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Short-term effects of L-citrulline supplementation on arterial stiffness in middle-aged men

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ABSTRACT

Background: Nitric oxide (NO) plays a key role in the maintenance of vascular tone, contributing to the functional regulation of arterial stiffness. Although oral L-citrulline could become the effective precursor of L-arginine (substrate for endothelial NO synthase) via the L-citrulline/L-arginine pathway, little is known about the efficacy of L-citrulline application on arterial stiffness.
Objective: We examined the short-term effects of L-citrulline supplementation on arterial stiffness in humans.
Methods: In a double-blind, randomized, placebo-controlled parallel-group trial, 15 healthy male subjects (age: 58.3 ± 4.4 years) with brachial–ankle pulse wave velocity (baPWV; index of arterial stiffness >1400 cm/sec) were given 5.6 g/day of L-citrulline (n = 8) or placebo (n = 7) for 7 days. baPWV and various clinical parameters were measured before (baseline) and after oral supplementation of L-citrulline or placebo.
Results: Compared with the placebo group, baPWV was significantly reduced in the L-citrulline group ($p<0.01$). No significant differences in blood pressure (BP) were found between the two groups, and no correlation was observed between BP and baPWV. The serum nitrogen oxide (NOx, the sum of nitrite plus nitrate) and NO metabolic products were significantly increased only in the L-citrulline group ($p<0.05$). Plasma citrulline, arginine and the ratio of arginine/asymmetric dimethylarginine (ADMA), an endogenous inhibitor of NO synthase (arginine/ADMA ratio) were significantly increased in the L-citrulline group compared with the placebo group ($p<0.05$, $p<0.01$, $p<0.05$, respectively). Moreover, there was a correlation between the increase of plasma arginine and the reduction of baPWV ($r = -0.553$, $p<0.05$).
Conclusion: These findings suggest that short-term L-citrulline supplementation may functionally improve arterial stiffness, independent of blood pressure, in humans.

1. Introduction

Nitric oxide (NO), which is formed from the amino acid precursor L-arginine and oxygen by NO synthase (NOS), plays a key role in maintaining the function and integrity of the endothelium, including vascular tone (as the mediator of endothelium-dependent vasodilation) and structure (through the prevention of leukocyte filtration and thrombus formation, and angiogenesis) [1]. NO deficiency in the vascular endothelium has been strongly associated with cardiovascular diseases, including hypertension [2], atherosclerosis [3], and diabetic vascular disease [4], suggesting that increased NO production is one of the keys to slowing the progression of these diseases or their component processes.

Arterial stiffness is suggested to be a powerful predictor of cardiovascular diseases. It generally reflects the degree of atherosclerosis of an elastic artery such as the aorta, and it can directly accelerate the atherosclerotic process [5]. Arterial stiffness is regulated by numerous factors such as NO. Mean arterial pressure and structural changes in the components of the arterial wall were once thought to be the main determinants of arterial stiffness, however it is now recognized that arterial stiffness is also regulated by vascular tone and that endothelium derived mediators, such as NO, contribute to the functional regulation of arterial stiffness [6].

It has been reported that L-citrulline, a byproduct of NO formation from L-arginine and oxygen, is effectively recycled via the enzymes (argininosuccinate synthase and argininosuccinate lyase) of the citrulline-NO cycle to L-arginine, and plays an important role in the metabolism and regulation of NO [7]. Recently, we and others demonstrated that oral supplementation of L-citrulline upregulated endothelial NO synthase (eNOS) expression, improved endothelial function and played an atheroprotective role in animal models [8–10]. Interestingly, a recent clinical trial showed that oral intake of L-citrulline dose-dependently increased the plasma L-arginine level more effectively than an equivalent dose of L-arginine in healthy

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human volunteers [11]. Due to substantial intestinal and hepatic metabolism of L-arginine to L-ornithine and urea by arginase I (and partially II), oral delivery of L-arginine is thought to be ineffective [12,13]. On the other hand, L-citrulline is not cleared to a large extent by the intestine and liver, instead being taken up by the kidney and other tissues to be converted to L-arginine [14]. Therefore, L-citrulline may be considered an effective L-arginine and NO supplier and thus is expected to be involved in the regulation of arterial stiffness. However, there are few reports regarding the efficacy of L-citrulline consumption on arterial stiffness.

