Effect of L-citrulline, L-leucine, and multicomponent exercises on body compositions, physical activity, and amino acid concentrations in older Japanese women with low body mass index: A randomized double-blind placebo-controlled study

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<ABSTRACT>

Background: The intake of citrulline (CIT) and leucine (LEU) can stimulate protein synthesis. However, the efficacy of the combined intervention of CIT and LEU intake with exercise on body composition and physical activity (PA) remains unclear. This study aimed to investigate the combined effect of CIT and LEU intake and weight-bearing exercises (WBE) and square stepping exercise (SSE) for 20 weeks on body composition, PA, and amino acid concentration in older women with low body mass index (BMI) (16 to 21 kg/m²).

Methods: A total of 23 participants practiced WBE and SSE once a week for 75 minutes and were administered supplement (Ex + CIT·LEU group: CIT 0.8 g and LEU 1.6 g; Ex + Placebo group: 3.5 g carbohydrate) twice a day for 20 weeks. Body composition was measured using dual-energy X-ray absorptiometry. PA, including leisure-time, household, and occupational PA, was assessed using the Physical Activity Scale for the Elderly. Amino acid concentrations in the blood were analyzed by high-performance liquid chromatography.

Results: Significant interactions were observed in the body weight, BMI, lean mass, body mass, household and total PA, tyrosine, and phenylalanine. The within-group analysis showed that tyrosine of post-intervention measurement (Post) significantly in both groups (p < 0.05). Body weight, BMI, lean mass, body mass, household PA, total PA, and phenylalanine of Post increased significantly in the Ex + CIT·LEU group (p < 0.05). Additionally, significant positive correlations were observed between the intake rate of supplements and bone mass (r = 0.80) and between the practice rate of WBE at home and bone mineral density (r = 0.66) in the Ex + CIT·LEU group.

Conclusion: Our findings suggest that the intake of CIT and LEU, with the practice of WBE and SSE, could improve body weight, muscle mass, bone mass, and PA in older women with low BMI, which may prevent sarcopenia and frailty.
Keywords: Sarcopenia; Leucine; Citrulline; Weight-bearing exercise; Older adults; Amino acids

Background

Aging strongly affects the body’s composition, particularly body cells, and bone and muscle mass [1]. Muscle mass begins to decrease at age 25, further accelerating at age 50, and by 80 years when it has decreased by an average of 40% compared with age 20 [2]. Asian women (-1.3 kg/m²) and men (-1.4 kg/m²) have a lower body mass index (BMI) than Europeans, which may lead to several consequences [3]. A previous study reported that low BMI may lead to poor physical performance in Japanese older adults [4]. In this population, abnormal BMI, either high or low, is a major cause of mortality, and a previous study found a higher mortality rate in older adults with low BMI than in those with normal BMI [5]. The low BMI of older individuals is strongly associated with sarcopenia, and sarcopenia is paired with phenotypes such as decrease in muscle mass [6]. Sarcopenia has been associated with disabilities in the performance of instrumental activities of daily living, mobility limitation [7], poor physical functioning [8], high risk of falls or fractures [9], and mortality [10]. To prevent the loss of body composition, the importance of exercise and a well-balanced diet have been proven for Japanese adults with low BMI. The combined therapy of exercise and amino acid intake has been proven to increase muscle protein synthesis [11] and leg muscle mass [12] in older adults. Therefore, exercise programs such as weight-bearing exercises (WBE), which can be easily performed without equipment at any time, are necessary for older adults.

Previous studies have shown that different exercise programs offer different benefits for older adults. The WBE program has been proven to improve bone mineral density [13] and promote muscle activation [14]. By contrast, the square stepping exercise (SSE), an aerobic exercise, has been reported to improve both lower-extremity [15] and cognitive functioning [16] and prevent falls [17] in older adults. Furthermore, the combined intervention of exercise and nutrition has shown more significance in the improvement of the body composition of underweight older adults compared with the single use of either one.

Leucine (LEU) is a well-known essential amino acid for protein metabolism and is composed of branched-chain amino acids (BCAA), including isoleucine and valine. Additionally, a unique characteristic of LEU is the stimulation of protein synthesis among BCAA. Through the activation of the mammalian target of rapamycin (mTOR), LEU regulates the signaling pathway of insulin PI 3-kinase and stimulates the translational control of protein synthesis [18]. In addition, citrulline (CIT), a non-protein amino acid, is a potent endogenous precursor of arginine. Direct intake of arginine can cause gastrointestinal distress; however, intake of CIT can reduce gastrointestinal distress and promote arginine production [19]. CIT is converted to arginine in the kidneys after ingestion, which is converted into nitric oxide in the ornithine circuit [20]. Arginine increases nitric oxide causing vasodilatation, which helps the circulation of both central and peripheral blood vessels, and increases growth hormone secretion, which promotes protein anabolism and wound healing [21-23]. A previous animal study showed that CIT intake increased protein synthesis and content in muscle [24]. Another study reported that the intake of both CIT and LEU could stimulate muscle protein synthesis, which resulted from the activation of the mTOR complex 1 (mTORC1) signaling pathway [25]. Therefore, the intake of both CIT and LEU, combined with WBE and SSE, can be expected to prevent the decrease in body composition, particularly body cells, and bone and muscle mass.
composition of older adults with low BMI.

To our knowledge, no study has examined the effect of the combined intake of CIT and LEU with exercise practice on older women with low BMI. We hypothesized that the simultaneous administration of CIT and LEU with exercise practice may increase amino acid concentration in the blood and improve the body composition and physical activity (PA) of older women. This study aimed to examine the effect of multicomponent exercises (WBE and SSE) and amino acid intake (CIT and LEU) on body compositions, PA, and amino acid concentrations of older Japanese women with low BMI using a randomized, double-blind, placebo-controlled trial.

