Citrulline Does Not Prevent Skeletal Muscle Wasting or Weakness in Limb-Casted Mice\textsuperscript{1–3}

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Abstract

Background: Increasing Arg availability reduces atrophy in cultured skeletal muscle cells. Supplementation with its metabolic precursor Cit is more effective at improving skeletal muscle Arg concentrations.

Objective: We tested the hypothesis that Cit supplementation would attenuate skeletal muscle atrophy and loss of function during hindlimb immobilization in mice.

Methods: Male C57BL/6J Arc mice underwent 14 d of unilateral hindlimb immobilization/plaster casting and were supplemented with −0.81 g Cit/kg per day (CIT group) or Ala (ALA group) mixed into their food. The uncasted contralateral limb (internal control) and an uncasted group (CON) served as controls. Muscle atrophy was evaluated with mass, fiber area, and in situ muscle function.

Results: Tibialis anterior (TA) muscle mass [ALA: 37.6 ± 0.92 mg; CIT: 38.3 ± 1.25 mg] and peak tetanic force [ALA: 1150 ± 38.5 mN; CIT: 1150 ± 52.0 mN] were lower (\(P < 0.001\)) in the ALA (53.9 ± 0.42 mg) and CIT (1760 ± 28.5 mN) groups than in the CON group. No difference was found between ALA and CIT groups for TA mass, fiber area, or peak force. The mRNA expression of the nitric oxide synthase 2, inducible (Nos2; ~15-fold) and B-cell chronic lymphoid leukemia/lymphoma 2/adenovirus E1B 19 kDa interacting protein 3 (Bnip3; ~17-fold) genes and the ratio of microtubule-associated protein light chain 3BII to 3BI (LC3BII/LC3BI) (60.5% ± 17.7%) were higher (\(P < 0.05\)) in the ALA group than in the CON group, suggesting increased autophagy. In the CIT group, Bnip3 mRNA was lower (~70%; \(P < 0.05\)) and Nos2 mRNA tended to be lower (~45%; \(P = 0.05\)) than in the ALA group, whereas LC3BII/LC3BI was not different from the CON group.

Conclusions: Cit treatment of male mice did not affect therapeutically relevant outcome measures such as skeletal muscle mass and peak muscle force after 14 d of hindlimb immobilization. \textit{J Nutr} doi: 10.3945/jn.114.203737.

Keywords: disuse, immobilization, amino acids, muscle atrophy, force function, autophagy, muscle breakdown

Introduction

Skeletal muscle wasting, the loss or atrophy of skeletal muscle, is a serious complication in many diseases and conditions, including chronic heart failure, sepsis, and cancer (1). Prolonged periods of inactivity as with illness (bed rest) or injury (immobilization) also results in a substantial loss of skeletal muscle mass and function. In humans, just 2 wk of immobilization or unloading reduces lean muscle mass by ~5% (2–4). The effect of inactivity on functional capacity is even more profound. Indeed, a 20% loss of muscle strength is experienced after 2–3 wk of forced inactivity (2, 3). The recovery of muscle mass and function can be slow, even with exercise rehabilitation (4). In at-risk populations, such as the elderly, the loss of muscle mass and function can affect mobility and reduce quality of life. As such, the development of strategies to prevent muscle wasting during disuse atrophy is of major importance.

Ultimately, muscle wasting results from a chronic imbalance between rates of muscle protein synthesis and breakdown, with breakdown exceeding synthesis (5). Both increases in protein breakdown and reductions in protein synthesis contribute to disuse-induced atrophy (6). Basal and postprandial protein synthesis are reduced during immobilization, and changes in the expression of muscle-specific ubiquitin ligases (Atrogin-1, muscle-specific RING-finger protein-1) and factors involved in autophagy are reported (6). Because food intake and amino acids, in particular, effectively stimulate protein synthesis and reduce protein breakdown, they have received considerable attention for the treatment of muscle-wasting conditions. Of the amino acids, the essential amino acid Leu is identified as the most potent regulator of muscle protein metabolism, both in

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\end{itemize}
vitro and in vivo. However, studies of prolonged Leu treatment have produced disappointing results. As such, there is a need to identify other nutrients that may regulate skeletal muscle mass during atrophic conditions.

