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NO EFFECT OF L-ARGININE SUPPLEMENTATION ON NITRIC OXIDE PRODUCTION AND MUSCLE RECOVERY

LA SUPLEMENTACIÓN DE LA L-ARGININA NO AFECTA LA PRODUCCIÓN DE ÓXIDO NÍTRICO NI LA RECUPERACIÓN MUSCULAR

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SUMMARY

Introduction: L-arginine (L-Arg) is a semi-essential amino acid precursor to nitric oxide (NO) synthesis. Recently, nutritional supplements containing L-Arg have been marketed with the purpose of promote vasodilation, due to an increased production of NO in the exercising muscle. The resulting vasodilation would elevate blood perfusion, leading to a higher nutrient and oxygen delivery, which may enhance exercise performance and muscular recovery.
Purpose: Identify the acute effect of L-Arg supplementation on work recovery ratio (WRR), average power, total work (TW3S) and indicators of NO production, plasma nitrite and nitrate (NOx), during a resistance exercise protocol.
Methods: Seventeen healthy and resistance-trained males participated in a randomized, double-blind, placebo-controlled study. Blood samples were collected before and 90 min (immediately post-exercise) after ingestion of oral 16g of L-Arg or placebo. The exercise protocol (3 sets of 10 maximal voluntary contractions of isokinetic concentric elbow extension at $60^{o}.s^{-1}$ with 2-min of rest between sets) was initiated 80 min after supplementation. NOx measurements were made by a traditional Griess reaction colorimetric method using a spectrophotometer monitoring absorbance at 540 nm.
Results: No significant difference between L-Arg versus placebo supplemented groups was observed on WRR, average power and TW3S (2630.4 ± 758.0 versus 2573.1 ± 669.9 Joules). Furthermore, no significant difference was observed in plasma NOx at any time point between L-Arg versus placebo supplemented groups at baseline (9.8 ± 2.3 vs. 9.5 ± 1.4 μmol/L) and immediately post-exercise (11.9 ± 5.5 vs. 10.2 ± 2.3 μmol/L).
Conclusion: Our data indicates that acute ingestion of L-Arg does not increaseNO production nor enhances muscle performance and recovery. Based on this fact, it is still premature to recommend nutritional supplements containing L-Arg as an ergogenic aid to optimize muscle recovery after resistance exercise bouts in healthy and resistance-trained subjects.

Key words: Nutritional supplements. Amino acids. Nitric oxide. Exercise.

RESUMEN

Introducción: La L-arginina (L-Arg) es un aminoácido semi esencial y precursor de la síntesis de óxido nítrico (NO). Recientemente, los suplementos nutricionales que contiene L-Arg son comercializados con la pretensión de promover vasodilatación, debido al aumento de la producción de NO en músculo. El resultado de la vasodilatación elevaría la perfusión sanguínea, promoviendo un mayor aporte de nutrientes y oxigeno, los cuales pueden mejorar el rendimiento y la recuperación muscular.
Propósito: Identificar el efecto agudo de la suplementación con L-Argsobre la ratio de recuperación del trabajo (WRR), potencia media, trabajo total (TW3S) y los indicadores de producción de NO, nitrito y nitrato plasmático (NOx), durante el ejercicio de contra-resistencia.
Métodos: Diecisiete hombres sanos y entrenados, participaron en un estudio, doble ciego, controlado con placebo. Se tomaron muestras de sangre antes y 90 min después de la ingesta de 6 g de L-Arg o placebo. El protocolo de ejercicio (3 series de 10 repeticiones máximas de extensión de codo isocinético concéntrico en $60^{o}.s^{-1}$ con 2 min de descanso entre las series) se inició 80 minutos después de la suplementación. Mediciones de NOx se realizaron por el método de Griess usando un espectrofotómetro de absorción a 540 nm.
Resultados: No se encontraron diferencias significativas entre el grupo suplementado con L-Argvs placebo en lo referente parámetros de WRR, potencia media y TW3S (2630,4 ± 758,0 vs 2573,1 ± 669,9 Joules). Además, no se observó diferencia significativa en el NOx plasmático, en ningún momento, entre el grupo suplementado con L-Arg vs placebo, antes de la suplementación (9,8 ± 2,3vs 9,5 ± 1,4 μmol/L) o inmediatamente después del ejercicio (11,9 ± 5,5 vs 10,2 ± 2,3 μmol/L).
Conclusión: Nuestros resultados indican que la ingesta aguda de L-Arg no aumenta la producción de NO, ni mejora el rendimiento recuperación muscular. En base a estos resultados, es precipitado recomendar suplementos nutricionales que contienen L-Arg como ayuda ergogénica para optimizar la recuperación muscular después del ejercicio de contra-resistencia en individuos sanos y entrenados.