Methods

Ethical approval and participants

This randomized, double-blind, placebo-controlled study was approved by ethical committees of the University of Tsukuba (reference no. Tai 27-144) and was registered in the University Hospital Medical Information Network center (UMIN no. 000022385). To ensure the reliability of double-blind trial, the recruitment and data management of the participants were entrusted to a business consignment agency (THF Co., Ltd., Tsukuba, Japan). Older women residing in Tsukuba City, Japan, were recruited through regional information magazines (Joyo Living Co., Ltd., Tsukuba, Japan). A screening survey was conducted through telephone interview using self-reported, general health questionnaires. The inclusion criteria were as follows: 1) age ranging from 65 to 80 years, 2) BMI ranging from 16 to 21 kg/m² [3, 26], 3) no exercise prohibition from doctors, and 4) independent mobility and active participation in the exercise classes of the study. As regards the exclusion criteria, participants were ineligible if they 1) take neurological medicine; 2) have a medical history of comorbid diseases such as diabetes, brain, liver, kidney, heart, and peripheral vascular diseases; 3) qualify to at least one question of the physical strength section (questions 6 to 10) of the “Kihon Checklist” [27], which is a measure used to identify frailty; 4) have excessive intake of alcohol (>60 g/day) [28], 5) smoking, 6) have allergies to the supplements administered in this study; 7) have undergone blood collection of more than 400 mL within 10 weeks, or 200 mL within 4 weeks, or have donated blood in the past 2 weeks; and 8) have participated in other clinical studies in the past 10 weeks. A total of 43 older women applied for this study, but 15 applicants were excluded according to the criteria; further, two applicants dropped out because of conflicting schedules. Eligible participants were fully informed face-to-face about the study objectives, design, criteria of inclusion and exclusion, intervention of exercise program and supplements, assessments, insurance compensation for injury, withdrawal of consent and privacy protection. Finally, written consent to participation and data publishing was obtained from 26 participants.

Study design

We commissioned THF Co., Ltd. to divide 26 participants into two groups by the simple randomization method: 13 female participants were assigned to the Ex + CIT·LEU group (exercises and L-CIT and L-LEU) and 13 to the Ex + Placebo group (exercises and placebo). First, random numbers were generated by a computer and distributed to the participants. Random numbers less than 0.5 were determined as A (Ex + CIT·LEU group), and numbers greater than 0.5 were determined as B (Ex + Placebo group). No difference in age and BMI was found between the two groups. However, three participants in the Ex + CIT·LEU group withdrew their consent to participate because of personal
reasons. Finally, 23 participants were analyzed for this study (Fig. 1). To ensure the efficacy of the double-blinding method, all data (class attendance records, distribution and collection of food, diary, and measurement results) were managed by THF Co., Ltd. Members of each group remained completely anonymous to both participants and researchers until the key codes were revealed after completion of the trials at 20 weeks. Measurements were obtained at baseline (pre-intervention measurement: Pre), after 10 weeks (mid-intervention measurement: Mid), and after 20 weeks (post-intervention measurement: Post).

Within the 20-week intervention period, the two groups equally performed WBE and SSE with the guidance of a professional instructor once a week. Exercise sessions lasted for 75 min (10-min warm-up, 25-min SSE, 30-min WBE, and 10-min cool down). WBE consisted of two training patterns (pattern A, five exercises on the chair including knee extension, knee raise, squat, heel raise, and abdominal roll-up; pattern B, five exercises on the mat including pressing towel between palms, supine bridge, side-lying leg raise, pressing towel between knees, and sit-up), and only one set of WBE was performed with each exercise repeated 10 times. In addition, to reduce possible burden of this exercise intervention, which lasts for 6 months, participants were required to practice pattern A or pattern B alternately on a daily basis at home. Then, their performance was evaluated at four levels, i.e., 1, certainly; 2, moderately; 3, slightly; 4, could not. Following this, their daily records were checked and feedback provided once every 2 weeks. SSE, detailed description of which has been introduced in previous studies [15], was practiced on a thin mat measuring 250 cm × 100 cm with 40 squares (25 × 25 cm). SSE contained step patterns of forward, backward, lateral, and oblique movements; in addition, step patterns increasingly became more complex and were categorized into six levels: junior, basic, semiregular, regular, senior, and master. Participants were required to memorize the patterns and then step forward continuously without treading on the front and side lines of squares.

All participants were required to take 4.1 g of supplements (KYOWA HAKKO BIO Co., Ltd., Tokyo, Japan) with 100 mL of water twice a day (8.2 g) for the duration of the trial. The 16.0 kcal total calorie supplementation in the Ex + CIT·LEU group consisted of 0.8 g CIT, 1.6 g LEU, 0.3 g valine, 0.3 g isoleucine, and 1.1 g carbohydrate. The 16.1 kcal total calorie supplementation in the Ex + Placebo group consisted only of 0.3 g valine, 0.3 g isoleucine, and 3.5 g carbohydrate. These supplements were distributed to all participants once every 2 weeks; their supplement intake was confirmed using self-reported diaries.

We calculated the participation rate of exercise classes, practice rate of WBE at home, and intake...
rate of amino acid supplement.

