Regular Article

Anti-stress Effect of Green Tea with Lowered Caffeine on Humans: A Pilot Study

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Theanine, an amino acid in tea, has significant anti-stress effects on animals and humans. However, the effect of theanine was blocked by caffeine and gallate-type catechins, which are the main components in tea. We examined the anti-stress effect of green tea with lowered caffeine, low-caffeine green tea, on humans. The study design was a single-blind group comparison and participants (n=20) were randomly assigned to low-caffeine or placebo tea groups. These teas (≥500 mL/d), which were eluted with room temperature water, were taken from 1 week prior to pharmacy practice and continued for 10 d in the practice period. The participants ingested theanine (*ca*. 15 mg/d) in low-caffeine green tea. To assess the anxiety of participants, the state-trait anxiety inventory test was used before pharmacy practice. The subjective stress of students was significantly lower in the low-caffeine-group than in the placebo-group during pharmacy practice. The level of salivary α-amylase activity, a stress marker, increased significantly after daily pharmacy practice in the placebo-group but not in the low-caffeine-group. These results suggested that the ingestion of low-caffeine green tea suppressed the excessive stress response of students. This study was registered at the University Hospital Medical Information Network (ID No. UMIN14942).

Key words green tea; anti-stress effect; clinical study; salivary α-amylase; theanine

Modern life causes stress in many people. The accumulation of stress can increase the risk of mood and anxiety disorders.[1] Intervention of stress-induced alterations with dietary supplements is thought to be helpful for preventing the accumulation of stress, and to be a potential therapeutic strategy for a healthy life. Green tea (*Camellia sinensis* (L.) Kuntze) is the most popular drink in Japan and Asian countries. Theanine (L-theanine), the major amino acid in tea leaves and an important sweet umami component of green tea, has significant anti-stress effects on animals and humans.[2–5] However, the anti-stress effect of green tea is not yet known. Green tea is mainly composed of catechins, caffeine, and amino acids. Catechins, mainly epigallocatechin gallate (EGCG), have potent antioxidative and anti-inflammatory activities that fortify the beneficial effect of green tea on health.[6–8] Caffeine is a non-selective antagonist of the adenosine receptor. A number of studies have indicated that regular daily dietary caffeine intake is associated with disturbed sleep.[9] We recently found that caffeine and EGCG suppressed the anti-stress effect of theanine while epigallocatechin (EGC) and arginine (Arg), other components in green tea, retained these effects.[10] This suggests that balances among theanine, caffeine, catechins and Arg are crucial for green tea to express its anti-stress effect. Therefore, we prepared green tea with lowered levels of caffeine, low-caffeine green tea. Caffeine decreased to 1/4–1/5 of the level of non-treated tea leaves.[10] Next, to reduce EGCG, we assessed the temperature–sensitive kinetics of water elution of each tea component. The solubility of EGCG and caffeine is low in room temperature water (EGCG <5 mg/mL and caffeine 22 mg/mL, respectively), whereas that of theanine is high (370 mg/mL). When green tea is eluted with room temperature water, the composition in eluate is changed from that eluted with hot water.[11,12] The ingestion of low-caffeine green tea that was steeped in room temperature water significantly suppressed the stress response in wild-type mice.[10]

In this study, we examined the effect of low-caffeine green tea on stress responses in 5th-year college students of the school of pharmaceutical sciences. They were assigned to practice outside the university such as in a hospital or a dispensing pharmacy. The commitment in new environments provides a stressful condition for young students. Salivary α-amylase activity (sAA), an oral cavity enzyme, was measured as the stress marker.[13] Two main body systems are involved in the stress response, the autonomic nervous system (ANS) and the hypothalamus–pituitary–adrenal axis. Measurement of sAA has been demonstrated as a useful tool for monitoring ANS reactivity to stress.[13] This enzyme rapidly increases in response to physiological and psychosocial stress.[14–17] We have previously clarified that ingestion of theanine effectively suppresses sAA and subjective stress during pharmacy practice.[5] In this study, we examined the anti-stress effect of low-caffeine green tea on humans to clarify the function of green tea.

