

## Research Paper

## Does L-citrulline supplementation improve exercise blood flow in older adults?

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## New Findings

- **What is the central question of this study?**  
Does short-term supplementation with L-citrulline in order to increase L-arginine improve exercise blood flow and peripheral dilatation responses to exercise in older adults?
- **What is the main finding and its importance?**  
L-Citrulline increased femoral blood flow by 11% and vascular conductance by 14% during lower-limb exercise in older men, whereas no changes were observed in older women. This modest improvement in bulk muscle blood flow in older men has implications for altering muscle metabolism that may result in enhanced exercise tolerance in older adults.

L-Citrulline (Cit) increases L-arginine (Arg), the primary substrate for nitric oxide biosynthesis. We tested the hypothesis that muscle blood flow during exercise would be enhanced by Cit supplementation in older adults. Femoral artery blood flow was measured during calf exercise using Doppler ultrasound, and vascular conductance (FVC) was calculated in 25 older adults (13 women and 12 men) before and after 14 days of Cit ( $6 \text{ g day}^{-1}$ ) and placebo (maltodextrin) in a randomized, double-blind, crossover study. Plasma [Arg] and resting blood pressure were also measured before and after each condition. Women and men were analysed separately because of significant sex-by-condition interactions for the change in exercise blood flow and FVC. Plasma [Arg] was increased by 30 and 35% after Cit ( $P < 0.01$ ) in women and men, respectively, with no change after placebo. Citrulline lowered diastolic blood pressure in men ( $75 \pm 9$  versus  $71 \pm 6$  mmHg,  $P = 0.02$ ), but this variable remained unchanged in women. Blood flow and FVC during exercise at higher workloads were increased after Cit in men (flow,  $521 \pm 134$  versus  $584 \pm 166 \text{ ml min}^{-1}$ ,  $P = 0.04$ ; FVC,  $5.0 \pm 1.5$  versus  $5.8 \pm 1.7 \text{ m, min mmHg}^{-1}$ ,  $P = 0.01$ ) but were not different after placebo. These variables were not altered by Cit in women. Adjusting for baseline diastolic blood pressure removed ( $P = 0.10$ ) the difference in FBF and FVC following Cit in men. These results indicate that L-citrulline has a modest effect of improving muscle blood flow during submaximal exercise in older men.

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## Introduction

The term ‘vascular ageing’ has often been used to encompass adverse changes in arterial structure and function with advancing age. Endothelial dysfunction is a hallmark characteristic of vascular ageing and is an

important contributor to lower peripheral vasodilatation (Crecelius *et al.* 2010) and blood flow responses to exercise in older compared with younger adults (Poole *et al.* 2003; Parker *et al.* 2008). A major deficit in endothelial function is reduced production and bioactivity of nitric

oxide (NO), a signalling molecule important for regulating blood flow and metabolism during exercise (Heinonen *et al.* 2011). A growing area of study is the effectiveness of dietary supplements to enhance NO production, one of which is L-arginine (Arg). Oxidation of endogenous Arg in a reaction catalysed by NO synthase results in NO production. Oral supplementation with Arg in humans increases plasma concentrations of Arg and NO biomarkers (Schwedhelm *et al.* 2008). In addition, accompanying changes in vascular function are seen with Arg supplementation in older adults, including improved peripheral flow-mediated vasodilatation (Bode-Böger *et al.* 2003) and reflex cutaneous vasodilatation (Holowatz *et al.* 2006).

L-Citrulline (Cit) is a non-essential amino acid that converts to argininosuccinate then Arg in reactions catalysed by argininosuccinate synthase and lyase enzymes, respectively. Unlike oral Arg supplementation, which is subject to presystemic elimination by hepatic metabolism (Morris, 2004), oral Cit is not taken up by the liver and is transported to the kidneys, where most of the Cit is catabolized to Arg (van de Poll *et al.* 2004). As a result, supplementation with Cit increases plasma concentrations of Arg (Churchward-Venne *et al.* 2014; Kim *et al.* 2015), with evidence that oral Cit elevates plasma Arg concentrations more than an equivalent dose of oral Arg in older adults (Moinard *et al.* 2016). Supplementation with Cit has been shown to reduce resting blood pressure (Figuroa *et al.* 2017) and lower peripheral arterial stiffness (Ochiai *et al.* 2012; Figuroa *et al.* 2015) in middle-aged to older adults. Few studies have investigated the impact of Cit on blood flow during exercise in older adults. Using an acute dose of Cit (10 g), Churchward-Venne *et al.* (2014) did not observe an additional benefit above an amino acid mixture on femoral blood flow after unilateral knee-extension resistance exercise in older men. Likewise, Kim *et al.* (2015) found no change in forearm reactive hyperemia after acute Cit (3 g) despite increasing the whole-body NO synthesis rate in older adults. These findings are in contrast to what is observed after chronic oral Cit in young men. Bailey *et al.* (2015) demonstrated that taking Cit (6 g day<sup>-1</sup>) for 7 days lowers the level of deoxygenated haemoglobin in active muscle during moderate-intensity exercise. Bailey *et al.* (2016) also reported a higher tissue oxygenation index during exercise when providing Cit (11.4 g day<sup>-1</sup>) in the form of watermelon juice for 16 days. Both these reports indicate that muscle perfusion during exercise may be improved after chronic supplementation with Cit, but whether a similar positive influence on exercise blood flow can be found with Cit in older adults has yet to be examined.

