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Anti-stress effect of theanine on students during pharmacy practice: Positive correlation among salivary α-amylase activity, trait anxiety and subjective stress

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ABSTRACT

Purpose: Theanine, an amino acid in tea, has significant anti-stress effect on experimental animals under psychosocial stress. Anti-stress effect of theanine on humans was evaluated in 5th-year university students during pharmacy practice.

Method: The study design was a single-blind group comparison and participants (n = 20) were randomly assigned to theanine or placebo groups. Theanine or placebo (lactose) tablets (200 mg, twice a day, after breakfast and lunch) were taken from 1 week prior to the pharmacy practice and continued for 10 days in the practice period. To assess the anxiety of the participants, the state–trait anxiety inventory test was carried out before the pharmacy practice. Salivary α-amylase activity (sAA) was measured as a marker of sympathetic nervous system activity.

Results: In the placebo-group, sAA in the morning (pre-practice sAA) was higher than in theanine-group during the pharmacy practice ($p = 0.032$). Subjective stress was significantly lower in the theanine-group than in the placebo-group ($p = 0.020$). These results suggest that theanine intake had anti-stress effect on students. Furthermore, students with higher pre-practice sAA showed significantly higher trait anxiety in both groups ($p = 0.015$). Similarly, higher pre-practice sAA was correlated to shorter sleeping time in both groups ($p = 0.41 \times 10^{-3}$).

Conclusion: Stressful condition increased the level of sAA that was essentially affected by individual trait anxiety. The low levels of pre-practice sAA and subjective stress in the theanine-group suggest that theanine intake suppressed initial stress response of students assigned for a long-term commitment of pharmacy practice.

1. Introduction

Chronic psychosocial stress is associated with the development of depression, mood disorders, as well as cardiovascular and other age-related diseases (McEwen and Magarinos, 1997; Pedersen et al., 2001; Gareri et al., 2002; Bellinger et al., 2008; Thayer et al., 2010). Intervention of stress-induced alterations with dietary supplements is a potential therapeutic strategy for a healthy life. We have previously reported that the intake of theanine (γ-glutamylethylamide) suppressed the stress-derived malfunctions in aged mice that were chronically stressed under the confrontational housing (Unno et al., 2011, 2013). Theanine (L-theanine) is the most abundant amino acid in tea. The sweet umami taste of green tea is due to amino acids, especially theanine. Several studies have reported that theanine exerts neuroprotective effects (Nagasawa et al., 2004; Egashira et al., 2004, 2007, 2008; Cho et al., 2008; Kim et al., 2009), modulates the activity of neurotransmitters (Yamada et al., 2007; Kakuda et al., 2008) and reduces psychological stress (Kimura et al., 2007). In this study, we aimed to investigate the effect of theanine supplementation 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 drug store, for 11 weeks. Such a long-term commitment in new environments provides a stressful condition for young students. Salivary α-amylase activity (sAA), an oral cavity enzyme, was measured as a stress marker (Nater and Rohleder, 2009). Two main body systems

Abbreviations: ANS, autonomic nervous system; HPA, hypothalamus–pituitary–adrenal; sAA, salivary α-amylase activity; pre-practice sAA, sAA in the morning; post-practice sAA, sAA in the evening; STAI, the state–trait anxiety inventory; VAS, visual analog scales.

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are involved in the stress response, the autonomic nervous system (ANS) and the hypothalamus–pituitary–adrenal (HPA) axis. Measurement of sAA has been demonstrated as a useful tool for monitoring ANS reactivity to stress (Nater and Rohleder, 2009). This enzyme is increased rapidly in response to physiological and psychosocial stress (Almela et al., 2011; Nater et al., 2005, 2006; Rohleder et al., 2004). The secretion of salivary amylase is directly stimulated by innervation followed by hormonal regulation in response to changes in serum noradrenalin levels. Therefore, the salivary gland acts more quickly and sensitively responds to the psychological stress than cortisol (Yamaguchi et al., 2004). The measurement of sAA is an efficient and non-invasive assessment to study the effect of psychosocial stress. In the present study, considering possible individual variability in responding to the same stressful condition, trait anxiety, physical condition, subjective stress, achievement emotion and sleeping time were scored and integrated with the changes in sAA in each participant during pharmacy practice. Our results suggest that the theanine supplementation is beneficial in suppressing psychosocial stress in humans.

2. Methods

2.1. 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: theanine ($n = 10$, 7 men and 3 women; average age 22.5 ± 0.2 yr) and placebo ($n = 10$, 7 men and 3 women; average age 22.2 ± 0.1 yr) via sealed envelopes to receive theanine or placebo tablets. The students were assigned to practice outside the university, in a hospital or a drug store for 11 weeks. The first 10 days 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 water, and not to take theanine- and caffeine-rich beverages such as green tea, coffee, and black tea throughout the experiment. The study was conducted in accordance with the Declaration of Helsinki. The study protocol was approved by the Ethics Committee of the University of Shizuoka. 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 clinicaltrials.gov (registration ID no. NCT01361204). The study period was from March to September in 2011.

