

Review Article

# Intracellular Mechanistic Role of Nitric Oxide: A Comparative Analysis of the Effectiveness of L-Arginine and L-Citrulline Supplementation on Nitric Oxide Synthesis and Subsequent Exercise Performance in Humans

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**Received Date:** November 19, 2014

**Accepted Date:** January 07, 2015

**Published Date:** January 09, 2015

**Citation:** Darryn, S.W., et al. Intracellular Mechanistic Role of Nitric Oxide: A Comparative Analysis of the Effectiveness of L-Arginine and L-Citrulline Supplementation on Nitric Oxide Synthesis and Subsequent Exercise Performance in Humans (2015) J Food Nutr Sci 2(1): 1-8.

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**Keywords:** Nitric oxide; L-arginine; L-citrulline; Vasodilation; Exercise; Nutra-ceutical

## Abstract

Of late, there has been a major increase in the marketing of sport nutraceuticals claiming to augment exercise performance and recovery due to their ability to increase nitric oxide (NO) release. NO is a labile molecule that is known to play a major role in regulating vasodilation, blood flow, platelet adhesion, immune system function, and mitochondrial respiration. Endogenous NO synthesis is carried out by pathways that are NO-synthase (NOS)-dependent and NOS-independent. L-arginine, a semi-essential conditional amino acid is known as the primary precursor to up-regulate the production of NO alongside L-citrulline within the NOS-dependent pathway. Recently, L-citrulline has also been identified as a secondary NO donor due to its potential to augment NO synthesis through the recycling of this substrate and subsequent conversion into L-arginine within the NOS-dependent pathway. Based on the significance of these two major molecules for NO synthesis, sport supplements have highlighted both L-arginine and L-citrulline as ergogenic agents capable of aiding vasodilation due to incurring nitric oxide stimulation. The primary premise behind these ergogenic claims is that exogenous administration of L-arginine or L-citrulline may increase both vasodilation properties and blood flow towards the active muscles. This would increase oxygen and nutrient delivery, which is suggested to improve muscular performance and oxygen efficiency during various types of exercises, as well as promote a greater clearance of metabolites such as lactate during periods of recovery. While some studies have presented significant positive effects of human performance attributed to L-arginine or L-citrulline supplementation, there have also been major conflicted findings with no significant effect noted. Therefore, the purpose of this review is to discuss the intracellular mechanisms of action of the NO-inducing nutraceuticals containing L-arginine and L-citrulline and whether they are capable of incurring positive effects towards human performance.

## Introduction

In recent years, there has been a major paradigm shift through which the “hype” of novel ergogenic agents has focused upon a type of nutraceutical sport supplement known as a “nitric-oxide stimulator”. Nitric oxide (NO) was originally referred to as endothelium-derived relaxing factor, since it was shown to assist in the regulation of vascular tone within mammals<sup>[1,2]</sup>. NO is a labile lipid-soluble gas that is known to play a significant role in blood flow regulation through the act of vasodilating and subsequently decreasing peripheral vascular resistance within various tissues and organs<sup>[3,4]</sup>. Furthermore, this mechanism attributing to the effects of both relaxation of vascular smooth muscle cells and vasodilation of blood vessels can also be carried over to skeletal muscle fibers as well. NO is also suggested to regulate important physiological functions through its potential role in mediating noradrenergic and non-cholinergic neurotransmission for learning and memory, synaptic plasticity, neuroprotection, decreased platelet and leukocyte adhesion, carrying regulatory function for skeletal muscle contractility and

satellite cell activation, as well as enhanced blood flow<sup>[4-7]</sup>.

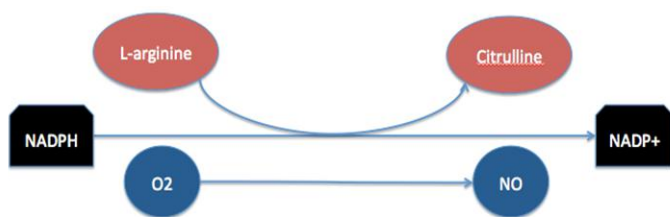
Due to evidence showing that NO has a role in mediating blood flow within muscle resistance vessels during exercise, it has been suggested that supplementation with alleged NO-inducing products can be a form of ergogenic aid<sup>[4,8]</sup>. The vasodilation of blood vessels can allow for greater efficiency in oxygen and nutrient delivery to the metabolically active muscles during exercise sessions. In addition, it is suggested that NO synthesis can augment blood perfusion towards the active muscles for improvements in muscle recovery and protein synthesis, as well as promote a greater removal of metabolites such as lactate or ammonia, which can carry an association to muscle fatigue during intense levels of exercise<sup>[4,6,9]</sup>.