**Body compositions**

We measured systolic and diastolic blood pressure plus heart rates (OMRON HEM-7111, Kyoto, Japan). Participants were required to roll up their sleeves, and the sensor of the blood pressure monitor was placed on the area of their left arm where the brachial artery was located; thereafter, systolic blood pressure, diastolic blood pressure, and heart rate were measured. The unit of blood pressure was millimeters of mercury (mmHg) and that of the heart rate was beats per minute (bpm). To measure height, participants were required to stand on the height scale with bare feet and look straight. Centimeter was used as the unit of height, and the value was specified to only one decimal place. As for the body weight, a weighing scale (MC-980A, TANITA, Tokyo, Japan) was used and weight was measured in kilograms. Participants were required to wear light clothes and take off their shoes before measurement. Considering the weight of the clothes, 0.5 kg was subtracted from the obtained body weight and specified to only one decimal place. BMI (kg/m²) is calculated as body weight divided by height squared. Using a dual-energy X-ray absorptiometry (DXA, QDR Discovery Wi, Hologic, Tokyo, Japan) performed by a clinical technician, we obtained data regarding body fat, bone area, bone mineral density, lean mass, fat mass, bone mass, and body mass (the sum of the lean mass, fat mass, and bone mass) at baseline and after intervention. Participants were required to wear light clothes, take off their shoes, and lie in a supine position on the DXA machine for 7 min.

**Physical activity**

PA was assessed using the Japanese version of the Physical Activity Scale for the Elderly (PASE), the validity and reliability of which have been analyzed by previous studies [29]. PASE is a 12-item questionnaire that measures the average time spent on daily physical activities during the past 7 days. The 12 items comprised sections on leisure time PA (such as walking, recreational activities, and strength training), household PA (such as home repair and garden maintenance), and occupational PA (such as job and volunteer work). These items are weighted based on the intensity of each activity, and the PASE (total PA) score is the sum of the 12 weighted items [29].

**Amino acid concentrations**

We entrusted the analysis of blood samples to a microbiological institute (Kotobiken Medical Laboratories Inc., Tsukuba, Japan). Participants were instructed to fast for 10 h in preparation for venipuncture of the brachial veins. Blood was collected using the EDTA-2NA containing tubes by nurses. To separate the plasma, 2 mL of blood was collected and centrifuged at 3000 rpm for 10 min at 4°C, and 0.5 ml of plasma was stored at -80°C until analysis. Amino acid concentrations were analyzed using high-performance liquid chromatography (HPLC) with an Acquity Ultra Performance Liquid Chromatography mass spectrometer system with a Mass Trak AAA amino acid analysis solution kit (Waters Co., Ltd., Tokyo, Japan) [30].

**Statistical analysis**

Student’s t-test was used to compare the mean of the characteristics at baseline between the two groups, and two-way repeated measures analysis of variance was used to evaluate differences in the effects of the intervention on body composition, PA, and amino acid concentrations. Covariates included height, body weight, and baseline values of each variable. Model I was adjusted for baseline values, model II for height and body weight, and model III for baseline values of each variable based on model II. Based on the significant interactions (time × groups), we conducted post hoc analyses
with Bonferroni correction. In addition, the effect sizes of all variables were evaluated by Cohen’s $d$ value (small, $d = 0.2$; medium, $d = 0.5$; large, $d = 0.8$) [31]. The change rates of all parameters were calculated using the formula “post intervention value - baseline value) / baseline value) × 100.” Body fat and CIT change amount was calculated using the formula “post intervention value - baseline value.” Spearman’s rank correlation test was used to examine the correlations among intake rate of supplementation, participation rate of exercise, body composition, PA, and amino acids. All statistical analyses were performed using SPSS version 26.0 (IBM Corp., Armonk, NY, USA), with the level of significance set at $p < 0.05$.

**Results**

**Characteristics of participants**

Characteristics of participants at baseline are shown in Table 1. Significant differences in systolic blood pressure, height, and body weight were observed between the two groups. No significant differences were found in other variables. In addition, the intake rate of amino acid supplementation and the participation rate of exercise classes of both groups were >90%.

### Table 1. Characteristics of the participants on baseline

| Variables                        | Ex + CIT·LEU (n = 10) | Ex + Placebo (n = 13) | $P$-value |
|----------------------------------|-----------------------|-----------------------|-----------|
| Age (year)                       | Mean ± SD             | Mean ± SD             |           |
| Height (cm)                      | 151.1 ± 4.0           | 155.4 ± 5.1           | 0.04      |
| Body weight (kg)                 | 42.4 ± 2.4            | 45.7 ± 3.5            | 0.02      |
| BMI (kg/m$^2$)                   | 18.6 ± 1.5            | 18.9 ± 1.1            | 0.56      |
| Systolic blood pressure (mmHg)   | 119.1 ± 12.2          | 132.5 ± 13.3          | 0.02      |
| Diastolic blood pressure (mmHg)  | 68.0 ± 7.0            | 72.9 ± 10.4           | 0.21      |
| Heart rate (bpm)                 | 75.8 ± 5.8            | 79.8 ± 11.0           | 0.31      |
| Intake rate of amino acid suppl. | 96.5 ± 5.0            | 96.9 ± 5.0            | 0.86      |
| Participation rate of exercise   | 96.1 ± 5.3            | 91.5 ± 9.2            | 0.17      |
| Practice rate of WBE at home     | 90.1 ± 9.2            | 86.7 ± 14.7           | 0.53      |
| Practice rate of WBE (slightly)  | 10.6 ± 9.9            | 14.1 ± 15.7           | 0.54      |

Note: student’s $t$-test, $P < 0.05$, n: number of participants, SD: standard deviation

Ex: exercise, CIT: citrulline, LEU: leucine, BMI: body mass index, WBE: weight-bearing exercises