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MATERIALS AND METHODS

Preparation of Low-Caffeine Green Tea Tea (*Camellia sinensis* (L.) Kuntze) leaves were collected in Shizuoka, Japan. Fresh tea leaves were treated with a hot water shower at 95°C for 180 s as described previously.[10] Then, the tea leaves were dried through a standard manufacturing process. We termed this low-caffeine green tea.

One tea bag of low-caffeine or placebo (barley) tea (3 g of tea in a bag) was steeped in 500 mL of room temperature water. Tap water was used in this experiment. Barley tea has no caffeine and very little catechins.[18–20] In addition, theanine is a unique amino acid that contained primarily in tea plant (*Camellia sinensis*).[21] The participants prepared low caffeine or placebo tea every morning and ingested it until the evening. Tea bag was left in water until the evening. Similarly, after each day's pharmacy practice, the participants drank these teas.

For the measurement of tea component in the eluate, tea leaves of low-caffeine green tea (3 g) were steeped in 500 mL of room temperature water for 0.5 or 5 h and stirred sometimes.

Measurement of Tea Components by HPLC The eluates of low caffeine green tea were measured by HPLC as described previously.[10] In brief, catechins and caffeine in the eluates were measured by HPLC (SCL-10Avp, Shimadzu, Japan; Develosil packed column ODS-HG-5, 150×4.6 mm, Nomura Chemical Co., Ltd., Japan) according to the method of Horie *et al.*[22] Catechins and caffeine were measured at 280 nm. Free amino acids in tea leaves were measured by HPLC as described above using homoserine as an internal standard.[23] Amino acids were detected at excitation wavelength of 340 nm and at 450 nm of emission wavelength (RF-535 UV detector, Shimadzu, Kyoto, Japan). The relative standard deviation (RSD%) of precision and repeatability were $<$5.0%. The recoveries of catechins, caffeine, and free amino acids were 99±4, 98±4, and 98±3%, respectively.

Participants Twenty healthy 5th-year students of the University of Shizuoka, who participated in the experiment, were randomly divided into two groups with matching sex: low-caffeine (n=10, 5 men and 5 women; average age 23.2±0.6 y) and placebo (n=10, 5 men and 5 women; average age 22.4±0.2 y) *via* sealed envelopes to receive low-caffeine or placebo (barley) tea bags. The participants were assigned to practice outside the university, in a hospital or a dispensing pharmacy, for 11 weeks. The first 10 d of the practice program were analyzed, because these days were assumed to be the most stressful. None of the participants indicated acute or chronic disease, regular medication intake, or habitual smoking. They were instructed to drink mainly the test tea, and not to take theanine- and caffeine-rich beverages such as green tea, coffee, and black tea throughout the experiment. They could drink water freely, but they did not consume alcohol at night. The study was conducted in accordance with the Declaration of Helsinki and Ethical Guidelines for Medical and Health Research Involving Human Subjects (Public Notice of the Ministry of Education, Culture, Sports, Science and Technology and the Ministry of Health, Labour and Welfare, 2008). The study protocol was approved by the Ethics Committee of the University of Shizuoka (No. 26-8). All the participants received verbal and written information about the study and signed an informed consent form before entering the study. This study was registered at the University Hospital Medical Information Network (UMIN) (registration ID No. UMIN14942). The study period was from August to September, 2014.

Procedure This study was a group comparison design and participants were randomly assigned to low-caffeine or placebo tea groups. The participants did not know whether they were consuming low-caffeine or placebo tea, because they have no information about low-caffeine green tea such as color, aroma and taste. The intake of low-caffeine or placebo tea taken from 1 week prior to pharmacy practice and continued for 10 d into the practice period, for a total of 17 d. To assess the anxiety of participants, the state-trait anxiety inventory (STAI) test (Japanese STAI Form X-1, Sankyobo, Kyoto, Japan) was carried out before pharmacy practice.