With respect to these physiological effects from the synthesis of NO, many researchers have carried out experimental studies examining potential ergogenic effects of intermediates within this enzymatic NO synthesis pathway such as L-arginine and L-citrulline. However, the results from these studies of the supposed relationship of NO-inducing “vasodilators” am-

plifying human performance warrant further elucidation. There is a lack of consensus in observing significant differences being attributed solely to the administration of the NO-inducing supplements. Some studies in the literature have observed increases in strength gains, hypertrophic adaptations, efficiency in oxygen consumption, tolerance levels to fatigue, and enhanced total power output in both acute and chronic studies<sup>[4,10-15]</sup>. However, there is great controversy noted with stark contrasted findings demonstrating no significant positive effects from the use of these NO-inducing supplements<sup>[16-18]</sup>. Therefore, it is of utmost importance to critically analyze the most current studies in the literature to gain a better understanding of whether the so-called “NO-inducing” ingredients, L-arginine and L-citrulline, can actually instigate positive effects in human exercise performance. Moreover, this particular review will expatiate on the intracellular signaling pathway associated with these NO donor intermediates as well as elucidate whether these NO-inducing supplements have any significant effects towards human performance. Due to the appreciable number of scientific studies analyzing NO-inducing supplements on a variety of variables such as clinical diseases, different exercise factors, or other physiological mechanisms this particular review will mainly focus on comparing NO-inducing supplements containing L-arginine and L-citrulline towards changes in exercise performance within healthy humans through aerobic- and anaerobic-based exercise studies.

#### NO Synthesis: NOS-Dependent Pathway section:

Nitric oxide (NO) is a gaseous, diffusible signaling molecule that can affect various physiological functions such as vascular tone, cellular calcium handling, skeletal muscle glucose uptake, neurotransmission, mitochondrial respiration, and skeletal muscle force production<sup>[19]</sup>. The synthesis of NO is highly dependent on the presence of specific nitric oxide synthase (NOS) enzymes through which a catalyzing enzymatic reaction can be carried out with the substrates, L-arginine and molecular oxygen<sup>[4,8,9,19,20]</sup> (Figure 1).



**Figure 1:** The biosynthesis of nitric oxide (NO) from substrates L-arginine, NADPH and oxygen (O<sub>2</sub>). Modified from<sup>[20]</sup>.

This catalytic reaction has two separate mono-oxygenation steps:

- (1) L-arginine is hydroxylated by O<sub>2</sub> and NADPH to form N<sup>ω</sup>-hydroxy-L-arginine<sup>[21,22]</sup>
- (2) N<sup>ω</sup>-hydroxy-L-arginine is oxidized to yield products L-citrulline and NO<sup>[21,22]</sup>

There are three types of NOS isoforms, which include neuronal NOS (nNOS or type I), inducible NOS (iNOS or type II), and endothelial NOS (eNOS or type III). In addition, both nNOS and eNOS isoforms are known to be constitutively-expressed. For the purpose of this review on vasodilator mechanisms from NO production, the eNOS enzyme is of high sig-

nificance since the eNOS-derived NO is associated with effects upon the cardiovascular system<sup>[23]</sup>. Even though eNOS is constitutive in nature, mechanisms such as endothelial wall shear stress as well as receptor-ligand interactions at the endothelial cell surface can activate this enzyme<sup>[24]</sup>. These mechanisms of shear stress and receptor-ligand binding can result in calcium influx into the endothelial cell through which calcium-facilitated electron transfer can reduce NOS and consequently allow for the oxidation of L-arginine to NO and L-citrulline<sup>[24]</sup>. The enzymatic formation of NO also requires cofactors such as nicotinamide adenine dinucleotide phosphate-oxidase (NADPH), flavin adenine dinucleotide phosphate (FAD), flavin mononucleotide (FMN), calmodulin (CaM), and tetrahydrobiopterin (BH<sub>4</sub>)<sup>[2,19]</sup>.