2.2. Procedure

This study was a group comparison design and participants were randomly assigned to theanine or placebo groups. The participants did not know whether they were consuming theanine or placebo. To assess the anxiety of the participants, the state–trait anxiety inventory (STAI) test (Japanese STAI Form X-1, Sankyobo, Kyoto, Japan) was carried out before the pharmacy practice.

Theanine or placebo (lactose) tablets (200 mg, twice a day, after breakfast and lunch; Lyon et al., 2011; Kimura et al., 2007; Lu et al., 2004) were taken from 1 week prior to the pharmacy practice and continued for 10 days in the practice period, in total for 17 days.

The placebo tablet of lactose was in a similar color to a theanine tablet. A questionnaire including physical condition, subjective stress and achievement emotion was assigned for 10 days after each day's practice. The physical condition of the participant was assigned an ordinal scale (5, very good; 4, good; 3, normal; 2, a little bad; 1, bad). Subjective stress was evaluated using visual analog 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). Sleeping hours were also recorded.

2.3. Measurement of sAA

To assess the physiological stress response, sAA was measured using a colorimetric system (Nipro Co., Osaka, Japan; Yamaguchi et al., 2004). 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 a color of a reagent strip from yellow to white, which change is 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-IUBNB, 1992).

Saliva was collected twice a day, in the morning after waking up and in the evening after practice, for 10 days during the practice. Prior to sampling, participants washed their mouths with water. After saliva was collected for 30 s using a sampling tip, each participant measured own sAA immediately every morning and evening for 10 days (including unassigned days, (i.e., a weekend), which measurement was excluded in the analyses).

To establish a no-stress and no-medication baseline of sAA, the participants measured sAA every morning and evening for 10 days during routine daily life at the university. The measurement was carried out before the pharmacy practice.

2.4. Statistical analysis

All results are expressed as mean ± SEM. The influence of stress on sAA was evaluated by two-way ANOVA and the Bonferroni test for differences between means. Correlation coefficients were obtained using a statistical analysis program (StatPlus, AnalystSoft Inc., online version). The comparison of correlation coefficients between placebo- and theanine-groups was carried out using Fisher's z-test. In each analysis, a p value < 0.05 was considered to be statistically significant.

3. Results

3.1. Changes of sAA

There was no significant difference in sAA levels between in the morning and in the evening during routine daily life at the university

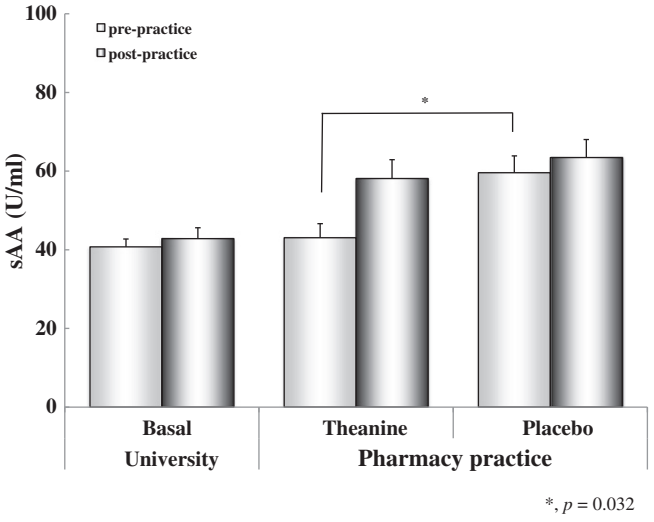

Fig. 1. Salivary α-amylase activity (sAA) of the participants during pharmacy practice was measured in the morning after waking up (pre-practice, gray bar) and in the evening after practice (post-practice, black bar). Theanine or placebo (lactose) tablets (200 mg, twice a day, after breakfast and lunch) were taken from 1 week prior to the pharmacy practice and continued for 10 days in the practice period. To assess a basal level, the participants measured sAA during daily life at the university with no-medication. The levels of sAA in unassigned days were not included in the analysis. Data are expressed as mean + SEM (*, $p = 0.032$).