A questionnaire that included feedback on physical condition, subjective stress and achievement emotion was assigned for 10 d after each day's practice. The physical condition of participants was assigned an ordinal scale (5, very good; 4, good; 3, normal; 2, a little bad; 1, bad). Subjective stress was evaluated using visual analogue scales (VAS: 0–10) from very relaxed to highly stressed. Achievement emotion was assigned an ordinal scale (5, completely; 4, better; 3, a little better; 2, a little worse; 1, much worse).[5] Sleeping hours were also recorded.

Measurement of sAA To assess the physiological stress response, sAA was measured using a colorimetric system (Nipro Co., Osaka, Japan).[24] Briefly, a substrate 2-chloro-4-nitrophenyl-4-*O*-β-D-galactopyranosylmaltoside is hydrolyzed by salivary amylase in the presence of maltose, a competitive inhibitor. This reaction turns the color of a reagent strip from white to yellow, and changes are quantified using a salivary amylase monitor. One unit activity (U) per mass of enzyme is defined as the production of 1 μmol of the reduction sugar, maltose, in 1 min (NC-IUBMB, 1992).

Saliva was collected twice a day, in the morning after waking up (pre-stress) and in the evening after pharmacy practice (post-stress), for 10 d during the practice. To establish a baseline of sAA before the pharmacy practice, the sAA of participants was measured every morning and evening for 7 d during routine daily life at the university. The measurement was carried out before pharmacy practice. Prior to sampling, participants washed their mouths with water. After saliva was collected for 30 s using a sampling tip, each participant measured their own sAA immediately every morning and evening for 17 d (including unassigned days (*i.e.*, weekends), whose measurements were excluded from the analyses).

Statistical Analysis All results are expressed as mean±standard error of the mean (S.E.M.). The influence of stress on sAA was evaluated using one-way ANOVA followed by Bonferroni's *post hoc* test for multiple comparisons. ANOVA and correlation coefficients were obtained using a statistical analysis program (StatPlus, AnalystSoft Inc., online version). In each analysis, a p value $<$0.05 was considered to be statistically significant.

RESULTS

The Contents of Theanine, Caffeine and Catechins in Low-Caffeine Green Tea The content of caffeine was very low in the eluate derived from steeping low-caffeine green tea

for 0.5 and 5.0 h while the content of catechins and theanine was not significantly different, nor affected by steeping period (Table 1). EGC was the most abundant catechin in the eluate, followed by EGCG that is a major gallate-type catechin. The other non-gallate type catechins such as epicatechin (EC) and catechin ((+)C) were also found in the eluate. The amount of theanine in low-caffeine green tea accounted for half of the total amino acids, about 21 mg/L, followed by Arg (5.0 mg/L). The participants ingested low-caffeine green tea (714±79 mL/d) or placebo tea (729±111 mL/d). The participants that ingested low-caffeine green tea consumed about 15 mg of theanine and 3.6 mg of Arg per day.

Changes of sAA and Subjective Stress in Students Since one participant of the low-caffeine-group did not record subjective stress, the data of 19 participants was evaluated.

Whereas the level of sAA is usually low at the time of waking up but becomes high as a result of sympathetic excitement during the day,[15,25] if stress is small, then no significant change is observed. There was no significant difference in sAA levels in the morning and evening during routine daily life at the university (Fig. 1a), suggesting that the stress level of the participants was not high in the university. During pharmacy practice, however, the level of post-stress sAA (*i.e.*, sAA in the evening after daily pharmacy practice) was significantly higher in the placebo-group than the level of pre-stress sAA (*i.e.*, sAA in the morning before daily pharmacy practice) (p=0.001; one-way ANOVA) (Fig. 1b). In the low-caffeine-group, the level of post-stress sAA tended to be higher, but not significantly, than the level of pre-stress sAA. Subjective stress was evaluated by each participant at the end of daily practice using VAS (0–10). The average score was significantly lower in the low-caffeine-group than the placebo-group (p=0.0003; one-way ANOVA; Fig. 1c). Physical condition was not different between both groups during pharmacy practice (placebo 3.36±0.14, low-caffeine 3.61±0.11).

