Bioscience, Biotechnology, and Biochemistry, 2017
Vol. 81, No. 2, 372–375

Note

The effects on plasma L-arginine levels of combined oral L-citrulline and L-arginine supplementation in healthy males

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Received June 21, 2016; accepted August 17, 2016
http://dx.doi.org/10.1080/09168451.2016.1230007

We investigated the effects of combining 1 g of L-citrulline and 1 g of L-arginine as oral supplementation on plasma L-arginine levels in healthy males. Oral L-citrulline plus L-arginine supplementation more efficiently increased plasma L-arginine levels than 2 g of L-citrulline or L-arginine, suggesting that oral L-citrulline and L-arginine increase plasma L-arginine levels more effectively in humans when combined.

Key words: oral intake; combination effect; L-arginine, L-citrulline, human

L-Arginine is a conditionally essential amino acid that has a variety of effects, including improved endothelial function,[1–3] enhanced sport performance,[4,5] improved insulin resistance,[6,7] and improved erectile function.[8] However, relatively large doses of L-arginine are required to produce these effects in humans. Approximately 40% of oral L-arginine is metabolized by arginase on the first pass,[9,10] and a further 15% of systemic L-arginine is abstracted and metabolized by the liver.[11]

L-Citrulline is present in the body and is a potent endogenous precursor of L-arginine as part of the recycling system within the L-citrulline–NO cycle.[12,13] NO synthase catalyzes a complex enzymatic reaction that leads to NO formation from L-arginine and oxygen, generating L-citrulline as a by-product. Interestingly, a clinical trial has shown that oral intake of L-citrulline dose dependently and more effectively increases plasma L-arginine levels than L-arginine supplementation alone in healthy human volunteers.[14] It is thought that L-citrulline is not extracted from the systemic circulation and is poorly metabolized in the intestine and liver,[15,16] therefore, oral supplementation with L-citrulline might be more effective in increasing plasma L-arginine than simply raising the dose of L-arginine.

Hayashi et al. demonstrated that long-term administration of a combination of L-citrulline and L-arginine has a better therapeutic effect on high cholesterol-induced atherosclerosis in rabbits than arginine alone.[17] Intriguingly, we recently discovered, in an animal study, that oral supplementation with a combination of L-citrulline and L-arginine efficiently increases plasma L-arginine concentration and peripheral circulation.[18] However, the effects of this combination on humans have not as yet been investigated. We hypothesized that plasma L-arginine would be increased to a greater extent with a combination of L-citrulline and L-arginine, each at half dosage, than by ingesting each of them singly at full dosage. The objective of the present study was to investigate the effects of a combination of oral L-citrulline and L-arginine on plasma L-arginine levels in humans.

Forty-five healthy Japanese male volunteers, aged 20–49 and 25 kg/m^2 ≤ body mass index < 30 kg/m^2, were included in this double-blind, randomized, placebo-controlled trial. Randomization was conducted using SAS 9.3 (SAS Institute Inc., Tokyo, Japan). Current smokers and subjects taking medication or dietary supplements for chronic conditions were excluded. Three males were excluded from the analysis because they dropped out for non-medical reasons. We therefore analyzed a final total of 42 males (Placebo: $n = 11$, L-citrulline: $n = 11$, L-arginine: $n = 10$, L-citrulline + L-arginine: $n = 10$). The protocol was conducted in accordance with the Declaration of Helsinki and was approved by the Ethics Committee of Fukuda Clinic (Osaka, Japan). All subjects gave their prior written informed consent. The study was conducted at Fukuda Clinic (Osaka, Japan). The subjects received either L-citrulline (2.0 g/day), L-arginine (2.0 g/day), a combination of each at half dosage (1.0 g/kg) or placebo (cornstarch), orally in capsule form for eight days. L-Citrulline and L-arginine were obtained from KYOWA HAKKO BIO CO., LTD. (Tokyo, Japan) and cornstarch was obtained from Nippon NSC Co., Ltd. (Tokyo, Japan). The daily dose was taken after breakfast from Days 1–7. On Day 1, blood samples were collected from the brachial vein before supplementation (baseline). On Day 8, 15-mL blood samples were collected from the brachial vein at 0 (pre-value), 0.5, 1, 2, and 4 h after oral supplementation. Plasma samples were prepared using an EDTA-2Na-containing tube and kept on ice until centrifugation