Palabras clave: Suplementos nutricionales. Aminoácidos. Óxido nítrico. Ejercicio.

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INTRODUCTION

Many nutritional supplements have been introduced in the market with the purpose of enhancing athletes' performance[1]. Most of these supplements allegedly help athletes tolerate a higher degree of heavy training by helping athletes recover faster during intense sport training[2]. Supplements containing L-arginine (L-Arg) are the latest ergogenic product to become commercially available with the purpose of increasing muscle performance. The semi-essential amino acid, L-Arg, is the only substrate for endogenous synthesis of nitric oxide (NO) in which, among other functions, its vasodilator effect takes center stage in sports nutrition. Theoretically, this vasodilator effect should favor an increase in blood perfusion, as well as a higher nutrient and oxygen delivery to the active muscles during exercise, enhancing muscle recovery.

Recently, Alvares *et al.*[2] published a review article about the ergogenic effect of L-Arg supplementation in healthy subjects and observed that from five acute studies that evaluated exercise performance after L-Arg supplementation, three studies reported significant improvements in exercise performance. Stevens *et al.*,[3] observed significant increase inpeak torque, total work and fatigue index, usingan isokinetic dynamometer after supplementing 13 subjects orally with a product comprised of L-Arg (6 g) or 9.46 g sucrose isocaloric control in three equalaliquots at 45, 30 and 10 minutes before exercise. By using a similar supplement protocol, Buford and Koch[4] observed asignificant improvement in average power during repeated sets of supra-maximal exercise during cycle ergometer. Although the authors observed improvements in physical performance, they did not measure the underlying mechanism that could explain how the results obtained may have been due to increased NO production.

Oxidation of NO via several metabolic reactions results in the formation of nitrite and nitrate as the two major end products[5]. The principal oxidation product of NO synthesis in aqueous solutions (in the absence of biological constituents such as hemoproteins) is nitrite. The further oxidation to nitrate requires the presence of additional oxidizing species such as oxyhemoproteins[6]. For example, NO is quickly oxidized to nitrite via autoxidation in aqueous solutions, such as biological fluids, and may react with superoxide anions to produce peroxynitrites. In the presence of heme groups in proteins, such as hemoglobin and myoglobin, NO reacts with oxyhemoglobin to produce metahemoglobin and nitrate. Therefore, measurement of nitrite and nitrate in various biological fluids turned out to be the most suitable, practical and reliable non-invasive method to assess systemic NO synthesis in vivo[5].

In order to test the notion that L-Arg supplementation enhances muscle performance and recovery in response to increased NO production, the present study was conducted to identify the acute effects of oral L-Arg supplementation on indicators of muscle performance and recovery – average power, total work (TW3S),work recovery ratio (WRR), and markers of NO synthesis – nitrite and nitrate (NOx). Based on the evidence at hand, it was hypothesized that there would be significant enhancement of muscle performance and recovery, accompanied by increased plasma levels of NOx, as a result of L-Arg supplementation when compared to the placebo condition.

MATERIALS AND METHODS

Subjects

Seventeen healthy males were recruited to participate in the study. All subjects were fully informed of the nature and purpose of the investigation and gave their written consent to participate. The exclusion criteria for participation in the study were any known cardiovascular, pulmonary or metabolic diseases (asthma, diabetes, hypertension, dyslipidemia, etc.), and the use of nutritional and pharmacological ergogenics. All experimental procedures were performed in accordance with the ethical standards of the Helsinki Declaration and were approved by the Institutional Ethics Committee of the Hospital

Universitário Clementino Fraga Filho (protocol #0118.0.197.000-10) from Rio de Janeiro, Brazil.