Recently, we demonstrated that Arg plays a direct role in the regulation of rapamycin-sensitive mechanistic target of rapamycin complex 1 signaling and protein synthesis and attenuates skeletal muscle wasting in C2C12 myotubes during nutrient and growth factor withdrawal. In vivo, Arg is considered a semi-essential amino acid and is critically involved in a number of physiologic processes, including providing substrate for the production of NO by nitric oxide synthase (NOS) (9). Within skeletal muscle, NO plays a role in satellite cell activation (10) and is necessary for overload-induced skeletal muscle hypertrophy (11). NO may also play a role in nutrient delivery to the muscle through its vasodilatory effects (12). Because mechanistic studies in vitro show that Arg modulates both protein synthesis (8) and breakdown (13) in muscle cells, increasing skeletal muscle Arg availability represents a promising treatment option for muscle wasting. Interestingly, Arg feeding is not the most effective way to increase muscle Arg availability. Intake of Cit, a non-proteinogenic amino acid with a unique interorgan metabolism, effectively increases plasma Cit, Arg, and ornithine concentrations in a dose-dependent manner, without affecting the concentration of other amino acids (14). Oral Cit administration is more efficient at increasing plasma and muscle concentrations of Arg compared with Arg feeding, because Cit is not metabolized in the gut (15) and could represent an effective anabolic treatment (16).

Although it is established that Cit supplementation reduces muscle wasting on a background of Arg deficiency (17–19), it is currently unclear whether Cit supplementation can protect skeletal muscle mass and function during other catabolic conditions. To examine the effect of Cit supplementation on inactivity-induced muscle wasting and loss of function, mice underwent unilateral hindlimb immobilization for 14 d and were supplemented with −0.81 g Cit or Ala (amino acid control) per day mixed into normal unpurified diet. We hypothesized that Cit supplementation would partially prevent the loss of muscle mass and function in the immobilized limb.

Methods

Animals. All experiments were approved by the Animal Ethics Committee of The University of Melbourne and were conducted in accordance with the Australian code of practice for the care and use of animals for scientific purposes as stipulated by the National Health and Medical Research Council (Australia). Ten-week-old male C57BL/6JArc mice, with a mean body mass of 26.7 ± 0.3 g (range: 24.5–29.8 g) were allocated into 1 of 3 treatment groups as follows: 1) an uncasted control group (CON; n = 10), 2) a casted group supplemented with Ala (ALA; n = 15), and 3) a casted group supplemented with Cit (CIT; n = 15). All mice remained in their original box of 5, and groups were matched for body mass. Animal numbers were based on previous work in our laboratory by using this model of casting (20). All mice were obtained from the Animal Resources Centre and housed in the Biological Research Facility at The University of Melbourne under a 12:12-h light-dark cycle. Mice were monitored daily for adverse signs and symptoms.

Casting/immobilization. To induce muscle wasting, mice underwent 14 d of unilateral hindlimb casting. Mice were anesthetized with an intraperitoneal injection of a mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg) such that they were unresponsive to tactile stimuli. The right hindlimb was shaved and wrapped in plaster (Vet-lite bandage; DLC) with the foot positioned in plantar flexion to induce maximal atrophy of the lower hindlimb muscles (21). Growth restriction (22) and hypotrophy of the contralateral limb (21) were observed with immobilization. Therefore, we used an additional uncasted group (CON) and the uncasted contralateral limb (internal control) as controls to account for potential alterations that resulted from growth restriction or changes to the mass of the contralateral limb. In our hands, unilateral plaster casting in mice leads to substantial (20–25%) muscle wasting and weakness within 14 d (20). The atrophy with plaster casting is relevant to clinical conditions such as prolonged bed rest and joint immobilization.