To date, little is known regarding the effect of chronic Cit in older adults, a condition that increases muscle oxygenation patterns in young adults (Bailey *et al.* 2015, 2016). Thus, the purpose of this study was to test the

hypothesis that chronic Cit will enhance muscle blood flow and peripheral dilatation during exercise in older adults.

## Methods

### Ethical approval

All participants provided written informed consent before data collection. The Human Research Protection Program at Texas Tech University provided ethics approval for this study (#504985). This study and its procedures conformed to standards set by the latest version of the *Declaration of Helsinki* and is registered on ClinicalTrials.gov (identifier NCT03127917).

### Participants

Thirteen older women (61–80 years old) and 12 older men (63–78 years old) took part in this study after giving informed consent to participate. Participants had no history of cardiovascular disease or pulmonary disease and were not taking medication for high blood pressure or cholesterol. Three women were on oestrogen-related therapy (two taking Evista, one taking Premarin). Removal of these women did not change any result, so they were included in the analysis. Participants were included in the study if they had a body mass index <30 kg m<sup>-2</sup>, resting seated brachial blood pressure <160/100 mmHg, fasting blood glucose <115 mg dl<sup>-1</sup>, and ankle-brachial index values >0.90.

### Study design and protocol

This study was a randomized, double-blind, crossover study involving Cit (BioKyowa, Cape Girardeau, MO, USA) and maltodextrin (NOW Foods, Bloomingdale, IL, USA) that was given as a placebo control. Supplements in the form of capsules were taken orally for 2 weeks, with a 2 week washout period. This duration of supplementation and washout is consistent with recent work that finds Cit supplementation improves muscle oxygenation patterns during exercise (Bailey *et al.* 2015, 2016). The dose of Cit was 6 g day<sup>-1</sup> (3 g taken twice daily), and the last dose of Cit or placebo was consumed the night before the study visit. This dose of Cit has been shown to increase plasma [Arg] significantly (Schwedhelm *et al.* 2008; Bailey *et al.* 2015). The order of supplements was randomized and equally balanced within each sex. All visits were scheduled in the morning, and participants were instructed to arrive after refraining from food, caffeine, vitamins and other supplements/medications for at least 8 h. An initial visit was made by participants for the purpose of written informed consent and screening. The remaining four experimental visits were similar in procedures, but surrounded the Cit and placebo conditions (i.e. before and

after visits for each condition). First, blood was collected for measurement of plasma concentrations of Arg, nitrite and nitrate (NO<sub>x</sub>) and fasting blood glucose. Height and weight were measured using a standard scale, followed by applanation tonometry for central arterial stiffness. Lastly, leg vascular function was then assessed at rest and during unilateral calf exercise.

### Central arterial stiffness

Participants rested in a supine position for 10 min before two measurements of blood pressure were taken, with a 1 min rest period between measurements, on the left arm using an automated device (HEM-907XL; Omron Healthcare, Lake Forest, IL, USA). Brachial blood pressure was used to calibrate an aortic pressure waveform derived from a composite of consecutive radial artery pressure waveforms measured at the left wrist using applanation tonometry and a general transfer function (SphygmoCor PVx system; Atcor Medical, Sydney, NSW, Australia). Pulse wave analysis of the aortic pressure waveform provided pulse wave transit time. Carotid–femoral pulse wave velocity, an index of central aortic stiffness, was calculated using an online standardization tool that uses blood pressure, carotid–femoral distance and pulse wave transit time to calculate pulse wave velocity (Londono *et al.* 2015).

### Exercise blood flow

Participants rested in a semi-recumbent position. Diameter and mean blood velocity were measured in the right superficial femoral artery using Doppler ultrasound (Vivid 7; GE Healthcare, Waukesha, WI, USA) with a 5–13 MHz linear transducer probe at rest and during single calf muscle exercise. The participant's right leg was extended, raised to a horizontal level, and the right foot was attached to a custom pulley system. Participants moved their right foot through dorsiflexion and plantarflexion in a full range of motion while lifting an external load of 0.5 and 1.5 kg at a cadence of 40 contractions min<sup>-1</sup>. Each workload lasted 3 min, and the order of workloads (light to heavier) was the same for all visits. Blood pressure was measured at rest and every minute during exercise (HEM-907XL; Omron Healthcare). Blood velocity waveforms were sampled in real time at 1000 Hz through a Doppler audio transformer (Herr *et al.* 2010) that connected the Doppler system to a data acquisition system (Powerlab 8SP; ADInstruments, Colorado Springs, CO, USA). Blood velocity and diameter were analysed offline from samples recorded at rest and during exercise. Mean blood velocity was derived from averaging across cardiac cycles for 60 s at rest and at the end of exercise (i.e. last minute of exercise). Average diameter was measured using an automated edge-detection system (Brachial Analyzer; Medical Imaging Applications, Coralville, IA, USA) from 20 s video clips recorded at

rest and during exercise immediately after blood velocity waveforms were sampled. Blood flow was calculated by multiplying the cross-sectional area ( $\pi r^2$ ) of the femoral artery by mean blood velocity. Femoral vascular conductance (FVC) was calculated by dividing mean blood flow by mean blood pressure.