Experimental Design

The study was conducted in a randomized, double-blind and placebo controlled fashion. All subjects reported to the laboratory on two occasions with at least a one-week interval between visits. The first visit was used to explain the experimental procedures, collect anthropometric data and familiarize the subjects with the exercise protocol. In the second visit, blood samples were drawn from an antecubital forearm vein at baseline after a ten minute period of quiet rest in the supine position. Thereafter, subjects were randomly divided into either a placebo (PLA) or a L-Arg group (ARG). The exercise protocol was initiated 80 minutes after supplementation, lasting approximately 10 minutes. Blood samples were drawn again at 90 minutes after supplementation, which was immediately post-exercise. During this 90-minute period, the subjects consumed no food or drink.

Dietary control

One day before conducting the study, the subjects were oriented as to the nitrite and nitrate content of foods and were requested to restrict their diets from foods rich in nitrite and nitrate. A list describing foods and groups of food to be avoided and to be preferred was distributed to the subjects, in order to simplify their dietary choices for low nitrite and nitrate foods for the 24-hour period prior to the study. In short, the subjects were advised to avoid vegetable products, such as spinach and squash, which contain the highest amounts of nitrate per serving. Sweets, nuts, fats and oils contain very little nitrate per serving and were thus permitted. Red meat (beef, pork, lamb, mutton, and liver) and bean products contain the highest amounts of dietary nitrite per serving and were to be avoided. Negligible sources of dietary nitrite are found in cottage cheese, fats such as butter or margarine, and various fruit juices. This dietary orientation was based on a list developed to estimates of dietary nitrite and nitrate[7]. Adherence to the diet was controlled by twenty-four-hour recall conducted upon arrival for the study, in which each subject was interrogated as to their dietary intake for the 24h period prior to arrival for the study.

Supplementation

Upon arrival at the laboratory, all subjects were orally administered either 6g of encapsulated L-Arg hydrochlorideor placebo (as corn starch) in identical forms with 400 mL of water in a double-blind and randomized manner. We chose to provide 6 g of L-Arg, because such a dose would be well-tolerated when consumed orally, and has been reported to increase vasodilation[8].

Exercise Protocol

The subjects performed unilateral elbow flexion and extension exercise in dominantlimbwith an isokinetic dynamometer (Cybex Norm®, Cybex International Inc., Ronkonkoma, NY, USA). Each subject lay down in a supine position, with the legs flexed and supported by a footplate. The body was stabilized in the bed and strapped with Velcro in order to minimize movements other than the elbow flexion and extension. The elbow flexion/extension adapter was adjusted to the semi-prone position, according to the body dimensions of each subject. These adjustments were recorded so they could be repeated accurately in each subsequent visit. Prior to the exercise test, the subjects performed a warm-up that consisted of a five-repetition set at a velocity of $60^{o} \cdot s^{-1}$, during which the subjects were advised not to perform at maximal effort. After two minutes of rest, three sets of ten maximal voluntary contractions were performed at a velocity of $60^{o} \cdot s^{-1}$ in both the extension and flexion phases, with a recovery period of two minutes between sets.

The exercise performance variables analyzed were: total work over the tree sets (TW3S), average power and work recovery ratio (WRR). Total work is the sum of the work for all of the repetitions for each direction of movement and TW3S is the sum of total work of each set. Total work performed is dependent on the person's

muscular power capability at the test speed, as well as available anaerobic energy stores and pH tolerance in the working muscles. The WRR represents the ratio of the total work performed in the second set to the total work performed in the first set. The Cybex Norm® system calculates the work recovery ratio by dividing the set total work done in the second set by the set total work performed in the first set multiplying by 100.This type of test gives important information about the person's ability to recover from exercise bout.

Nitric Oxide Production

Blood was drawn from antecubital veins and collected in EDTA-containing tubes, and immediately centrifuged at 3000 g for 10 min at 4ºC in order to separate the plasma, before storing it at –80ºC for posterior analysis. Before analysis, the plasmawas filtered using a 10-kDa cutoff ultrafilter membrane (Vivaspin 2, GE Healthcare®) at 14000 g for 15 min to remove high-molecular weight substances. NO production was determined by using a commercially available nitrate/nitrite colorimetric assay kit (Cayman, Ann Arbor, MI). The analysis is based in two-step process: the first step is the conversion of nitrate to nitrite utilizing the enzyme nitrate reductase; the second step is the addition of the Griess Reagents which converts nitrite into a deep purple azo compound. Absorbancewas measured at 540 nm by a microplatereader (VICTOR X4®, PerkinElmer). The results were compared with a standard curve constructed with known concentrations of nitrite. All samples were made in duplicate and at room temperature.