Amino acid administration. Casted mice were administered Ala or Cit (Sigma-Aldrich Co.) via their food, a method that was previously shown to result in elevated concentrations of Cit and Arg in plasma and skeletal muscle (18). Five mice from the same group were housed together and given crushed unpurified diet, supplemented with 0.5% Cit or Ala, mixed into a paste with water. The unpurified diet (Barastoc; Ridley AgriProducts) contained 9.0% fat, 25% starch, 3.8% sugar, 21% protein, and 1.5% Arg. Food was prepared fresh daily and placed in a dish on the cage floor to allow unrestricted access. On the basis of the typical food consumption of 3 g of dry food per day for an ~25-g C57BL/6Arc mouse, this equated to a dose of ~1 g/kg per day. However, dry food consumption across the 14-d treatment period (4.3 ± 0.1 g/d per mouse) was slightly lower than anticipated, resulting in Cit and Ala intakes of 0.81 ± 0.02 g/kg per day. Similar doses of Arg were shown to preserve soleus muscle mass during hindlimb suspension (23). Moreover, because of a favorable metabolism, Cit is more effective at increasing skeletal muscle concentrations of Arg than Arg supplementation (14, 18, 24, 25). No difference in food consumption was observed between the ALA, CIT, or CON (uncasted, unpurified diet fed) groups. It is important to note that dietary protein intake in mice is ~27.2 g/kg per day, and the given dose represents a higher amino acid intake of only ~3.0%.

Plasma amino acid concentration. In a preliminary experiment, we delivered Ala or Cit at 1 g/kg as a single bolus via oral gavage to confirm that in C57BL/6Arc mice Cit intake resulted in higher plasma concentrations of Cit and its metabolite Arg. Exactly 1 h after amino acid administration, 18 mice (9 Ala and 9 Cit) were killed by cervical dislocation, and blood was collected from the abdominal aorta and centrifuged at 1000 × g and 4°C for 10 min in heparin-containing tubes. Plasma was frozen in liquid nitrogen and stored at −80°C. Amino acid analysis was performed as described previously (26).

In situ skeletal muscle function. At the end of treatment (i.e., 14 d after casting) mice were anesthetized with sodium pentobarbitone (Nembutal; 60 mg/kg; Sigma-Aldrich) via intraperitoneal injection, and the function of the right tibialis anterior (TA) muscle was assessed in situ. The methods used for evaluating the functional properties (maximal strength, fatigability, and recovery) of muscles in situ were described by us in detail elsewhere (27, 28). Specific force (kN/m²), which represents peak force normalized to muscle cross-sectional area (CSA) was calculated with the following formula: peak force (mN)/[muscle mass (g) × 0.636 × muscle length (mm)] (28). After functional analyses, the TA, gastrocnemius, soleus, and quadriceps muscles were carefully excised, blotted on filter paper, and weighed on an analytic balance. Right and left TA muscles were mounted in embedding medium and frozen in thawing isopentane, whereas the other muscles were frozen directly in liquid nitrogen and stored at −80°C for subsequent analyses. Because of the emphasis on functional measures in this study and the laborious nature of these measures, the logistics of collecting muscle tissue at an appropriate time point to measure changes in muscle Cit and Arg.
concentrations induced by the supplemental diet that contained 0.5% Cit were not feasible.

**Skeletal muscle histology.** Serial sections (5 μm) were cut transversely through the right (casted) and left (uncasted) TA muscles of mice from the CIT and ALA groups by using a refrigerated (−20°C) cryostat (Cryostat; IEC). Sections were reacted with antibodies raised against laminin (Sigma-Aldrich) and myosin heavy chain Ila (N2.261; developed by Prof. Helen M. Blau; obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the National Institute of Child Health and Human Development and maintained by the University of Iowa Department of Biology) to enable the determination of mean myofiber type-specific CSA and minimum fiber diameter (Feret’s μm) and succinate dehydrogenase (SDH) activity to determine fiber oxidative enzyme capacity (29). Digital images of stained sections were obtained with an upright microscope with camera (Axio Imager D1; Carl Zeiss), controlled by AxioVision AC software (AxioVision AC Rel. 4.8.2; Carl Zeiss Imaging Solutions). Images were quantified with AxioVision 4.8.2 software as described previously (29).

**RNA extraction and PCR.** mRNA expression of tumor necrosis factor α gene; IL-6 gene; nitric oxide synthase 1 (Nos1); nitric oxide synthase 2 (Nos2); cluster determinant 68 gene; epidermal growth factor-like module containing, mucin-like, hormone receptor-like sequence 1 gene; tripartite motif-containing 63 gene (or muscle-specific RING-finger protein-1 gene); F-box protein 32 (Fbxo32 or atrogin-1); forkhead box O1 (Foxo1); forkhead box O4 (Foxo4); B-cell chronic lymphoid leukemia/lymphoma 2 adenovirus E1B 19 kDa interacting protein 3 (Bnip3); microtubule-associated protein 1 light chain 3 β gene; B-cell leukemia/lymphoma 2 (Bcl2); and Bcl2-like 1 (Bcl2l1; Bcl-x isoform) was determined by real-time RT-PCR as described previously (30). Primer sequences are listed in [Supplemental Table 1](#). Gene expression was quantified by normalizing to the cDNA content of each sample and expressed as arbitrary units.

