Regular Article

Anti-stress Effect of β-Cryptoxanthin in Satsuma Mandarin Orange on Females

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Beta-cryptoxanthin (β-CRX, (3R)-β, β-caroten-3-ol) is an oxygenated carotenoid and a potent antioxidant that is abundant in Satsuma mandarin orange (*Citrus unshiu* Marc.), which is the most popular fruit in Japan. Since our preliminary data suggested that the ingestion of β-CRX had an anti-stress effect in female participants, the effect was evaluated in another set of female participants. The study design was a double-blind group comparison and participants ($n = 23$) were randomly assigned to β-CRX-rich orange juice or placebo (β-CRX was removed from orange juice) groups. β-CRX or placebo juice (125 mL, after breakfast) were consumed from 1 week prior to pharmacy practice and continued for 5 d into the practice period. Salivary α-amylase activity (sAA), a marker of sympathetic nervous system activity, was significantly higher in the evening than in the morning in the placebo-group during pharmacy practice, but not in the β-CRX-group. This result supports the anti-stress effect of β-CRX. The dose-dependency of β-CRX was observed in male mice that were loaded with stress. These results indicate that the ingestion of β-CRX is helpful to reduce stress.

Key words stress; clinical study; salivary α-amylase; orange juice

INTRODUCTION

Intervention of stress-induced alterations with dietary supplements is thought to be helpful for preventing the accumulation of stress and to be beneficial for a healthy life. For example, theanine, an amino acid unique to green tea, has been confirmed to exhibit an excellent stress-reducing effect in clinical and animal experiments.[1–4] Although caffeine in green tea counteracts the action of theanine,[5] green tea with lowered caffeine has been found to show an excellent stress-reducing effect in individuals in their 20 s, 40–50 s and 80–90 s.[6–8] There is a demand for these foods and dietary supplements in a modern stressful life. Beta-cryptoxanthin [β-CRX, (3R)-β, β-caroten-3-ol] is an oxygenated carotenoid and a potent antioxidant that is abundant in Satsuma mandarin orange (*Citrus unshiu* Marc., approx. 1 mg/100 g). This is the most popular fruit in Japan.[9] β-CRX is one of the major carotenoids present in the plasma along with lycopene, α- and β-carotene, lutein and zeaxanthin, whose plasma level was found to be 0.14–0.40 μM in males of five European countries[10] and 0.17 μM in Japanese males.[11] In Japan, β-CRX in the serum is mainly derived from Satsuma mandarin orange in Japan.[12] Therefore, people who eat many Satsuma mandarin oranges have higher levels of β-CRX in the serum (1.46 μM in females and 1.03 μM in males).[13] Previous studies have demonstrated that serum β-CRX is inversely associated with the risks of several lifestyle-related diseases such as metabolic syndrome,[14–16] low bone mineral density,[17] liver dysfunction,[15,18] and insulin resistance.[19–23] The high level of β-CRX in serum/plasma may have other beneficial functions for a healthy life.

The anti-stress effect of β-CRX on humans was preliminarily evaluated in fifth-year university students ($n = 20$; 12 females, 8 males) during their routine daily lives at the university and at a pharmacy practice (UMIN 000017165). In the β-CRX-group, the increase of post-practice salivary α-amylase activity (sAA) was suppressed in females. To confirm the effect of ingesting β-CRX, we examined the possibility of an anti-stress effect of β-CRX in another group of female participants. Furthermore, we examined the dose-dependence of β-CRX using mice that had been loaded with territorially-based stress. This model is suitable for evaluating the anti-stress effect of various compounds.[2,3] Based on these data, we explored the possibility of a new function of β-CRX on stress reduction.

MATERIALS AND METHODS

Preparation of Test and Placebo Juices The Satsuma mandarin orange juice with enhanced β-CRX (β-CRX juice) was a commercially available product (Ashitanokarada, Ehime Beverage Inc., Ehime, Japan) containing β-CRX derived from three oranges in a bottle (125 mL). The concentration of β-CRX in the β-CRX juice was 3.30 mg/125 mL. Placebo juice was prepared by removing β-CRX from the β-CRX juice by centrifugation and ultrafiltration membrane treatment (Ehime Beverage Inc.). The concentration of β-CRX in the placebo juice was below the detection limit (0.008 mg/100 g). However, the placebo juice still retained the orange juice taste and color. The measurement of β-CRX and removal of β-CRX from or-

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ange juice were conducted by Ehime Beverage Inc. The measurement of β-CRX was performed according to the method of Sumida *et al.*[24] with some modifications. The calorie and ingredients in β-CRX and placebo juices were compared using the methods of Ohshima *et al.*[18] In brief, the water content was measured by reduced pressure heating and a drying method. Protein, lipid and ash were measured by the Kjeldahl method, the ether extraction method and the direct ashing method, respectively. Carbohydrate content was obtained as follows: 100 − (water + protein + lipid + ash). Sodium and potassium contents were measured by atomic absorption. Total ascorbic acid was measured by high performance liquid chromatography. In this test, β-CRX and placebo juices were placed in white containers so that subjects could not distinguish between juices.