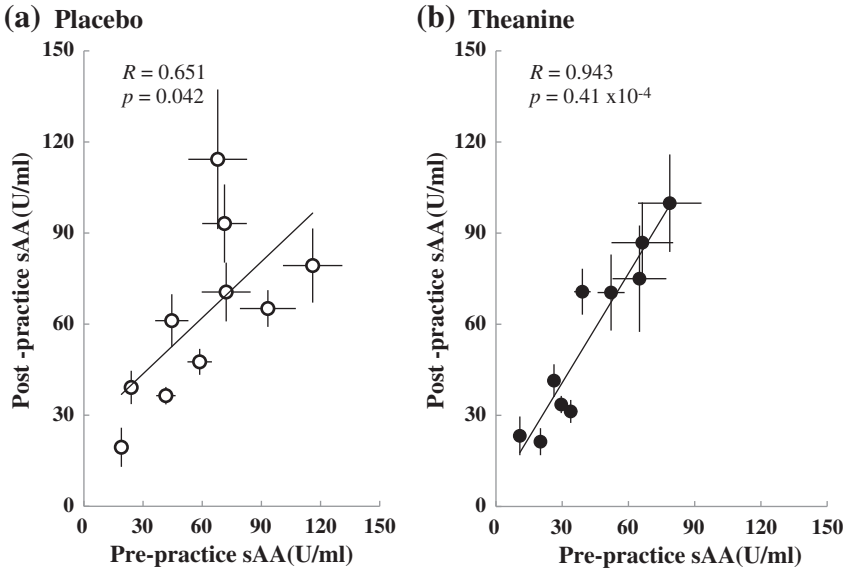

Fig. 2. Correlation between pre- and post-practice sAAs. (a), Placebo-group; (b), theanine-group. Each point of sAA represents the mean value of each participant that was calculated from sAA during pharmacy practice. Data are expressed as mean ± SEM.

(Fig. 1). During the practice, the level of pre-practice sAA (i.e., in the morning) (40 U/ml) was considered to be a baseline of sAA in the participants. The pre-practice sAA was significantly higher in the placebo-group than in the theanine-group ($p = 0.032$; two-way ANOVA) (Fig. 1). The theanine-group maintained the baseline observed during routine activity at the university. There was no significant difference in post-practice sAA (i.e., in the evening), however, the placebo-group showed a tendency of higher levels compared to the theanine-group ($p = 0.491$). In the theanine-group, the post-practice sAA tended to be higher than the pre-practice sAA ($p = 0.056$).

Considering individual variability, the mean values of pre- and post-practice sAA of each participant were analyzed. Participants of higher pre-practice sAA exhibited higher post-practice sAA (placebo, $R = 0.651$ and $p = 0.042$; theanine, $R = 0.943$ and $p = 0.41 \times 10^{-4}$; Fig. 2). The correlation between pre- and post-practice sAAs tended to be higher in the theanine-group than in the placebo-group ($p = 0.069$, Fisher-z test).

3.2. STAI value

The average STAI values were examined to assess anxiety based on the appraisal standard and there was no difference between two groups (Table 1). However, a positive correlation was observed between the values of STAI and pre-practice sAA in the placebo-group ($R = 0.742$, $p = 0.014$; Fig. 3a). Participants with high STAI value exhibited the high level of pre-practice sAA. In the theanine-group, a positive correlation between STAI and pre-practice sAA was similarly observed ($R = 0.560$, $p = 0.092$; Fig. 3b). The correlation coefficients between STAI value and post-practice sAA were low in both the placebo-group ($R = 0.505$, $p = 0.137$) and the theanine-group ($R = 0.580$, $p = 0.079$) (Fig. 3c and d).

Table 1
Characteristics of the placebo-group and the theanine-group.

	Placebo	Theanine
Age	22.5 ± 0.2	22.2 ± 0.1
Men/women	7/3	7/3
STAI value	41.0 ± 2.9	41.9 ± 2.4
Physical condition (score: 1–5)	3.65 ± 0.19	3.69 ± 0.23
Subjective stress (VAS: 0–10)	4.07 ± 0.33	3.10 ± 0.25*
Achievement emotion (score: 1–5)	3.26 ± 0.07	3.19 ± 0.06
Sleeping time (h)	6.20 ± 0.19	6.22 ± 0.25

* $p = 0.020$.

3.3. Subjective stress

Psychosocial stress was evaluated by each participant at the end of daily practice using VAS (0–10). The average score was significantly lower in the theanine-group than in the placebo-group ($p = 0.020$; one-way ANOVA; Table 1), which trait is notable from the first day of pharmacy practice (Fig. 4).

A positive correlation between subjective stress and post-practice sAA was observed both in the theanine-group ($R = 0.771$, $p = 0.0090$; Fig. 5b) and in the placebo-group ($R = 0.582$, $p = 0.077$: Fig. 5a). The correlation coefficients were not significantly different ($p > 0.05$, Fisher's z-test). Next, the participants were divided into three grades based on the subjective stress score (low: 0–3.4, middle: 3.5–6.4, and high: 6.5–10). The participants with low subjective stress exhibited significantly lower post-practice sAA in the theanine-group than in the placebo-group ($p = 0.0023$; one-way ANOVA; Table 2). No theanine-group participants showed high subjective stress. A close relationship between subjective stress and STAI was observed in theanine-group ($R = 0.866$ and $p = 0.0012$; Fig. 5d) but not in placebo-group (Fig. 5c).