Considering individual variability, the mean values of pre- and post-stress sAA of each participant were analyzed. Participants of higher pre-stress sAA exhibited higher post-stress sAA in the placebo-group than in the low-caffeine-group (placebo, y=1.35x+2.45, R=0.900 and p=0.0004; low-caffeine, y=1.09x+6.86, R=0.805 and p=0.009; Fig. 2a), while the distribution of pre-stress sAA was not different between the low-caffeine and placebo groups. This suggests that ingestion of low-caffeine green tea suppressed the increase of post-stress sAA. The correlation between post-stress sAA and subjective stress tended to be high in the placebo-group but not in the low-caffeine-group (Fig. 2b, placebo, p=0.085; low-caffeine, p=0.908). The ingestion level of theanine was estimated from the drinking volume of low-caffeine green tea in each participant. The participants that ingested a higher level of theanine tended to show lower post-stress sAA. However, no correlation between subjective stress and theanine ingestion was

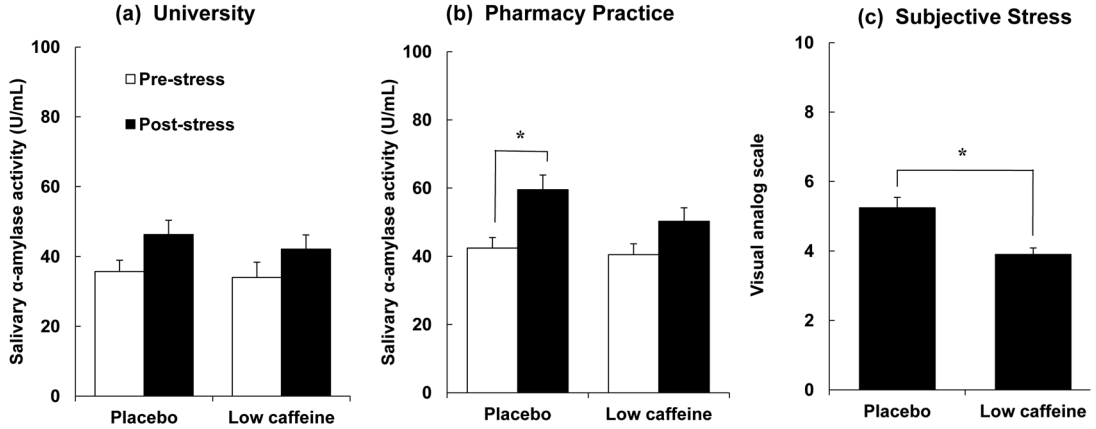

Fig. 1. Salivary α-Amylase Activity (sAA) of the Participants Was Measured in the Morning after Waking Up (Pre-stress sAA, White Bar) and in the Evening (Post-stress sAA, Black Bar)

The participants consumed low-caffeine or placebo (barley) teas during daily life at the university (a), and during the pharmacy practice (b). Daily score of subjective stress during pharmacy practice was compared between the placebo and low-caffeine groups (c). Data are expressed as mean±S.E.M. (*p<0.05; one-way ANOVA).

Table 1. The Contents of Caffeine, Catechins and Amino Acids in the Eluate of Low-Caffeine Green Tea

Low-caffeine green tea (3 g/500 mL)	Caffeine (mg/L)	Catechins (mg/L)							
		EGCG	EGC	ECG	EC	CG	(+)C	Total	
0.5 h	0.43	13.1	210	0.06	6.1	0	5.8	235	
5.0 h	1.08	14.9	236	0.04	9.5	0	7.1	268	
Low-caffeine green tea (3 g/500 mL)	Free amino acids (mg/L)								
	Theanine	Glu	Arg	Asp	Gln	Ser	Ala	Asn	GABA
0.5 h	20	4.6	5.5	3	3.7	1.5	0.6	0.2	0.4
5.0 h	22	4.5	4.5	3	5.3	1.3	0.6	0.3	0.5

Low-caffeine green tea (3 g) was steeped in 500 mL of room temperature water for 0.5 or 5.0 h. EGCG, (−)-epigallocatechin gallate; EGC, (−)-epigallocatechin; ECG, (−)-epicatechin gallate; EC, (−)-epicatechin; CG, (−)-catechin gallate; (+)C, (+)-catechin; Glu, glutamic acid; Arg, arginine; Asp, aspartic acid; Gln, glutamine; Ser, serine; Ala, alanine; Asn, asparagine; GABA, γ-aminobutyric acid.