Participants The number of cases was decided by considering the results of the analysis of sAA, which was the main item used to evaluate the effectiveness of β-CRX in previous clinical research.[25] In this study, there were about twice as many female participants as in the previous study. Twenty-three healthy 5th-year female students (average age 22.4 ± 0.6 y) of the University of Shizuoka, who participated in the experiment, were randomly divided into two groups: the β-CRX group ($n = 12$) and the placebo group ($n = 11$). The participants were assigned to practice outside the university, in a hospital or a pharmacy, for 11 weeks. The first 5 d of the practice program were analyzed, because these days were assumed to be the most stressful. The juice intake period was about the same as the previous study. They were instructed to drink one bottle (125 mL) of β-CRX or placebo juice after breakfast, and were reminded not to forget to drink juice. One bottle of β-CRX juice contains β-CRX corresponding to three Satsuma mandarin oranges. The participants did not consume any β-CRX-rich fruits and confectioneries such as other citrus fruit, orange juice, and gummy or candy containing orange throughout the experiment. β-CRX or placebo juice were consumed from 1 week prior to pharmacy practice and continued for 5 d into the practice period.

A questionnaire that included feedback on physical condition, subjective stress and achievement emotion was assigned for 5 d after each day's practice. The physical condition of participants was assigned an ordinal scale (5, very good; 4, good; 3, normal; 2, slightly bad; 1, bad). Subjective stress was evaluated using visual analogue scales (VAS: 0–10) from very relaxed to highly stressed. Achievement emotion of each participant was assigned an ordinal scale (5, completely; 4, better; 3, a little better; 2, a little worse; 1, much worse) relative to the level of professional pharmacist. Sleeping hours were also recorded. None of the participants indicated acute or chronic disease, regular intake of medication, or habitual smoking. 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 of Japan, 2008). This study was approved in Japan, and the study protocol, which was approved by the Ethics Committee of the University of Shizuoka (No. 27–51), was registered at the University Hospital Medical Information Network (UMIN) (registration No. UMIN 000021775). The study period was from April to May 2016.

Measurement of sAA To assess the physiological stress response, sAA was measured using a colorimetric system (Nipro Co., Osaka, Japan).[26] One unit of 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 sAA) and in the evening after pharmacy practice (post-stress sAA), for 5 d during the 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 her own sAA immediately. Although the juice intake period was about the same as in a previous study,[25] the measurement of sAA was limited exclusively to pharmacy practice and was taken in triplicate to reduce measurement errors. Median data was used for analysis.

Measurement of STAI The state-trait anxiety inventory (STAI) is a self-survey method used to study a participant's anxiety status that consists of 40 questions on a self-report basis. State anxiety is anxiety about an event, and trait anxiety is the anxiety level as a personal characteristic. Higher scores are positively correlated with higher levels of anxiety. The STAI test (Japanese STAI Form X-1, Sankyobo, Kyoto, Japan) was carried out before pharmacy practice (pre-practice) and on the 5th day of pharmacy practice (post-practice). Both state and trait anxiety in pre- and post-practice were examined in this study, whereas only state anxiety in pre-practice was measured in a previous study.[25]

Animals and Stress Experiment Male ddY mice (Slc: ddY, 4 weeks old) were purchased from Japan SLC Co., Ltd. (Shizuoka, Japan) and kept under conventional conditions in a temperature- and humidity-controlled environment with a 12/12 h light/dark cycle (light period, 8:00 a.m.–8:00 p.m.; temperature, 23 ± 1°C; relative humidity, 55 ± 5%). Four-week-old mice were housed in a group of six in a cage for five days to allow them to adapt to co-habitation. Mice were fed a normal diet (CE-2; Clea Co., Ltd., Tokyo, Japan) and water *ad libitum*. All experimental protocols were approved by the University of Shizuoka Laboratory Animal Care Advisory Committee (approval No. 166197) and were in accordance with the guidelines of the U.S. National Institute of Health for the Care and Use of Laboratory Animals. To apply psychosocial stress to mice, confrontational housing was established in a standard polycarbonate cage that was divided into two identical subunits by a stainless steel partition as described previously.[3] In brief, two male mice were housed in a partitioned cage for six days (single housing) to establish territorial consciousness. Then, the partition was removed to expose the mice to confrontational stress for 24 h (confrontational housing). Since this experiment is based on male territoriality, no significant adrenal hypertrophy was observed in female mice under confrontational housing.[3] Therefore, the animal experiment was performed in male mice. Each cage was placed in a styrofoam box (width 30 cm, length 40 cm, height 15 cm) to avoid visual social contact between cages. At the end of the 24 h of confrontational housing, mice were sacrificed and adrenal glands were weighed.

