post-Run	79.21 ± 8.65	78.6 ± 8.55	0.89	75.5 ± 10.97	78 ± 9.42	0.56
Mean arterial BP (mmHg)						
Pre-Run	87.26 ± 9.05	92.2 ± 7.05	0.08	91.92 ± 8.93	92.6 ± 8.46	0.71
Post-Run	92.26 ± 6.62	93.51 ± 7.83	0.62	90.86 ± 8.39	92.8 ± 8.29	0.58

PLA = placebo, L-Arg = L-arginine, L-Cit = L-citrulline, BP = blood pressure.

4. Discussion

The aim of this study was to determine the effects of a single dose of a combined L-Arg (6 g) and L-Cit (6 g) (L-arginine/L-citrulline) beverage on NO bioavailability, physiological and performance responses to exercise, and RPE following a repeated bout of high-intensity

exercise among university, Tier Three, soccer players. The current study showed a marginal, yet statistically insignificant, 3.6% increase in NOx bioavailability following acute consumption of the L-arginine/L-citrulline beverage. The $\dot{V}O_2$ peak and AT increased by 8.6% and 13.5%, respectively, after supplementation with the L-arginine/L-citrulline beverage. Following the L-arginine/L-citrulline beverage supplementation, the TTE saw an 8.5% increase in the second bout of exercise performance. There was a 7.1% difference in TTE between the groups; the L-arginine/L-citrulline group ran 65 s longer, with a mean TTE of 979 s compared to 914 s for the placebo group. Supplementation may have enhanced other pathways or mechanisms contributing to endurance performance, such as improved mitochondrial efficiency, reduced oxygen cost during exercise, or delayed onset of muscle fatigue. Additionally, even small, non-significant changes in NO levels can still have a cumulative effect on vascular function, leading to improved blood flow and oxygen delivery to the muscles, which could explain the observed performance improvement.

Previous studies that assessed the influence of standalone L-arginine or L-citrulline showed that chronic supplementation was more favorable than acute treatment [23,40–42], while other studies supported combining L-arginine and L-citrulline to enhance NO synthesis and exercise performance markers [15,19]. Specifically, seven days of supplementation in university soccer players increased NO synthesis and power output, and reduced muscle soreness [15]. While such protocols provide valuable insights into the supplement's efficacy, they may encounter limitations in real-world usage patterns, including issues of low adherence, reflecting the broader challenge of low adherence to chronic interventions [43]. This underscores the importance of exploring effective acute supplement regimens to increase the chances of making a practical contribution to improving athletic performance. The current study showed that a single dose of combined L-arginine and L-citrulline did not increase the concentration of NOx in university-trained soccer players. A previous study indicated that supplementing with a combination of 1.2 g of L-arginine and 1.2 g of L-citrulline increased the NO bioavailability of collegiate soccer players, at least after a short-term supplementation protocol lasting seven days [15]. In support of this, intake of 6 g L-arginine daily for seven days improved vasodilation and maximal oxygen uptake, suggesting increased NO synthesis [44]. The effectiveness of supplementation may require low-dose supplementation over several days, rather than a single high dose. However, some evidence suggests that continued L-arginine supplementation may increase the concentration of asymmetric dimethylarginine (ADMA), thereby inhibiting NOS function [45]. Elevated ADMA can impair nitric oxide production by inhibiting the enzyme responsible for its synthesis, potentially reducing the overall benefit of the supplementation. Therefore, it is important to balance the dosage and duration of L-arginine supplementation to avoid such adverse effects and achieve optimal outcomes. Furthermore, the current study showed no significant change in NOx concentration before and after the first exercise bout. While L-citrulline consumption supports continued NO synthesis by recycling L-arginine in kidneys and liver, L-arginine is the direct precursor to producing NO; if eNOS becomes saturated, further increases in L-arginine may not lead to increased NO synthesis [46]. When eNOS becomes saturated, it means that the enzyme is working at its maximum capacity. At this point, increasing the amount of L-arginine might not lead to a proportional increase in NO production because the enzyme is already fully engaged.

Optimal NOx levels hinge on the correct dosage and precise supplementation timing [47]. In the current study, the 40-min timing was based on previous research that indicated supplementing with L-citrulline alone effectively improved markers of NO bioavailability and exercise performance markers within 60 min in trained athletes [48,49]. Even better, combined L-arginine and L-citrulline reportedly led to a more rapid increase in circulating biomarkers than supplementing with L-citrulline alone [19]. This was echoed in a study showing that a combination of L-arginine and L-citrulline supplementation elevated plasma L-arginine levels, reaching a peak approximately half-an-hour post-supplementation, followed by a gradual decline towards 60 min [17]. However, NO markers only reached peak concentration 60 min post-supplementation with combined L-

arginine and L-citrulline [17]. Similarly, supplementation timing of at least 60 min improved NO markers and exercise performance in studies that adopted short-term supplementation treatment [15,48–52]. As observed in the short-term supplementation protocols, it appears that a single dose of L-arginine/L-citrulline supplementation requires 60 min to achieve peak NOx levels. In the current study, the 40 min might not have allowed sufficient time to achieve peak NOx concentration, resulting in only a marginal 3.6% increase following L-arginine/L-citrulline consumption.