3.4. Achievement emotion

Achievement emotion was evaluated by participants as an ordinal scale at the end of daily practice (Table 1). It was negatively correlated with post-practice sAA both in the placebo-group ($R = -0.440$, $p = 0.204$) and in the theanine-group ($R = -0.315$, $p = 0.376$) but not with STAI (Fig. 6).

3.5. Influence of physical condition and sleeping time

As pre-practice sAA was significantly lower in the theanine-group than in the placebo (Fig. 1), effects of physical condition and sleeping time were examined. No serious disturbance was reported in either theanine- or placebo-groups. The average values of physical condition and sleeping time were not different between the two groups (Table 1). No direct correlation between sAA and sleeping time was observed in each group (Fig. 7). However, when the sleeping time was compared between participants with low and high pre-practice sAA (with the cutoff

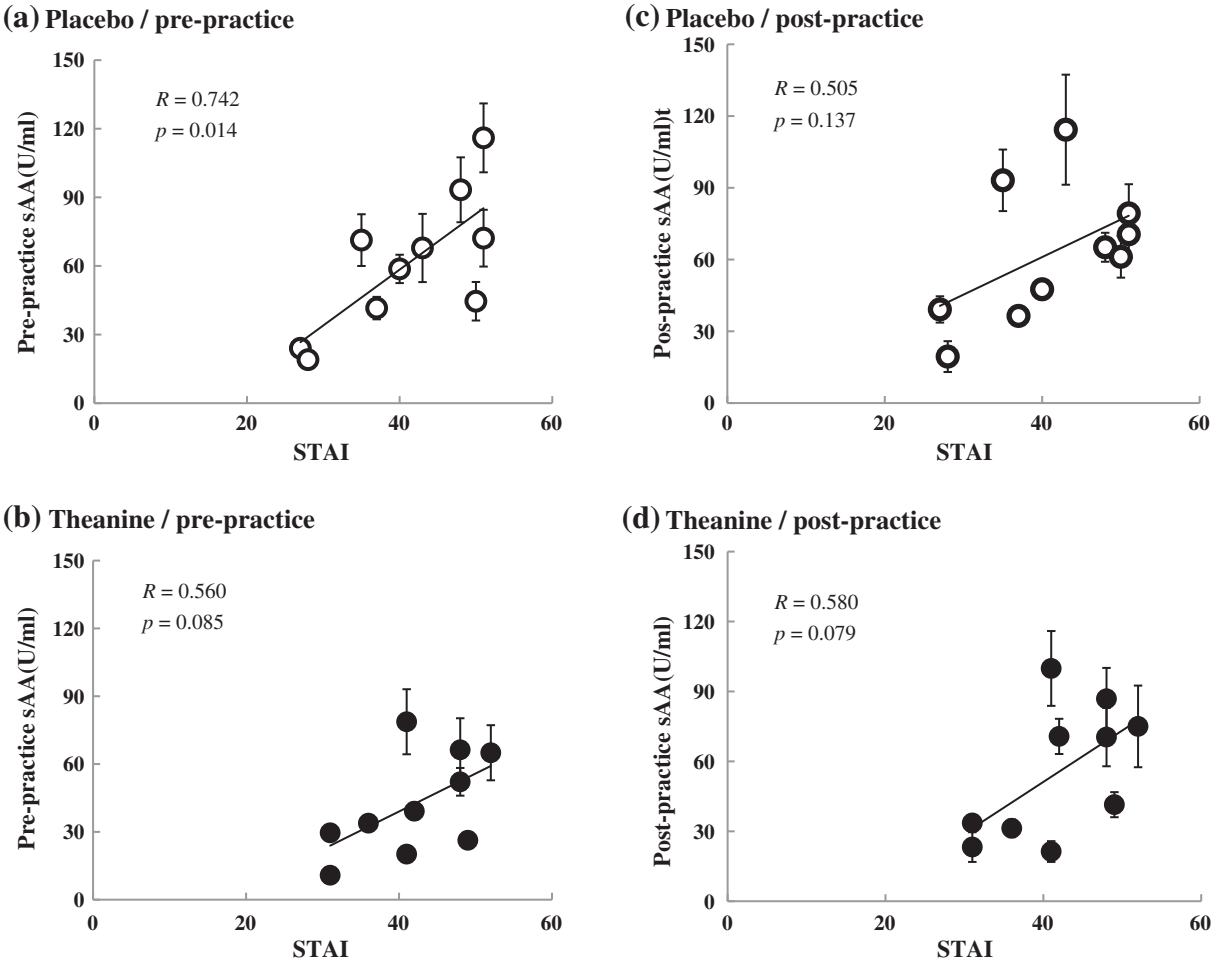

Fig. 3. Correlation between STAI and sAA. (a), STAI and pre-practice sAA of the placebo-group; (b), STAI and pre-practice sAA of the theanine-group; (c), STAI and post-practice sAA of the placebo-group; and (d), STAI and post-practice sAA of the theanine-group. Data are expressed as mean ± SEM.

value of 50 U/ml), it was significantly shorter in the participants with high pre-practice sAA ($p = 0.41 \times 10^{-3}$; one-way ANOVA). STAI value was also significantly higher in the participants with high pre-practice sAA ($p = 0.015$; one-way ANOVA; Table 3).