**Body compositions**

In Table 2, both the unadjusted model and model I showed that interactions in body weight and BMI were significant. According to the post hoc analysis, the increase in both body weight and BMI at Post was more significant compared with that at Pre and Mid in the Ex + CIT·LEU group. In model II, a significant interaction was observed in body mass; the increase in the body mass at Post was more significant compared with that at Pre in the Ex + CIT·LEU group. In model III, significant interactions were observed in both lean mass and body mass. Only an increase in body mass at Post was significant compared with that at Pre in the Ex + CIT·LEU group. The effect sizes of body weight ($d = 0.37$), BMI ($d = 0.29$), body mass ($d = 0.25$), and lean mass ($d = 0.18$) were negligible in the Ex + CIT·LEU group.
### Table 2. Effects of multicomponent exercises and amino acids (L-citrulline and L-leucine) intake on body compositions

| Variables          | Unit | Time | Ex + CIT·LEU (n = 10) | Ex + Placebo (n = 13) | Main effect of time P-value (Pre-Mid-Post) | Interaction P-value (Time*Groups) | Post hoc analysis with Bonferroni correction | Main effect of time P-value (Pre-Mid-Post) | Interaction P-value (Time*Groups) | Post hoc analysis with Bonferroni correction | Main effect of time P-value (Pre-Mid-Post) | Interaction P-value (Time*Groups) | Post hoc analysis with Bonferroni correction |
|--------------------|------|------|------------------------|-----------------------|------------------------------------------|-----------------------------------|------------------------------------------|------------------------------------------|-----------------------------------|------------------------------------------|------------------------------------------|-----------------------------------|------------------------------------------|
| Height (cm)        |      | Pre  | 151.1 ± 4.0            | 155.4 ± 5.1           | .04                                      | .08                               | Ex + CIT·LEU: Pre < Mid < Post             | .09                                      | Ex + CIT·LEU: Pre < Mid < Post             | .09                                      | Ex + CIT·LEU: Pre < Mid < Post             | .09                                      | Ex + CIT·LEU: Pre < Mid < Post             | .09                                      |
| Bodyweight (kg)    |      | Pre  | 42.4 ± 2.4             | 45.7 ± 3.5            | .09                                      | .06                               | Ex + CIT·LEU: Pre < Mid < Post             | .05                                      | Ex + CIT·LEU: Pre < Mid < Post             | .05                                      | Ex + CIT·LEU: Pre < Mid < Post             | .05                                      | Ex + CIT·LEU: Pre < Mid < Post             | .05                                      |
| Body fat (%)       |      | Pre  | 18.6 ± 1.5             | 18.9 ± 1.1            | .04                                      | .01                               | Ex + CIT·LEU: Pre < Mid < Post             | .05                                      | Ex + CIT·LEU: Pre < Mid < Post             | .05                                      | Ex + CIT·LEU: Pre < Mid < Post             | .05                                      | Ex + CIT·LEU: Pre < Mid < Post             | .05                                      |
| Bone area (cm²)    |      | Pre  | 150.0 ± 125.2          | 1617.5 ± 151.5        | .68                                      | .06                               | Ex + CIT·LEU: Pre < Mid < Post             | .05                                      | Ex + CIT·LEU: Pre < Mid < Post             | .05                                      | Ex + CIT·LEU: Pre < Mid < Post             | .05                                      | Ex + CIT·LEU: Pre < Mid < Post             | .05                                      |
| Bone mineral density (g/cm²) |      | Pre  | 0.9 ± 0.15             | 0.9 ± 0.10            | .01                                      | .01                               | Ex + CIT·LEU: Pre < Mid < Post             | .05                                      | Ex + CIT·LEU: Pre < Mid < Post             | .05                                      | Ex + CIT·LEU: Pre < Mid < Post             | .05                                      | Ex + CIT·LEU: Pre < Mid < Post             | .05                                      |
| Bone mass (g)      |      | Pre  | 149.1 ± 217.3          | 1584.5 ± 264.2        | .02                                      | .01                               | Ex + CIT·LEU: Pre < Mid < Post             | .05                                      | Ex + CIT·LEU: Pre < Mid < Post             | .05                                      | Ex + CIT·LEU: Pre < Mid < Post             | .05                                      | Ex + CIT·LEU: Pre < Mid < Post             | .05                                      |
| Fat mass (kg)      |      | Pre  | 107 ± 3.3              | 123 ± 2.5             | .04                                      | .01                               | Ex + CIT·LEU: Pre < Mid < Post             | .05                                      | Ex + CIT·LEU: Pre < Mid < Post             | .05                                      | Ex + CIT·LEU: Pre < Mid < Post             | .05                                      | Ex + CIT·LEU: Pre < Mid < Post             | .05                                      |
| Lean mass (kg)     |      | Pre  | 29.1 ± 1.3             | 30.4 ± 2.2            | .01                                      | .01                               | Ex + CIT·LEU: Pre < Mid < Post             | .05                                      | Ex + CIT·LEU: Pre < Mid < Post             | .05                                      | Ex + CIT·LEU: Pre < Mid < Post             | .05                                      | Ex + CIT·LEU: Pre < Mid < Post             | .05                                      |
| Body mass (kg)     |      | Pre  | 413 ± 2.6              | 443 ± 3.4             | .04                                      | .01                               | Ex + CIT·LEU: Pre < Mid < Post             | .05                                      | Ex + CIT·LEU: Pre < Mid < Post             | .05                                      | Ex + CIT·LEU: Pre < Mid < Post             | .05                                      | Ex + CIT·LEU: Pre < Mid < Post             | .05                                      |

Note: Two-way repeated measures analysis of variance, P < 0.05. Body mass = sum of the bone mass, fat mass and lean mass; N.S: not significant. Effect size (Cohen's d): Pre NS: Post 0.2 small; 0.5 medium; 0.8 large.  
Model I: adjusted for baseline value of each variable. Model II: adjusted for height and body weight. Model III further adjusted for baseline value of each variable in the form of Model II.
Physical activity

In Table 3, the unadjusted model showed significant interactions in household PA and total PA. According to the post hoc analysis, the increase at both Mid and Post was more significant compared with that at Pre in the Ex + CIT·LEU group. Large effect sizes were observed in leisure time PA (Ex + CIT·LEU group; $d = 1.15$, Ex + Placebo group; $d = 1.37$), household PA (Ex + CIT·LEU group; $d = 1.61$), and total PA (Ex + CIT·LEU group; $d = 1.46$).