The purpose of this present study was to evaluate the short-term effects of L-citrulline supplementation on arterial stiffness in healthy human volunteers using a brachial–ankle pulse wave velocity (baPWV), a non-invasive method and device for arterial stiffness measurement.

2. Materials and methods

2.1. Subjects

We studied 15 healthy middle-aged men (mean age, 58.3 ± 4.4 years). They were recruited from a group of 100 healthy middle-aged male volunteers (range, 45–64 years) screened for stable measurements of both baPWV and waist circumference on two occasions 7 days apart prior to the study. Subjects were eligible if they had both baPWV > 1400 cm/sec, the cutoff value for risk prediction of developing cardiovascular diseases [15], and waist circumference >85 cm, one of the diagnosis criteria of metabolic syndrome in Japan [16]. Subjects meeting the follow criteria were excluded in order to obtain accurate baPWV data: (1) an ankle/brachial systolic blood pressure index <0.95, to exclude the substantial effect of existing atherosclerosis, including arteriosclerosis thrombosis; (2) a history of cardiovascular diseases, diabetes mellitus, liver diseases, renal diseases; (3) serious anemia; (4) an arrhythmia such as arterial fibrillation; (5) a smoking habit; (6) drug and supplement consumption, including amino acids, vitamins, and other nutritional supplements. Subjects were instructed to refrain from any unusual changes in physical activity and nutritional habits throughout the study period. The study protocol was conducted according to the Declaration of Helsinki, approved by the ethics committee of Nagoya University and Nihonbashi cardiology clinic Institutional Review Board, and written informed consent was obtained from all subjects.

2.2. Study design

In a double blind, randomized, placebo-controlled parallel-group trial, subjects received either L-citrulline 5.6 g (n = 8, Kyowa Hakko Bio Co., Ltd. Tokyo, Japan) or placebo (n = 7) for 7 days. On day 1, 5.6 g was only administered once to evaluate the acute effect of oral supplementation at 60 min. For the remaining 6 days, samples were administered twice a day at doses of 2.4 g (before breakfast) and 3.2 g (before sleeping). baPWV measurements and blood sampling were performed at the baseline, 60 min after oral supplementation on day 1 and at the end of the 7-day follow-up period. Every study was conducted from 9:00 AM to 12:00 AM. Subjects were measured at the same time throughout the study period. On the day before the measurement day, subjects were asked to refrain from alcohol and caffeine after 10:00 PM. On the morning of the measurement day, they were instructed to eat breakfast (two rice balls, 300 kcal) 2 hours before the measurement in order to limit fasting stress.

2.3. Measurements

The waist circumference used for the screening was measured according to the criteria of the Japan Society for the Study of Obesity. The measurement was taken between the lowest rib margin and iliac crest while the subject was in a standing position and recorded to the nearest 0.1 cm. baPWV as an index of arterial stiffness was evaluated as described below in detail.

Non-fasting blood samples were drawn from the antecubital vein while in a seated position. Samples were collected into vacuum tubes containing sodium EDTA as plasma or serum separator gel as serum. Plasma or serum samples were transported to Kotobiken Medical Laboratories Inc. (Tokyo, Japan) and stored at −80 °C until analysis. Serum concentrations of glucose, lipids (total cholesterol, triglycerides, LDL cholesterol, HDL cholesterol), parameters for liver and renal function (aspartate aminotransferase, alanine aminotransferase, lactate dehydrogenase, total protein, γ-glutamyltranspeptidase, albumin, creatinine, blood urea nitrogen) and electrolytes, and hematological markers (lymphocytes, erythrocytes, Hb) were determined by their standard laboratory methods. Serum NOx (nitrite + nitrate) was assayed by using an NO-detector-HPLC system (ENO10; Eicom, Kyoto, Japan) [17].

