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Effect of Combined Ingestion of L-Theanine and L-Arginine for Short-Term Psychological Stress in Young Adults: A Randomized Placebo-Controlled Study

Daisuke FURUSHIMA[1,2], Ibuki SUGIYAMA[2], Yuzuki NOMURA[2], Keiko UNNO[3] and Hiroshi YAMADA[2]

[1] *Graduate School of Health Sciences, Kagoshima University, Kagoshima 890–8544, Japan*
[2] *Department of Drug Evaluation and Informatics, University of Shizuoka Graduate School of Pharmaceutical Sciences, Shizuoka 422–8526, Japan*
[3] *Tea Science Center, Graduate Division of Nutritional and Environmental Sciences, University of Shizuoka, Shizuoka 422–8526, Japan*

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Summary L-Theanine, the most abundant amino acid component in green tea, has anti-stress effects and refreshes the mental state. A recent study demonstrated that L-arginine, the second most abundant amino acid in green tea, might enhance the anti-stress effects of L-theanine. The aim of this study was to evaluated the effects of combined ingestion of L-theanine and L-arginine on psychological stress in humans. A randomized placebo-controlled trial was conducted including 120 healthy young adults (mean age 22.4 y, 63.3% female). Subjects were randomly assigned to theanine (200 mg L-theanine), combined theanine/arginine (200 mg L-theanine, 50 mg L-arginine), or placebo groups. After consuming a test beverage, we administered a stress-loading test (Uchida-Kraepelin performance test) and performed salivary alpha-amylase activity (sAA) measurements to assess the physiological stress response at 0 min (immediately after), 5 min, and 15 min. The changes in sAA at 15 min after the stress-loading test were −2.75 (11.2) kIU/L in the theanine/arginine group, −0.40 (11.5) kIU/L in the theanine group, and 6.95 (18.6) kIU/L in the placebo group. The values in the theanine/arginine (p=0.007) and theanine (p=0.02) groups differed significantly from those in the placebo group. However, the difference between theanine/arginine and theanine groups, was not statistically significant (p=0.74). From this study, no clear conclusion could be drawn regarding the potentiating effect of theanine and arginine combined ingestion on anti-stress effects in human.

Key Words L-theanine, L-arginine, short-term psychological stress, salivary alpha-amylase activity, randomized placebo-controlled study

The beneficial health effects of green tea and its components are well known (*1–3*). The ingredient L-theanine (γ-glutamylethylamide), which affects the taste of the tea, is the most abundant water soluble non-proteinous amino acid component in green tea and accounts for more than 50% of the total free amino acids (*4*). L-Theanine constitutes ~25–60 mg per 200 mL of green tea, ~1%–2% of the dry weight of green tea leaves, with the L isomer being the predominant form of theanine in green tea (*5*). L-Theanine is easily absorbed into the bloodstream and small intestine, then transports through the blood-brain barrier into the brain. Its various effects in the brain occur within 30 min (*6–9*).

Several biomedical studies have demonstrated that L-theanine has neuroprotective and regulatory effects (*10*), reduces blood pressure (*11*), antioxidative properties (*12*), and immunomodulatory properties (*13*). L-Theanine has thus been used historically for calming or refreshing the mental state, and some studies report that L-theanine has beneficial effects for psychological stress (*14*). Studies in mice revealed that L-theanine decreases serum corticosterone levels after stress and improves behavior under stressful conditions (*15*). L-Theanine also suppresses the deterioration of learning ability and behavioral depression under stressful conditions (*16*); decreases serotonin synthesis and enhances the degradation of serotonin, leading to changes in brain 5-hydroxyindole concentrations (*17*); and refreshes the mental state by promoting alpha brain waves (*18*).