Amino acid concentrations in plasma

Table 4 shows the amino acid concentrations in plasma. In both the unadjusted model and model I, interactions with tyrosine and phenylalanine were significant. According to the post hoc analysis, a more significant increase in tyrosine at Post was observed compared with that at Pre in both groups; however, the increase in phenylalanine concentrations at Post was more significant than that at Pre in the Ex + CIT·LEU group. In both groups, large effect sizes can be observed in arginine (Ex + CIT·LEU group; $d = 1.42$, Ex + Placebo group; $d = 1.33$), glutamic acid (Ex + CIT·LEU group; $d = 0.85$, Ex + Placebo group; $d = 1.00$), and methionine (Ex + CIT·LEU group; $d = 1.36$, Ex + Placebo group; $d = 0.79$). In the Ex + CIT·LEU group, large effect sizes can be observed in glutamine ($d = 0.81$), alanine ($d = 0.76$), and tyrosine ($d = 1.12$). In the Ex + Placebo group, large effect sizes can be observed in glycine ($d = 0.90$), cystine ($d = 0.88$), and lysine ($d = 0.93$).
Table 4. Effects of multicomponent exercises and amino acids (L-citrulline and L-leucine) intake on amino acid concentrations

| Variables       | Unit | Time | Mean ± SD | Main effect of time P-value | Interaction P-value | Post hoc analysis with Bonferroni correction | Mean ± SD | Main effect of time P-value | Interaction P-value | Post hoc analysis with Bonferroni correction |
|-----------------|------|------|-----------|----------------------------|---------------------|---------------------------------------------|-----------|----------------------------|---------------------|---------------------------------------------|
| Arginine (nmol/mL) | Pre  | 63.3 ± 14.2 | 0.19 | 65.2 ± 16.0 | 0.01 | 69.1 ± 19.9 | 1.33 | 0.01 | 0.37 |
|                | Effect | 62.5 ± 16.3 |           | 65.1 ± 16.3 |           | 69.1 ± 19.9 |           | 0.80 |           |
| Citrulline (nmol/mL) | Pre  | 113.5 ± 19.0 | 0.35 | 107.3 ± 13.1 | 0.05 | 107.5 ± 13.1 | 0.09 | 0.05 | 0.11 |
|                | Effect | 116.0 ± 19.8 |           | 109.2 ± 13.4 |           | 107.5 ± 13.1 |           | 0.03 |           |
| Leucine (nmol/mL) | Pre  | 106.8 ± 35.1 | 0.04 | 107.7 ± 17.8 | 0.04 | 107.5 ± 17.8 | 0.04 | 0.54 |           |
|                | Effect | 113.5 ± 35.6 |           | 107.7 ± 17.8 |           | 107.5 ± 17.8 |           | 0.10 |           |
| Valine (nmol/mL) | Pre  | 230.2 ± 39.7 | 0.10 | 230.5 ± 40.1 | 0.12 | 230.5 ± 40.1 | 0.12 | 0.01 | 0.07 |
|                | Effect | 230.5 ± 39.7 |           | 230.5 ± 40.1 |           | 230.5 ± 40.1 |           | 0.01 |           |
| Lysine (nmol/mL) | Pre  | 62.5 ± 13.5 | 0.05 | 62.5 ± 13.5 | 0.05 | 62.5 ± 13.5 | 0.05 | 0.01 | 0.07 |
|                | Effect | 63.2 ± 13.8 |           | 63.2 ± 13.8 |           | 63.2 ± 13.8 |           | 0.01 |           |
| Histidine (nmol/mL) | Pre  | 60.0 ± 9.2 | 0.43 | 57.6 ± 9.2 | 0.43 | 57.6 ± 9.2 | 0.43 | 0.01 | 0.07 |
|                | Effect | 61.5 ± 9.2 |           | 57.6 ± 9.2 |           | 57.6 ± 9.2 |           | 0.01 |           |
| Threonine (nmol/mL) | Pre  | 78.7 ± 12.3 | 0.57 | 81.7 ± 12.3 | 0.57 | 81.7 ± 12.3 | 0.57 | 0.01 | 0.07 |
|                | Effect | 80.0 ± 12.6 |           | 81.7 ± 12.3 |           | 81.7 ± 12.3 |           | 0.01 |           |
| Glycine (nmol/mL) | Pre  | 110.8 ± 23.7 | 0.76 | 110.8 ± 23.7 | 0.76 | 110.8 ± 23.7 | 0.76 | 0.01 | 0.07 |
|                | Effect | 113.5 ± 24.0 |           | 110.8 ± 23.7 |           | 110.8 ± 23.7 |           | 0.01 |           |
| Alanine (nmol/mL) | Pre  | 225.0 ± 47.0 | 0.19 | 225.0 ± 47.0 | 0.19 | 225.0 ± 47.0 | 0.19 | 0.01 | 0.07 |
|                | Effect | 225.0 ± 47.0 |           | 225.0 ± 47.0 |           | 225.0 ± 47.0 |           | 0.01 |           |
| Methionine (nmol/mL) | Pre  | 51.8 ± 4.7 | 0.06 | 51.8 ± 4.7 | 0.06 | 51.8 ± 4.7 | 0.06 | 0.01 | 0.07 |
|                | Effect | 51.8 ± 4.7 |           | 51.8 ± 4.7 |           | 51.8 ± 4.7 |           | 0.01 |           |
| Glutamine (nmol/mL) | Pre  | 68.5 ± 16.1 | 0.06 | 68.5 ± 16.1 | 0.06 | 68.5 ± 16.1 | 0.06 | 0.01 | 0.07 |
|                | Effect | 68.5 ± 16.1 |           | 68.5 ± 16.1 |           | 68.5 ± 16.1 |           | 0.01 |           |
| Tyrosine (nmol/mL) | Pre  | 78.7 ± 12.3 | 0.57 | 78.7 ± 12.3 | 0.57 | 78.7 ± 12.3 | 0.57 | 0.01 | 0.07 |
|                | Effect | 78.7 ± 12.3 |           | 78.7 ± 12.3 |           | 78.7 ± 12.3 |           | 0.01 |           |
| Phenylalanine (nmol/mL) | Pre  | 57.6 ± 9.2 | 0.43 | 57.6 ± 9.2 | 0.43 | 57.6 ± 9.2 | 0.43 | 0.01 | 0.07 |
|                | Effect | 57.6 ± 9.2 |           | 57.6 ± 9.2 |           | 57.6 ± 9.2 |           | 0.01 |           |
| Lysine (nmol/mL) | Pre  | 69.1 ± 19.9 | 0.19 | 69.1 ± 19.9 | 0.19 | 69.1 ± 19.9 | 0.19 | 0.01 | 0.07 |
|                | Effect | 69.1 ± 19.9 |           | 69.1 ± 19.9 |           | 69.1 ± 19.9 |           | 0.01 |           |
| Tryptophane (nmol/mL) | Pre  | 45.5 ± 9.1 | 0.15 | 45.5 ± 9.1 | 0.15 | 45.5 ± 9.1 | 0.15 | 0.01 | 0.07 |
|                | Effect | 45.5 ± 9.1 |           | 45.5 ± 9.1 |           | 45.5 ± 9.1 |           | 0.01 |           |