Plasma arginine and citrulline were measured by an amino acid analyzer (JLC-500/V; JOEL, Tokyo, Japan). Serum asymmetric dimethylarginine (ADMA) was determined by high-performance liquid chromatography, as previously described [18]. Serum high-sensitivity C-reactive protein (hs-CRP) as a prototypic marker of inflammation was measured by a highly sensitive nephelometric assay using a monoclonal antibody to CRP coated on polystyrene beads (Dade Behring, BN II nephelometric method).

2.4. Assessment of baPWV

baPWV was measured using an automatic waveform analyzer, Form PWV/ABI (BP-203RPEII, Colin Co., Komaki, Japan), according to a previously described methodology [19]. In brief, electrocardiographic electrodes were set on both wrists, and a microphone for the phonocardiogram was attached on the left side of the chest. The occlusion cuffs, which were connected to plethysmographic and oscillometric sensors, were tied around both the upper arms and ankles. The measurement of baPWV took about 5 min. baPWV was calculated according to the following equation: $baPWV = (La - Lb)/\Delta Tba$. *La* is the distance between the heart and ankle, *Lb* is the distance between the heart and brachium, and *ΔTba* is the time interval between the brachium and ankle. These distances were calculated automatically according to body height. The measurements were performed after the subjects had rested for at least 10 min in the supine position in an air-conditioned room (24–26 °C). Besides baPWV, blood pressure (BP), heart rate (HR) and ankle–brachial pressure index (ABI) were simultaneously recorded by this apparatus and the mean values of them on both the left and right sides were used for statistical analysis.

2.5. Statistics

Data are expressed as mean ± S.E.M. Continuous variables were evaluated using an unpaired Student's *t*-test and a paired Student's *t*-test. Correlations between baPWV and others were analyzed with Spearman's correlation test. Differences with $p<0.05$ were considered significant.

3. Results

The baseline clinical characteristics of all eligible subjects are presented in Table 1. There were no differences between both groups in terms of body weight, body height, body mass index (BMI), waist circumference, BP and baPWV.

Table 2 shows the effects of oral L-citrulline on baPWV, ABI, BP and HR. Although there was no acute change of baPWV at 60 min, significant reduction was observed at 7 days in the L-citrulline group. baPWV in the placebo group did not change throughout the study period. No significant differences in the levels of ABI were found in the two groups. Small changes in BP and HR were observed in each group; however, there were no significant differences between the groups.

As shown in Fig. 1, there was no correlation between changes in BP and changes in baPWV.

Table 3 shows the effects of oral L-citrulline on serum or plasma biochemical parameters. Serum glucose at 60 min and LDL-cholesterol at 7 days significantly decreased only in the placebo group, while triglycerides at 7 days decreased only in the L-citrulline group. However, they did not differ significantly between groups. A significant increase in NOx was observed in the L-citrulline group but not in the placebo group. Both plasma citrulline and arginine were significantly increased in the L-citrulline group compared with the placebo group. Although plasma ADMA was increased in both groups, the plasma arginine/ADMA ratio was significantly increased only in the

Table 1
Baseline clinical characteristics. Data are mean ± S.E.M. BMI: body mass index, and BP: blood pressure. No significant difference was observed between the placebo and L-citrulline groups.

Characteristics	Placebo	L-Citrulline	p-value
	n = 7	n = 8	
Age (year)	58 ± 3.9	58.5 ± 5.0	0.834
Height (cm)	167.1 ± 5.5	172.5 ± 5.0	0.068
Weight (kg)	69.6 ± 7.8	75 ± 8.9	0.235
BMI	24.9 ± 2.2	25.2 ± 2.4	0.840
Waist circumference (cm)	91.8 ± 5.1	93.4 ± 5.2	0.550
Systolic BP(mm Hg)	130.9 ± 9.3	135.5 ± 13.3	0.454
Diastolic BP (mm Hg)	84.6 ± 6.8	82.4 ± 7.5	0.578
Heart rate (beats/min)	71.9 ± 10.8	64.8 ± 10.0	0.208
baPWV (cm/sec)	1,600.8 ± 99.5	1,577.8 ± 77.7	0.623

Data are mean ± S.E.M. BMI: body mass index, and BP: blood pressure.