### Blood analysis

Fasting blood glucose was measured by finger prick (Accu-Chek Active; Roche Diagnostics, Indianapolis, IN, USA). Blood was collected anaerobically by venipuncture from an antecubital vein into tubes (BD Vacutainer, Franklin Lakes, NJ, USA) containing K<sub>2</sub>-EDTA (10.8 mg). Immediately upon obtaining the blood sample, tubes were centrifuged at 650 g for 10 min. Subsequently, the supernatant was collected and immediately frozen at –80°C until analysis for plasma NO<sub>x</sub>. Plasma concentrations of NO<sub>x</sub> were assessed by a commercially available colorimetric assay that uses a Griess reagent in a microtitre plate (Cayman Chemical, Ann Arbor, MI, USA). Plasma [Arg] was measured using liquid chromatography (LC)–tandem mass spectrometry (MS) following derivatization procedures described by Mao *et al.* (2010) and LC MS conditions for analysis described by Zheng *et al.* (2015).

### Statistical analysis

Descriptive statistics were calculated for participant characteristics, and Student's unpaired *t* test was used to compare the means between women and men. For crossover data, the primary outcome variables examined were pre-to-post changes in resting vascular function and blood flow for each condition (Cit and placebo). Consistent with methods detailed by Senn (2002), we established a general linear model to test the effect of condition on primary outcome variables after adjusting for sequence (Cit then placebo *versus* placebo then Cit) and period (first 2 weeks *versus* second 2 weeks) effects of the crossover design. The analyses were stratified by sex based on the results of preliminary analysis showing a significant sex-by-condition interaction effect on changes in blood flow. For each sex-specific general linear model, we additionally included resting diastolic blood pressure and body mass index as covariates, as they were significantly different by sex (see Table 1). The normality of primary outcome variables as well as residuals estimated from the models was examined using Kolmogorov–Smirnov test to ensure the use of a parametric linear model. In addition, given that the use of change scores as primary outcome variables in the model limited us to examine the within-condition differences, Student's paired *t* tests were used to compare pre-to-post values within each condition. Statistical significance was considered  $P \leq 0.05$ .

**Table 1. Participant characteristics**

Characteristic	Women ( <i>n</i> = 13)	Men ( <i>n</i> = 12)	<i>P</i> value
Age (years)	70 ± 5	71 ± 5	0.67
Height (cm)	163 ± 5	176 ± 5	<0.01
Weight (kg)	59 ± 6	79 ± 9	<0.01
Body mass index (kg m <sup>-2</sup> )	22.0 ± 2.8	25.5 ± 2.1	<0.01
Lowest ankle–brachial index	1.13 ± 0.07	1.19 ± 0.14	0.20
Seated systolic BP (mmHg)	130 ± 13	137 ± 16	0.23
Seated diastolic BP (mmHg)	65 ± 8	77 ± 9	<0.01
Seated heart rate (beats min <sup>-1</sup> )	58 ± 3	59 ± 11	0.75
Fasting blood glucose (mg dl <sup>-1</sup> )	96 ± 6	93 ± 15	0.57

Values are means ± SD. Abbreviation: BP, blood pressure.

## Results

### Participant characteristics

Table 1 shows participant characteristics at the first study visit before starting Cit or placebo. Men were taller, weighed more and had a higher body mass index and higher diastolic blood pressure than women ( $P < 0.01$ ). No sex difference ( $P > 0.05$ ) was found for age, ankle–brachial index, resting systolic blood pressure, heart rate or fasting blood glucose.

### Resting vascular function

Table 2 shows the effect of Cit on resting blood pressure and arterial stiffness. The change in diastolic pressure was significantly different between Cit and placebo in women and men ( $P \leq 0.05$ ). Diastolic blood pressure decreased after Cit by 5% in men, but was not significantly different ( $P = 0.23$ ) from pre- to post-Cit in women. Adjusting for baseline blood pressure removed ( $P = 0.17$ ) the difference in the change in diastolic blood pressure between Cit and placebo in men. The change in carotid–femoral pulse wave velocity and plasma [NOx] were not different between Cit and placebo ( $P > 0.05$ ), but plasma [Arg] was significantly different between Cit and placebo in women ( $P \leq 0.05$ ) and men ( $P = 0.01$ ). Plasma [Arg] increased by 30 and 35% in women and men, respectively, after Cit ( $P \leq 0.01$ ), with no significant change after placebo ( $P > 0.05$ ). Adjusting for body mass index and baseline blood pressure did not alter the significant ( $P \leq 0.05$ ) increase in plasma [Arg] after Cit compared with placebo in women or men.

### Exercise blood flow

Table 3 shows the effect of Cit on femoral blood flow and vascular conductance during calf muscle exercise. The change in mean blood pressure, blood flow and FVC was not different ( $P > 0.05$ ) between Cit and placebo in women at rest or during exercise at either workload. In contrast, men showed lower mean blood pressure from pre- to

post-Cit at rest ( $P < 0.01$ ) and during exercise (0.5 kg,  $P = 0.04$ ; 1.5 kg,  $P = 0.02$ ), although the change in mean blood pressure after Cit was not significantly different compared with placebo at any time point ( $P > 0.05$ ). The change in blood flow after Cit was different ( $P = 0.01$ ) from placebo only for exercise at the higher workload such that blood flow increased by 11% after Cit ( $P < 0.05$ ), with no difference after placebo ( $P = 0.57$ ). The change in FVC was also different ( $P = 0.01$ ) after Cit compared with placebo, with FVC increasing by 14% with Cit ( $P = 0.01$ ), with no difference after placebo ( $P = 0.78$ ). Adjusting for baseline blood pressure, but not body mass index, removed the significant difference between Cit and placebo in blood flow ( $P = 0.10$ ) and FVC ( $P = 0.10$ ) during exercise at the higher workload.