Statistical Analysis

A two-way analysis of variance with repeated measures was used to identify differences in the variables WRR, average power and plasma NOx values between ARG versus PLA conditions. For identify differences on TW3S between groups, an unpaired student t-test was used. When a significant *F* was found, additional post hoc tests with Bonferroni adjustment were performed. Statistical significance was set at the 0.05 level of confidence. All analyses were performed using a commercially available statistical package (GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California USA). The statistical power (1-β err prob) was performed in order to detect the probability of type II error to occur. For this analysis, the G*Power (version 3.1.3) software was used.

RESULTS

Subject characteristics

At the study onset there were no significant differences ($P > 0.05$) between the randomly assigned ARG versus PLA groups with respect to age (26.0 ± 4.6 versus 24.9 ± 1.7 yr), height (175.4 ± 7.7 versus 177.7 ± 7.6 cm), body weight (79.3 ± 12.5 versus 78.1 ± 8.4 kg), body mass index (25.7 ± 2.4 versus 24.9 ± 2.3 kg.m-2) and body fat (14.4 ± 5.6 versus 16.4 ± 2.5%), respectively.

Dietary control

Based on the evaluation of the twenty-four-hour recall, all subjects of both the L-Arg and placebo groups had apparently adhered to the dietary orientation and avoided all foods listed as high in nitrite and nitrate.

Nitric oxide production

Plasma nitrite + nitrate (NOx) concentrations at each time point are depicted in Table 1. No significant differences were found between ARG versus PLAgroups at baseline (9.8 ± 2.3 versus 9.5 ± 1.4 μmol/L; $P > 0.05$) and immediately post-exercise (11.9 ± 5.5 versus 10.2 ± 2.3 μmol/L; $P > 0.05$), respectively.

Muscle performance and recovery

Muscle performance and recovery changes during L-Arg and placebo supplementation over the three sets are presented in Table 2 and Figure 1, respectively. There were no significant differences between ARG versus PLA groups after the supplementation period with regard to average power (46.5 ± 13.1; 45.5 ± 12.2; 45.9 ±

14.6 watts for sets 1, 2 and 3 versus 47.8 ± 12.7 watts; 43.7 ± 12.0 watts; 43.2 ± 9.3 watts for sets 1, 2 and 3, respectively), TW3S (2630.4 ± 758.0 joules versus 2573.1 ± 669.9 joules), and WRR (95.9 ± 6.5; 94.6 ± 8.4 %; for sets 2-1 and sets 3-2 versus 92.6 ± 6.2; 97.8 ± 17.0 for sets 2-1 and sets 3-2, respectively) over the three sets of the exercise protocol.

TABLE 1. Values of NOx (μmol/L) at baseline and immediately post-exercise (90 min after L-arginine supplementation)

Group	Baseline	Post-exercise
ARG	9.8 ± 2.3	11.9 ± 5.5
PLA	9.5 ± 1.4	10.2 ± 2.3

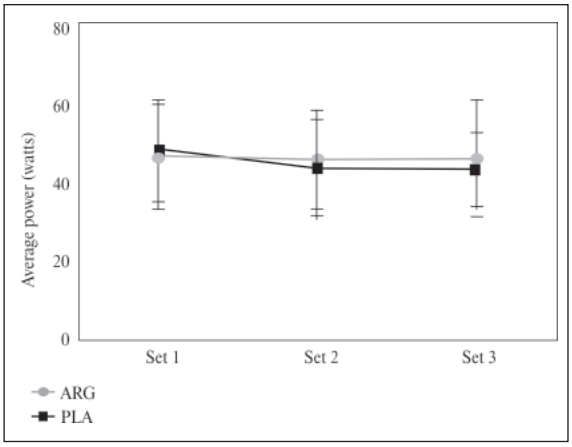
The values are mean ± standard deviation. NOx= Nitrite plus nitrate; ARG= L-arginine supplemented group; PLA= placebo supplemented group.