Within the endothelium, NO is produced via the enzymatic reaction with eNOS in order to induce vasodilation of the arteries leading to increases in blood flow. Since endothelial NO is lipid soluble, this molecule rapidly diffuses into the vascular smooth muscle of the tunica media in order to bind to the enzyme guanylatecyclase<sup>[2,24,25]</sup>. This leads to the respective increase in cyclic guanosine monophosphate (cGMP) and consequent decrease in the intracellular Ca<sup>2+</sup> concentrations. The molecule cGMP will then act as a second messenger, through which the activation of various kinases in a signaling cascade can stimulate calcium pump activity, decrease sarcoplasmic calcium activity, and consequently induce smooth muscle relaxation (maintenance of vascular tone) and subsequent vascular dilation<sup>[23,25]</sup>. Further discussion into the complex intracellular signaling steps of this NO/cGMP signaling pathway can be found in two comprehensive reviews<sup>[2,20]</sup>.

With respect to the physiological significance of NO, the implementation of acute sessions of exercise is known to raise NO levels<sup>[6,26-28]</sup>. However, it is important to understand that the half-life of circulating NO is only 3 to 4 seconds<sup>[6]</sup>. Therefore, comparisons of specific circulating NO levels during conditions of rest and exercise within sedentary and well-trained individuals may be of potential difficulty; therefore, the assessment of the NO metabolites, nitrate and nitrite, are typically determined to evaluate NO activity. Nevertheless, the continual exposure to conditions of exercise training is known to place drastic changes within cardiovascular hemodynamics and respective adaptations. The implementation of acute exercise can augment blood flow towards the metabolically active muscles through which enhances in cardiac output can still be maintained relative to the training status of the individual. Furthermore, the mechanical pattern of contractions and relaxations upon the skeletal muscle (muscle pump) can also cause immediate increases in blood flow towards the muscle, which releases vasoactive metabolites (K<sup>+</sup>, adenosine, NO) to subsequently act upon the blood vessels<sup>[25]</sup>. Moreover, the transportation of NO in circulation bound by hemoglobin can induce vasodilation at the terminal arterioles<sup>[29]</sup>. It is suggested that increases in local blood flow during sessions of exercise may be due to the principle of “ascending vasodilation,” through which the contact of by-products of metabolism upon the terminal vessels may lead to increases in blood-flow velocity<sup>[30]</sup>.

It is also possible that the release of NO is due to the increased stimulation of eNOS activity due to mechanisms of shear stress, which is related to the changes in blood flow and viscosity<sup>[31]</sup>. Shear stress is defined as the force placed upon the endothelial vessel wall due to the sliding actions of blood

flow. During sessions of exercise, shear stress will increase due to both increases in cardiac output and blood flow towards the active muscle. Thus, flow-mediated vasodilation will occur due to subsequent NO production by this increase in shear stress (leading to exercise hyperemia). It is important to note that NO activity can be diminished during conditions of high oxidative stress through the production of superoxide radicals. The reaction of NO with these superoxide radicals can produce peroxynitrate, which would reduce the bioavailability of NO to induce vasodilation<sup>[20]</sup>. Peroxynitrate is a potential mediator for endothelial cell injury within cardiovascular disease due to its role as a potent oxidizer of lipoproteins, thus promoting atherogenesis<sup>[32]</sup>. Furthermore, the impairment of the NOS-isoform dependent pathway can signal progression towards atherogenesis as absences of NO substrates such as L-arginine or cofactors like BH4 can propagate NOS to synthesize superoxide instead of NO<sup>[33]</sup>. Thus, the promotion of NOS-mediated ROS production can raise the risk level for developing cardiovascular diseases such as atherosclerosis.

#### **NO Synthesis: NOS-Independent Pathway section:**

According to the literature, NO can also be produced by the reduction of both nitrate and nitrite, which are also precursors towards NO synthesis<sup>[4,34]</sup>. Increases in nitrate and nitrite levels can also be accomplished via dietary means through green leafy vegetables or beetroot and cured meat products respectfully<sup>[34,35]</sup>. According to the literature, a diet high in these vegetables rich with nitrate may induce cardiovascular benefits and lead to marked reductions in blood pressure (a form of cardioprotection)<sup>[36]</sup>. Physiologically, the ingested nitrate can be quickly absorbed within the upper gastrointestinal tract or be extracted by salivary glands and be secreted within saliva<sup>[34]</sup>. Within the mouth, the secreted saliva-containing nitrate can be reduced to nitrite by facultative anaerobic bacteria and consequently swallowed in order to enter the systemic circulation<sup>[4,35]</sup>. Then a portion of the swallowed levels of nitrite can be subsequently reduced to form NO in an NOS-dependent pathway typically during conditions of low molecular oxygen within the blood and tissues<sup>[4,35]</sup>. In order to increase and maintain levels of circulating plasma nitrite, the remainder of the swallowed nitrite will be absorbed and metabolically converted into NO when necessary under certain conditions<sup>[37]</sup>. It is wise to note that the NOS-dependent pathway can also contribute to increased levels of nitrate and nitrite from the oxidation of product NO within the blood and targeted tissues<sup>[7]</sup>.

