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Citrulline in health and disease. Review on human studies

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Citrulline in health and disease

Abstract

The amino acid L-citrulline (CIT) is safely used from the neonatal period onwards in those with urea cycle defects and carbamyl phosphate synthetase or ornithine transcarbamylase deficiencies, but several lines of enquiry indicate that it might have a much wider therapeutic role.

When protein intake is low and there is a catabolic state, endogenous arginine (ARG) synthesis cannot fully be met and its supplementation can prove challenging, particularly in patients with critical and multisystem illness. Supplementary CIT could constitute a safer but still focused means of delivering ARG to endothelial and immune cells as CIT is efficiently recycled into these cells and as kidneys can convert CIT into ARG. Unlike ARG, CIT is efficiently transported into enterocytes and bypasses liver uptake. It also appears to prevent excessive and uncontrolled nitric oxide (NO) production. Animal studies and early human data indicate positive effects of CIT on protein synthesis, in which its contribution is thought mediated through the mTOR pathway.

It apperas that CIT is an anabolic pharmaconutrient that can be safely administered even in critically ill patients. Promising results in cardiovascular diseases and in disease-related malnutrition can now be considered sufficient to justify formal clinical exploration in these areas and in sarcopenia in general.

Introduction and background

Citrulline (CIT) is an amino acid which is an end product of glutamine metabolism and a metabolite of arginine (ARG).

Its name is derived from citrullus the Latin name for watermelon. It was extracted in 1914 by Koga&Odake from watermelon[1] and identified by Wada in 1930. Of note, this aminoacid is not incorporated into proteins[2].

Glutamine is a precursor of ornithine, which can be converted to CIT by the intestine. Arginine is also metabolized in CIT into enterocytes[2] . Because enterocytes do not possess argininosuccinate synthase, CIT is released in the portal vein [3]. Since there only small net CIT uptake by the liver[3], in the presence of normal liver function, it enters the systemic circulation and is then transformed in the kidney to ARG (fig.1). CIT net production at the whole body level is therefore almost exclusively from the epithelium of small intestine (Fig 1) [2] .

CIT can act as ARG regulator as it can control delivery of ARG to the liver. Intestinal arginase and ornithine carbamoyl transferase yield citrulline in proporsion to delivery of dietary protein. Fasting leads to less CIT and proportionally more ARG reaching liver. ARG has a major impact on hepatic enzymes and up to 5 fold increase in ureagenesis. This effect needs to be regulated, hence the important role of CIT in this physiological context[2,3]

These metabolic considerations explain why administration of CIT has been proposed to increase systemic ARG concentrations. Of note, CIT is almost absent in food. Only

watermelon contains significant amounts of CIT; all parts of watermelon, rind, flesh and seeds contain CIT in greatest amount, on a dry weight basis in the rind, which might offer a convenient source of natural citrulline[4]

In healthy individuals the plasma concentration of citrulline is about 40 µmol/L with some racial variation (less in Chinese Asians) [5].

CIT plasma concentration has been proposed as clinical tool for identification of small bowel absorptive mass. Statistical significant correlations between plasma CIT concentration and small bowel length as well as villous atrophy have been demonstrated. CIT has been considered a reliable marker of intestinal malabsorption and its role in clinical practice in currently under investigation[6].

CIT as an organic supplement appears to be a powerful pharmaconutrient, and early experimental studies have suggested its therapeutic potential to restore ARG metabolism in critically ill patients with sepsis [7, 8].

CIT exhibits good bioavailability [9], thanks to its ability to be handled by a wide number of amino acid transporters [10]. In the liver CIT is a metabolic intermediate involved in the elimination of a toxic component (ammonia) through another which is non-toxic (urea) (Fig.1). Of note, CIT recycling in the urea cycle is mainly separated meaning that there is neutral /balanced flux in the liver [3] (Fig 1). The brain and some leukocytes can also produce ARG from CIT [11] to a limited extent.