Clinical studies, although few in number, have demonstrated an association of L-theanine with anti-stress effects. Kimura et al. reported that 2 indices of psychological stress, heart-rate and salivary immunoglobin A level, are reduced under stress conditions after ingesting 200 mg L-theanine compared with placebo (*19*). Unno et al. conducted randomized controlled trials using theanine-rich matcha green tea, typically produced from shade-grown green tea leaves, and cookies containing matcha green tea, and found that salivary amylase activity, a commonly used stress biomarker, is

E-mail: dfuru@health.nop.kagoshima-u.ac.jp

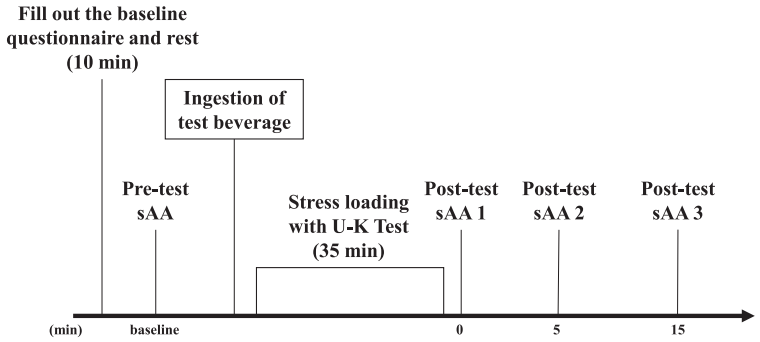

Fig. 1. Procedures of intervention in the study. Saliva collection for measurement of sAA was performed before administering the Uchida-Kraepelin (U-K) test as a baseline value, and again at 0 (immediately after), 5, and 15 min after the U-K test.

significantly reduced in response to social stress compared with placebo (*20*, *21*). In studies using low-caffeine green tea, Unno et al. found a stress-reducing effect of a lower dose (60 mg/d) of L-theanine, suggesting the possibility of other stress-reducing components in green tea (*20*).

L-Arginine, the second most abundant amino acid in green tea, contained about 0.85 to 3.14 mg/g, also exhibits a high stress-reducing effect and thus might enhance the anti-stress effects of L-theanine (*22*). The various diverse metabolites of L-arginine, such as nitric oxide, L-ornithine, polyamines, L-proline, L-glutamate, L-glutamine, creatine, and agmatine, produce its metabolic effects (*23*). With regard to the anti-stress effects of L-arginine, Pervin et al. reported that L-arginine suppresses several genes associated with the oxidative stress response and neuronal excitotoxic cell death, which increase in response to psychosocial stress, and reduces stress in stress-loaded mice (*24*). Thus, while findings from basic research support the anti-stress effects of L-arginine and L-theanine, few human studies have been performed and the effects remain largely unexplored.

We hypothesized that the combined ingestion of L-arginine and L-theanine could have beneficial clinical effects for psychological stress compared with ingestion of theanine alone. To evaluate this hypothesis, we conducted a randomized, single-blinded, placebo-controlled, parallel-group clinical trial in healthy young adults.

MATERIALS AND METHODS

Study design and subjects. This was a randomized, placebo-controlled, single-blinded study conducted at the University of Shizuoka (Shizuoka, Japan) from November 27, 2019, to October 31, 2020. Subjects were recruited through an on-campus advertisement, such as internet postings and posters, and comprised healthy, young volunteers. The criteria for inclusion were 1) >20 y of age; and 2) able to ingest barley tea orally. The criteria for exclusion were 1) taking antihypertensive drugs such as captopril, valsartan, and furosemide; 2) taking stimulants such as diethylpropion and epinephrine; 3) taking anxiolytics such as etizolam and diazepam; 4) and ingested beverages containing caffeine and L-theanine, such as green tea, coffee, and energy drinks the day before or on the day of the test.

The study subjects were assigned to one of the following groups by balanced block randomization at a ratio of 1 : 1 : 1 L-theanine alone ingestion group (T group), combined L-theanine and L-arginine ingestion group (T/A group), or placebo ingestion group (placebo group). Group allocation was concealed by a computer-generated randomized number code.