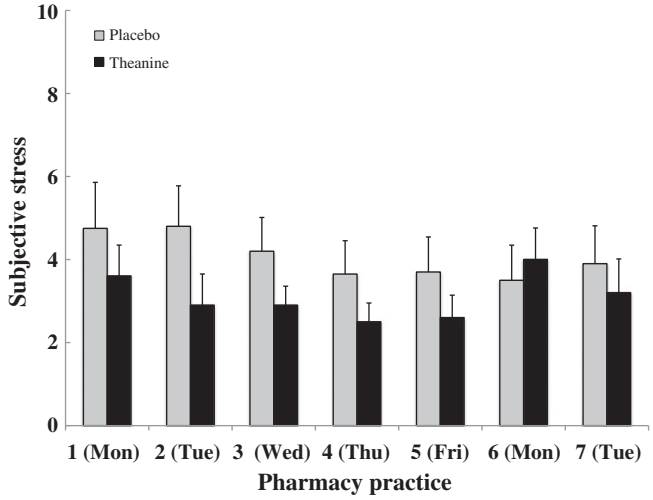

Fig. 4. Daily score of subjective stress for 7 days in placebo- (gray bar) and theanine-group (black bar). Data are expressed as mean ± SEM.

4. Discussion

4.1. Pharmacy practice during initial weeks is a stressful condition for students

It has been reported that sAA levels, a biomarker of ANS excitation, were low at the time of waking up (Adam et al., 2011) and the levels increased during the course of the day (Nater et al., 2007; Wingenfeld et al., 2010; Out et al., 2013). However, the circadian rhythm of sAA remains relatively constant throughout the day under a stress-free environment (Yamaguchi et al., 2006). Thus, in this study, the daily life at university was considered to be a stress-free state for the participants, which reflects the baseline of pre-practice sAA.

On the other hand, diurnal profile of sAA secretion has been reported to be altered by chronic stress or stress-related diseases. For example, levels of sAA after awakening increased rapidly in patients with posttraumatic stress disorder compared to healthy controls (Thoma et al., 2012). Diurnal sAA was associated with chronic stress and mood in healthy volunteers (Nater et al., 2007). In our study, increased levels of sAA in the morning and evening were observed in the participants of placebo-group during pharmacy practice. It suggests that pharmacy practice outside the university was a stressful condition for the participants, at least during the initial several weeks. While all the participants reported that they felt less stressed toward the end of the 11 week-practice (data not shown), theanine intake should be beneficial for reducing psychological burden during the initial weeks under stressful condition.