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at 1700 g for 10 min at 4 °C. To assess amino acids, the plasma sample was deproteinized with 4% sulfosalicylic acid (plasma to 20% sulfosalicylic acid ratio = 0.3:0.075 mL) for 30 min on ice and then centrifuged at 1700 g for 10 min at 4 °C. The supernatant was stored at -80 °C until analysis. The concentrations of L-arginine in the plasma were measured using an amino acid analyzer (L-8900, Hitachi High-Technologies Corporation, Tokyo, Japan).[19,20] Values are shown as mean ± SD. One-way ANOVA with Bonferroni correction was used to evaluate the significance of any differences between each group. Analyses were performed using SPSS Statistics 22 (IBM Japan, Ltd., Tokyo, Japan) with p values of below 0.05 regarded as statistically significant.

The physical characteristics of the subjects are shown in Table 1. No significant changes in body weight, body mass index, or systolic and diastolic blood pressure were observed in this study. None of the subjects experienced clinically relevant or any other side effects during the study.

The time-course of changes in plasma L-arginine concentrations is shown in Fig. 1(A). Plasma L-arginine in the combination group was significantly higher at 30 min after supplementation than seen in the single-dose L-citrulline group, and tended to be higher at 1 h after supplementation than in the single-dose L-arginine group ($p = 0.066$). It is reported that in humans, the normal range of blood arginine is 21–137 μM.[21] We therefore conducted a subgroup analysis in which we excluded the subjects whose plasma L-arginine level was over 137 μmol/L at baseline. Fig. 1(B) shows the changes in plasma L-arginine levels in the subgroup at 1 h after supplementation on Day 8 (Placebo group: 5.4 ± 16.3 μM; L-citrulline group: 66.3 ± 33.6 μM; L-arginine group: 72.3 ± 31.5 μM; combination group: 121.9 ± 46.7 μM). Compared with the single amino acids, simultaneous oral supplementation of L-citrulline and L-arginine raised plasma L-arginine levels at 1 h after supplementation. Table 2 shows $AUC_{0–1h}$ and $AUC_{0–4h}$ of plasma L-arginine in all subjects in the analysis and subgroup analysis. All supplemented groups, in both analyses, showed higher $AUC_{0–4h}$ than the placebo group. $AUC_{0–1h}$ was significantly higher in the L-arginine and combination groups than in the placebo group, and, in both analyses, the combination group showed significantly higher $AUC_{0–1h}$ than the L-citrulline group.

As previously reported in animal studies,[17,18] this study was useful in terms of confirming the combination effects on plasma L-arginine increase in humans as well as in animals. One possible reason for this combination effect might be due to the inhibiting effect of L-citrulline on arginase, which metabolizes L-arginine to urea and L-ornithine. Orally ingested L-arginine is strongly trapped in the gastrointestinal tract and the hepatic tissue, where it is extensively catabolized by arginase,[9–11,15] leading to very limited oral bioavailability.

Interestingly, previous research has demonstrated that L-citrulline suppresses arginase activity *in vitro* and *in vivo* by acting as a strong allosteric inhibitor.[22] This is one possible mechanism for the significant increase seen in plasma L-arginine levels and high $AUC_{0–1h}$ of plasma L-arginine in combination group, suggesting that L-arginine can pass through the gastrointestinal tract and liver without being influenced by intestinal or hepatic first-pass effects, probably due to the inhibition of arginase activity by L-citrulline. Moreover, several studies have demonstrated that L-citrulline is an effective precursor of L-arginine, thus contributing to a sustained L-arginine supply. The plasma L-arginine levels in the L-citrulline group increased slowly and remained high for several hours, indicating that L-citrulline alone is useful for long-acting enhancement of L-arginine availability. On the other hand, the significantly high plasma L-arginine levels at 1h and $AUC_{0–1h}$ in the combination group indicate that a combination of L-citrulline plus L-arginine is useful for short-acting effects. There was no significant difference in the $AUC_{0–4h}$ of plasma L-arginine between the groups,

Table 1. Baseline characteristics and physical parameters during the study period.