**Protein extraction and Western blot analysis.** Quadriceps muscle samples (~20 mg) were homogenized (Polytron 2100; Kinematica) on ice in a 1:10 dilution with homogenizing buffer [50 mmol/L Tris-HCl, pH 7.5, 1 mmol/L EDTA, 0.1% protease inhibitor cocktail (P8340; Sigma-Aldrich), 0.1% phosphatase inhibitor cocktail (P2850; P7526; Sigma-Aldrich)]. Samples were centrifuged at 10,000 × g for 10 min at 4°C, and the resulting supernatant fluid was analyzed for total protein content (Bio-Rad DC Protein Assay; Bio-Rad) with BSA as the standard. Samples were standardized to a protein content of 2 g/L in homogenizing buffer. Western blot analysis was performed with the same methods as reported in detail previously (8). Primary antibodies [microtubule-associated protein light chain 3B (LC3B) and eukaryotic initiation factor 5F] were purchased from Cell Signaling Technologies and diluted 1:1000 in 5% BSA/Tris-buffered saline and Tween 2.0. Results were normalized to total protein as determined by BLOTFastStain according to the manufacturer’s instructions (G-Biosciences). No differences were observed for protein loading between samples.

**Statistical analyses.** All values are expressed as means ± SEMs unless stated otherwise. Data were tested for normality and homogeneity of variance by using Shapiro-Wilk and Levene’s tests, respectively.

Body mass, fatigue, and frequency-force data were compared between and within groups by using a repeated-measures ANOVA with Fisher’s least significant difference post hoc test. A factorial ANOVA with Tukey’s post hoc test was used to compare histologic analyses (e.g., fiber oxidative capacity) within and between groups by using a repeated-measures ANOVA with the least squares method (LSM) (30).

## Results

**Plasma amino acid concentrations (preliminary experiment).** One hour after an oral gavage of 1 g Cit/kg, plasma concentrations of Cit and its metabolites Arg and ornithine were 8-fold, 2.6-fold, and 2.3-fold higher, respectively, than in mice that received an oral gavage of 1 g Ala/kg (P < 0.001; [Table 1](#)). In mice gavaged with Cit, plasma Glu (−14%; P < 0.05) was also lower, and plasma Ile (P = 0.05), Phe (P = 0.09), and Lys (P = 0.08) all tended to be lower than in mice gavaged with Ala.

**Body mass and skeletal muscle mass.** Data for skeletal muscle mass are presented in Figure 1. Compared with the CON group, the mass of the quadriceps, gastrocnemius, TA, and soleus muscles were significantly lower in both the ALA and CIT groups (P < 0.001; Figure 1A). No differences were found in muscle mass between the ALA and CIT groups for either the casted or uncasted internal control limb. Interestingly, compared with the CON group, the mass of the quadriceps, gastrocnemius, and TA muscles of the internal control limb were lower (P < 0.05) in the CIT group and were either lower (quadriceps; P < 0.05) or tended to be lower (gastrocnemius and TA; P = 0.06) in the ALA group (Figure 1B). Starting body mass was not different between groups. Over the 14-d treatment period, body mass increased in the CON group (P < 0.001) but decreased in the ALA group (P < 0.005) and tended to decrease in the CIT group (P = 0.10). As a result, final body mass was significantly greater (P < 0.05) in the CON group than in the ALA and CIT groups (Figure 1C).