Oral CIT supplementation raises plasma ARG concentrations and augments nitric oxide (NO)-dependent signalling proportionally [12] . Since CIT is not subject to pre-systemic

elimination in the liver whereas ARG is largely extracted there [13] (Fig. 1) CIT serves as an ARG precursor more productively than ARG itself [2, 3, 12]. In most human studies, CIT has been used as a supplement, intending this as a substrate from which ARG can be synthesized or as a NO precursor.

CIT is indeed one of the key organic compound leading to production of NO in most cells, NO synthase (NOS) enzymes catalysing the conversion of ARG into CIT, producing NO in an internally conservative cycle[13] (fig 2). Although intracellular ARG concentrations are sufficient to saturate NOS, therapy with excess ARG can enhance NO production because the CAT-2 ARG transporter is closely associated to NOS within the cell membrane and both are co-stimulated by signals such as pro-inflammatory cytokines [3]. In the situation of low protein intake, it is possible that the alternative pathway is activated. To inhibit ARG derived ureagenesis and thus loss of proteins, intestinal arginase and ornithine carbamoyl transferase are activated. This results in an increase in prehepatic conversion of ARG to CIT, which (unlike ARG) passes more or less freely through the liver and is released to the systemic circulation[2,13,14].

CIT as dietary supplement appears to be a powerful pharmaconutrient, and early experimental studies have suggested its therapeutic potential to restore ARG metabolism in critically ill patients with sepsis [7, 8].

Safety of citrulline administration

CIT is considered as safe for oral use [15]. It has no identified toxicity and is used as long-

term replacement therapy for children with urea cycle defects. In contrast to ARG and ornithine, which induce gastrointestinal side effects at moderate dosage (e.g. 10g in one bolus) [16], no side effects have been reported from CIT administration as an oral supplement at doses up to 15g [9]. Additional safety data come from interventional studies.

In a randomized placebo-controlled double-blind trial of orally administered CIT in 40 children undergoing repair of congenital cardiac defects no adverse events were noted [17]. In a pharmacokinetic study of intravenously administered CIT no side effects or adverse events were noted [18]. This pharmacokinetic study formed the basis for an on-going randomized, placebo controlled, double-blind trial of intravenous CIT in children at above average postoperative risk after surgery for congenital heart disease [19] .

CIT supplementation is now raising clinical interest for the treatment of paediatric pulmonary hypertension however further controlled clinical trials are needed to drawn an impactful conclusion [20] .

Effects on immunity, oxidative stress and related parameters

CIT administration reduces the number of total leukocytes and of neutrophils in circulation [9] and might induce ARG-derived NO-mediated vasoprotection, with inhibition of cell adhesion and leukocyte activation, and suppression of endothelial damage (Fig. 2) [21].

In red blood cells NO is oxidized to nitrate. Nitrite and nitrate are excreted in the urine. PRMT (protein arginine methyl transferase) methylate L-arginine in proteins and methylated proteins are hydrolysed to L-arginine derivate including ADMA (asymmetric dimethylarginine) that is hydrolysed by DDAH (dimethylarginine dimethylaminohydrolase) to L-citrulline. CIT acts as scavenger for oxidative lipoproteins and ADMA[22] (Fig 2).

Citrulline mediated vasoprotection has now been demonstrated in a phase 2 study of sickle cell disease [23] and further trials are on-going.

As further NO mediated effect with major changes in markers of oxidative stress was demonstrated by supplementing a group of professional cyclists[21] with a single pre-race dose of 6g CIT malate. Higher concentrations of neutrophil nitrite suggested that these effects were mediated by NO (Fig. 2), and there was no evidence of oxidative damage (levels of malondialdehyde and creatine kinase, for example, remaining normal).

However, there is no definitive evidence that effects of CIT on immunity are mediated through NO synthesis only. Polyamines derived from ARG and ornithine could also be involved. Also, CIT has anti-oxidant properties, which could be involved in these effects.