There are several studies implemented to investigate whether nitrate supplementation can induce increases in NO synthesis via the NOS-independent pathway and demonstrate positive effects on human performance. One study explored whether the supplementation of nitrate can reduce the oxygen cost during sessions of walking and running<sup>[36]</sup>. The investigators administered dietary supplementation of beetroot juice, which is high in nitrate, to 9 healthy male subjects for 6 days while examining changes in blood pressure, mitochondrial, oxidative capacity and varied intensity sessions of running and walking<sup>[36]</sup>. Following beetroot juice supplementation, the authors found positive physiological results with reductions in systolic blood pressure, increases in both the plasma levels of nitrite and the time to fatigue in high-intensity running, as well as in the O<sub>2</sub> cost of walking. The implementation of nitrate supplementation may also induce positive benefits towards increasing NO levels as

well as improving the efficiency of oxygen consumption (VO<sub>2</sub>). Interestingly, another study found that the administration of sodium nitrate (0.1 mmol/kg/day) to 9 healthy individuals prior to a maximal incremental exercise test of both arm and leg ergometers reduced the maximum oxygen consumption (VO<sub>2</sub><sup>max</sup>) levels, but increased the time until exhaustion<sup>[35]</sup>.

There are other studies that examined nitrate supplementation in the form of sodium nitrate or beetroot juice, which goes beyond the scope of this review<sup>[4,38]</sup>. Nevertheless, some conclusions drawn from these studies may include a greater need for further research on the assessment of sodium nitrate supplementation towards untrained and trained individuals. Lastly, further specific analysis of the intracellular signaling mechanistic activity within studies involving beetroot juice supplementation may also shed further light on confirming the increase in NO production via the NOS-independent pathway.

#### **L-Arginine Supplementation: Effects on Exercise Performance section:**

L-arginine (Arg) is known as a conditional semi-essential proteinogenic amino acid found in many dietary proteins. Arg can be obtained by ingesting seafood, watermelon juice, nuts, seeds, algae, meat, rice protein concentrate and soy protein isolate<sup>[4,39]</sup>. Arg plays an important role in its conversion into L-ornithine and urea, which is mediated by arginase enzymes for the purpose of eliminating nitrogen compounds through the urea cycle<sup>[9]</sup>. The infusion of Arg within conditions of rest can also raise concentration levels of hormones such as plasma insulin, glucagon, GH, prolactin, and catecholamines<sup>[4]</sup>. Arg is significant with regards to endothelial vasodilatation due to its role as a precursor for the synthesis of NO. An enzymatic reaction with eNOS can convert Arg into L-citrulline and NO. The supposed rationale behind marketing of supplements containing Arg has been due to the enhancement of vasodilation and blood perfusion towards the metabolically active muscle, which would theoretically augment muscle recovery and protein synthesis both during and after exercise. Furthermore, recently various supplements containing Arg have been created to draw the population on the marketed claims of these ergogenic aids being capable of enhancing vasodilation and promoting adaptive improvements in skeletal muscle function.

However, there has been a lack of solidarity in these alleged beneficial claims within the scientific literature. In recent years, various studies utilizing Arg-based supplementation have observed positive effects towards human performance. Researchers in a study administered 13 subjects with an oral dose of a multi-nutrient compound containing Arg (6 g) with glycine (2 g) and alpha-ketoisocaproic acid (3.2 g) at three time periods prior to exercise (10, 30, 45 minutes) and observed significant gains in peak torque, total work, and fatigue index through the utilization of an isokinetic dynamometer in comparison to a control group<sup>[40]</sup>.