The study flow is shown in Fig. 1. The trial was conducted on weekdays from 1:00 p.m. to 5:00 p.m., avoiding immediately after meals. On the day of the trial, subjects were seated in a quiet room (22–25°C), and asked to provide responses to a baseline questionnaire and then rest for 10 min. The questionnaire included age, sex, smoking habits, green tea drinking habit, regular use of supplements, breakfast and lunch contents on the test day, sleeping time (h) on the test day, subjective assessment of physical condition, and state–trait anxiety inventory form (STAI) test score (Japanese STAI Form X-1, Sankyobo, Kyoto, Japan) (*25*). After the 10-min rest, a test beverage according to the assigned group was provided to each subject under blinded conditions, and the beverage was consumed under the supervision of a researcher. After the test beverage ingestion, the Uchida-Kraepelin performance test (U-K test) was administered to load psychological/mental stress onto the subjects. The U-K test is a standardized, paper-based arithmetic task that is widely used in research as a psychological stressor (*26–28*). In the present study, subjects were given a printed paper containing 34 rows of random, single-digit, horizontally aligned numbers and then given 15 min to perform the required calculations as quickly as possible. The test was given twice with a 1 min interval.

Salivary alpha-amylase activity (sAA) was measured to assess the physiological stress response using a colorimetric salivary amylase monitor (Nipro Co., Osaka, Japan) as previously reported (*20*). The saliva of each subject was collected using a saliva sampling tip at 4 time-points: before the start of the U-K test (Pre-test sAA), immediately after the U-K test (0 min, Post-test sAA 1), 5 min after the U-K test (Post-test sAA 2), and

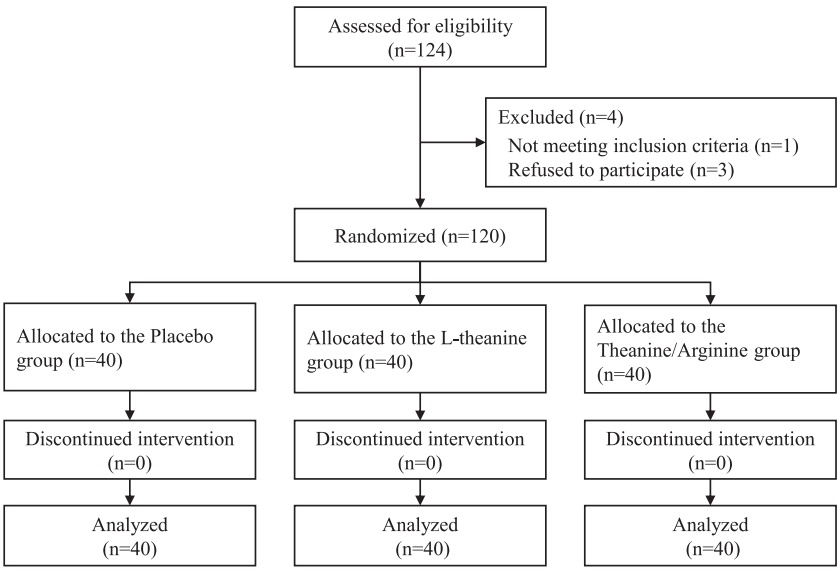

Fig. 2. Study flow diagram.

Table 1. Baseline characteristics of the study subjects.

Variables	Placebo (*n*=40)	T group (*n*=40)	T/A group (*n*=40)
Sex, *n* (%)			
Male	16 (40.0)	13 (32.5)	16 (40.0)
Female	24 (60.0)	27 (67.5)	24 (60.0)
Age (y), mean (SD)	22.1 (0.96)	22.3 (1.11)	22.8 (1.78)
Non-smoker, *n* (%)	39 (97.5)	38 (95.0)	40 (100.0)
Green tea drinking habits (more than once a day), *n* (%)	32 (80.0)	37 (92.5)	34 (85.0)
Regular use of supplements, *n* (%)	6 (10.8)	3 (7.5)	4 (10.0)
Breakfast on the test day, *n* (%)	27 (67.5)	27 (67.5)	27 (67.5)
Lunch on the test day, *n* (%)	33 (82.5)	34 (85.0)	37 (92.5)
Sleeping time (h) on the test day, mean (SD)	6.75 (1.05)	6.81 (0.86)	6.96 (0.88)
Subjective assessment of physical condition, *n* (%)			
Very good	15 (37.5)	16 (40.0)	13 (32.5)
Good	21 (52.5)	23 (57.5)	26 (65.0)
Not good, bad	4 (10.0)	1 (2.5)	1 (2.5)
STAI test score, mean (SD)	39.2 (7.1)	39.0 (8.0)	38.3 (7.6)