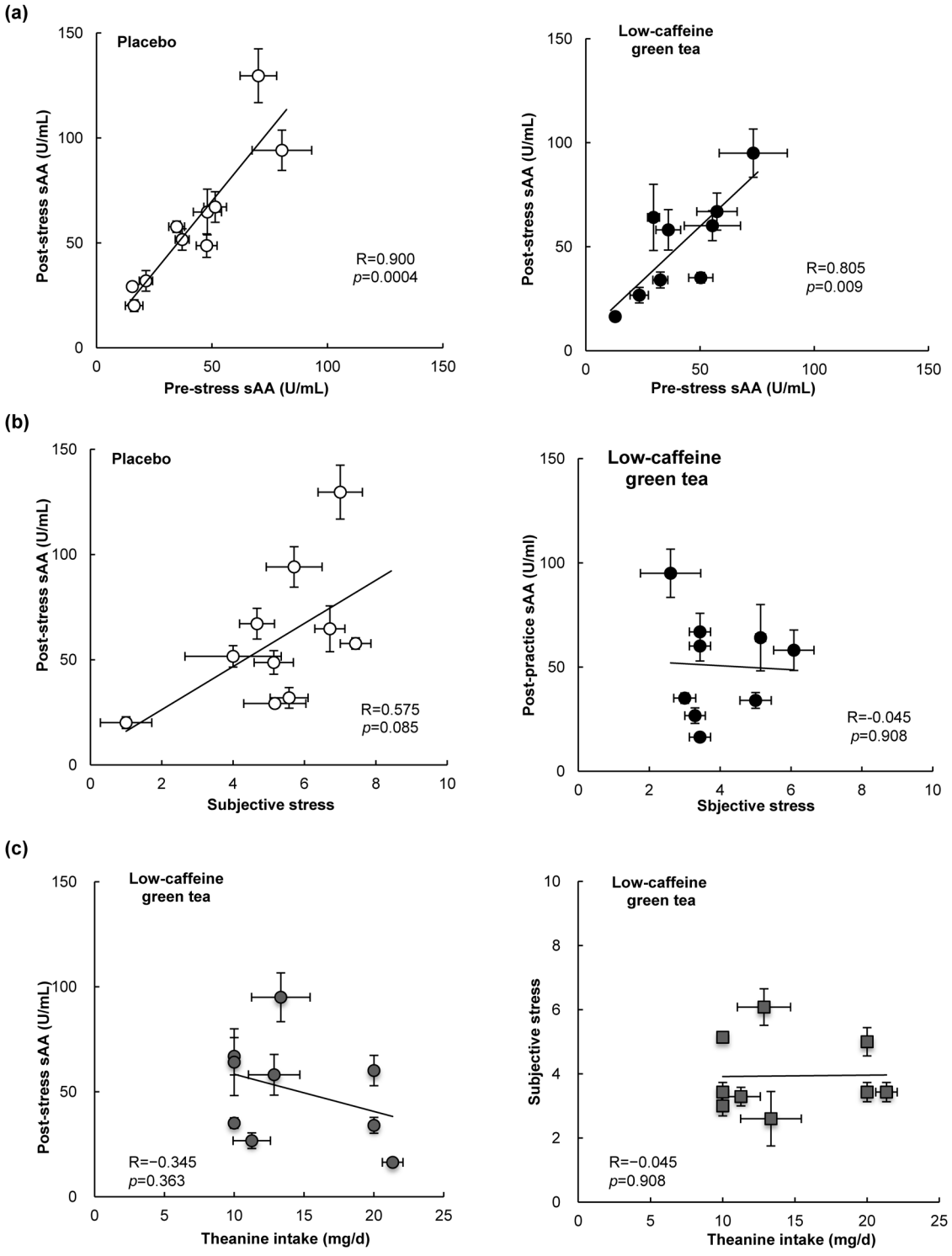

Fig. 2. Correlation between sAA Activity, Subjective Stress and Theanine Ingestion in the Placebo and Low-Caffeine Groups