Another study supplemented 10 men in a randomized, double-blinded, placebo-controlled experimental study with Arg (6 g) with glycine (2 g) and alpha-ketoisocaproic acid (3.2 g) and observed significant improvements in attenuating declines in mean power during repeated sets of anaerobic cycling performance<sup>[41]</sup>. However, there were no significant differences in lactate levels between the experimental and placebo groups within this study. Also, another study on healthy, postmenopausal wom-

en observed that higher doses of Arg (14.2 g) daily for a period of 6 months led to significant increases solely in relative maximal power through the test of peak jump power in comparison to other jump testing variables<sup>[42]</sup>. One study administered arginine aspartate (3g) or a placebo to 16 trained males for a duration of 3 weeks in order to assess whether chronic supplementation can have any positive adaptive effects towards metabolic and cardiovascular responses during bouts of submaximal exercise<sup>[43]</sup>. The authors found that 3 weeks of supplementation led to positive improvements such as lower plasma lactate concentrations as well as decreased ventilation and CO<sub>2</sub> production during exercise within the treatment group. Although the studies discussed above presented positive performance adaptations following ingestion of Arg-based compounds, none of these research studies have identified the underlying mechanism that may explicate the enhancements noted. Furthermore, none of these studies have also examined circulating levels of NO or its oxidized counterparts nitrate or nitrite, which may present a lack of evidence in confirming whether Arg supplementation augments NO bioavailability. Another study examined whether acute Arg supplementation may reduce the O<sub>2</sub> cost as well as improve both exercise efficiency and tolerance at various intensity levels<sup>[44]</sup>. Nine subjects ingested a beverage containing either Arg (6g) or a placebo and implemented a series of cycle ergometer exercise sessions of both moderate- and high-intensity sessions one hour after the ingestion of the supplement. The results of this study showed marked reductions in the O<sub>2</sub> cost of moderate intensity exercise, increases in both plasma nitrite and the time to exhaustion at high-intensity exercise within the Arg-treated groups in comparison to the placebo group<sup>[44]</sup>. However, a few methodological limitations within this study may include the lack of measurements on baseline nitrite levels, which may have varied between subjects prior to the induction of the study. There were also no restricted dietary guidelines through which variances may occur in subjects due to nitrite- or nitrate-rich foods<sup>[44]</sup>.

In contrast to the findings above observing positive enhancements following the ingestion of Arg containing supplements, there exists disagreement from recent studies within the literature. Alvares et al.<sup>[10]</sup> administered Arg (6 g/daily) to 15 experienced runners (11 males and 4 females) over the course of 4 weeks and evaluated whether there would be improvement in exercise performance by changes in metabolic parameters such as markers of NO production [nitrate and nitrite (NOx)], cyclic guanine monophosphate (cGMP), and hormone levels of insulin, growth hormone, cortisol, and IGF-1. This study also assessed whether any changes within these metabolic and hormonal parameters would have a positive effect on running performance. Following the 4-week study, the authors found no significant differences in NOx or changes within hormonal parameters including insulin, growth hormone, IGF-1, and cortisol following Arg supplementation. There were alleged increases in these hormonal parameters, which may have been attributed to the sessions of exercise alone. The Arg supplementation did not appear to enhance running performance within the subjects. The authors mentioned some methodological limitations within this study, which included no baseline evaluation on each subject's exercise performance prior to the supplementation period as well as a potential weakness in statistical power due to a small sample size of only 15 subjects. Greer & Jones<sup>[45]</sup> carried out a study analyzing the acute effect of L-arginine-alpha-ketogluta-

rate (AAKG) supplementation on the variable of muscular endurance and blood pressure levels. In brief, this study recruited 12 resistance trained subjects to ingest 3,700 mg of AAKG or placebo at two separate time intervals before testing muscular endurance through chin ups, reverse chin-ups, and push-ups to failure<sup>[45]</sup>. The results of this study found that the AAKG treatment group did not have significant differences in performance due to supplementation ingestion towards muscular endurance performance, as well as changes in blood pressure activity in comparison to the placebo group. However, a potential limitation to this study would include a lack of power due to a small sample size as well as the small dosage of AAKG supplementation.

Wax et al.<sup>[46]</sup> explored whether the acute ingestion of AAKG (3g/daily) prior to a resistance exercise protocol would show increases in muscular strength and endurance within untrained and resistance-trained men. Alpha-ketoglutarate is known as a metabolite that is produced by the oxidative decarboxylation of isocitrate, which is an intermediate within the Krebs cycle<sup>[46]</sup>. Furthermore, AAKG may potentially increase the rate of the common denominator acetyl-CoA oxidation within the Krebs cycle<sup>[8,46]</sup>. The authors of that study carried out a counterbalanced, double blind study and assessed changes in measures of upper and lower body muscle strength and total load volume over the course of 1 week. There were no significant differences accounted towards the supplementation of AAKG towards strength and total load volume gains. Furthermore, this study concluded that there were no ergogenic benefits of AAKG towards both resistance-trained and untrained men in this study. Interestingly, this study did not investigate the underlying mechanisms of NO synthesis possibly explaining why AAKG may confer ergogenic benefits towards gains in muscular force. In addition, some limitations noted by the authors included the questionable efficacy of a single dose of AAKG or one bout of exercise, as well as the lack of classification on baseline strength differences between the subjects<sup>[46]</sup>.