15 min after the U-K test (Post-test sAA 3).

Intervention materials. The intervention was a beverage containing L-theanine alone (200 mg/150 mL barley tea), combined L-theanine (200 mg) and L-arginine (50 mg) in 150 mL barley tea, or placebo containing no L-theanine or L-arginine (150 mL barley tea), which was provided to each subject under blinded conditions according to the assigned group. Theanine and arginine contents were set based on previous studies (*22*). As for L-arginine, previous studies reported that 100 mg/150 mL of L-arginine exhibits anti-stress effects, however, adding 100 mg/150 mL to barley tea would produce a strong bitter taste characteristic of arginine, which made it difficult to drink and maintain the blind of the study. Therefore L-arginine content was set at halved to 50 mg/150 mL.

The barley tea used in each test beverage contained 0 mg caffeine, 0.24 g protein, and 1.0 g carbohydrates, and the taste and color of the beverages were indistinguishable among the groups. All products used for intervention were commercially available, the L-arginine powder was manufactured by Nichie (Nagoya, Japan), and L-theanine and barley tea were manufactured by ITO EN, Ltd. (Tokyo, Japan).

Statistical analysis. A sample size calculation was performed according to previous reports on the effects of L-theanine on psychological stress (*20*), with a statistical power of 0.8, assuming 3 comparison groups, an estimated standard deviation (SD) of 25.0 and a significance level of 0.05 (1-way analysis of variance ANOVA).