To characterize the magnitude of the primary effects of limb immobilization on skeletal muscle atrophy independent of growth impairment, we compared muscle mass of the casted leg with that of the internal control leg (Figure 1D). In the CON group, muscle

**TABLE 1** Plasma amino acid concentration 1 h after an oral gavage with saline or Cit at 1 g/kg in male mice.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Saline, μM</th>
<th>Citrine, μM</th>
<th>P1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tau</td>
<td>491 ± 86.9</td>
<td>422 ± 72.2</td>
<td></td>
</tr>
<tr>
<td>Asp</td>
<td>8.50 ± 2.00</td>
<td>7.45 ± 1.76</td>
<td></td>
</tr>
<tr>
<td>Thr</td>
<td>96.8 ± 5.62</td>
<td>90.8 ± 6.79</td>
<td></td>
</tr>
<tr>
<td>Ser</td>
<td>93.3 ± 4.22</td>
<td>85.6 ± 4.44</td>
<td></td>
</tr>
<tr>
<td>Asn</td>
<td>35.8 ± 1.70</td>
<td>32.0 ± 2.11</td>
<td></td>
</tr>
<tr>
<td>Glu</td>
<td>41.4 ± 4.62</td>
<td>41.2 ± 5.29</td>
<td></td>
</tr>
<tr>
<td>Gin</td>
<td>640 ± 25.6</td>
<td>550 ± 17.2</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Gly</td>
<td>276 ± 21.6</td>
<td>236 ± 14.5</td>
<td></td>
</tr>
<tr>
<td>Ala</td>
<td>327 ± 27.3</td>
<td>334 ± 19.4</td>
<td></td>
</tr>
<tr>
<td>Cit</td>
<td>842 ± 5.18</td>
<td>753 ± 52.7</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Val</td>
<td>189 ± 18.8</td>
<td>194 ± 14.2</td>
<td></td>
</tr>
<tr>
<td>Cys</td>
<td>41.3 ± 4.59</td>
<td>39.4 ± 3.54</td>
<td></td>
</tr>
<tr>
<td>Met</td>
<td>28.9 ± 1.32</td>
<td>27.9 ± 2.87</td>
<td></td>
</tr>
<tr>
<td>Ile</td>
<td>120 ± 8.61</td>
<td>87.1 ± 12.6</td>
<td>0.05</td>
</tr>
<tr>
<td>Leu</td>
<td>166 ± 16.9</td>
<td>121 ± 21.8</td>
<td></td>
</tr>
<tr>
<td>Tyr</td>
<td>33.5 ± 2.87</td>
<td>32.6 ± 2.38</td>
<td></td>
</tr>
<tr>
<td>Phe</td>
<td>53.5 ± 2.71</td>
<td>44.2 ± 4.31</td>
<td>0.09</td>
</tr>
<tr>
<td>Orn</td>
<td>47.2 ± 3.00</td>
<td>158 ± 12.2</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>His</td>
<td>53.0 ± 2.54</td>
<td>53.9 ± 1.70</td>
<td></td>
</tr>
<tr>
<td>Lys</td>
<td>166 ± 10.8</td>
<td>126 ± 18.0</td>
<td>0.08</td>
</tr>
<tr>
<td>Arg</td>
<td>713 ± 3.64</td>
<td>255 ± 27.6</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Pro</td>
<td>63.9 ± 3.11</td>
<td>63.5 ± 3.54</td>
<td></td>
</tr>
</tbody>
</table>

1 Values are means ± SEs, n = 8 per group. Orn, ornithine; Tau, taurine.
2 Data were analyzed with Student’s t tests; P values are presented for trends (P < 0.1) and significant differences (P < 0.05) between groups.
mass was not different between the right and left legs for any of the muscles investigated. In both the ALA and CIT groups, the mass of the casted quadriceps, gastrocnemius, TA, and soleus muscles was significantly lower than the mass of the corresponding muscle of the internal control limb (P < 0.001), but the measured muscle atrophy was reduced. For example, TA muscle mass in the ALA and CIT groups was 30% ± 1.7% and 29% ± 2.3% lower compared with the CON group but was only 22% ± 2.0% and 18% ± 1.6% lower compared with the internal control, respectively. Adjusting TA muscle mass for final body mass by using the LSM resulted in a similar magnitude of muscle atrophy for both the ALA (−19% ± 1.8%) and CIT (−17% ± 2.0%) groups.