Effects on sports performance and recovery

In addition to the study reported above [21], several others are of interest in this field. Oral CIT supplementation given for a week reduced the time needed to complete a cycle ergometer exercise trial in healthy trained men in a double-blind randomized placebo-

123 controlled 2-way crossover study [24] CIT supplementation significantly increased plasma
124 ARG levels and reduced the exercise time by 1.5 % ($p < 0.05$). This was associated with
125 subjective improvements in muscle fatigue and ability to concentrate immediately after
126 exercise.
127 The effects of CIT on NO biomarkers, pulmonary O_2 uptake ($\dot{V}O_2$) kinetics, and exercise
128 performance were studied in a randomized, placebo-controlled, crossover study. Short-
129 term CIT, but not ARG supplementation can improve $\dot{V}O_2$ kinetics as expressed by $\dot{V}O_2$
130 mean response time (59 ± 8 and 53 ± 5 s with placebo and CIT respectively, $p<0.05$)
131 during severe-intensity exercise, improving the tolerance (duration: 589s ± 101 vs 661s ±
132 107), and increasing the whole volume of work completed [25].
133 A further preliminary study suggested that consuming CIT malate before competition has
134 the potential to improve some elements of performance in masters level female tennis
135 players [26]. In this lab-based study CIT yielded improved grip strength, peak and
136 explosive power compared to placebo. Direct application to “on court performance” is
137 requested to validate results.
138 A randomized double-blind cross-over study [27] examined the effect of a single 8g dose
139 of CIT malate on the performance of flat barbell bench presses (pectoral training) as an
140 anaerobic exercise and to test muscle soreness after this exercise. The study showed a
141 significant increase in the number of repetitions achieved (52% more in the 4th set than in
142 the equivalent placebo session where 40mL lemon juice, 10 g powdered sugar, 60 mg
143 sodium saccharine, and tap water 200 mL were used) and there was a 40% decrease in

muscle soreness at 24 and 48 hours [27]. A further randomized double-blind study [28] examined the effect of a pre-exercise dose of CIT (6g), watermelon juice (to provide CIT 1g), or placebo (7.5% sucrose placebo drink) on the total number of repetitions completed over 5 sets, time to exhaustion, maximal oxygen consumption (VO_2max), anaerobic threshold, and flow-mediated vasodilation. In this study pre-exercise supplementation appeared to be ineffective in improving exercise performance [28].

It thus appears that CIT may improve exercise performance in young healthy adults under some conditions, but these acute effects still need further investigation [29].

Of note, in several of these studies, CIT has been used as a malate salt, not as the native amino acid. As malate is an intermediary of the Krebs cycle increasing cellular energy production it is unclear whether the observed effects are due to malate, to CIT or to both. There is no study comparing the effects of CIT malate and CIT. In Table 2 the doses of citrulline from citrulline malate have been corrected to subtract the contribution of malate in those cases where this salt was used.

MELAS syndrome

As CIT plays a key role in the production of NO in most cells, due to its great ability to increase intracellular ARG availability, it has been used in children with the Mitochondrial Encephalomyopathy, Lactic Acidosis, and Stroke-like episodes (MELAS) syndrome [30]. In a recent clinical study stable isotope infusion techniques were used to assess NO production in children with MELAS syndrome and in healthy controls. In

children with MELAS syndrome, CIT supplementation resulted in important increases in NO production, ARG flux, plasma ARG, and CIT flux, which were greater than obtained with ARG supplementation [31]. In an earlier clinical trial [32], the effect of ARG or CIT on lactic acidaemia had been studied in adults with MELAS syndrome. Plasma lactate decreased significantly after CIT supplementation, whereas the effect of ARG supplementation did not reach statistical significance. These promising results justify additional controlled trials to assess the therapeutic effects of CIT on clinical features and complications of MELAS syndrome.

Cardiovascular diseases

In most of the work in the cardiovascular area, CIT has been given with the intention of boosting levels of ARG and as a NO precursor. The conditions studied have therefore included those where absolute or relative deficiencies of ARG and NO are known or suspected, and include arteriosclerosis, pulmonary and systemic hypertension and cardiac failure. In general, oral CIT is seen to improve cardiac performance with exercise. A causal link is supported by the study on professional cyclists already referred to above [19]. In a double-blind, randomized, placebo-controlled trial [33] 15 otherwise healthy middle-aged male subjects with evidence of early arteriosclerosis were given 5.6 g/day of CIT (n=8) or placebo (n=7) for 7 days. Their initial arterial stiffness was abnormal, as indicated by a brachial-ankle pulse wave velocity (baPWV) of >1400 cm/s. CIT supplementation increased plasma CIT ($p<0.05$), plasma ARG ($p<0.01$), and the ratio of