Table. 2
Changes in variables before and after oral L-citrulline or placebo supplementation. Data are mean ± S.E.M. ABI: ankle–brachial index. ## $p<0.01$ versus placebo group in day 7. baPWV. * $p<0.05$ versus before supplementation, ** $p<0.01$ versus before supplementation.

	Group	n	Baseline	60 min		Day 7	
baPWV (cm/min)	Placebo	7	1.600.8 ± 37.6	1.604.9 ± 25.7		1.570.9 ± 20.8	
	L-Citrulline	8	1.577.8 ± 27.5	1.549.4 ± 48.8		1.442.0 ± 31.7	** ##
ABI	Placebo	7	1.17 ± 0.02	1.21 ± 0.02		1.20 ± 0.02	
	L-Citrulline	8	1.21 ± 0.02	1.22 ± 0.02		1.21 ± 0.03	
Systolic BP (mm Hg)	Placebo	7	130.9 ± 3.5	134.2 ± 4.6		127.9 ± 4.2	
	L-Citrulline	8	135.5 ± 4.7	137.8 ± 4.7		131.6 ± 4.3	
Diastolic BP (mm Hg)	Placebo	7	84.6 ± 2.6	83.9 ± 3.5		81.8 ± 3.0	*
	L-Citrulline	8	82.4 ± 2.7	84.9 ± 3.4		81.9 ± 2.8	
Mean BP (mm Hg)	Placebo	7	103.0 ± 3.1	104.4 ± 3.4	*	100.9 ± 3.0	
	L-Citrulline	8	101.7 ± 4.0	105.4 ± 3.9		102.8 ± 4.0	
Heart rate (beats/min)	Placebo	7	71.9 ± 4.1	64.6 ± 3.6	**	71.9 ± 4.6	
	L-Citrulline	8	64.8 ± 3.5	58.0 ± 2.8	**	64.3 ± 3.3	

L-citrulline group. Other markers including total cholesterol, HDL cholesterol and hsCRP, a prototypic marker of inflammation were unchanged. As shown in Fig. 2, there was a significant correlation between the increase of plasma arginine and the reduction of baPWV ($r=-0.553$, $p<0.05$). None of the subjects suffered any adverse effects throughout the study period (data not shown).

4. Discussion

In the present study, L-citrulline, but not placebo, significantly reduced baPWV without changing BP levels. We evaluated the mechanism of this effect based on the hypothesis that L-citrulline increases NO bioavailability by increasing L-arginine via the L-citrulline/L-arginine pathway. The increased production of L-arginine due to L-citrulline in cultured endothelial cells has been reported [20]. L-arginine-depleted endothelial cells did not form urea or metabolize L-ornithine but converted L-citrulline to L-arginine through the formation of L-argininosuccinic acid.

Serum NOx, plasma citrulline and arginine were significantly increased in the L-citrulline group compared with the placebo group. NOx are stable oxidation products of NO and represent markers of NO production. In the present study, the baPWV value was reduced by L-citrulline treatment without any change of blood pressure. Interestingly, there was a correlation between the increase of plasma arginine and the reduction of baPWV ($r=-0.553$, $p<0.05$). That is why baPWV may be a marker of atherosclerosis of an elastic artery if the effect on blood pressure is adjusted for. The present study might show the role of L-citrulline in increasing NO bioavailability in middle-aged males.