TABLE 2. Values of muscle performance

Group	WRR (%)		TW3S (Joules)
	Sets 2-1	Sets 3-2	
ARG	95.9 ± 6.5	94.6 ± 8.4	2630.4 ± 758.0
PLA	92.6 ± 6.2	97.8 ± 17.0	2573.1 ± 669.9

The values are mean ± standard deviation. ARG= L-arginine supplemented group; PLA= placebo supplemented group; WRR= work recovery ratio; TW3S= total work over the three sets of exercise.

FIGURE 1. Changes in average power (watts) between ARG and PLA groups



It is important to point out that the relevant statistical test in the present study had virtually a very small power to detect a "high" size difference between the ARG and PLA groups regarding: 3STW (1-β err prob = 0.33), average power (1-β err prob = 0.23) and WRR (1-β err prob = 0.33).

DISCUSSION

Dietary supplements based on the semi-essential amino acid L-Arg (the only substrate of NOS) have been introduced in the market, claiming to promote vasodilatation by increasing production of NO in the exercising muscle. The present study was designed to test our hypothesis that oral L-Arg supplementation would enhance: 1) indicators of muscle performance and recovery – average power, TW3Sand WRR; and 2) markers of NO production – NOx in healthy male subjects.

In the present study, we found that in healthy subjects 6g of oral L-Arg supplementation neither improved muscle performance and recovery nor stimulated an increase in NO production when compared with the PLA group. The results of present study are contrary to previous studies that had observed increased on exercise performance after L-Arg supplementation. Stevens *et al.*[3] supplemented 13 subjects orally with a product comprised of L-Arg (6g) plus glycine (2g) plus α-ketoisocaproic acid (3.2g) or 9.46g sucrose isocaloric control in three equal aliquots at 45, 30 and 10-min period before exercise, which consisted of 35 continuous isokinetic concentric/eccentric knee extension repetitions at 90°•s^{-1}. They observed a significant increase in peak torque and total work as well as a significant decrease in the fatigue index. By using a similar supplement protocol, Buford and Koch[4] observed significant improvement of average power during repeated sets of supra-maximal exercise during cycle ergometer.In another study, Santos *et al.*[9] observed a significant decrease in the work fatigue indexes after 15 days of L-Arg supplementation (3 g) in healthy volunteers who completed an exercise test protocol that consisted of isokinetic dynamometry at an angular velocity of 180°•s^{-1} using 15 reps of knee extension and flexion. Campbell *et al.*[10] observed significant increases in Wingate peak power and 1RM bench press after supplementing 35 resistance-trained healthy males

during three weeks with 12 g of oral L-arginine alpha-ketoglutarate (AAKG).

The present study has shown no significant difference in WRR – the indicator of a person's ability of recover after an exercise bout – or muscle performance (average power and TW3S) between ARG and PLA groups. However, there are some possible limitations that may be addressed: it is important to note that the number of repetitions used in the exercise protocol of the present study was smaller when compared to the study of Stevens *et al.*[3] and Santos *et al.*[9], which had found a significant tolerance to fatigue. Furthermore, both Stevens *et al.*[3] and Santos *et al.*[9] submitted the subjects to a fatigue-inducing exercise protocol, whereas Campbell *et al.*[10] submitted the subjects to whole-body resistance training. Moreover, Santos *et al.*[9] and Campbell *et al.*[10] evaluated the effects of chronic supplementation – contrary to the protocol of the present study.

The apparent controversy between the results of these studies could be explained in part by differences in the exercise protocols. L-Arg supplementation may only be effective for exercises with a large number of repetitions (> 30 reps), or for exercises that induce complete muscle fatigue (the point at which an individual can no longer withstand the load) or for applied multiple-joint exercises. Another important factor to be considered regarding the ergogenic effect of L-Arg supplementation is the training status of the subjects. Studies involving untrained or moderately trained healthy subjects showed that L-Arg supplementation could improve tolerance to aerobic and anaerobic exercise[3,9,10]. However, it cannot be assumed that the positive results for exercise performance were due to increased NO production via L-Arg supplementation, since none of these reports investigated the underlying mechanisms, such as measurements of NO production. Furthermore, when trained subjects were supplemented, no positive effect on performance was observed[11,12]. It is important to point out that the factor that would explain the absence of L-arginine effect in well-trained subjects could be explained by the physiological and metabolic adaptation derived from physical training.