The blood flow and FVC response to exercise were also calculated as the increase from rest ( $\Delta$ , Exercise – Rest). Similar to the other results, the change in  $\Delta$ blood flow (Cit,  $64 \pm 85$  ml min<sup>-1</sup> versus placebo,  $-10 \pm 69$  ml min<sup>-1</sup>,  $P = 0.02$ ; Fig. 1A) and  $\Delta$ FVC (Cit,  $0.75 \pm 0.77$  ml min mmHg<sup>-1</sup> versus placebo,  $-0.09 \pm 0.87$  ml min mmHg<sup>-1</sup>,  $P = 0.01$ ; Fig. 1B) was different after Cit compared with placebo for exercise at 1.5 kg in men. It was found that  $\Delta$ blood flow increased by 13% ( $P = 0.02$ ; Fig. 2A) and  $\Delta$ FVC by 16% ( $P < 0.01$ ; Fig. 2B) after Cit, whereas both variables were not different after placebo ( $P > 0.05$ ). Adjusting for baseline blood pressure, but not body mass index ( $\Delta$ blood flow,  $P = 0.03$ ;  $\Delta$ FVC,  $P = 0.02$ ), removed the difference between Cit and placebo in  $\Delta$ blood flow ( $P = 0.14$ ) and  $\Delta$ FVC ( $P = 0.07$ ) at 1.5 kg in men. The change in  $\Delta$ blood flow ( $P = 0.72$ ) and  $\Delta$ FVC ( $P = 0.94$ ) was not different between conditions in women for either workload (see Figs 1 and 2 for 1.5 kg; data not shown for 0.5 kg), or for exercise at 0.5 kg for men (data not shown).

## Discussion

The aim of this study was to determine whether short-term (14 days) Cit supplementation enhances peripheral blood

**Table 2. Effect of L-citrulline on resting vascular function**

Parameter	Placebo			L-Citrulline			P value <sup>†</sup>	Adjusted P value
	Pre	Post	Change	Pre	Post	Change		
<b>Women</b>								
Seated systolic BP (mmHg)	129 ± 15	130 ± 10	1.0 ± 8.0	127 ± 12	128 ± 15	0.1 ± 9.7	0.73	0.76, 0.89
Seated diastolic BP (mmHg)	64 ± 7	66 ± 6	2.0 ± 5.0	65 ± 7	64 ± 7	−1.4 ± 4.2	0.05	<0.01, <0.01
Carotid–femoral PWV (m s <sup>−1</sup> )	8.9 ± 1.5	9.1 ± 1.7	0.2 ± 1.1	9.0 ± 1.5	9.0 ± 1.4	0.1 ± 0.4	0.77	0.69, 0.74
Plasma NOx (μmol l <sup>−1</sup> )	34 ± 11	36 ± 15	1.2 ± 10.2	34 ± 16	31 ± 10	−3.3 ± 13.9	0.50	0.46, 0.31
Plasma L-arginine (μmol l <sup>−1</sup> )	96 ± 47	105 ± 56	9.2 ± 33.2	101 ± 36	144 ± 44*	43.3 ± 38.8	0.02	0.03, 0.05
<b>Men</b>								
Seated systolic BP (mmHg)	135 ± 11	132 ± 11	−2.7 ± 12.1	135 ± 14	132 ± 13	−2.5 ± 5.3	0.87	0.73, 0.64
Seated diastolic BP (mmHg)	74 ± 9	73 ± 6	−0.5 ± 7.6	75 ± 9	71 ± 6*	−3.9 ± 5.3	0.04	0.05, 0.17
Carotid–femoral PWV (m s <sup>−1</sup> )	8.8 ± 1.2	8.8 ± 1.0	−0.1 ± 0.4	8.7 ± 1.2	8.8 ± 1.2	0.1 ± 0.7	0.26	0.31, 0.67
Plasma NOx (μmol l <sup>−1</sup> )	32 ± 10	32 ± 12	−0.1 ± 5.7	33 ± 8	33 ± 10	0.2 ± 11.0	0.87	0.95, 0.72
Plasma L-arginine (μmol l <sup>−1</sup> )	96 ± 54	94 ± 37	−2.3 ± 39.1	78 ± 29	119 ± 50*	37.4 ± 31.9	0.01	<0.01, 0.03

Values are means ± SD. Abbreviations: BP, blood pressure; NOx, nitrite and nitrate; and PWV, pulse wave velocity. <sup>†</sup>Comparison of the change value between conditions after controlling for sequence and period effects in a crossover design. \*Difference ( $P < 0.05$ ) between pre and post values within condition. Model 1 adjusted for order, period and baseline body mass index. Model 2 adjusted for model 1 plus baseline seated diastolic BP.

flow and dilatory responses to dynamic calf exercise in older adults. This study was the first to assess the impact of Cit on volumetric blood flow during active contractions. The new finding is that Cit increases the femoral blood flow response to exercise by enhancing vascular conductance in older men. This finding was observed during exercise at the higher workload, indicating that the effect may be dependent on intensity. Importantly, the significant augmentation in exercise blood flow by Cit was removed by adjusting for baseline blood pressure, suggesting that the improvement in blood flow with Cit was related to the basal vascular state of older adults.