Ingestion of β-CRX by Mice The effect of β-CRX was examined in mice (6–12 mice/group, $n = 54$). Mice consumed β-CRX juice diluted with regular water *ad libitum* for 7 d (single housing for six days and confrontational housing for 1 d). Mouse body weight was measured on the last day of the

experiment. The volume of β-CRX that was ingested was calculated from the drinking volume. The stress-reducing effect of β-CRX was compared among the six groups as follows: group 1 was group housing mice that drank water; group 2 was group housing mice that drank 5-times diluted β-CRX (5.3 μg/mL), corresponding to about 1.75 mg/kg; group 3 was confrontational housing mice that drank water; group 4 was confrontational housing mice that drank 5-times diluted β-CRX, corresponding to about 1.75 mg/kg; group 5 was confrontational housing mice that drank 16-times diluted β-CRX (1.65 μg/mL), corresponding to about 0.5 mg/kg; and group 6 was confrontational housing mice that drank 160-times diluted β-CRX (0.17 μg/mL), corresponding to about 0.05 mg/kg.

Table 1. Components of Consumption of Test- and Placebo-Juice

Juice (in 125 mL)	β-CRX	Placebo
Energy (kcal)	54	54
Water (g)	116.5	116.8
Protein (g)	0.9	0.5
Lipids (g)	—	—
Ash (g)	0.4	0.3
Carbohydrate (g)	13	13
Sodium (mg)	—	1.3
Potassium (mg)	179	165
Total ascorbic acid (mg)	38	39
β-CRX (mg)	3.3	$<$0.008

Statistical Analysis All results are expressed as mean $\pm$ standard error of the mean (S.E.M.). Differences in sAA were evaluated using one-way ANOVA followed by a Tukey–Kramer *post hoc* test for multiple comparisons, as well as simple linear regression analysis. All statistical analyses in the clinical study were carried out using SAS 9.4 (SAS Institute Inc., Cary, NC, U.S.A.). ANOVA and correlation coefficients in the animal experiment were obtained using a statistical analysis program (StatPlus, AnalystSoft Inc., online