Skeletal muscle CSA and fiber type. On the basis of muscle mass data, the internal control limb was used for comparisons of fiber CSA and minimal Feret’s diameter by using a 2-factor ANOVA (casting × treatment). Fourteen days of hindlimb immobilization resulted in a shift in TA muscle fiber CSA distribution (toward smaller fibers) in the casted limb, with a significantly lower mean fiber CSA (−22% ± 1.9%; P < 0.001) and minimal Feret’s diameter (−11% ± 1.2%; P < 0.001) than the internal control for all fibers combined (Figure 2A, B). No difference was observed in fiber CSA or minimal Feret’s diameter between the ALA and CIT groups. The difference in fiber CSA between the casted limb and internal control tended (P = 0.06) to be greater in type IIb/x (−22.8% ± 2.5%) than type IIa fibers (−11.5% ± 5.1%), but was not different between the ALA and CIT groups. The percentage of type IIa fibers was not different between the casted limb and the internal control. However, a treatment effect for fiber type composition was found, with muscles in the CIT group having fewer type IIa fibers (12.4% ± 2.1%) than in the ALA group (21.8% ± 3.5%; Figure 2C). SDH reactivity was significantly higher in type IIa fibers than in IIb/x fibers but was not different between the ALA and CIT groups. In addition, a trend was found toward lower SDH activity in type IIa fibers of the casted leg than of the internal control (P = 0.07; Figure 2D).

Skeletal muscle function. Muscle contractile parameters are presented in Table 2. Compared with the ALA and CIT groups, the CON group recorded a higher peak twitch force and rate of force development but not time-to-peak twitch or half-relaxation time. Likewise, peak tetanic force, tetanic force across the full frequency-force relation, and specific force were higher in the CON group than in both the ALA and CIT groups (Figure 3A–C). No difference between the ALA and CIT groups was observed for any peak functional measure. Final body mass was identified as a significant (P < 0.001) covariate for peak force. After adjustment for body mass by using LSM, peak tetanic force in both the ALA (1190 ± 34.2 mN) and CIT (1200 ± 36.1 mN) groups was still lower than in the CON group (1630 ± 49.8 mN; P < 0.001).

Consistent with the pronounced reduction in type IIb/x fiber area, TA muscles in both the ALA and CIT groups were more fatigue resistant than in the CON group (P < 0.001), producing a higher percentage of maximal force after 1, 2, 3, and 4 min (Figure 3D). Despite the greater fatigability, the CON group recovered more rapidly than both the ALA and CIT groups. Three minutes after the fatigue protocol, force (expressed as a percentage of initial contraction force) was lower in the CIT group (74.3% ± 3.22%; P < 0.001) and tended to be lower in the ALA group (82.3% ± 2.29%; P = 0.07) than in the CON group (91.0% ± 1.62%). In addition, a trend was found toward better recovery of peak tetanic force in the ALA group than in the CIT group at 3 min (P = 0.08).

Skeletal muscle degradation and inflammation. The mRNA expression of the proinflammatory cytokine genes IL-6 and tumor necrosis factor α; the macrophage marker genes cluster determinant 68 and epidermal growth factor-like module containing, mucin-like, hormone receptor-like sequence 1; and the neuronal NOS isoform (Nos1) was not different between groups (Supplemental Figure 1). Compared with the CON group, mRNA expression of the inducible NOS isoform (Nos2) were −15-fold higher in the ALA group and −7-fold higher in the CIT group (Supplemental Figure 1). A tendency for Nos2 expression was found to be lower in the CIT group than in the ALA group (P = 0.05). No differences in mRNA expression of genes involved in the ubiquitin-proteasome muscle protein breakdown system (Fbxo32, tripartite-motif-containing 63 gene, Foxo1, and Foxo4) were observed between the CON, ALA, and CIT groups. Consistent with unaltered Fbxo32 expression, protein expression type of the eukaryotic initiation

![FIGURE 1](https://example.com/figure1.png)

Cit does not prevent casting-induced loss of muscle mass in male mice. Muscle mass in the (A) casted and (B) uncasted internal control leg of CON mice (n = 10) and mice treated with Ala (n = 15) and Cit (n = 15). (C) Body mass before and after 14 d of casting. (D) Casted muscle mass normalized to the uncasted internal control leg. Values are means ± SEs. (A, B, and D) Comparisons were made with a 1-factor ANOVA. *Different from CON, P < 0.05. †Different for trend from CON, < P < 0.1. (C) Comparisons were made with a repeated-measures ANOVA with Fisher’s least significant difference post hoc test. Means without a common letter differ between groups, P < 0.05. ALA, alanine group; CIT, citrulline group; CON, control group; GAST, gastrocnemius; QUAD, quadriceps; SOL, soleus; TA, tibialis anterior.
factor 3\(\text{I}\), an important regulator of protein synthesis and major target of atrogin-1, was unaltered. However, autophagic signaling was higher in the ALA group than in the CON group. The mRNA expression of the proautophagic gene Bnip3 (but not microtubule-associated protein 1 light chain 3 \(\beta\) gene) was higher in the ALA (\(-17\text{-fold}; P < 0.01\)) and CIT (\(-4\text{-fold}; P < 0.01\)) groups than in the CON group. Bnip3 was significantly lower in the CIT group (\(-75\%; P < 0.01\)) than in the ALA group. A significantly higher protein ratio of LC3BII to LC3BI (LC3BII:LC3BI), indicating increased autophagosome formation, was seen in the ALA group than in the CON group (\(P < 0.05\)). LC3BII:LC3BI in the CIT group was not different from the CON group (Supplemental Figure 1).