### Effect of L-citrulline on blood pressure

Older men had higher resting diastolic blood pressure than older women and experienced a modest blood-pressure-lowering effect after Cit. The change in resting diastolic blood pressure after Cit in men was inversely correlated ( $r = -0.72$ ,  $P < 0.001$ ) with baseline diastolic blood pressure, suggesting that the reduction in absolute diastolic pressure after Cit supplementation was likely due to men having higher basal blood pressure. Hart *et al.* (2009) have shown that diastolic (but not systolic) blood pressure is positively related to resting muscle sympathetic nerve activity in older men. Supplementation with Cit might have reduced sympathetic vasomotor tone by enhancing smooth muscle relaxation. This is plausible considering that past work finds chronic Cit increases urinary excretion of cyclic GMP (Schwedhelm *et al.* 2008), a byproduct of NO-stimulated guanylyl cyclase in smooth muscle that results in vasodilatation. Also, evidence exists that acute nitrate supplementation is effective at reducing

muscle sympathetic nerve activity at rest (Notay *et al.* 2017).

### Effect of L-citrulline on plasma biomarkers

Plasma [NOx] was not altered with Cit in the present study. Other studies in middle-aged adults have reported elevated biomarkers of NO production after chronic Cit (Schwedhelm *et al.* 2008; Ochiai *et al.* 2012), whereas some studies in younger adults have not (Bailey *et al.* 2015; Suzuki *et al.* 2016). The lack of change in plasma [NOx] in the present paper indicates that Cit was unable to augment systemic NO production; however, it is possible that Cit still had an influence on NO bioactivity within tissue. Resting diastolic blood pressure was lowered and blood flow responses to exercise were improved in men; evidence that Cit had a positive vascular effect in men despite no change in plasma [NOx].

The goal of Cit supplementation is to increase Arg concentrations. Arginine is substrate for NO production, and therefore, may counter low NO bioavailability associated with ageing and disease (El Hattab *et al.* 2012; Kim *et al.* 2015). Both women and men showed significantly elevated plasma [Arg] after Cit supplementation, indicating successful intestinal absorption of Cit and subsequent production of Arg in the kidney. This is an interesting observation considering that participants took their last dose the night before testing (9–12 h before). Moinard *et al.* (2016) has reported that plasma [Arg] returns to basal concentrations about 5–8 h after an acute dose of Cit (10 g) in older men. Therefore, our results indicate that Arg accumulates in blood over time with chronic Cit in older adults.

**Table 3. Effect of L-citrulline on blood flow during exercise**

	Placebo			L-Citrulline			P value <sup>†</sup>	Adjusted P value
	Pre	Post	Change	Pre	Post	Change		
<b>Women</b>								
Mean blood pressure (mmHg)								Model 1, 2
Rest	86 ± 8	88 ± 6	1.9 ± 5.6	86 ± 8	85 ± 8	-0.8 ± 5.3	0.16	0.09, 0.15
0.5 kg	95 ± 8	96 ± 7	1.4 ± 6.6	96 ± 10	95 ± 9	-1.1 ± 6.9	0.23	0.35, 0.46
1.5 kg	95 ± 7	97 ± 7	2.1 ± 6.0	97 ± 9	96 ± 8	-0.4 ± 5.3	0.22	0.24, 0.36
Mean blood flow (ml min <sup>-1</sup> )								
Rest	80 ± 28	75 ± 36	-5.4 ± 38.5	84 ± 32	73 ± 35	-10.8 ± 27.5	0.58	0.12, 0.19
0.5 kg	374 ± 80	364 ± 90	-9.8 ± 65.9	381 ± 99	340 ± 102	-41.4 ± 96.3	0.28	0.38, 0.23
1.5 kg	452 ± 117	454 ± 101	2.4 ± 67.6	430 ± 71	415 ± 98	-15.5 ± 68.2	0.55	0.40, 0.25
FVC (ml min mmHg <sup>-1</sup> )								
Rest	0.9 ± 0.3	0.8 ± 0.3	-0.08 ± 0.40	0.9 ± 0.3	0.8 ± 0.4	-0.11 ± 0.31	0.79	0.25, 0.40
0.5 kg	3.9 ± 0.9	3.7 ± 0.9	-0.18 ± 0.86	3.9 ± 1.1	3.5 ± 1.0	-0.40 ± 0.91	0.43	0.49, 0.20
1.5 kg	4.7 ± 1.2	4.6 ± 0.9	-0.10 ± 0.69	4.5 ± 1.0	4.3 ± 1.0	-0.17 ± 0.69	0.85	0.64, 0.30
<b>Men</b>								
Mean blood pressure (mmHg)								
Rest	94 ± 9	92 ± 6	-1.8 ± 7.4	95 ± 9	91 ± 7*	-3.7 ± 3.8	0.17	0.13, 0.55
0.5 kg	102 ± 10	101 ± 10	-0.2 ± 9.2	105 ± 12	100 ± 9*	-4.1 ± 6.3	0.32	0.24, 0.58
1.5 kg	102 ± 11	102 ± 11	-0.6 ± 7.9	103 ± 11	100 ± 10*	-3.4 ± 4.5	0.56	0.47, 0.70
Mean blood flow (ml min <sup>-1</sup> )								
Rest	95 ± 45	94 ± 59	-0.7 ± 50.9	85 ± 33	84 ± 29	-1.8 ± 20.3	0.84	0.99, 0.94
0.5 kg	448 ± 132	439 ± 127	-8.5 ± 70.9	465 ± 131	463 ± 140	-1.6 ± 81.3	0.55	0.70, 0.98
1.5 kg	565 ± 121	555 ± 160	-10.5 ± 62.6	521 ± 134	584 ± 166*	62.6 ± 98.0	0.01	0.02, 0.10
FVC (ml min mmHg <sup>-1</sup> )								
Rest	1.0 ± 0.4	1.0 ± 0.6	0.02 ± 0.55	0.9 ± 0.3	0.9 ± 0.3	0.01 ± 0.20	0.74	0.89, 0.97
0.5 kg	4.3 ± 1.0	4.3 ± 1.2	-0.04 ± 0.71	4.4 ± 1.2	4.6 ± 1.4	0.17 ± 0.69	0.31	0.40, 0.83
1.5 kg	5.5 ± 1.0	5.4 ± 1.4	-0.06 ± 0.82	5.0 ± 1.5	5.8 ± 1.7*	0.77 ± 0.93	0.01	0.02, 0.10