Note: Two-way repeated measures analysis of variance; * p < 0.05, Effect size (Cohen's d) Pre VS. Post, 0.2: small, 0.5: medium, 0.8: large, †Model adjusted for baseline value of each variable

Correlations among intake rate, participation rate, practice rate, and change rate of parameters

Table 5 shows the results of the rank correlation coefficient among the intake rate of supplementation, participation rate of exercise classes, practice rate of WBE at home (certainly, moderately), change rate of body compositions, PA, and amino acids between the two groups. In the Ex + CIT·LEU group, the intake rate of supplementation showed significantly positive correlations with bone mass (r = 0.80), asparagine (r = 0.72), methionine (r = 0.68), phenylalanine (r = 0.65), histidine (r = 0.80), and lysine (r = 0.64). In addition, significantly positive correlations were found in the participation rate of exercise classes with lysine (r = 0.70) and in practice rate of WBE at home

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with both bone mineral density \((r = 0.66)\) and CIT change amount \((r = 0.72)\). In the Ex + Placebo group, significantly negative correlations were shown in the intake rate of supplementation with valine \((r = -0.59)\), and practice rate of WBE at home with both LEU \((r = -0.70)\) and isoleucine \((r = -0.64)\). In addition, the participation rate of exercise classes showed significantly positive correlations with arginine \((r = 0.59)\), asparagine \((r = 0.73)\), glutamine \((r = 0.81)\), and histidine \((r = 0.57)\), but significantly negative correlations with height \((r = -0.60)\), leisure time PA \((r = -0.72)\), and LEU \((r = -0.57)\).

Table 5. Rank correlations among rate of intake, practice and change rate of variables

| Change rate of variables | Intake rate of amino acid supplementation | Participation rate of exercise classes | Practice rate of WBE at home (certainly, moderately) | Intake rate of amino acid supplementation | Participation rate of exercise classes | Practice rate of WBE at home (certainly, moderately) |
|--------------------------|------------------------------------------|---------------------------------------|-------------------------------------------------|------------------------------------------|---------------------------------------|-------------------------------------------------|
| Height                   | -0.58                                    | 0.21                                  | -0.49                                           | -0.14                                    | -0.60†                                | -0.18                                           |
| Body weight              | -0.08                                    | 0.19                                  | -0.16                                           | -0.11                                    | -0.05                                 | -0.08                                           |
| BMI                      | -0.04                                    | 0.19                                  | -0.08                                           | -0.16                                    | 0.01                                  | -0.15                                           |
| Body fat                 | 0.08                                     | -0.03                                 | -0.09                                           | 0.07                                     | 0.18                                  | -0.09                                           |
| Bone area                | 0.24                                     | -0.06                                 | -0.41                                           | 0.23                                     | 0.32                                  | -0.10                                           |
| Bone mineral density     | 0.54                                     | 0.41                                  | 0.66†                                            | 0.24                                     | 0.20                                  | 0.06                                            |
| Bone mass                | 0.80†                                    | 0.24                                  | 0.38                                            | 0.39                                     | 0.36                                  | 0.01                                            |
| Fat mass                 | 0.29                                     | -0.03                                 | 0.18                                            | -0.06                                    | 0.07                                  | -0.23                                           |
| Lean mass                | -0.16                                    | -0.14                                 | -0.07                                           | -0.06                                    | -0.24                                 | -0.04                                           |
| Body mass                | 0.05                                     | 0.10                                  | 0.02                                            | -0.11                                    | 0.06                                  | 0.04                                            |
| Leisure time PA          | 0.35                                     | 0.20                                  | -0.53                                           | 0.10                                     | -0.72‡                                | -0.25                                           |
| Household PA             | 0.52                                     | 0.27                                  | 0.61                                            | -0.48                                    | -0.15                                 | 0.01                                            |
| Occupational PA          | 0.06                                     | 0.46                                  | -0.29                                           | 0                                        | 0                                     | -0.16                                           |
| Total PA                 | 0.37                                     | 0.44                                  | 0.28                                            | -0.38                                    | -0.20                                 | 0.14                                            |
| Arginine                 | 0.61                                     | 0.07                                  | 0.28                                            | -0.07                                    | 0.59†                                 | 0.11                                            |
| Citrulline               | 0.22                                     | 0.03                                  | 0.60                                            | -0.26                                    | 0.28                                  | -0.03                                           |
| Citrulline change amount | 0.37†                                    | 0.19                                  | 0.72‡                                            | -0.23                                    | 0.31                                  | -0.003                                          |
| Leucine                  | 0.22†                                    | -0.01                                 | 0.08                                            | -0.49                                    | -0.57‡                                | -0.70‡                                           |
| Valine                   | 0.18                                     | 0.31                                  | 0.32                                            | -0.59†                                    | -0.26                                 | -0.27                                           |
| Isoleucine               | -0.14‡                                   | 0.18                                  | 0.43                                            | -0.48                                    | -0.47                                 | -0.64‡                                           |
| Threonine                | 0.04                                     | 0.49                                  | 0.19                                            | -0.13                                    | 0.35                                  | 0.10                                            |
| Serine                   | 0.58                                     | 0.53                                  | 0.44                                            | -0.32                                    | 0.38                                  | -0.05                                           |
| Asparagin               | 0.72‡                                    | 0.45                                  | 0.54                                            | -0.02                                    | 0.73‡                                 | 0.31                                            |
| Glutamic acid            | -0.17‡                                   | 0.46                                  | -0.21                                           | 0.03                                     | -0.08                                 | 0.24                                            |
| Glutamine                | 0.60                                     | 0.17                                  | 0.27                                            | 0.03                                     | 0.81‡                                 | 0.31                                            |
| Glycine                  | 0.18                                     | 0.32                                  | 0.18                                            | -0.09                                    | 0.23                                  | 0.25                                            |
| Alanine                  | -0.24‡                                   | 0.06                                  | 0.04                                            | 0.52                                     | 0.27                                  | 0.52                                            |
| Cystine                  | 0.46                                     | 0.17                                  | 0.08                                            | -0.35                                    | 0.30                                  | -0.23                                           |
| Methionine               | 0.68‡                                    | 0.42                                  | 0.48                                            | 0                                        | 0.22                                  | 0.27                                            |
| Tyrosine                 | 0.44                                     | 0.29                                  | 0.12                                            | -0.02                                    | -0.10                                 | 0.26                                            |
| Phenylalanine            | 0.65‡                                    | 0.25                                  | 0.28                                            | -0.31                                    | -0.03                                 | -0.03                                           |
| Histidine                | 0.80‡                                    | 0.40                                  | 0.48                                            | 0.09                                     | 0.57‡                                 | 0.23                                            |
| Lysine                   | 0.64‡                                    | 0.70‡                                 | 0.48                                            | 0.09                                     | 0.48                                  | 0.11                                            |
| Tryptophan               | 0.10                                     | 0.39                                  | 0.28                                            | 0.33                                     | -0.24                                 | 0.003                                           |