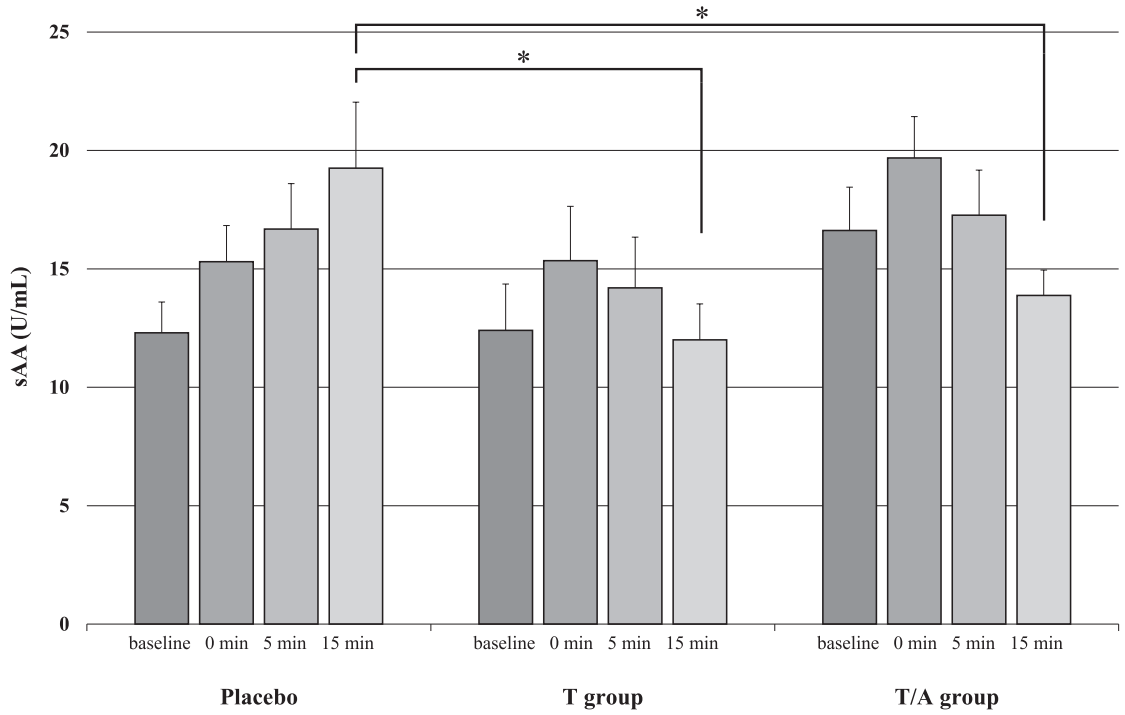

Fig. 3. Change in saliva alpha-amylase activity (sAA) after the U-K test at 0 min (immediately after), 5 min, and 15 min, by allocated group. Data are expressed as mean with SE (error bar). Lower scores indicate lower stress levels. *$p<0.05$, p-values are vs placebo.

As a result, the sample size was estimated to be 40 subjects for each group (total of 120 subjects). Descriptive statistics for all analyses are expressed as means and standard deviations (SD) for continuous variables, and as numbers and percentages (%) for categorical variables. Comparisons of temporal changes in sAA among the 3 groups were analyzed using a general linear model analysis of variance for repeated measures. Between-group comparisons of changes in sAA at each time-point were performed using analysis of covariance (ANCOVA) followed by Tukey honestly significant difference test for multiple comparisons. The intention-to-treat principle was used for all analyses. The statistical analyses were conducted using the program R (version 3.4.2, R Development Core Team 2018, R Foundation for Statistical Computing, Vienna, Austria). A p-value of <0.05 indicated a statistical significance.

Ethical considerations. The study followed the Declaration of Helsinki and Ethical Guidelines for Medical and Health Research Involving Human Subjects in Japan (https://www.mhlw.go.jp/). The study protocol and all procedures involving research study participants were approved by the Ethics Committee of the University of Shizuoka (No. 30-43, approved December 3, 2018). Written informed consent was obtained from all eligible subjects prior to their participation in the study. This trial was registered with the University Hospital Information Network, Japan, registration number UMIN000040967.

RESULTS

The flow diagram of the subjects is shown in Fig. 2. A total of 124 subjects were assessed for eligibility and 4 subjects were excluded because they did not meet the inclusion criteria ($n=1$) or refused to participate ($n=3$). Thus, 120 subjects were enrolled into the study and randomly assigned to 1 of the 3 groups. All enrolled subjects completed the trial without dropout or missing data, and were included in the analysis set. The baseline characteristics of the subjects per group are summarized in Table 1. Overall, the mean (SD) age was 22.4 (1.3) y and 75 of 120 subjects (62.5%) were female. No significant differences were detected among the groups for any variable.

The changes in sAA from baseline to 15 min for each group are shown in Fig. 3. The baseline value of sAA was slightly higher in the T/A group [mean 16.6 (12.4) kIU/L] than in the other 2 groups [T group: mean 12.4 (11.6) kIU/L, placebo group: 12.3 (8.3) kIU/L]. Before and after the U-K test was administered, the sAA values increased in all groups. After the U-K test, the sAA values gradually decreased over time in the T/A and T groups, whereas they increased in the placebo group, although the differences among groups was not statistically significant (repeated-measures ANOVA, $p=0.22$). Analysis by measurement time-point revealed no statistically significant difference among the groups at 0 min ($p=0.46$) or at 5 min ($p=0.59$) after the U-K test, but the values differed significantly between the T and placebo groups, and between the T/A and placebo groups at 15 min (ANCOVA, $p=0.01$; Tukey honestly significant difference test, T/A group vs placebo, $p=0.04$; T group vs placebo, $p=0.02$). The differences between the T/A group and T group were not statistically significant (Tukey honestly significant difference test, $p=0.96$).