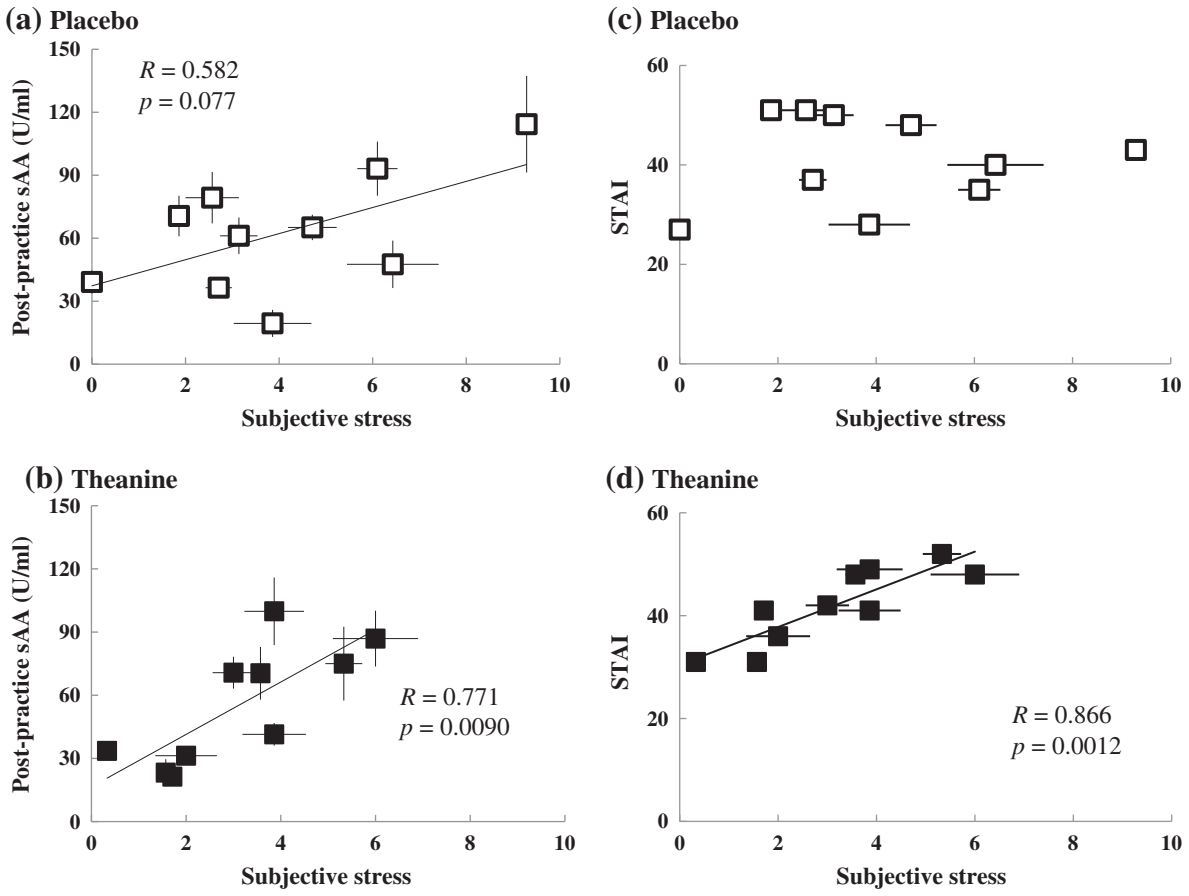

Fig. 5. Correlation between subjective stress and post-practice sAA (placebo, a; theanine, b), and between subjective stress and STAI (placebo, c; theanine, d). Data are expressed as mean ± SEM.

4.2. Interaction among sAA, trait anxiety and subjective stress

Although the levels of sAA considerably varied among the participants under the stressful condition, the pre-practice sAA was correlated with the STAI value, i.e., trait anxiety of each participant. The participants with low pre-practice sAA (<50 U/ml) exhibited significantly lower value of STAI than the participants of high pre-practice sAA (>50 U/ml) in both groups. These results indicate that trait anxiety of each participant essentially affects on the level of sAA. In addition, theanine intake had a role in suppressing the pre-practice sAA to the level of a non-stressful baseline under stressful condition. Although the post-practice sAA was increased, it was not statistically significant in the theanine-group. Moreover, the subjective stress was significantly lower in the theanine-group than in the placebo-group. The daily score of subjective stress was already low in the theanine-group from the first day of pharmacy practice, suggesting that the prior intake of theanine was effective for suppression of subjective stress.

In the participants with lower subjective stress (<3.4), post-practice sAA was significantly lower in the theanine-group than in the placebo-group. Some participants regarded the pharmacy practice as a relatively easy task, and reported low subjective stress. However, their nervous system could have been more excited than the self-reported stress level in the placebo-group. Thus, it is possible that they underestimated their true stress level. It has been reported that sAA was already high prior to the examination in dental school students with anticipation, while subjective distress was low (Robles et al., 2011). These results imply that the level of sAA is closely correlated with subjective stress, while excitation of the nervous system leads to higher sAA than the level of subjective stress.

As negative emotions such as anxiety, hopelessness and shame affect performance negatively (Pekrun et al., 2009), a participant with negative emotion could have evaluated his/her performance negatively. However, achievement emotion tended to correlate negatively to post-practice sAA but not to STAI.

Table 2
Relation between the level of subjective stress and post-practice sAA.

Group		Subjective stress		
		0–3.4	3.5–6.4	6.5–10
Placebo	n	5	4	1
	Post-practice sAA (U/ml)	57.3 ± 4.6	55.5 ± 6.4	114.3 ± 23.0
Theanine	n	5	5	0
	Post-practice sAA (U/ml)	37.7 ± 4.0*	68.1 ± 6.6	

* $p = 0.0023$.

4.3. Role of theanine in stress response

Orally consumed theanine is easily absorbed from the intestinal tract and partially transported into the brain competitively via the L-system at the blood–brain barrier (Yokogoshi et al., 1998; Terashima et al., 1999). The level of theanine intragastrically administered reached to the highest in the brain of rats after 5 h, and completely disappeared within 24 h (Terashima et al., 1999). In the theanine-group, the level of theanine in the brain might have been high from noon to evening and gradually decreased toward the next morning.