	Experiment	Day 1 (baseline)	Day 8
Age (years)	Placebo	43.5 ± 7.0	–
	L-Citrulline	43.0 ± 3.9	–
	L-Arginine	43.3 ± 4.0	–
	L-Citrulline + L-Arginine	42.9 ± 5.8	–
BMI (kg/m^2)	Placebo	27.1 ± 1.1	27.0 ± 1.3
	L-Citrulline	27.2 ± 1.1	27.2 ± 1.3
	L-Arginine	27.1 ± 1.2	27.2 ± 1.2
	L-Citrulline + L-Arginine	27.5 ± 1.7	27.6 ± 2.0
Systolic blood pressure (mmHg)	Placebo	117.8 ± 12.0	119 ± 10.3
	L-Citrulline	119.2 ± 9.9	119.5 ± 10.6
	L-Arginine	121.7 ± 10.3	118 ± 5.0
	L-Citrulline + L-Arginine	116.6 ± 11.6	117.1 ± 10.0
Diastolic blood pressure (mmHg)	Placebo	73.9 ± 7.5	77.1 ± 9.6
	L-Citrulline	77.8 ± 6.8	80.0 ± 6.1
	L-Arginine	79.2 ± 7.6	79.6 ± 3.9
	L-Citrulline + L-Arginine	75.7 ± 6.9	77.3 ± 8.6
Heart rate (Beats per minute)	Placebo	74.5 ± 9.0	72.2 ± 7.4
	L-Citrulline	69.8 ± 8.1	69.5 ± 4.7
	L-Arginine	66.4 ± 6.3	67.0 ± 6.2
	L-Citrulline + L-Arginine	70.8 ± 7.8	68.4 ± 5.8

Notes: All values are expressed as mean ± SD. (Placebo: $n = 11$, L-Citrulline: $n = 11$, L-Arginine: $n = 10$, L-Citrulline + L-Arginine: $n = 10$).