(a), Pre-stress sAA (sAA in the morning before daily pharmacy practice) and post-stress sAA (sAA in the evening after daily pharmacy practice); (b), post-stress sAA and subjective stress. (c) Correlation between theanine intake that was estimated from the ingestion volume and post-stress sAA or subjective stress in the low-caffeine group. Each point of sAA represents the mean value of each participant that was calculated from sAA during pharmacy practice. Data are expressed as mean±S.E.M.

observed (Fig. 2c).

STAI Value, Achievement Emotion and Sleeping Time The average STAI values were examined to assess anxiety based on the appraisal standard. There was no difference between both groups (placebo 42.0±3.2; low-caffeine 41.0±2.3). Although the levels of sAA varied considerably among the participants under stress, pre- and post-stress sAA were correlated with the STAI value (Figs. 3a, b). The participants with a higher STAI value exhibited higher post-stress sAA in the placebo than in the low-caffeine-group (placebo, $p=0.064$; low-caffeine, $p=0.489$). Theanine intake did not correlate with STAI value (Fig. 3c).

Achievement emotion was evaluated by participants as an ordinal scale at the end of daily practice. There was no difference between the average of both groups (placebo 3.53±0.20; low-caffeine 3.59±0.22). However, participants with lower

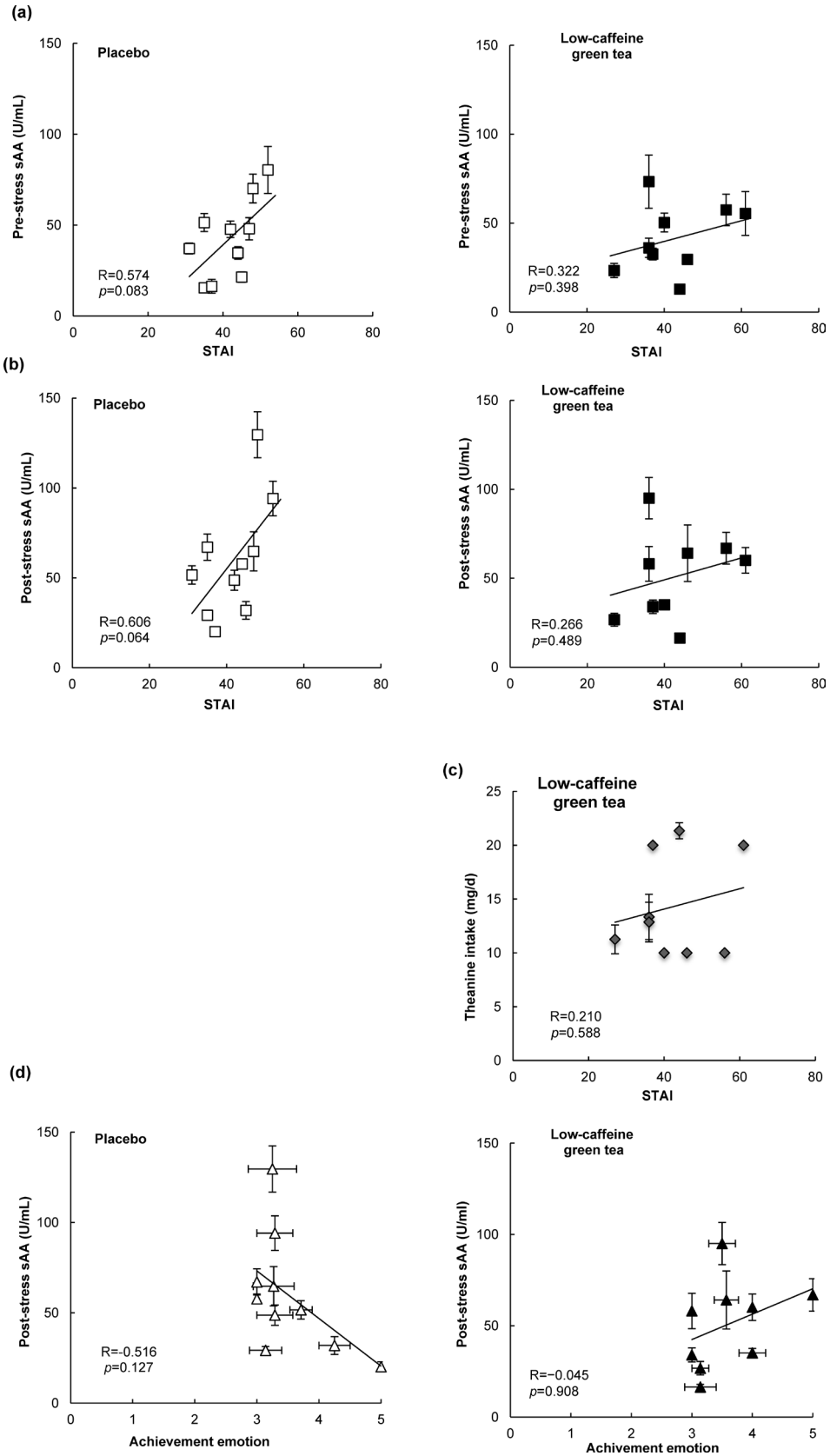

Fig. 3. Correlation between sAA Activity, STAI and Achievement Emotion in the Placebo and Low-Caffeine Groups

(a), Pre-stress sAA and STAI; (b), post-stress sAA and STAI; (c), theanine intake and STAI; (d), post-stress sAA and achievement emotion. Each point of sAA represents the mean value of each participant that was calculated from sAA during pharmacy practice. Data are expressed as mean±S.E.M.

achievement emotion tended to show higher post-stress sAA in the placebo-group (Fig. 3d), suggesting that negative emotion also influenced sAA. The average sleeping time was not different between both groups (placebo 6.45±0.14; low-caffeine 6.36±0.11) during pharmacy practice.

The difference of pharmacy practice at a hospital or at a dispensing pharmacy had no effect on these parameters (data not shown).

DISCUSSION