Discussion

The main finding of this study was that Cit supplementation did not prevent loss of muscle mass, fiber area, or peak force-producing capacity in a mouse model of unilateral hindlimb immobilization.

**Cit does not prevent the casting-induced loss of muscle mass and fiber area.** On the basis of previous findings (17–19) we hypothesized that Cit supplementation during immobilization would reduce skeletal muscle wasting. In our preliminary experiment, we showed that oral administration of Cit at 1 g/kg resulted in higher plasma availability of Cit (8-fold) and Arg (2.6-fold) in C57BL/6\(\text{Arc}\) mice. Therefore, we expected a protection against wasting as we have previously observed in C2C12 myotubes (8). In this study we focused on clinically relevant functional measures. The laborious nature of these measures did not permit collection of plasma and muscle tissue at an appropriate time point to verify that our supplemental diet that contained 0.5–1 g/kg per day) were frequently observed (18, 19, 23). Contrary to our hypothesis, we did not observe any differences in muscle mass or fiber CSA between the ALA and CIT groups.

Short periods of unloading, bed rest, immobilization, and inactivity activate muscle protein breakdown and have profound effects on skeletal muscle mass and functional capacity (2–4, 31). Likewise, limb immobilization in rodents activates muscle protein breakdown and reduces muscle mass and strength, although because of higher metabolic rates and protein turnover the extent of muscle wasting is more dramatic (20, 32, 33). In line with previous studies, we observed a number of alterations in autophagic signaling after 14 d of hindlimb immobilization in the ALA group compared with the CON group (33, 34). LC3BII:LC3BI (\(P = 0.03\)), a reliable marker of autophagosome number (35), and the mRNA expression of Bnip3, which mediates the recruitment of the growing autophagosome to damaged mitochondria (36), were both higher in the ALA group than in the CON group. In the CIT group, LC3BII:LC3BI was not different from the CON group, and Bnip3 mRNA expression was significantly lower than the ALA group, but still 4-fold higher than the CON group. Similarly, the mRNA expression of Nos2, a key mediator of muscle breakdown pathways (37), was 15-fold higher in the ALA group than in the CON group and tended to be higher than the CIT group (\(P = 0.05\)), which

<table>
<thead>
<tr>
<th>TABLE 2</th>
<th>Peak in situ twitch properties of the tibialis anterior muscle after 14 d of casting in male mice(^1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CON</td>
<td>ALA</td>
</tr>
<tr>
<td>(uncasted; (n = 9))</td>
<td>(casted; (n = 14))</td>
</tr>
<tr>
<td>Peak twitch, mN</td>
<td>514 ± 21.7</td>
</tr>
<tr>
<td>Time to peak twitch, ms</td>
<td>14.0 ± 0.2</td>
</tr>
<tr>
<td>Half-relaxation time, ms</td>
<td>13.6 ± 0.9</td>
</tr>
<tr>
<td>(dP/dt), mN/ms</td>
<td>85.1 ± 3.4</td>
</tr>
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\(^1\) Values are means ± SEs. Data were analyzed with 1-factor ANOVA with Tukey’s post hoc test. ALA, alanine group; CIT, citrulline group; CON, control group; \(dP/dt\), rate of force development during a twitch contraction.