Values are means ± SD. Abbreviations: FVC, femoral vascular conductance. <sup>†</sup>Comparison of the change value between conditions after controlling for sequence and period effects in a crossover design. \*Difference ( $P < 0.05$ ) between pre and post values within condition. Model 1 adjusted for order, period and baseline body mass index. Model 2 adjusted for model 1 plus baseline seated diastolic BP.

### Effect of L-citrulline on exercise blood flow

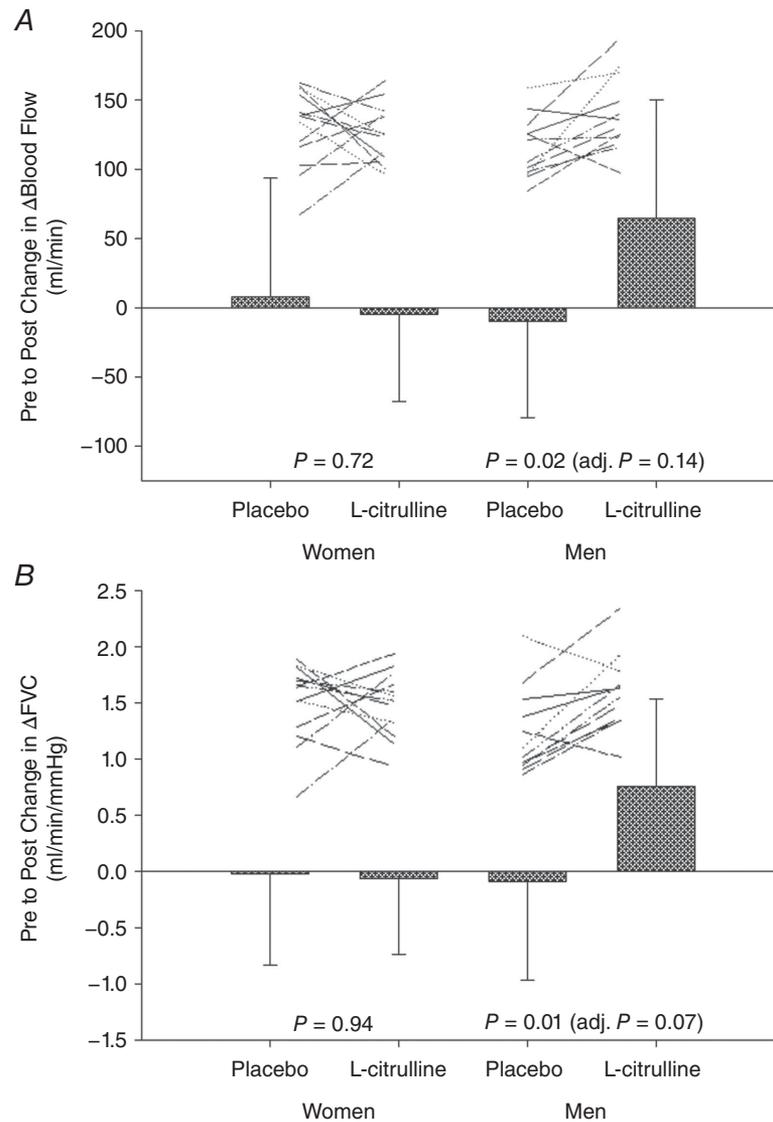
The protocol used in this study involved exercise at two workloads performed in succession. The first workload was set at an external resistance of 0.5 kg, which was enough weight to provide tautness to the pulley system, allowing for a semi-unloaded stage of exercise. At this light intensity, there was no influence of Cit on exercise blood flow or FVC in either sex. As this stage of exercise required low tension development, it can be interpreted that Cit was unable to influence the response of the endothelium to stimuli such as shear stress and mechanical distortion of vessels that largely drive local resistance arteriolar dilatation during passive to light exercise (Wray *et al.* 2005). The second workload was set at an external resistance of 1.5 kg, because our pilot work found that weight above this amount led to some older adults struggling to complete 3 min of exercise. At this intensity of exercise that involved greater contractile work, and thus metabolism, we found that Cit increases both absolute and relative ( $\Delta$  from rest) blood flow responses to exercise