ARG to ADMA ($p<0.05$). The circulating concentrations of nitrogen oxides (the sum of nitrite and nitrate) and other metabolic products of NO also rose significantly ($p<0.05$) [33]. Associated with these biochemical changes there was a clinically significant fall in the baPWV in the CIT group ($p<0.01$) with no change in systemic blood pressure, suggesting the potential for functional improvements in arterial stiffness from CIT supplementation, independent of blood pressure.

Other studies in patients and in healthy volunteers do however demonstrate effects of CIT on systemic blood pressure. In the cold pressor test (CPT), it is normal to see substantial increases in systemic blood pressure, pulse pressure and a number of other haemodynamic parameters. After 4 weeks of daily CIT supplementation (6 g orally) these effects were all attenuated (each by 4 to 6 mmHg) [34].

Beneficial effects of CIT and ARG on endothelial function are shown by their normalization of the MAT/TT index in patients with early cardiac failure. This index is the ratio of the maximum amplitude time (MAT) on finger plethysmography to the total time (TT) of the curve. After 60 days during which 3 g oral CIT malate was given daily, the basal MAT/TT had fallen from a mean of 41.1 (± 13.47) to 23.6 (± 6.74) ($p=0.007$) (where 30 is the upper limit of normal). Testing was repeated after brief, experimentally induced, digital ischaemia, and when ARG was given rather than CIT, and in both cases similar improvements were seen at 60 days [35], which suggests a common mechanism of action, likely through NO production.

In a second paper [36], on the same patients, the authors recorded the effects of CIT on

pulmonary artery pressure, which fell by 16% (56.7 ± 7.96 mmHg to 47.7 ± 8.59 mmHg; $p<0.05$) over 60 days in association with an improvement in right ventricular ejection fraction, blood pressure and treadmill tolerance. ARG was equally effective, but required the higher dose of 8 g [36]. This may be explained by the fact that ARG is metabolized in splanchnic area whereas CIT is not (see above for details).

Another group of investigators recently reported that adults with heart failure had improvements in left ventricular ejection fraction, functional class, and endothelial function as assessed by photoplethysmography after treatment with oral CIT for 4 months [37].

Intensive CIT supplementation (oral or intravenous) was previously proposed as a possible means of preventing post-operative pulmonary hypertension, with a subsequently suggested target plasma CIT concentration in excess of 37μmol/L [37]. This has been partially tested in children undergoing surgical procedures for congenital heart lesions. Oral CIT supplements safely increased plasma CIT and ARG concentrations compared with placebo, and improved NO production [17]. The expected decreases in plasma CIT and ARG concentrations after cardiopulmonary bypass seen in the placebo group were prevented by CIT. This was associated with a decreased risk of postoperative pulmonary hypertension (15% in those treated with CIT compared to 30% in the controls). It was thought that this effect was causally derived from the production of L-ARG from CIT, and to stimulation of NO pathway in the hepatic and pulmonary tissues. The cytosolic portion of the urea cycle was thought to be enabling localized, intracellular production of

L-ARG from CIT within the pulmonary endothelium as well as in hepatocytes. Curiously these papers, which report on work from more than a decade ago, do not seem to have been followed-up by their authors or others in the field [38,39].

Electrophysiological mechanisms may also be important. A study in healthy individuals given CIT (3.2g 60-90 minutes before testing) demonstrated a reduction in QT interval on electrocardiography, indicative of a shortening of the time required to de/repolarize the myocardium [40].

Taken as a whole these results are impressive but most enrolled a limited number of subjects and only surrogate markers were studied. The time appears ripe for study of larger cohorts of patients and evaluating the effects of CIT on morbidity and mortality.