Researchers also investigated whether the acute ingestion of AAKG (3.7g/daily) can have an ergogenic benefit during resistance exercise within trained ROTC cadets<sup>[47]</sup>. This double-blind, crossover study recruited 19 active subjects who ingested either the placebo or AAKG supplement prior to a session of muscle strength and muscular endurance testing on both the bench press and leg press exercises. Blood lactate measures were also obtained following both the muscle strength and endurance assessments. The results showed significant increases in blood lactate from baseline to post-exercise in both groups in all exercises. This study also demonstrated that acute AAKG supplementation may not provide any ergogenic benefits towards augmentation of human performance or blood lactate concentration within trained individuals. Some major methodological limitations may include the post-test only study design through which variances in the inter-subject's strength and lean muscle mass can alter the findings as well as the lack in measuring Arg and biomarkers of NO production within the circulation. Liu et al.<sup>[17]</sup> also saw no significant differences within biomarkers of NO production (NOx) following the oral administration of arg (3g/daily) on 10 judo athletes for a period of 3 days<sup>[17]</sup>. Researchers also investigated whether the ingestion of 10 g of essential amino acids with Arg (10 g) would have any ergogenic effect towards NO synthesis, muscle blood flow, and skeletal muscle

protein synthesis following a bout of unilateral leg resistance exercise<sup>[48]</sup>. The investigators of that study found that plasma concentrations of biomarkers of NO synthesis (NOx, endothelin-1) had no significant differences between Arg and control groups. Although both growth hormone and Arg concentrations were greater within the Arg group post-exercise, there were no significant differences in femoral artery blood flow between both groups. Even though the mixed and myofibrillar levels of MPS were increased within the exercised leg, there were no significant differences between groups. Therefore, the oral supplementation of Arg did not show ergogenic benefits towards human performance or changes in NO synthesis, muscle blood flow, and MPS beyond the effects noted by the exercise session.

One study administered Arg (6g) to subjects and evaluated whether changes were seen in biceps strength performance, NOx, muscle blood volume and oxygenation during the inter-set recovery periods from 3 sets of resistance exercise<sup>[49]</sup>. The results showed significant increases in muscle blood volume within the Arg group compared to placebo during recovery periods of the resistance exercise session. However, no significant differences occurred with NOx, muscle oxygenation, and total work done between the Arg and placebo groups. Willoughby et al.<sup>[8]</sup> also examined whether the supplementation of AAKG (12g/day) had an effect on hemodynamics, increases in NO synthesis, brachial artery blood flow, as well as changes in the circulation levels of Arg, NOx, asymmetric dimethyl arginine (inhibitor of eNOS activity), and the L-arginine:ADMA ratio following resistance exercise sessions with the elbow flexors<sup>[8]</sup>. Similarly to other studies, there were no significant differences attributed to the alleged ergogenic benefit of AAKG supplementation within hemodynamic variables (heart rate, blood pressure), blood flow, NOx and Arg levels. Furthermore, the ratio of L-arginine:ADMA increased within the treatment group most likely due to the exogenous administration of Arg. Therefore, even though there were increases observed in plasma Arg levels, the changes observed in the hemodynamic variables, brachial-artery blood flow, and NOx levels were most likely due to the resistance exercise session alone.

A study was conducted to assess the hemodynamic and vascular responses to resistance exercise with L-arginine supplementation<sup>[50]</sup>. In this cross-over designed study, 18 college-age men performed two acute bouts of resistance exercise in which they orally ingested either placebo or L-arginine (7g) before each bout. Results demonstrated no beneficial effect in increasing forearm blood flow or attenuating central arterial stiffness from L-arginine. Bloomer et al.<sup>[51]</sup> used a randomized, double-blind, cross-over design with 19 resistance-trained men and performed tests of muscular power and endurance. A placebo and three AAKG-containing supplements were ingested prior to exercise with one week separating the conditions. Results demonstrated no preferential beneficial effects for the AAKG-containing supplements for exercise performance, blood levels of lactate and NOx, and skeletal muscle oxygen saturation.