In addition, all the abovementioned evidence is mainly based on a young male population. Further research in other populations (e.g.: elderly and female subjects) are needed to determine whether NO supplements can induce benefits in exercise capacity.

Several others studies had showed no significant difference in NO production (measured by nitrite and nitrate) after L-Arg supplementation[11-14]. Liu *et al.*[11] did not observe any significant differences in plasma nitrite and nitrate concentrations after orally supplementing ten healthy male athletes with 6 g of L-Arg (as free form) or placebo for 3 days. Koppo *et al.*[12] observed no significant difference in urinary nitrite and nitrate after 14 days of supplementing seven physically active healthy males with 7.2 g of L-Arg hydrochloride (3 × 3 capsules of 805 mg), and Tang *et al.*[13] also did not observed any significant difference on NO synthesis (measured by plasma nitrite and nitrate) in eight healthy young men after an single dose of 10 g of L-Arg. However, contrary to the present study, neither of the abovementioned studies controlled the intake of food rich in nitrite and nitrate from subject's diet before or during the study. Most nitrite and nitrate comes from diet (vegetable products contain the highest levels of nitrate; meat and bean products contain the highest levels of nitrite), which may alter the results of the analysis. Thus, endogenous synthesis of NO may not be adequately measured by nitrite and nitrate in plasma and urine if the diet is not controlled.

The lack of significant differences in NO observed in the present study may be explained by the fact of physiological L-Arg concentration far exceeds the Michaelis-Mentenconstant of endothelial NOS. Pollock *et al.*[15] reported that the in vitro Michaelis-Menten constant of endothelial NOS is ≈3 μmol/L, whereas the L-Arg concentrations in the plasma of both healthy and non-healthy individuals ranges from 40 to 100 μmol/L[16]. These data suggest that physiological concentrations of L-Arg are enough to saturate endothelial NOS, and that supplementary L-Arg does not promote increased enzyme activity. Therefore, there should theoretically be no need

for supplementary L-arginine to synthesize NO in healthy subjects.

However, evidence suggests that L-Arg supplementation may help treat individuals with atherosclerosis risk factors, such as hypercholesterolemia, hypertension, diabetes mellitus, kidney failure, hyperhomocysteinemia, smoking, and aging – all of which are conditions that are associated with reduced NO biosynthesis[17-21]. Dong *et al.*[22], recently publisheda meta-analysis investigating the effects of oral L-arginine supplementation on blood pressure. Eleven randomized, double-blind, placebo-controlled trials with a duration of 4 weeks or longer were investigated, which involved 387 participants supplemented with oral L-arginine (Doses of L-arginine ranging from 4 to 24 g/day). Compared with placebo, L-arginine intervention significantly lowered systolic blood pressure by 5.39 mm Hg and diastolic blood pressure by 2.66 mm Hg. The authors suggest that adopting a healthy diet containing L-arginine–rich foods may contribute to preventing hypertension. Another meta-analysis[23] of randomized controlled trials that evaluated the effect of oral L-arginine supplementation on endothelial function suggests that individuals with apparently impaired endothelial function (low baseline flow-mediated dilation) are likely to benefit from short-term oral L-arginine intake (dose of L-arginine ranges from 3 to 24 g/d).

Böger,[24] had shown that plasma levels of asymmetric dimethylarginine (ADMA), an endogenous NOS inhibitor, are increased approximately 2-3 fold in the pathophysiological conditions associated with cardiovascular disease. For this reason, elevated ADMA concentration may be one possible explanation for endothelial dysfunction and decreased NO synthesis in this disease cluster. Therefore, it appears that only in subjects with poor NO synthesis are more likely to benefit from L-Arg supplementation.

In conclusion, oral supplementation with 6g of L-Argwas not able to improve muscle recovery between sets of resistance exercise. In addition, L-Arg supplementation did not stimulate an increase in NO synthesis. This data did not support the notion of recommending supplements based on L-Arg amino acid in order to improve muscle performance in healthy subjects. Long-term studies investigating the effects of L-Arg supplementation on different populations (healthy and non-healthy subjects) are needed to better understand the underlying mechanism and physiological effects of L-Arg supplementation before making any recommendation about L-Arg utilization as a nutritional supplement.

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