Anabolic effects

Through various underlying mechanisms, CIT has the potential to affect protein metabolism [41]. Osowska et al [42] showed that when malnourished elderly rats were re-fed with a CIT-enriched diet, muscle protein synthesis was greater, while hepatic protein synthesis was less than in control rats fed an isonitrogenous supplement of non-essential amino acids (NEAA). These data are now being supported by human studies of muscle protein synthesis [43]. Eight healthy participants were investigated in a crossover study in which, following 3 days of standardised low protein intake, CIT or a NEAA mixture was given orally as small boluses over the course of 8 hours. Stable isotopes of phenylalanine [^{13}C] and tyrosine [^{15}N] were administered as tracers to assess protein metabolism. The

fractional synthesis rate (FSR) of muscle protein was measured using phenylalanine enrichment in muscle tissue fluid as the precursor pool. The FSR of mixed muscle protein was found to be higher after the period on CIT than when on NEAA (NEAA: 0.049 ± 0.005; CIT: 0.060 ± 0.006; p= 0.03). Muscle mitochondrial protein FSR and whole-body protein turnover did not differ between the two phases of the study [43].

In a randomized controlled study of 10 healthy subjects, oral CIT supplementation was associated with a 57% improvement in nitrogen balance at 12 hours (from 683 (±246) to 970 (±187) mmol nitrogen/12 h; p=0.0053 for the comparison with placebo) [15].

In a more recent study [44] on sixty-six healthy volunteers, supplementation with CIT and reduced glutathione were associated with an improvement in cGMP activity, suggesting direct effects on muscle protein synthesis and muscle performance. As in Jourdan's study [43], in a further study [45] of healthy, well-nourished volunteers, oral CIT could not be shown to affect whole-body protein kinetics in the post-absorptive state.

Muscle protein synthesis contributes only about 25% of whole body protein synthesis [34] and an increase of (for example) 20% in muscle protein synthesis would therefore contribute less than a 7% increment in whole body protein synthesis. Together with Osowska's data [42] of lower hepatic protein synthesis rates in rats fed with CIT, this may explain the apparent lack of a CIT effect at the whole-body level despite a statistically significant effect on muscle protein synthesis. Bouillanne et al [46] show, in a prospective randomized multicentre study, that 3-week's CIT supplementation (10 g/day) in 29 moderately malnourished elderly subjects led to higher muscle mass and fat free mass,

and lower fat mass than controls supplemented with NEAA, whereas whole body protein synthesis was similar in the two groups. In other words, the effects of CIT on nitrogen handling are neither ubiquitous nor uniform, and its anabolic effects are likely to be specific to muscle.

However, in one study [47] of 22 healthy, elderly subjects, an effect of CIT on myofibrillar protein synthesis was not confirmed. CIT co-ingestion with a low quantity (15 g) of protein was ineffective in augmenting anabolism compared with NEAA. Hyperargininaemia was interestingly demonstrated after ingestion of CIT in this study.

The mechanisms of action of CIT begin to be understood. Data suggest an involvement of the mTOR (mammalian/mechanistic target of rapamycin) pathway in the effect of CIT on protein synthesis [41, 48]. In general mTOR coordinates protein synthesis and mitochondrial functions by selectively modulating synthesis of a series of nuclear-encoded mitochondrial proteins as well as by regulating mRNA translation [48].

In addition, it has been shown that NOS activity is necessary for calcium-induced activation of the Akt pathway (involved in translation initiation and thus muscle protein synthesis) through a cGMP/PI3K-dependent pathway [49]. Nitrite has been shown to enhance mTOR activity and cell proliferation of myoblasts [50]. CIT has relevance in both of these contexts.

Conclusion

Exogenous CIT is a potent precursor for ARG and it functions as a donor of NO in many clinical contexts. Its administration appears safe but there are currently few clinical

studies from which to draw conclusions on its therapeutic efficacy. Preliminary data indicate that it could be of value in systemic and pulmonary hypertension, in cardiac failure, in the management of arteriosclerosis, and in sarcopenia in the elderly (Table 1and 2). Several new clinical research studies have been designed to address these interesting possibilities and are on-going.