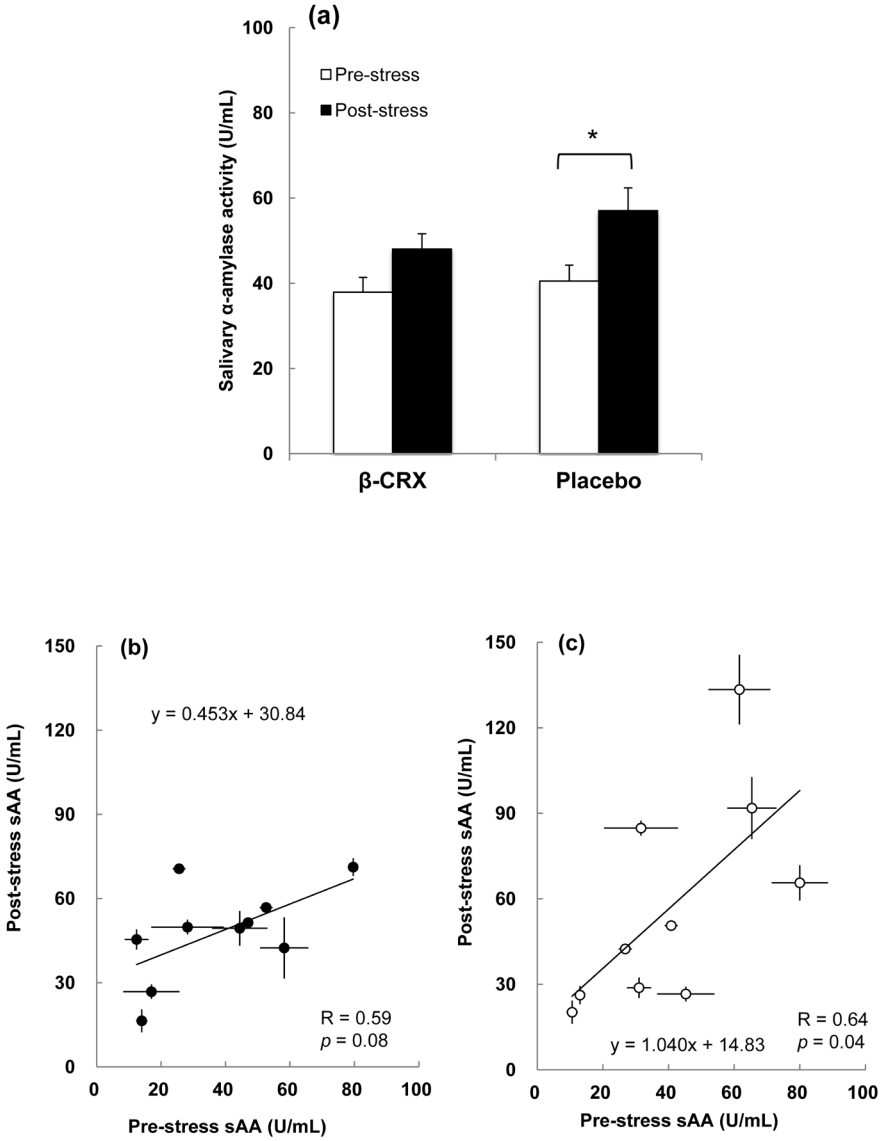

Fig. 1. Level of sAA in the Participants

(a) Open bar represents the level of pre-stress sAA that was measured in the morning after waking up, and the closed bar represents the level of post-stress sAA that was measured after a day of pharmacy practice. Each bar represents the mean $\pm$ S.E.M. (β-CRX, $n = 12$; placebo, $n = 11$; $^*p < 0.05$); (b) Correlation between pre-stress sAA and post-stress sAA in participants of the β-CRX group; (c) Correlation between pre-stress sAA and post-stress sAA in participants of the placebo group. Each point represents mean $\pm$ S.E.M. ($n = 5$).