The current study on L-arginine/L-citrulline supplementation's principal novelty was to determine the protocol's efficacy under conditions of theoretically depleted NOx levels following an insufficient rest period between two bouts of high-intensity exercise performance. A study that used a two-bout exercise performance, separated by four hours, to detect subtle performance changes in the second exercise bout showed that performance decreased by between 6% and 11% compared to the first performance [30]. High-intensity exercise induces physical stress and physiological changes due to hormonal disturbances that necessitate adequate rest to allow adaptation and recovery [53]. Ideally, 24 h of rest from intense exercise is recommended before the graded exercise test [54]. In the current study, the two-hour rest period between the two bouts of high-intensity exercises did not allow full recovery before the second exercise bout. L-citrulline supplementation has been shown to enhance recovery and potentially improve exercise tolerance. L-citrulline supplementation reportedly helps detoxify ammonia in the urea cycle and suppresses rising blood lactate levels to extend the duration of high-intensity exercise [55]. Research by Bailey et al. (2015) indicated that an intake of L-citrulline 90 min before severe exercise intensity performance increased exercise tolerance by about 72 s [50]. Similarly, the current study showed a 65-s improvement in TTE following supplementation, although this improvement was not significant. Furthermore L-arginine/L-citrulline supplementation had no effect on blood lactate. Another study showed that L-citrulline supplementation did not decrease blood lactate accumulation or increase TTE following high-intensity exercise [56]. A longer waiting period for total absorption of the amino acids may be necessary for their full potential to significantly increase NO synthesis, improve exercise performance markers, and reduce perception of exertion.

The current study showed a significant increase in $\dot{V}O_2$ peak and AT, determined by ventilatory gas analysis for the L-arginine/L-citrulline group. However, the absence of significant differences in NOx concentrations between the groups makes it difficult to attribute the improvement in the variables to NOx. Furthermore, supplementation did not change BP before or after completing the exercise bouts. Decreased BP is one of the fundamental indicators of increased NO synthesis [57]. NO activates guanylate cyclase, an enzyme that converts guanosine triphosphate (GTP) into cyclic guanosine monophosphate (cGMP), leading to the relaxation of smooth muscle [58]. Relaxation of blood vessels reduces vascular resistance and increases blood flow to active muscles. Similarly, L-citrulline supplementation increased blood flow in trained athletes [3]. The potential of L-arginine or L-citrulline to improve exercise performance, physiological markers, or perceptual markers of exercise stems from their influence on NO production [3]. Research from Bailey et al. (2015) reported that supplementing with L-citrulline improved oxygen kinetics during high-intensity exercise performance, yet NO_2^- only tended to increase without significant changes [50]. In the current study, the marginal increase in NOx seems to have impacted aerobic metabolism, although its effect mechanism is unclear given the complex nature of the L-arginine to NO pathway. While the statistical non-significance in NOx suggests caution in interpreting the findings, the observed marginal physiological changes may have had impact on practical implications for performance enhancement in $\dot{V}O_2$ peak and AT. Furthermore, previous studies have shown that NOx may not always provide the best measure of NO metabolism; instead, NO_2^- levels are considered a more sensitive indicator of NO synthesis [59,60].

Limitations

There were a number of limitations that may have impacted the final results of the study. Blood samples were drawn immediately after completing the exercise bouts and stored as per the manufacture's guidelines (R&D Systems, Inc. Minneapolis, MN 55413, USA). However, we acknowledge that NO is short-lived and highly reactive and, therefore, measurement of its metabolites is very sensitive. The process of blood sample collection could potentially influence NO measurement, while delays in running the assay could have affected the stability of NO within the sample. Unfortunately, the nitrite assay, deemed the most accurate to reflect human NOS activity compared with the nitrate reduction assay, was not successfully completed due to power outage issues that occurred in the laboratory on the day of testing [60]. The mean peak respiratory exchange ratio in the two exercise bouts was below 1.1, suggesting that not all the participants met the criteria for a maximal test [61]. Nevertheless, all the participants achieved heart rates exceeding 85% of their age predicted maximum heart rate during the test, with no significant differences observed between the two groups in terms of the maximum heart rate achieved across both tests.

5. Conclusions

This study demonstrated that acute consumption of a 500 mL beverage containing L-arginine and L-citrulline increase $\dot{V}O_2$ peak and AT, suggesting potential to improve exercise tolerance in Tier Three University of KwaZulu-Natal soccer players. However, this supplementation did not significantly enhance NOx levels or RPE. The main finding of this study is that while L-arginine and L-citrulline supplementation showed limited effects in NOx, it has potential to positively influence aerobic capacity and exercise tolerance. Future research is recommended to (1) study the efficacy of supplementing with combined L-arginine and L-citrulline at least 60 min or longer before assessment of NO metabolites, specifically NO_2^- and exercise performance markers, and (2) investigate short-term effects of combined L-arginine and L-citrulline on NO synthesis and exercise performance.

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