in men but not women. This finding indicates that Cit enhances blood flow when oxygen is at a greater demand and/or when the stimulus for vasodilatation is more appreciable. The improved blood flow response to exercise for men may be attributable to several mechanisms. First, mean blood pressure was lower during exercise in men, which might have reflected lower sympathetic vasomotor activity, allowing for greater conductance for blood flow. However, mean blood pressure was also lower at rest and during the first stage of exercise without accompanying improvements in FVC or blood flow, thus this mechanism alone is unlikely. Second, Cit could have improved NO bioavailability, which can enhance the accumulation of vasodilators supporting blood flow (Creelius *et al.* 2010) and/or NO improved the regulation of mitochondrial respiration by binding to cytochrome *c* oxidase (Brown, 2001), thereby facilitating greater blood flow (and oxygen) distribution to active muscle. This postulate is consistent with Bailey *et al.* (2015), who found that 7 days of Cit supplementation (6 g day<sup>-1</sup>) increases

vastus lateralis muscle oxygenation in young men during moderate-intensity cycling, indicating improved muscle perfusion.

Our finding that exercise blood flow is elevated with chronic Cit in older men is in contrast to what is reported after acute Cit administration. Postexercise hyperaemia (Churchward-Venne *et al.* 2014) and peak reactive hyperaemia (Kim *et al.* 2015) are unaltered by acute doses of Cit in older adults who are overweight with normal to elevated basal blood pressures. This indicates that chronic Cit administration, even in the short term,

is necessary to produce a positive benefit to exercise blood flow. The present study, for the first time, shows that Cit increases volumetric blood flow during exercise. However, this improvement was dependent on resting blood pressure, indicating that Cit may benefit only older adults with some degree of vascular dysfunction. The observation that Cit did not increase exercise blood flow in older women may be due to women having healthier vasculature than men as reflected by lower resting diastolic blood pressure and body mass index (Table 1).

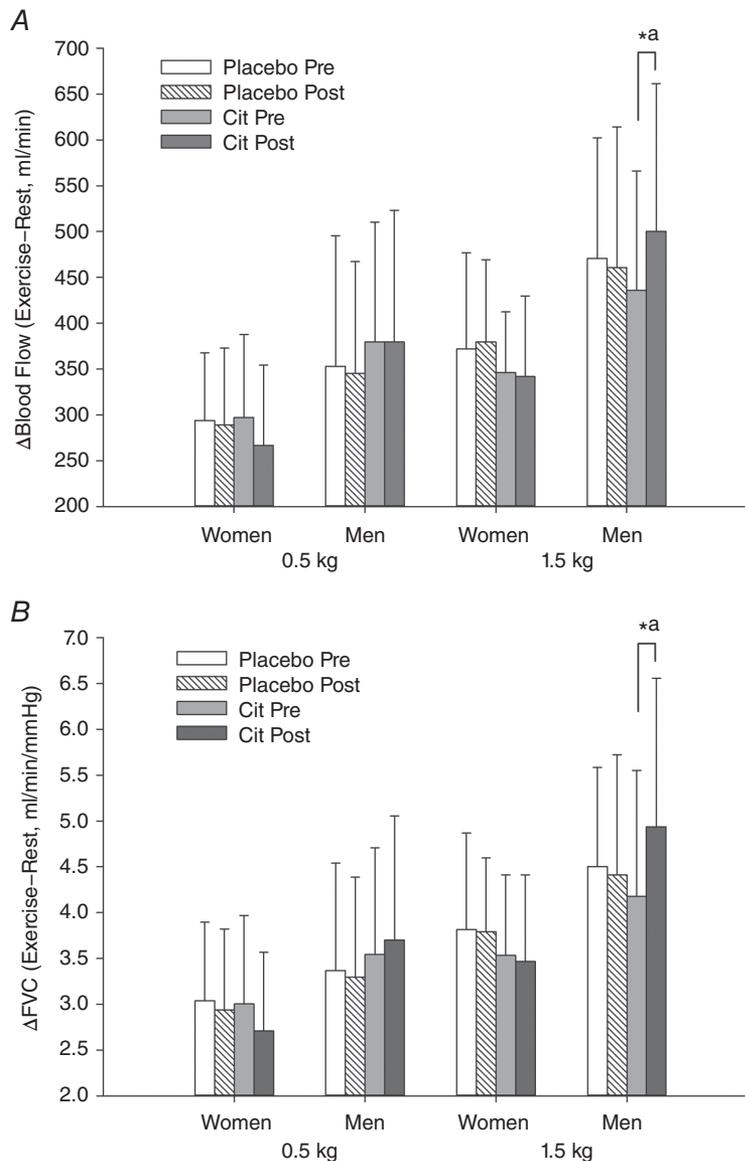


**Figure 1.** Change in blood flow and femoral vascular conductance (FVC) expressed as an increase from rest to exercise ( $\Delta$ ) after placebo and L-citrulline conditions

Only data for the 1.5 kg workload condition are presented. Data are means  $\pm$  SD. Lines represent non-scaled individual responses. The  $P$  values are from statistical comparisons of the change values between conditions after controlling for sequence and period effects in a crossover design. The  $P$  value in parenthesis is adjusted for body mass index and baseline diastolic pressure in addition to sequence and period effects.