Note: Spearman rank correlation, † P < 0.05, ‡ P < 0.001
Change rate of all parameters were calculated using the formula (post intervention value - baseline value) / baseline value *100
# Body fat and citrulline change amount were calculated using the formula (post intervention value - baseline value)
Discussion

Age-induced decreases in body composition are inevitable; furthermore, its management presents a challenge to physicians. However, our study demonstrated the effectiveness of the combined intervention of exercise (WBE and SSE) and supplement intake (CIT and LEU) in increasing body weight, muscle mass, and PA in older women with low BMI.

A longitudinal study reported that in a period of 10 years, muscle mass decreased by 12.9% and 5.3% in men and women, respectively; further, a decrease of 9.9–19.4 kg in body weight was observed [32]. Additionally, a 5-year longitudinal study of the Health Aging Body Composition Study (Health ABC study) reported that changes in body weight significantly affected both muscle mass and fat [33]. In our study, the Ex + CIT·LEU group demonstrated an increase in body weight, BMI, and lean mass; however, no significant changes in fat and bone mass were observed (Table 2). Our results were consistent with those of the Health ABC study [33] showing that an increase in body weight was strongly associated with an increase in muscle mass, but different results were observed in changes in fat mass. This may be due to the lower prevalence of obesity among Asians than among Americans; the participants’ fat mass in this study (11.5 kg) was lower than those in the Health ABC study (28.2 kg).

A prospective cohort study in Japan reported that both low BMI and high BMI (obesity) were strongly associated with mortality, showing a U-shaped curve [34]. High BMI and low PA are known to have an inverse relationship [35]; however, the relationship between low BMI and low PA has not been reported previously. However, low BMI may positively relate to low PA, because low BMI is a risk factor for sarcopenia, while a decreased PA is often a consequence of sarcopenia; therefore, the presence of sarcopenia may indirectly affect the relationship between the two variables [36]. In this study, household and total PA of older women with low BMI increased significantly as a result of CIT and LEU intake. A previous study reported that after 24 weeks of trial, total PA improved significantly and was maintained in the exercise (resistance exercise) and nutrition (structural proteins mainly) group when compared with the control group. Moreover, lower limb muscle mass and appendicular skeletal muscle mass were also increased [37], which is consistent with our findings. These results suggest that exercise practice and protein intake can improve both body composition and daily PA.