Changes in sAA from baseline values are shown in

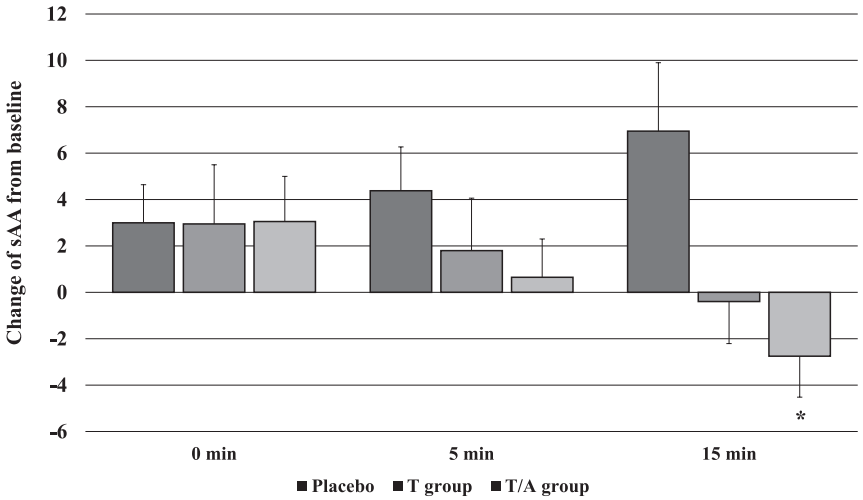

Fig. 4. Change in sAA from baseline at 0 min (immediately after), 5 min, and 15 min, by allocated group. Data are expressed as mean with SE (error bar). $*p<0.05$, p-values are vs placebo.

Fig. 4. In all groups, sAA values increased at 0 min (immediately after the U-K test; placebo group: 3.0 (10.3) kIU/L, T/A group: 3.1 (12.3) kIU/L, and T group: 3.0 (16.1) kIU/L). At 5 min, changes in sAA in the T and T/A groups tended to be smaller than in the placebo group (placebo group: +4.38 (12.0) kIU/L, T group: +1.8 (14.3) kIU/L, T/A group: +0.65 (10.4) kIU/L), but the difference was not statistically significant (ANCOVA, $p=0.39$). After 15 min, the difference became more pronounced; placebo group: +6.95 (18.6) kIU/L, T group: −0.4 (11.5) kIU/L, and T/A group: −2.75 (11.2) kIU/L. The changes in the sAA values between the T/A and placebo groups was significantly different (Tukey honestly significant difference test, T/A group vs placebo, $p=0.007$). The T/A group seemed to show a greater decrease than the T group, but the difference was not statistically significant ($p=0.74$ for Tukey honestly significant difference test).

DISCUSSION

In the present study, we evaluated if combined ingestion of L-theanine and L-arginine had greater anti-stress effects than ingestion of L-theanine alone or placebo. A randomized, single-blinded, placebo-controlled, parallel-group clinical trial was conducted in healthy young adults in their 20 s. As an indicator of stress, sAA values were used and measured at 0 (immediately after), 5, and 15 min after stress loading by the U-K test. The major findings of the present study were that combined ingestion of L-theanine and L-arginine tended to have greater stress reducing effects than ingestion of L-theanine alone, although the difference was not statistically significant.

There are several possible reasons that we could not show a clear anti-stress effect of combined ingestion of L-theanine and L-arginine. First, the overall sAA values of subjects were lower than expected. In similar clinical trials that we conducted previously, the observed mean sAA values were ~40–60 U/mL in young adults (mean age 23±0.9 y, $n=36$) and ~100 U/mL in older adults (mean age 89.3±4.2 y, $n=10$), and sAA values tend to increase with age (*20*, *21*, *29*, *30*). Although the measurement of sAA in the present study was performed in the same way as in the previous studies, the sAA values were lower. The subjects in this study were young university students. These low baseline levels might have resulted in a lower statistical power of detection.