Another consideration worthy of discussion is Arg transport into the vasculature. Although women were not limited in converting Cit to Arg as reflected by elevated plasma [Arg], transportation of Arg to membrane-bound endothelial nitric oxide synthase is accomplished by cationic amino acid transporter-1 (CAT-1), which can be inhibited through several mechanisms, including post-translational modulation by phosphorylated protein kinase  $C\alpha$  (PKC $\alpha$ ) and extracellular signal-regulated kinase (ERK). Older female rats are shown to have higher

phosphorylated PKC $\alpha$  protein levels than older male rats (Schwartz *et al.* 2009). Moreover, oestrogen is found to increase CAT-1 activity by decreasing phosphorylated ERK (Bentur *et al.* 2015), and oestrogen decreases arginase levels (enzyme that metabolizes arginine) in vascular cells (Hayashi *et al.* 2006), thus older women with low oestrogen levels may have impaired Arg transport and/or intracellular activity compared with older men. Although this study cannot address whether this was a contributing factor in our results, we cannot discount the possibility



**Figure 2. Femoral blood flow and vascular conductance (FVC) during calf muscle exercise expressed as an increase from rest to exercise ( $\Delta$ ) in older women and men**

The change in  $\Delta$ blood flow and  $\Delta$ FVC was different between L-citrulline (Cit) and placebo only for exercise at the higher workload in men. Data are means  $\pm$  SD. \*Difference between pre and post values within condition after controlling for sequence and period effects in a crossover design ( $P < 0.05$ ). <sup>a</sup>No longer significant ( $P > 0.05$ ) after adjusting for body mass index and baseline diastolic pressure in addition to sequence and period effects.

of sex differences in the ability of Cit to increase NO bioavailability in older adults.

### Limitations

Several limitations accompany this study. Although we were sufficiently powered to detect a significant increase in Arg after Cit supplementation based on past work (Schwedhelm *et al.* 2008), no other study has reported changes in bulk blood flow during exercise to perform a power analysis for our main outcome variable. However, the data presented here can be used by future researchers to perform such power analysis. The present sample of older adults had no history of cardiovascular disease, thus our results might not translate to clinical populations. We included older adults with normal to high blood pressure, with six men and four women having Stage I hypertension owing to elevated systolic blood pressure. Although this sample reflects vascular ageing, because systolic blood pressure significantly increases with advancing age (Franklin *et al.* 1997), it does not well represent a model of healthy ageing. Another limitation is that plasma [NOx] was measured rather than other NO biomarkers that might be more specific to endothelial function (e.g. nitrite; Lauer *et al.* 2001). We observed no change in plasma [NOx] in women or men, thus have no evidence that NO production was elevated. Although we attribute improved blood pressure and exercise blood flow after Cit in men to elevated endothelial NO bioavailability, consistent with other researchers (Bailey *et al.* 2015, 2016; Figueroa *et al.* 2017), we appreciate that there may be other pathways by which Cit might impart benefits to vascular function. For instance, Cit increases neuronal expression of NO synthase (Yabuki *et al.* 2013), which recent research shows to be an important regulator of blood pressure and vascular resistance in humans (Shabeeh *et al.* 2017). This central effect may be augmented by Arg-induced elevations in neuronal cyclic AMP levels (Sidorov *et al.* 1999) that can subsequently trigger adenosine-stimulated NO synthesis (Dubey *et al.* 1998). The present study cannot address whether this central pathway contributed to our results, thus requiring future research to uncover the specific pathway(s) responsible for improved blood flow responses to exercise in men after Cit supplementation.

### Conclusions

In this sample of older adults, Cit supplementation increased femoral blood flow and vascular conductance during lower-limb exercise in men. The improvement in exercise blood flow in men was removed when adjusting for baseline blood pressure, indicating that the benefit of Cit supplementation is more pronounced in older men with higher resting blood pressure. These results support the suggestion that arginine supplementation using Cit

can improve muscle blood flow during exercise in older men; however, the 10–15% increase in haemodynamics observed in this study is less than the ~25% age-related impairment in muscle blood flow reported for older men (Poole *et al.* 2003). Although this diminishes the clinical meaningfulness of our results, physiological implications of enhanced blood flow within the magnitude observed after Cit in this study have implications for improving exercise tolerance as demonstrated in older adults with vascular dysfunction (Sullivan *et al.* 1988).

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## Additional information

### Competing interests

None declared.

### Author contributions

This study was conducted in the Department of Kinesiology and Sport Management at Texas Tech University. J.U.G. conceptualized and designed the study. J.U.G., A.R. and J.A. collected and analysed the data and drafted the manuscript, providing individual intellectual contributions. Y.K. assisted with statistical analysis and interpretation of results and critically

revised the manuscript for important intellectual content. All authors approved the final version of the manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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