\(^2\) Different from CON, \(P < 0.001\).
FIGURE 3  Cit does not prevent casting-induced muscle weakness in male mice. (A) In situ frequency-force curve, (B) peak tetanic force, (C) specific force, and (D) fatigability were measured in the casted TA muscles of CON mice (n = 9) and mice treated with Ala (n = 14) and Cit (n = 12). Values are means ± SEs. Comparisons were made with a repeated-measures ANOVA with Fisher’s least significant difference post hoc test (A and D) and a 1-factor ANOVA with Tukey’s post hoc test (B and C). *Different from CON for both ALA and CIT, P < 0.05. #Different between CIT and CON, P < 0.05. ALA, alanine group; CIT, citrulline group; CON, control group; TA, tibialis anterior.

was still 7-fold higher than the CON group. However, despite lower autophagic signaling and Nos2 mRNA expression in the CIT group, the mass of the quadriceps, gastrocnemius, TA, and soleus muscles were all not different between the ALA and CIT groups and were significantly smaller than the CON group after 14 d of hindlimb immobilization. In developing rodents, growth restriction is commonly observed with casting. As such, comparisons between muscles from casted limbs and muscles from uncasted control mice represent the combined effect of immobilization-induced atrophy and growth restriction and may therefore overestimate the extent of muscle atrophy (22, 32, 38). We observed several signs of growth impairment in our casted mice, including a blunted accretion in body mass and reduced muscle mass in the uncasted limb. To account for growth impairment we used muscles from the contralateral, uncasted limb as an internal control. TA muscle mass was 21.6% ± 2.0% and 17.6% ± 1.6% smaller than the internal control in the ALA and CIT groups, respectively. A similar magnitude of muscle atrophy was observed if muscle mass was adjusted for final body mass by using LSM. The difference in muscle mass by using an internal control was ~30% lower than comparisons with the CON group. Independent of the comparison, muscle mass was not different between the ALA and CIT groups for any muscle. Compared with the internal control, mean TA muscle fiber CSA and minimal Feret’s diameter were lower in the casted limb of both the ALA and CIT groups. As previously reported during catabolic conditions (39), we observed a tendency for a greater difference in fiber CSA between the casted limb and internal control in faster type IIB/x than slower type IIA fibers (P = 0.06). In line with muscle mass data, we did not observe a difference in TA muscle fiber CSA or minimal Feret’s diameter in either type IIA or type IIB/x muscle fibers between the ALA and CIT groups. Curiously, we found a treatment effect for fiber type composition, with muscles in the CIT group having fewer type IIA fibers than in the ALA group. However, the physiologic relevance of the difference in fiber proportions is unclear because muscle fatigue was not different between the ALA and CIT groups.

Cit does not prevent the casting-induced loss of muscle function. Reductions in peak twitch force were similar to the reduction in mass and fiber area observed in the TA muscle with casting. In contrast, the difference in peak tetanic force exceeded that attributable to muscle atrophy, with the casted TA muscle producing 34.7% ± 1.8% less force than the uncasted CON group and 27.1% ± 2.1% less force than the uncasted CON group adjusted for body mass. It is well recognized that muscle mass is not the sole determinant of force production and that reductions in the relative force-producing capacity often accompanies muscle-wasting diseases and conditions (40, 41). Indeed, specific force of the TA muscle was significantly lower in both the ALA and CIT groups than in the CON group. The magnitude of muscle atrophy and loss of force in the present study is consistent with previous studies of limb immobilization in rodents (20, 42).

Our comprehensive evaluation of TA muscle function showed no difference between the ALA and CIT groups for any measure, indicating that Cit treatment is not effective at preserving skeletal muscle function during 14 d of hindlimb immobilization in mice. These findings are consistent with recent work showing no effect of Cit supplementation on muscle protein synthesis in elderly men at rest and after exercise (43). However, others have observed a significant NO-dependent protection of soleus muscle mass and fiber CSA with Arg supplementation (0.5 g/kg) in a 14-d hindlimb suspension model of unloading in rats (23). Because the soleus is a small, predominantly slow postural muscle that contributes little to overall hindlimb muscle mass, we chose to focus our evaluation of the effects of Cit treatment on casting-induced wasting on larger mixed fiber type muscles (i.e., quadriceps, gastrocnemius, TA) and concluded that Cit treatment during 14 d of immobilization did not significantly attenuate muscle wasting.

Conclusion. Cit treatment had no effect on therapeutically relevant outcome measures such as skeletal muscle mass and peak muscle force after 14 d of hindlimb immobilization in male mice.

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