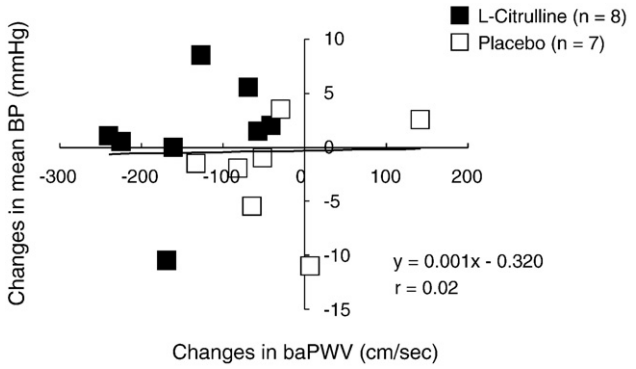

Fig. 1. Correlation between changes in baPWV and changes in mean BP at day 7. There is no correlation between these parameters. $n=15$, and $r=0.02$. L-citrulline ($n=8$), Placebo ($n=7$).

Here, we used baPWV as a promising parameter for assessing arterial stiffness. The stiffness of the central arteries is a predictor of a cardiovascular disease. Some clinical studies have demonstrated that baPWV is strongly related to aortic PWV [19,21], and reductions in aortic PWV with exercise training are significantly correlated with changes in baPWV [21]. Additionally, one novel clinical assessment has shown that the correlation coefficients of baPWV with (heart–femoral) hfPWV and (heart–carotid) hcPWV for the assessment of central arteries were 0.796 and 0.541, respectively, and the respective correlations of baPWV with (heart–brachial) hbPWV and (femoral–ankle) faPWV for the assessment of peripheral arteries were 0.581 and 0.705 [22]. These observations strongly suggest that baPWV is an index of arterial stiffness, which can reflect central arterial stiffness, showing characteristics similar to those of aortic PWV. Therefore, the significant reduction of baPWW in our study may have reflected an improvement in central arterial stiffness after L-citrulline intake.

The ratio of the plasma concentration of arginine/ADMA, an endogenous inhibitor of NO synthase (arginine/ADMA ratio), was significantly increased in the L-citrulline group compared with the placebo group in the present study. Plasma concentrations of L-arginine, a cationic amino acid precursor for NO synthesis, were reduced while levels of the endogenous L-arginine analogues, asymmetric and symmetric dimethylarginine and N (G)-monomethyl-L-arginine, were reported to be elevated in chronic renal and heart failure as well as endothelial dysfunction [23]. An increase in the ratio of arginine/ADMA also means an increase in NO bioavailability. Taken together, the present data may show that L-citrulline supplementation plays an atheroprotective role in middle-aged males.

Our previous study firstly showed the anti-atherosclerotic effect of supplementation of L-citrulline in the progression of atherosclerosis in the aorta of rabbits fed a high cholesterol diet [8]. The hallmark of diet-induced atherosclerosis in rabbits is vascular endothelial cell dysfunction, which is characterized by marked impairment of endothelium-dependent vasorelaxation in isolated arteries, as well as blood flow *in vivo* and atherosclerosis with distinct atheromatous lesions [24,25]. L-citrulline caused a marked improvement in endothelium-dependent vasorelaxation and rabbit ear blood flow, dramatic inhibition of the progression of atherosclerosis and a decrease in $O2^-$ production. The effect was slightly larger than the effect of L-arginine alone, and together, L-citrulline and L-arginine show additive atheroprotective effects [8]. These therapeutic effects were associated with concomitant increases in aortic eNOS expression. It is well known that the *Km* for L-arginine as a substrate for eNOS is 2–15 μM, whereas plasma L-arginine levels in mammals are 100–200 μM, thereby suggesting that eNOS may already be saturated with substrate. This enigma has been termed the "arginine paradox." Plasmalemmal caveolae may be the principal source of L-arginine available to eNOS [26,27]. Moreover, the L-citrulline to L-arginine recycling pathway is localized to caveolae and may be the principal source of available

Table 3
Changes in biochemical parameters before and after oral L-citrulline or placebo supplementation. Data are mean ± S.E.M. ADMA: asymmetric dimethylarginine #: $p<0.05$; ##: $p<0.01$ versus the placebo group. *: $p<0.05$; **: $p<0.01$ versus before supplementation.