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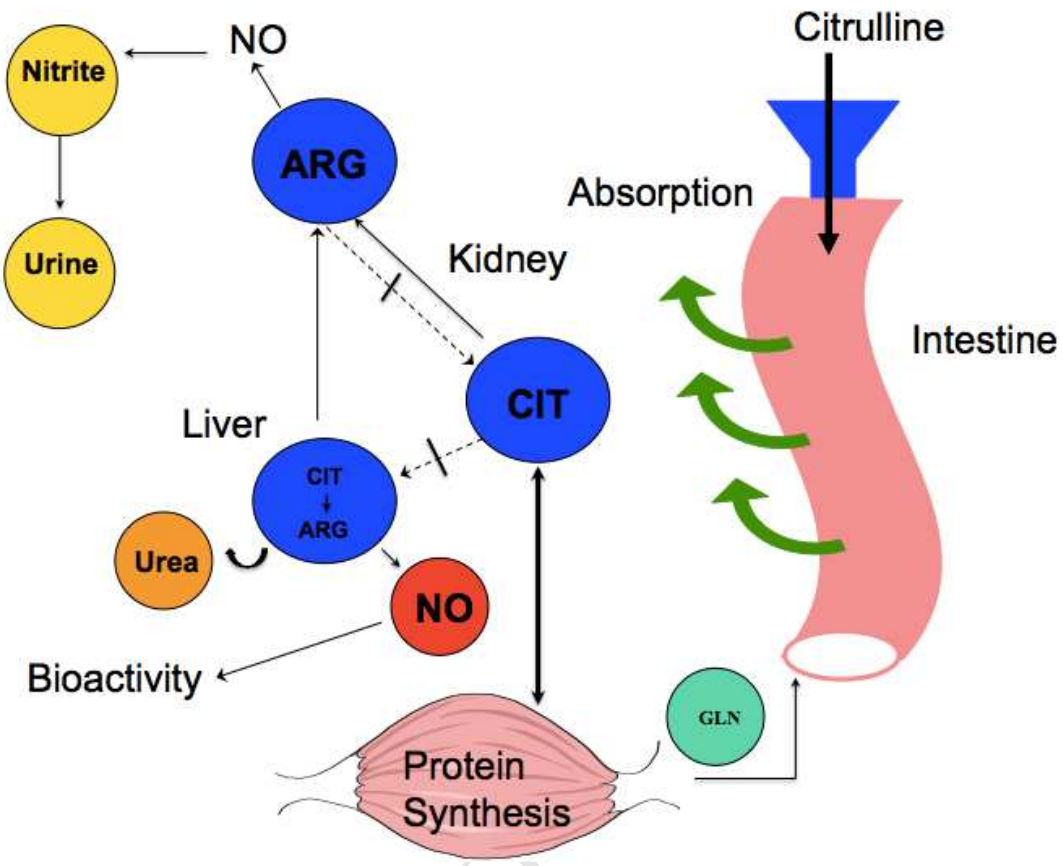
Table1. L- Citrulline: Interventional studies in humans

References	Conditions	Effects
8, 36, 39, 42, 43	Protein malnutrition	Increases fractional synthesis rate of muscle proteins, increases muscle mass and fat free mass, increases nitrogen balance
18-20	Healthy controls	Increases muscle performance
15, 17	Professional athletes	Reduces post-exercise oxidative stress
15, 17, 22, 26	Arteriosclerosis	Improves arterial stiffness, increases NO metabolites, decreases lipid oxidation
27	Hypertension	Reduces blood pressure on the cold pressor test
16	Sickle cell disease	Reduces hypertension in crises
28-30	Cardiac failure	Normalizes MAT/TT index, improves right ventricular ejection fraction and reduces hypertension
13, 29, 31	Pulmonary hypertension	Reduces pulmonary artery pressure