The incorporation of Arg in sports nutritional supplements seems to be premature due to the equivocal findings noted within the scientific literature. Although the exogenous administration of Arg may theoretically induce increases in circulating levels of NO, there are confounding variables such as dietary restrictions (in nitrate/nitrite rich foods), exercise intensity, training status of the subjects, and methodological approaches

for measuring biomarkers of NO and blood flow, which requires further research. The contrasted findings of Arg supplementation noted throughout the literature should propagate further meticulous investigations prior to agreeing with any manufacturer's claim of Arg supplementation inducing ergogenic benefits for performance.

### **L-Citrulline Supplementation: Effects on Exercise Performance section:**

L-citrulline is known as a non-essential alpha-amino acid that can be synthesized endogenously from the enzymatic NOS-dependent reaction with Arg as the substrate<sup>[4]</sup>. L-citrulline is known to have a specific metabolism through which it can bypass splanchnic extraction and avoid pre-systemic elimination while not being taken up by the liver<sup>[52,53]</sup>. L-citrulline is also known as an intermediate metabolite within the process of ureagenesis<sup>[52]</sup>. Furthermore, this by-product from the Arg/NO conversion reaction has recently developed popularity due to its supposed ability to bypass hepatic metabolism and elude degradation by arginase enzymes<sup>[4]</sup>. Therefore, it has been proposed that L-citrulline may serve as a second donor towards NO synthesis by theoretically raising extracellular levels of Arg<sup>[53]</sup>.

L-citrulline can also be synthesized by glutamine within enterocytes through the condensation reaction of ornithine and carbamyl phosphate that is catalyzed by the enzyme ornithine carbamyl-transferase<sup>[4,54]</sup>. Furthermore, exogenous levels of L-citrulline are released by these enterocytes within the portal circulation where it is directly transported to the kidneys to be catalyzed into Arg within the proximal tubules<sup>[4,52]</sup>. An animal study was carried out in which the supplementation of L-citrulline (250 mg/kg body weight) was administered to mice for 7 days. This study found that the L-citrulline-supplemented group was able to increase tolerance to fatigue levels in the swimming exercise protocol compared to the control group<sup>[55]</sup>. Furthermore, the blood lactate levels within the L-citrulline group were significantly lower in comparison to the control. Interestingly, the blood ammonia levels were significantly higher in the control group in comparison to the treatment group. The results of this study suggest that L-citrulline supplementation may assist in ammonia detoxification through the urea cycle as well as potentially play a role in increased NO synthesis (noted by the decrease in lactate levels)<sup>[55]</sup>. Therefore, the results of this study signify similar events within humans towards increasing NO synthesis and subsequently having a positive effect on human performance. In summary, with respect to the NO cycle, administration of L-citrulline may play a role in NO metabolism and regulation by being an Arg precursor for NO synthesis. Further discussion of the significance behind L-citrulline for metabolic and therapeutic usage can be found in a recent article by Bahri et al.<sup>[52]</sup>.

Presently, there are only a few scientific investigations into supplemental L-citrulline towards NO synthesis and effects towards human performance. A study involved the oral provision of either L-citrulline (3 g) or a placebo to 17 human subjects prior to incremental treadmill tests towards exhaustion<sup>[56]</sup>. The investigators hypothesized that the treatment group would have improvements in time towards exhaustion within the exercise session due to the alleged physiological effects of L-citrulline. In opposition to the proposed hypothesis, the results showed that there was a reduction in treadmill time to exhaustion within the

treatment group as well as normality in levels of plasma insulin. The proposed rationale of the results by the investigators may have been that L-citrulline ingestion brought about an increase in insulin clearance or reductions within NO-mediated pancreatic insulin secretion<sup>[56]</sup>. Nevertheless, this study showed that contrary to the theoretical effect of L-citrulline in raising NO synthesis, there might be other underlying physiological mechanisms that may be prioritized beyond the Arg/NO pathway.