Theanine incorporation into the brain is reported to reduce the release of glutamate from presynapse to the synaptic cleft by strongly acting as a glutamine transporter (Kakuda et al., 2008; Kakuda, 2011). It then inhibits the incorporation of extracellular glutamine into neurons,

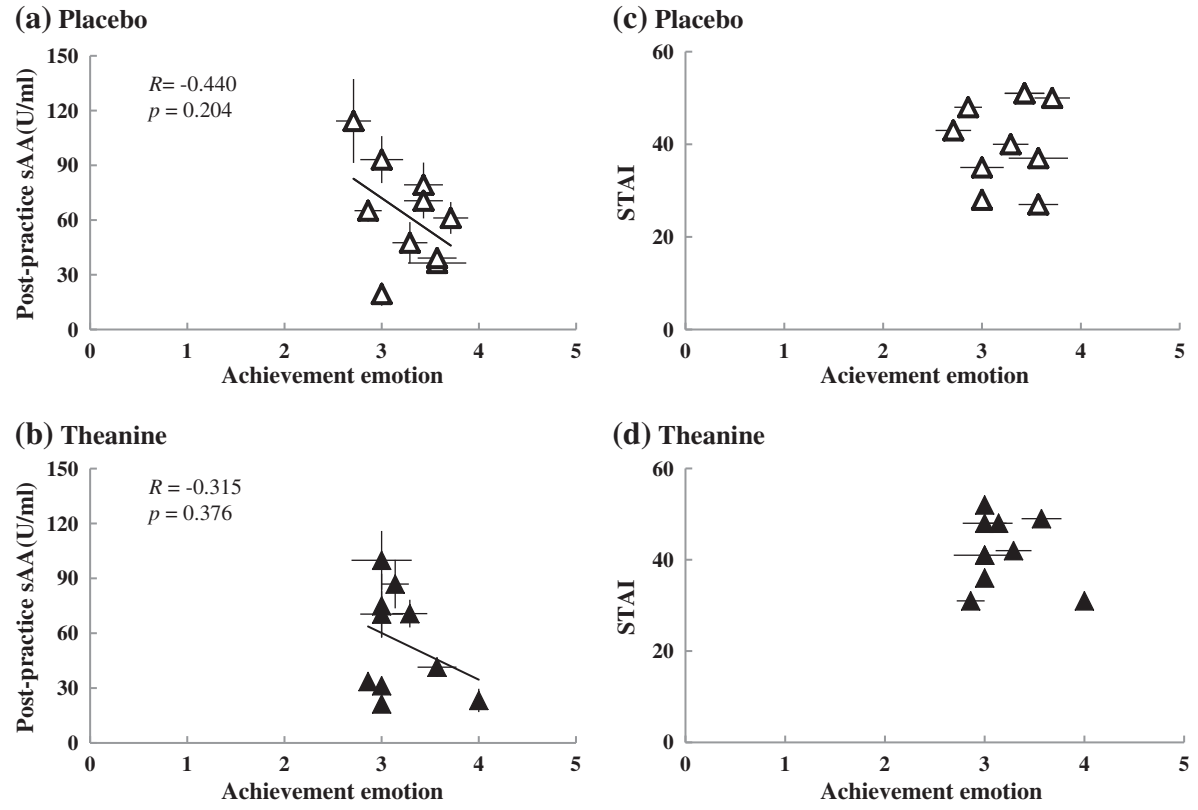

Fig. 6. Correlation between the achievement emotion and post-practice sAA (placebo, a; theanine, b), and between the achievement emotion and STAI value (placebo, c; theanine, d). Data are expressed as mean ± SEM.