Table 2. L-Citrulline: Design of human studies

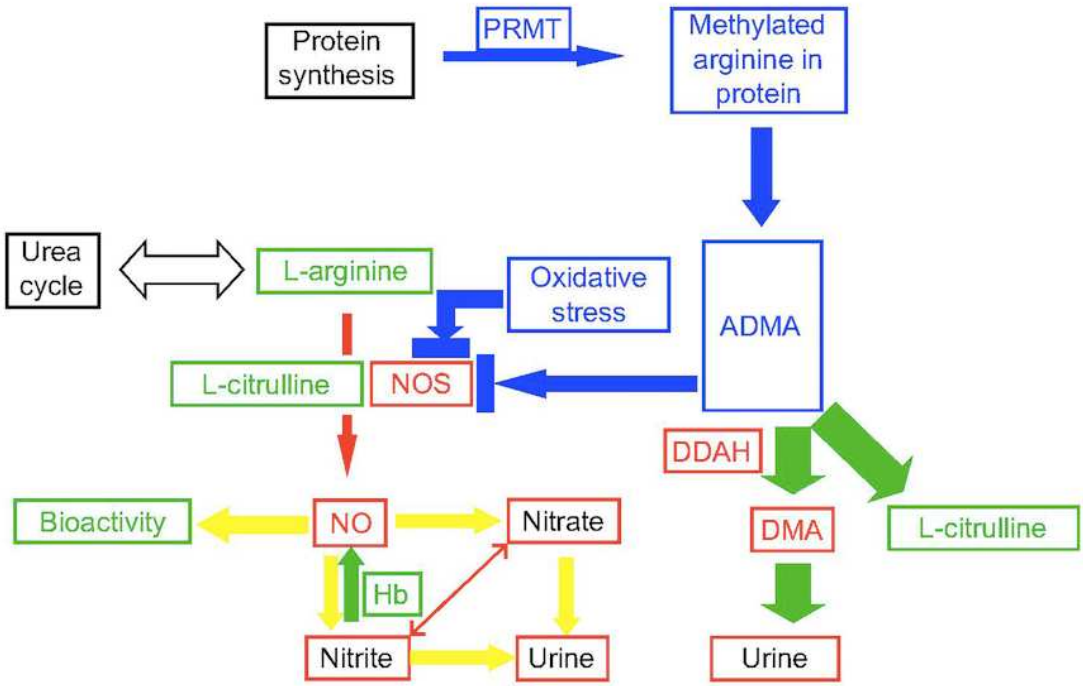
Author	References	Dose/day	Days	Subjects	Design
Schwedhelm E	8	0.75, 1.5, 3 g BD	7	20	Double-blind randomized placebo-controlled cross over
Rouge C	11	0.18 g/kg	3	10	Pilot randomized-controlled
Smith HA	13	9.5g / m^2	1	40	Randomized placebo controlled
Sureda A	15	3.4 g	1	16	Prospective, randomized single blinded study
Waugh WH	16	0.1 g/kg	28	5	Early phase II
Suzuki T	17	2.4 g	7	22	Double-blind randomized placebo-controlled 2-way crossover
Bailey SJ	18	6 g	7	10	Randomized placebo-controlled, crossover
Glenn JM	19	5.3 g	1	17	Double-blind, randomized
Pérez-Guisado J	20	4.5 g	1	41	Randomized, double-blind, 2-period crossover
Morita M		80 mg/day	60	22	Prospective pilot
Figueroa A	27	6 g/day	28	17	Randomized controlled crossover
Orea-Tejeda A	28	3g	60	30	Prospective controlled
Orozco-Gutiérrez JJ	29	3g	60	15	Prospective controlled
Balderas-Munoz K	30	3g	120	20	Prospective randomized placebo controlled
Jourdan M	36	11 – 24 g	1	8	Randomized cross-over pilot
Bouillanne O	39	10 g	21	29	Randomized multicentre

Abbreviations: BD, bis die (twice daily). In this table the citrulline doses have been corrected to subtract the contribution of malate in those cases where citrulline malate was used (15,19,20).



Abbreviations : ARG L-Arginine; CIT L-Citrulline, GLN Glutamine, NO Nitric Oxide

Figure 1: Effects of L-Citrulline supplementation



Abbreviations: ADMA: asymmetric dimethylarginine; DDAH: dimethylarginine dimethylaminohydrolase; DMA: dimethylamine; NOS nitric oxide synthase

Figure 2: Citrulline and Oxidative Stress