	Group	n	Baseline	60 min		Day 7	
Total cholesterol (mg/dl)	Placebo	7	196.9 ± 38.1	197.4 ± 37.4		190.4 ± 29.6	
	L-Citrulline	8	191.3 ± 30.6	190.5 ± 31.6		190.4 ± 26.5	
HDL cholesterol (mg/dl)	Placebo	7	50.0 ± 5.1	50.7 ± 6.2		49.4 ± 6.0	
	L-Citrulline	8	44.8 ± 6.1	45.6 ± 6.0		47.0 ± 6.8	
LDL cholesterol (mg/dl)	Placebo	7	127.1 ± 39.8	127.1 ± 40.6		113.6 ± 37.7	**
	L-Citrulline	8	118.6 ± 24.3	118.8 ± 25.2		119.6 ± 21.7	
Triglycerides (mg/dl)	Placebo	7	115.7 ± 38.1	105.3 ± 31.9		129.6 ± 84.9	
	L-Citrulline	8	143.4 ± 60.4	136.9 ± 56.0		100.6 ± 32.2	*
Serum glucose (mg/dl)	Placebo	7	114.7 ± 17.3	95.4 ± 14.7	**	115.4 ± 27.4	
	L-Citrulline	8	105.5 ± 13.3	97.3 ± 10.3		107.4 ± 18.7	
NOx (μM)	Placebo	7	53.55 ± 31.02	49.83 ± 27.92		74.19 ± 32.92	
	L-Citrulline	8	78.61 ± 53.84	75.02 ± 54.98		107.73 ± 66.17	*
Citrulline (μM)	Placebo	7	39.3 ± 12.2	–		34.8 ± 2.6	
	L-Citrulline	8	33.4 ± 3.9	–		56.9 ± 22.8	*#
Arginine (μM)	Placebo	7	101.3 ± 12.1	–		116.0 ± 11.2	**
	L-Citrulline	8	119.3 ± 26.8	–		162.6 ± 28.1	**##
ADMA (μM)	Placebo	7	0.36 ± 0.07	–		0.39 ± 0.07	*
	L-Citrulline	8	0.37 ± 0.06	–		0.42 ± 0.06	*
Arginine/ADMA Ratio	Placebo	7	294.6 ± 62.2	–		307.5 ± 51.6	
	L-Citrulline	8	324.0 ± 43.4	–		393.7 ± 66.9	*#
hsCRP (mg/dl)	Placebo	7	0.070 ± 0.068	–		0.069 ± 0.048	
	L-Citrulline	8	0.056 ± 0.027	–		0.062 ± 0.052	

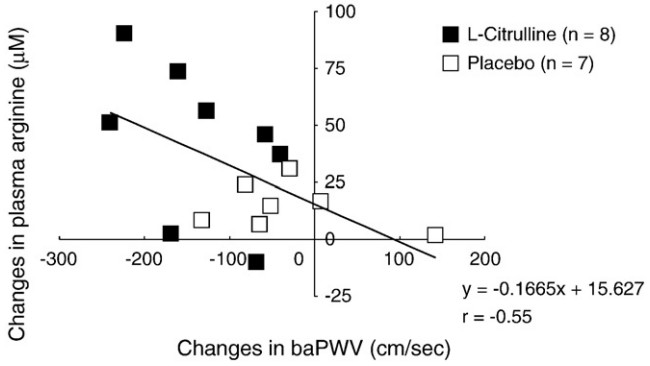

Fig. 2. Correlation between changes in baPWV and changes in plasma arginine at day 7. There is an inverse correlation between these parameters. $n = 15$, $r = -0.55$. L-citrulline ($n = 8$), Placebo ($n = 7$).

L-arginine [26,27]. The "arginine paradox" has also been explained by the presence of elevated levels of ADMA during atherosclerosis, as mentioned above [28,29]. Excess L-arginine could effectively compete with ADMA for binding sites on eNOS. A key observation was that regular administration of L-citrulline, in part or entirely, shows the NO-like pharmacological effects. In conclusion, the present study suggests that in humans, short-term L-citrulline supplementation may functionally improve arterial stiffness, independent of blood pressure.

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We complied with the Principles of Ethical Publishing in the *International Journal of Cardiology* [30].

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