Effect of Low-Caffeine Green Tea Ingestion on Humans This study was carried out to explore the anti-stress effect of low-caffeine green tea on humans but has several limitations. Firstly, this study is a single-blind group comparison and has a small participant size (n=20). Secondly, the effect of low-caffeine green tea is only compared with that of placebo (barley) tea. Thirdly, we did not measure the components of all the tea samples that were prepared daily by participants.

Barley tea has no caffeine, catechins or theanine,[18–21] thus it was a suitable placebo for the comparison of the effect of low-caffeine green tea. In addition, all participants were unfamiliar with the color, taste and aroma of low-caffeine green tea, and were unable to judge whether the tea they had drunk was low-caffeine green tea or placebo (barley) tea. Therefore, the placebo may have had little effect on the measured sAA data. One possible problem is that the concentration of tea components among participants may have been different. However, the eluate data indicates that catechins and amino acids had become fully eluted from tea leaves at 0.5 h (Table 1), suggesting that the content of tea components was similar under the same elution conditions, including the same volume of tea leaves and water, and a similar temperature. Thus, the content of tea components that was estimated from the volume ingested by each participant may be close to the actual value. Therefore, despite these limitations, the anti-stress effect of low-caffeine green tea obtained in this experiment is considered to be reliable.

We previously reported the anti-stress effect of theanine at 200 mg, twice a day in participants during pharmacy practice (400 mg/d, 7–9 mg/kg/d).[5] This dose had been decided from previous data.[3,26,27] However, in this study, we showed that a significant anti-stress effect was observed in participants that ingested *ca.* 15 mg/d (0.3 mg/kg/d) of theanine from low-caffeine green tea. In mice that ingested theanine, adrenal hypertrophy, a typical stress response in living organisms, was significantly suppressed at least at 10 μg/mouse/d (0.3 mg/kg) under stressful conditions.[4] These results indicate that a lower dose of theanine, at least 0.3 mg/kg/d, may exhibit an anti-stress effect in humans and mice.