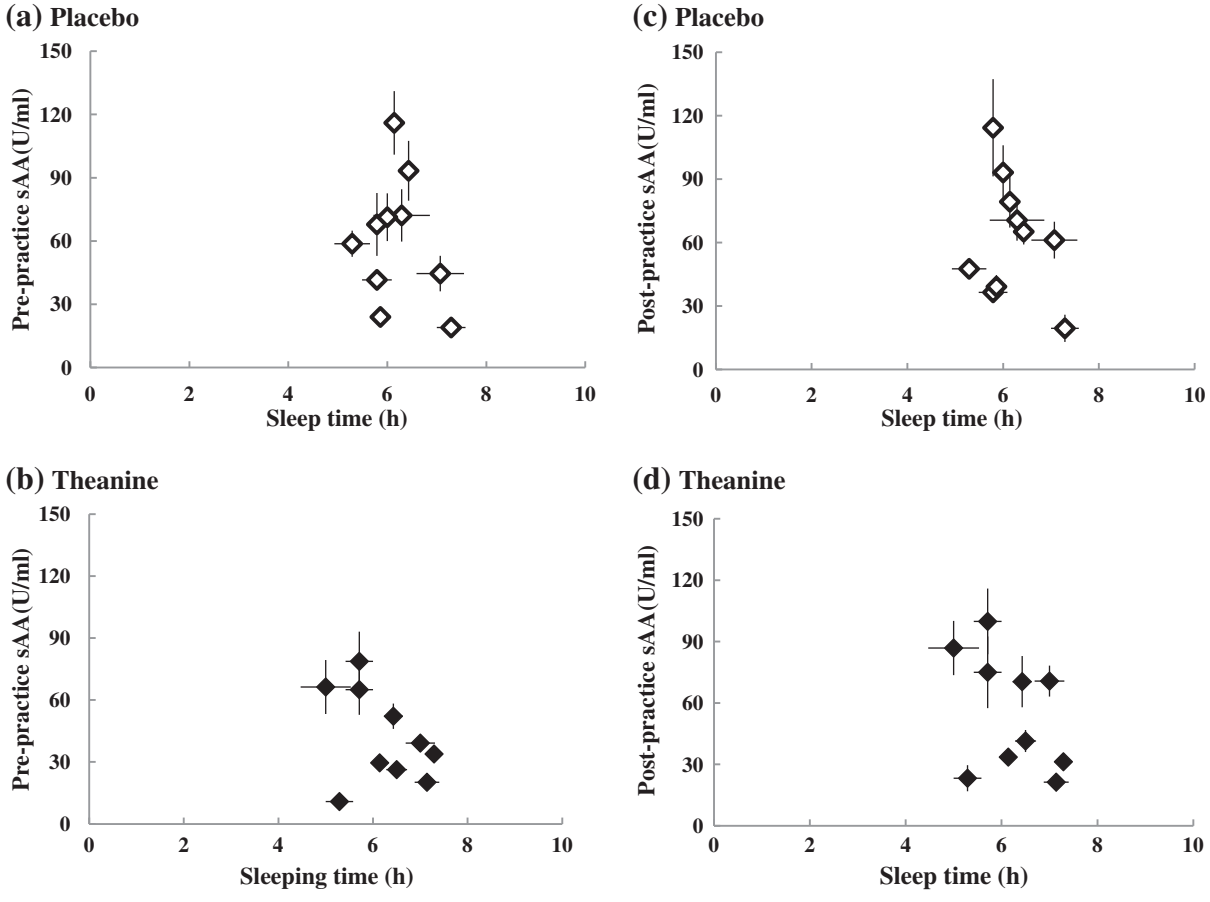

Fig. 7. Correlation between sleeping time and pre-practice sAA in the placebo-group (a and c) and in the theanine-group (b and d). Data are expressed as mean ± SEM.

Table 3
Relation between the level of pre-practice sAA and sleeping time.

Pre-practice sAA	Low		High	
	<50 U/ml		>50 U/ml	
Number of participants	Placebo 4	Theanine 6	Placebo 6	Theanine 4
Sleeping time (h)	6.54 ± 0.10		5.94 ± 0.13* (* $p = 0.41 \times 10^{-3}$)	
STAI	37.2 ± 2.6		45.7 ± 1.79* (* $p = 0.015$)	

which suppresses the conversion of glutamine to glutamate, a potent excitatory amino acid, by glutaminase. We have shown that theanine intake completely suppressed HPA-axis alteration and behavioral depression in mice (Unno et al., 2013). These results suggest that theanine has an anti-stress effect through the suppression of adverse alteration of the HPA axis under stressful condition. Therefore, theanine intake may suppress the ANS and HPA axis excitability by reducing glutamate release, and lead to the low subjective stress.

Theanine intake has been reported to be effective in improving sleep quality (Lyon et al., 2011). The sleeping time of participants with high sAA (>50 U/ml) was significantly shorter than participants with low sAA (<50 U/ml) in both groups (Table 3). It has been reported that in children with short sleeping time, sAA of the baseline and peak levels were higher after a standardized psychosocial stress test than in those with average sleep duration (Räikkönen et al., 2010). Taken together, it implies that a short sleeping period does not allow the excited nervous system to recover, not only in children but also in adults. While some individuals who slept for a short period of time exhibited low sAA in this study, short sleep may have overactivated the ANS. Theanine incorporated into the brain may be effective for resetting stress response to basal level during sleep. In a modern stressful life, excitation of the ANS and HPA axis is rather anticipated as a daily stress response. Therefore, it should be beneficial when one can suppress excessive excitation and reset it to a basal level by the next morning. It is of interest to examine effects of sleeping time and quality on excitation of the nervous system.

5. Conclusions

Anti-stress effect of theanine on humans was evaluated in the students during pharmacy practice. The levels of sAA were measured as a marker for sympathetic nervous system activity. Pre-practice sAA was significantly lower in the theanine-group than in the placebo-group. The level of pre-practice sAA was predominantly affected by trait anxiety. Post-practice sAA was positively correlated to subjective stress. Theanine ingestion significantly decreased subjective stress. Sufficient sleep would also be helpful for suppressing excessive excitation. Taken together, sAA is a useful biomarker for evaluating physical and psychological conditions and theanine intake has a significant anti-stress effect on humans.

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