Second, the mechanisms underlying the anti-stress effects of L-theanine and L-arginine in humans may require more time than allowed in the present study. The stress load test was started approximately 10 min after ingestion of the test beverage based on several previous studies reporting that L-theanine passes through the blood-brain barrier and exerts its effects directly on the brain within 30 min (*7*, *9*). Although previous animal and clinical studies demonstrated anti-stress effects of each L-theanine and L-arginine, the detailed mechanisms of the anti-stress effects induced by combined ingestion of L-theanine and L-arginine remain unclear. Therefore, the timing of the sAA measurements may have been insufficient to reflect the effects in humans. This point needs to be clarified through further research.

The strengths of the study were the test beverages contained no catechins or caffeine. In a study we conducted prior to this study, we used a sample of matcha green tea and found that caffeine and epigallocatechin gallate (EGCG), the main components in green tea, counteract the anti-stress effects of L-theanine and L-arginine (*20*). Furthermore, we found that the molar ratio of caffeine and catechin to L-theanine and L-arginine must be less than 2.0 to block the antagonistic effects of caffeine and EGCG on stress (*22*). To avoid these antagonistic effects, in the present study, we used barley tea, which contains no caffeine or catechins. Nevertheless, the anti-stress effects of the combined ingestion of L-theanine and L-arginine were smaller than those assumed on the basis of previous studies. Stress sensitivity varies by individual, age, sex, and other factors among the subjects in clinical trials, so it is highly likely that there is some divergence in the mechanisms of

action of L-theanine and L-arginine. Therefore, future studies to elucidate the mechanism of the effects of combined ingestion of L-theanine and L-arginine on the stress response in humans must take into account potential divergent anti-stress mechanisms between the 2 compounds.

The present study provides valuable information, but has some limitations regarding the interpretation of the results. First, the present study was a clinical trial conducted among young adult university students at a single site with a relatively small sample size. Therefore, it is possible that the baseline value of sAA was lower than that of the general population. Furthermore, the stress load caused by the U-K test had a smaller effect than in previous studies (*20*, *21*, *29*, *30*). The U-K test is widely used as a psychological stressor in various studies because it is an ethically acceptable method of stress loading, but it may not be sufficiently stressful in a population that does not struggle with mental arithmetic. Therefore, it is possible that it produced a low stress load for students engaged in learning and research activities on a daily basis. Thus, the generalizability of the findings must be examined in a larger sample composed of a wide age range and sexes.

Second, L-arginine contains was set as 50 mg/150 mL in present study, previous studies reported that 100 mg/150 mL of arginine exhibits anti-stress effects. However, adding 100 mg/150 mL to barley tea would produce a strong bitter taste characteristic of L-arginine, which made it difficult to drink and maintain the blind of the study. This may have resulted smaller than expected anti-stress effect of L-arginine. Also, the L-arginine alone ingestion group was not included in the present study because the bitter taste of arginine makes it difficult to maintain blinding. To clarify the anti-stress effects of L-theanine and L-arginine combined ingestion, a comparison with the L-arginine alone ingestion group should be made. We would like to consider this task as challenges for the future study.

Third, the present study evaluated the effects of L-theanine and L-arginine on an acute period of psychological stress, not on long-term psychosocial stress. Therefore, the results may not be directly applicable to the effects on those under chronic stress.

In conclusion, we could not demonstrate clear effect on promoting anti-stress effects of combined ingestion of L-theanine and L-arginine in human young adults with short-term stress. However, few clinical trials have studied the anti-stress effects of the combined ingestion of L-theanine and L-arginine, and, to our knowledge, our results are the first clinical results suggesting synergistic effects of these 2 ingredients against stress. Further studies are needed to provide more evidence of the clinical anti-stress effectiveness of the combination of these 2 ingredients. We hope that further research will be conducted based on the results of this study.

Authorship

Conceptualization, DF, KU and HY; data curation, YN and IS; formal analysis, DF, IS and YN; investigation, IS and YN; methodology, DF, KU and HY; project administration, DF; supervision, DF, KU and HY; visualization, DF; writing-original draft, DF; writing–review & editing, IS, KU and HY.

Disclosure of state of COI

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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