The risk factors of sarcopenia include underweight [6], female sex, low BMI, low PA, and poor mobility [36]. Therefore, older adults with low BMI are more likely to develop geriatric syndrome, such as sarcopenia or frailty, than healthy older adults. According to the EWGSOP systematic review, the prevalence of sarcopenia ranged from 1% to 29% [38]. Furthermore, a large cohort study in Japan reported that women (22.1%) had a higher prevalence of sarcopenia than men (14.2%) [39]. Therefore, the combination of exercise and nutrition intervention is more essential for older women with low BMI to prevent sarcopenia. The efficacy of the combined intervention of exercise and nutrition is controversial. Combined intervention of exercise and LEU-rich essential amino acid for 3 months was reported to increase leg muscle mass in Japanese women with sarcopenia [12]. They defined sarcopenia as BMI \( \leq 22 \) kg/m\(^2\) and found that exercise improved the muscle mass of older women with low BMI, which is consistent with our study. In another study, supplement intake (6 g CIT maleate, 5 g creatine, 3 g LEU, 3 g isoleucine, 1.5 g valine, etc.) and resistance exercise practice for 4 weeks significantly increased body mass and lean body mass compared with the placebo group (digestion-resistant maltodextrin) [40]. The participants of the previous study were recreationally active men, whereas our study focused on older women with low BMI, but the effects of CIT and LEU intake and exercise practice were consistent. However, other studies have reported conflicting results. A previous
study reported that a high dose of whey protein (45 g) intake, rather than combined intake of low dose
whey protein (15 g) and CIT (10 g), promoted postprandial muscle protein synthesis after resistance
exercise in older men [41]. Another study reported that CIT intake (0.18 g/kg/day) increased plasma
CIT and promoted arginine availability, but exerted no influence on LEU oxidation and whole-body
protein synthesis [42]. This finding contradicted a previous study reporting that CIT intake increased
arginine availability in the urea cycle, which promotes protein anabolism, indicating that CIT may
affect protein synthesis [24, 25].

These conflicting findings may result from various doses and durations of CIT and LEU intake.
According to a review study, an effective dose for daily CIT supplementation was recommended at a
minimum of 3 g to a maximum of 10 g [43]. The dose of CIT administered in our study was less than
that of the review study. In addition, the WHO recommended doses for daily intake of LEU (0.039
g/kg), isoleucine (0.020 g/kg), and valine (0.026 g/kg) [44]. Our study proved that LEU at a dose of
0.072 g per body weight (kg), calculated according to the average body weight of all participants (44.1
kg) and the daily intake of LEU (3.2 g), was effective compared with previous studies. Considering
caloric balance, we administered uniform doses of valine (0.6 g) and isoleucine (0.6 g) to both groups,
so their intake in the Ex + Placebo group may have influenced the results. Therefore, administration
of only carbohydrates to the Ex + Placebo group should be considered in future studies. Although the
concentrations of CIT and LEU in plasma showed no significant interaction between the two groups,
a tendency of interaction (p = 0.07) was observed in the CIT level of the unadjusted model and the
LEU level of model I. In addition, the effect size was larger in the Ex + CIT·LEU group (CIT: d =
0.66, LEU: d = 0.45) than in the Ex + Placebo group (CIT: d = 0.23, LEU: d = 0.10). In addition, the
intake rate of amino acid supplement in the Ex + CIT·LEU group was 96.5%. These results suggest
that CIT and LEU supplements were well tolerated by the participants, and a long-term intake of CIT
and LEU effectively increased their plasma levels. In addition, the concentration of tyrosine
significantly increased in both groups, but the effect size was larger in the Ex + CIT·LEU group (d =
1.12) than in the Ex + Placebo group (d = 0.51). The concentration of phenylalanine increased
significantly only in the Ex + CIT·LEU group. Phenylalanine is an essential amino acid converted to
tyrosine during protein metabolism, which plays a vital role in transmitting signals between the brain
and nerve cells. After a mixed diet (49% carbohydrate, 22% protein, 29% lipid), leucine and
phenylalanine-tyrosine tracers are produced in the plasma or intracellularly increased whole-body
protein synthesis [45]. With reference to previous studies, LEU intake may have increased
phenylalanine and tyrosine concentrations in our study; however, this requires further studies.

Our study has several limitations. First, there was sampling bias and a relatively small number of
participants. Because our participants were Japanese older women with low BMI, the results may not
be generalizable to the population of older women with low BMI. Furthermore, 23 of 35 participants
were included in this study based on the exclusion criteria and personal reasons. Therefore, a study
with a larger population that adheres to the same intervention design is warranted. Second, a nutritional
survey was not conducted among the participants. Results of the main effect of time showed significant
differences in most variables of amino acid concentrations, indicating that in both groups, amino acid
levels in the plasma increased after the intervention (Pre < Post). However, this may have resulted
from the leniency toward the participant’s lifestyle during the intervention. Diet, sleep, and other types
of exercises were not limited to verify the effect of the combined intervention without interfering with
the patient’s usual lifestyle. Therefore, rather than diet limitation, it is necessary to conduct a
nutritional survey to investigate the increase in amino acid concentrations of the placebo group. Third,
the amount of PA was measured using the PASE questionnaire, which is a reliable tool to evaluate
different types of PA in the elderly [29]. However, reporting bias may exist, and other methods to obtain objective data, such as using an accelerometer, should be considered in future research.

**Conclusion**

This study demonstrated that body weight, BMI, lean mass, bone mineral density, bone mass, household and total PA, tyrosine, and phenylalanine were increased significantly after 20 weeks in the Ex + CIT·LEU group. Our findings suggest that the combination practice of multicomponent exercises (WBE + SSE) and intake of L-citrulline and L-leucine can improve body weight, muscle mass, and PA in older women with low BMI, contributing to the prevention of sarcopenia and frailty.

**List of Abbreviations**

- PA – Physical Activity
- CIT – Citrulline
- LEU – Leucine
- WBE – Weight-bearing exercise
- SSE – Square stepping exercise
- PASE – Physical Activity Scale for the Elderly
- BCAA – Branched chain amino acids
- DXA - Dual-energy X-ray absorptiometry

**Declarations**

**Ethics and consent to participate:** All procedures of the study were approved by University of Tsukuba (Tai 27-144).

**Consent for publication:** Not applicable

**Availability of data and materials:** All data generated or analyzed during this study are included in this published article.

**Competing interests:** None of the authors has competing interests to declare.

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**Author’s Contributions:** HI and TO designed the experiments. MK and TO performed the experiments. MK analyzed the data and wrote the manuscript. All authors read and approved the final manuscript.

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