Other studies have utilized a combined supplement of L-citrulline and malate, which is known as an intermediate within the Krebs cycle<sup>[4]</sup>. Malate undergoes dehydrogenation within the Krebs cycle to form oxaloacetate, which is a rate-controlling step within this cycle. In addition, it is known that L-citrulline malate can assist in the elimination of ammonia during recovery periods from exhaustive bouts of exercise while serving as a precursor for Arg and creatine<sup>[53]</sup>. Lastly, the supposed rationale behind the addition of malate may also be due to its effect towards ultimately augmenting aerobic ATP production<sup>[57]</sup>. One study investigated whether the oral supplementation of L-citrulline-malate (6 g) in 17 well trained male cyclists could have an effect on metabolic levels of amino acids, NOS-dependent NO signaling levels of arginine, as well as its metabolites such as creatine, urea and nitrite<sup>[58]</sup>. The investigators observed that the blood samples taken 15 minutes after the race (137 kilometers) as well as 3 hours post-exercise within subjects showed significant increases in citrulline, arginine, ornithine, urea, creatinine, and nitrite levels<sup>[58]</sup>. Also, both growth hormone and insulin levels were raised within both groups after exercise with an even greater response of growth hormone increase noted within the treatment group. The authors found that L-citrulline-malate supplementation may raise the production of arginine's metabolites such as nitrite, creatinine, ornithine and urea during sessions of aerobic activity. However, various confounding factors may have affected the gathered data such as the variation in the athlete's endurance abilities, environmental conditions during the race, and nutritional inter-subject differences. Bloomer et al.<sup>[51]</sup> used a randomized, double-blind, cross-over design with 19 resistance-trained men and performed tests of muscular power and endurance. A placebo or L-citrulline- and L-citrulline malate-containing supplement were ingested prior to exercise with one week separating the conditions. Results demonstrated no preferential beneficial effects for the L-citrulline-containing supplements-containing supplements for exercise performance, blood levels of lactate and NOx, skeletal muscle oxygen saturation. A randomized, counterbalanced, double-blind study was performed to determine the effects of supplemental citrulline malate ingestion during repeated bouts of lower-body exercise in advanced weight lifters<sup>[59]</sup>. Twelve advanced resistance-trained men ingested either placebo or citrulline malate (8 g) and then performed repeated bout of multiple lower-body resistance exercise. The citrulline condition resulted in a higher number of performed repetitions; however, there were no differences between supplements for blood lactate levels or hemodynamic measures. One final study investigated whether the supplementation of citrulline malate had an effect on the performance of an anaerobic exercise as well as an alleviation of post-exercise muscle soreness<sup>[60]</sup>. This study was designed as a randomized, double blind, two-period crossover sessions in which 41 men ingested either citrulline malate (8 g) or a placebo, and carried out repetitions of high intensity (80% maximum strength) bench

press until fatigue. The results of the study showed that there was a significant decrease in muscle soreness (24 and 48 hours post-exercise) as well as increases in the number of repetitions within the treatment group. However, a limitation to the study would be the lack of evidence exploring the biomarkers of NO production with ingestion of L-citrulline-malate. Therefore, no clear association can be made with these positive findings of enhancement in anaerobic performance and mitigations of muscle soreness towards increased NO synthesis.

Based on the paucity of studies found within the literature, it is clear that further research is necessary in order to adamantly confirm any claim of L-citrulline supplementation inducing positive effects towards NO synthesis and subsequent human performance. The results of these few studies may primarily be due to other metabolic pathways activated independent of increases in NO synthesis. The utilization of malate alongside citrulline may potentially explain the observations of reductions in fatigue noted by subjects in the anaerobic performance study by Perez-Guisado & Jakeman<sup>[60]</sup>. Therefore, future investigations need to be carried out on supplements containing only L-citrulline on biomarkers of NO synthesis in order to determine if increases in vasodilation can both augment human performance and relieve sensations of fatigue.

## Conclusion and Future Perspectives section:

The investigation into recent literature demonstrates that there is a lack of confirmation as to whether supplements containing Arg or L-citrulline can indeed increase NO synthesis and promote vasodilatation for augmented human performance. Whereas the combination of other components with Arg such as glycine, alpha-ketoglutaric acid, AAKG, or malate with L-citrulline may have led to increase of performance in some studies discussed, the findings remain controversial and equivocal at best. The lack of explanation towards the underlying mechanisms attributing to the improvements in oxygen efficiency or overall human performance within the Arg or L-citrulline study weakens the suggested relationship of upregulation of NO synthesis and performance. Furthermore, the manufacturers' claim on Arg and L-citrulline ergogenic properties for human performance is premature at best without more unequivocal scientific findings. While some benefits have been noted with varied doses of Arg or L-citrulline supplementation such as improved time to failure in aerobic exercises or increases in lactate clearance, there is controversy in the methodological approaches within these studies. Confounding variables such as training status, gender differences, overall volume load, exercise intensity, environmental considerations, technological differences for data measurements, dietary considerations, and lack in biomarker analysis for NO synthesis or intermediates of Arg need to be considered in future studies for increasing the validations of ergogenic potentiation in these supplements. Therefore, the marketed claims allegedly regarding Arg and L-citrulline as ergogenic vasodilator supplements cannot be confirmed without further research incorporating more investigation into the activity levels of biomarkers for NO production.

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