Since Arg also has a high anti-stress effect in mice,[10] the cooperative action of theanine and Arg may cause an anti-stress effect at a lower dose than the single action of theanine. Arg is considered to be an important regulator in the central nervous system through the synthesis of nitric oxide[28] and has vital functions in physical stress and anxiety.[29] The vasoconstrictive effect of caffeine is eliminated when combined with theanine,[30] suggesting that both Arg and theanine cooperatively affect cerebral blood flow.

Relationship between Theanine and Other Tea Components Although the effect of theanine is antagonized by caffeine,[31] theanine may completely suppress the effect of caffeine because the content of theanine was ≥20 times higher than caffeine in the eluate of low-caffeine green tea (Table 1). The effect of caffeine was significantly suppressed by 10-fold higher of theanine in mice.[10] Theanine that is incorporated into the brain reduces the release of glutamate (Glu) from the presynapse to the synaptic cleft by acting with a glutamine (Gln) transporter and inhibiting the incorporation of extracellular Gln into neurons, which suppresses the conversion of Gln to Glu by glutaminase.[32,33] Indeed, in the hippocampus of mice that ingested theanine (6 mg/kg) in drinking water for 2 weeks, the level of Glu was significantly reduced, and conversely, the level of γ-aminobutyric acid (GABA) increased.[34] GABA is the chief inhibitory neurotransmitter in the mammalian central nervous system, therefore, theanine exerts an anti-stress effect, in part, through modulation between GABA and Glu, the main excitatory neurotransmitter.

EGCG has been reported to suppress over-expression of the GABA pathway in Down syndrome mouse models[35] and to inhibit GABA[36] by modulating the $GABA_A$ receptor.[37] However, the content of EGC was 16-fold higher than that of EGCG in the eluate created with room temperature water (Table 1). The effect of EGCG on the stress response was suppressed by 20-fold more EGC and two-fold more theanine,[10] suggesting that the effect of EGCG was limited when the participants drank green tea that had been eluted with room temperature water. Taken together, it may be important, when assessing the anti-stress effect of green tea, to consider that the contents of theanine, Arg and EGC were relatively higher than the contents of caffeine and EGCG. Further work that explores the potential modulatory mechanisms of green tea components is warranted.

Effect of Ingestion of Low-Caffeine Green Tea on sAA and Trait Anxiety Pre- and post-stress sAA varied considerably among participants under stress and participants with higher pre-stress sAA showed higher post-stress sAA. The STAI value, *i.e.*, trait anxiety of each participant, affected the level of sAA. Ingestion of low-caffeine green tea suppressed sAA but not STAI, resulting that a close correlation between sAA and STAI was observed in the placebo but not in the low-caffeine group. Response of sAA may be more sensitive to the ingestion of low-caffeine green tea than psychological responses such as subjective stress and achievement emotion.

The activity of sAA increased in response to stress and caffeine,[38] indicating the validity of low-caffeine green tea. These results suggest that the ingestion of low-caffeine green tea effectively suppresses an excessive stress response in participants of high pre-stress sAA and STAI who are sensitive to stress. In stress-vulnerable people, intervention with dietary supplements may be a potential strategy to suppress excessive stress and sustain a healthy life. An additional large-scale clinical trial would be required to determine the effect of low-caffeine green tea. In addition, since each stress-sensitivity is different among individuals, a detailed analysis that is based on individual differences is needed.

CONCLUSION

We examined whether the ingestion of low-caffeine green tea is able to suppress the stress-response in students. The subjective stress of students was significantly lower in the

low-caffeine-group than in the placebo-group during pharmacy practice. The level of sAA increased significantly after daily pharmacy practice in the placebo-group but not in the low-caffeine-group. The ingestion of green tea with lowered caffeine may suppress excessive stress in students.

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Conflict of Interest The authors declare no conflict of interest.

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