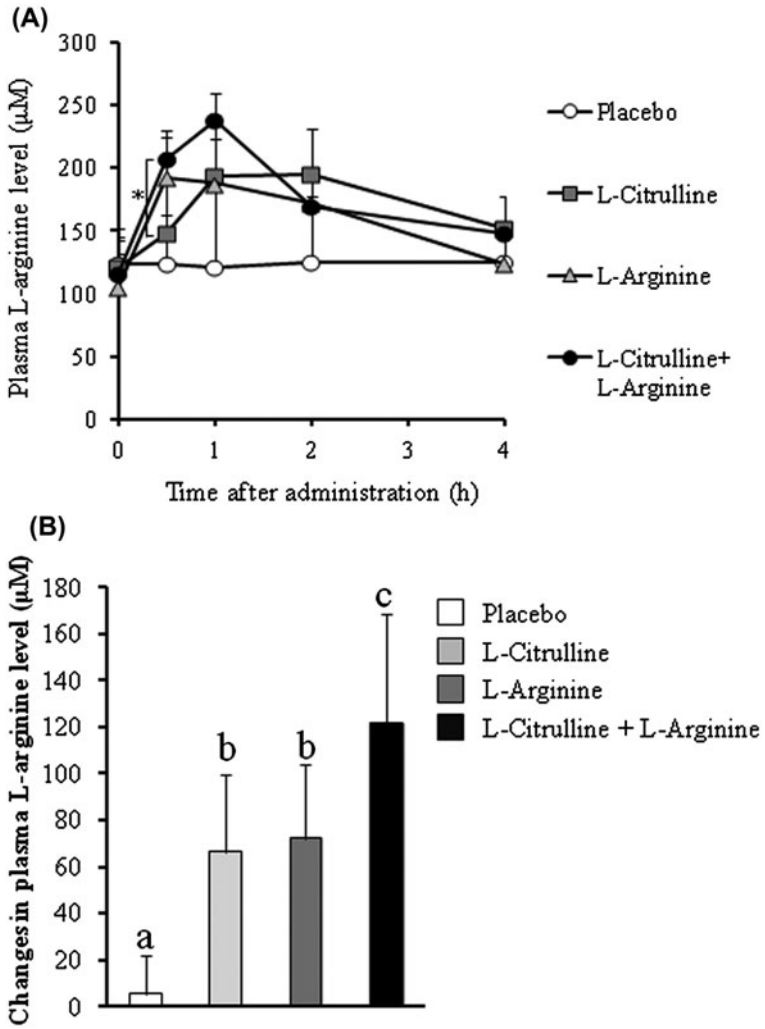

Fig. 1. Plasma L-arginine levels**.** (A) Time course of plasma L-arginine levels. Subjects orally took a placebo, L-citrulline, L-arginine, or a combination of L-citrulline and L-arginine at half-dosage each as an oral supplement. Blood samples were collected sequentially before and after supplementation to analyze plasma L-arginine concentration. All values are expressed as mean ± SD. * $p < 0.05$. (Placebo: $n = 11$; L-Citrulline: $n = 11$; L-Arginine: $n = 10$; L-Citrulline + L-Arginine: $n = 10$)**.** (B) Subgroup analysis of changes in plasma L-arginine levels at 1 hour after supplementation. All values are expressed as mean ± SD. Bars with different letters represent significantly different results with $p < 0.05$. (Placebo: n = 7; L-Citrulline: $n = 8$; L-Arginine: $n = 9$; L-Citrulline + L-Arginine: $n = 10$).

Table 2. The $AUC_{0–1h}$ and $AUC_{0–4h}$ (nmol•h/mL) of plasma L-arginine.

	All subjects analysis		Subgroup analysis	
	$AUC_{0–1h}$	$AUC_{0–4h}$	$AUC_{0–1h}$	$AUC_{0–4h}$
Placebo	123.1 ± 21.6	497.0 ± 67.0	113.9 ± 17.0	462.0 ± 67.0
L-Citrulline	152.4 ± 22.7	693.8 ± 81.6 **	145.4 ± 23.0	666.2 ± 78.4**
L-Arginine	169.3 ± 34.5*	645.3 ± 143.1*	163.5 ± 31.2*	622.4 ± 130.9*
L-Citrulline + L-Arginine	192.0 ± 38.6**#	713.3 ± 95.3**	192.0 ± 38.6**#	713.3 ± 95.3**

Notes: All values are expressed as mean ± SD.
*$p < 0.05$.
**$p < 0.01$ vs. Placebo group.
#$p < 0.05$ vs. L-Citrulline group.
All subjects analysis: Placebo: $n = 11$, L-Citrulline: $n = 11$; L-Arginine: $n = 10$; L-Citrulline + L-Arginine: $n = 10$. Subgroup analysis: Placebo: $n = 7$; L-Citrulline: $n = 8$; L-Arginine: $n = 9$; L-Citrulline + L-Arginine: $n = 10$

except for the placebo, but the combination group showed the highest mean value. Taken together, L-citrulline appears to work as an inhibiter of arginase and precursor of L-arginine: as a result, plasma L-arginine synergistically increases when it is simultaneously supplemented with L-arginine.

In this study, there were no significant effects on plasma nitrite and nitrate (NOx) (data not shown). To detect any changes in NOx, it might have been needed to strictly monitor food intake and any loading that might enhance NO, such as exercise.

Our study had several limitations. We recruited only male to avoid the influence of estrus cycle. Here, we conducted a double-blind randomized placebo-controlled study in 45 subjects; in future, an additional, larger-scale study will be needed to verify our findings.

In conclusion, our data shows that a combination of oral L-citrulline and L-arginine effectively increases plasma L-arginine levels in humans. This indicates that a combination of L-citrulline and L-arginine might have a more beneficial effect than a single dose of L-arginine alone.

Author Contributions

T Suzuki, M Morita, T Hayashi, A Kamaimura designed the study. T Suzuki wrote the manuscript, A Kamimura supervised manuscript preparation. All authors reviewed and approved the final manuscript.

Disclosure statement

T. Suzuki, M. Morita and A. Kamimura are employees of KYOWA HAKKO BIO CO., LTD.

Funding

This work was supported by a grant from KYOWA HAKKO BIO CO., LTD.

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