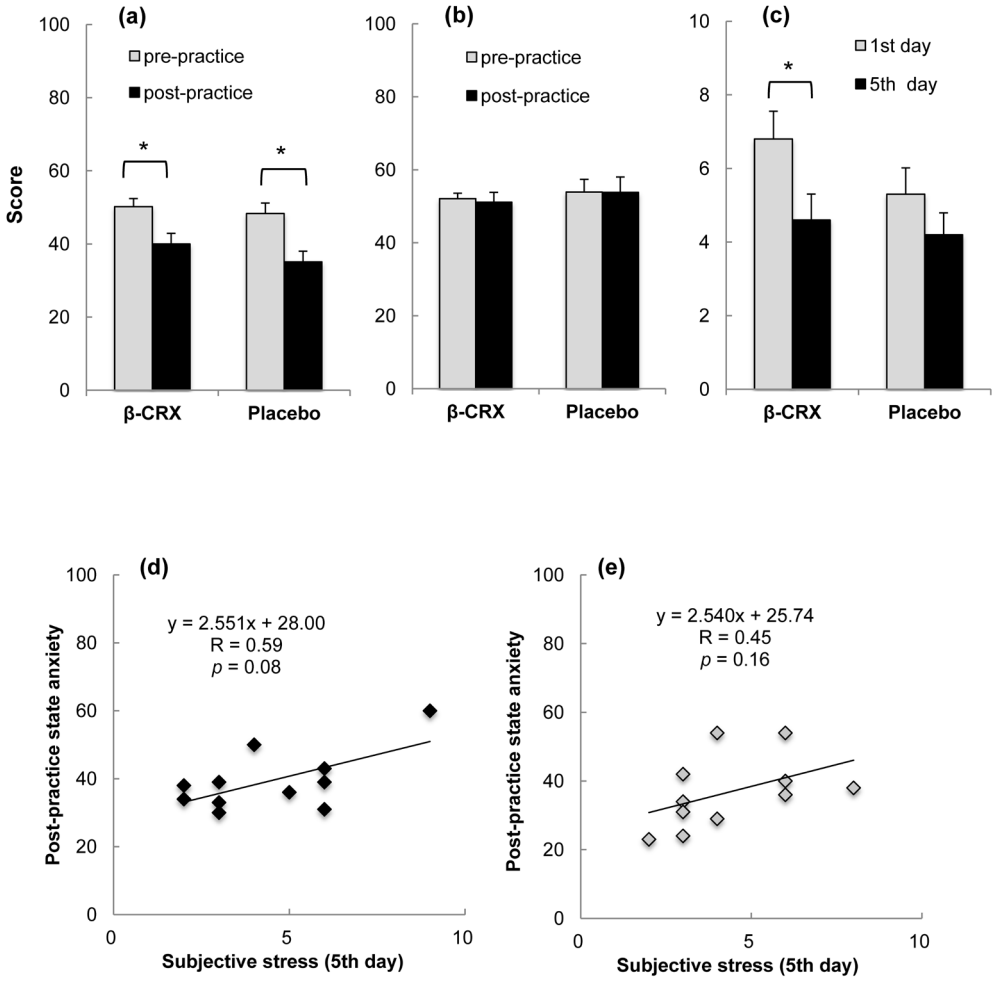

Fig. 2. The Levels of STAI and Subjective Stress Were Measured in the Participants of β-CRX and Placebo Groups

State anxiety (a) and trait anxiety (b) were measured one day before starting pharmacy practice (pre-practice) and on the fifth day of pharmacy practice (post-practice). Each bar represents the mean ± S.E.M. (β-CRX, $n = 12$; placebo, $n = 11$; $*p < 0.05$); (c) Subjective stress was evaluated using a visual analogue scale (VAS: 0–10) from very relaxed to highly stressed at the end of daily practice. The mean values on the first day and on the fifth day of pharmacy practice are shown. Each point represents the mean ± S.E.M. (β-CRX, $n = 12$; placebo, $n = 11$); correlation between subjective stress on the fifth day and the post-practice state of anxiety of the β-CRX group (d) and the placebo group (e).

version). Statistical analyses in animal studies were performed using a student's *t*-test and one-way ANOVA followed by Bonferroni's *post-hoc* test for multiple comparisons. In each analysis, a p value <0.05 was considered to be statistically significant.

RESULTS

Levels of sAA, Anxiety and Subjective Stress in Students There were few differences in calories and ingredients between β-CRX and placebo juices (Table 1). The placebo juice had the taste and color of orange juice. Thus, all the participants in both groups reported that the juice was delicious orange juice. During pharmacy practice, the level of post-stress sAA was significantly higher in the placebo-group than the level of pre-stress sAA ($p = 0.001$; one-way ANOVA) (Fig. 1a). In the β-CRX-group, the level of post-stress sAA tended to be higher, but not significantly, than the level of pre-stress sAA. Although there was a significant individual difference in sAA, the participants with low pre-stress sAA also had a low level of post-stress sAA. In the placebo group, the participants of high pre-stress sAA showed much higher post-stress sAA than the β-CRX group (Figs. 1b, c).

The average STAI values were examined to assess anxiety based on the appraisal standard. State anxiety was higher on the pre-practice day than on the post-practice day in both β-CRX and placebo groups (Fig. 2a). However, state anxiety on the pre- and post-practice days was not significantly different between β-CRX and placebo groups. Trait anxiety was not different between both groups (Fig. 2b).

Subjective stress in each participant was recorded at the end of daily practice using VAS (0–10). The average score tended to be lower on the fifth day than on the first day in both groups (Fig. 2c). Considering individual variability, the values of subjective stress on the fifth day and the post-practice state anxiety of each participant were compared. Participants with higher subjective stress exhibited higher post-practice state anxiety in both groups (Fig. 2d, e). Achievement emotion was evaluated in participants with an ordinal scale at the end of daily practice. There was no difference between the average of both groups (Table 2). The physical condition and average sleeping time were not different between both groups during pharmacy practice.

Anti-stress Effects of β-CRX in a Mouse Model of Psychosocial Stress The relationship between the intake of β-CRX and the suppression of stress was examined in a male

Table 2. Effect of Consumption of Test- and Placebo-Juice by Students on Psychosocial Responses, as Assessed from a Questionnaire

Questionnaire item	β-CRX-group	Placebo-group
Physical condition (1–5)	3.42 ± 0.14	3.64 ± 0.21
Achievement emotion (1–5)	3.25 ± 0.09	3.40 ± 0.16
Sleeping time (h)	6.20 ± 0.28	6.27 ± 0.19

The physical condition of participants was assigned an ordinal scale (5, very good; 4, good; 3, normal; 2, slightly bad; 1, bad). Achievement emotion was assigned an ordinal scale (5, completely; 4, better; 3, a little better; 2, a little worse; 1, much worse).

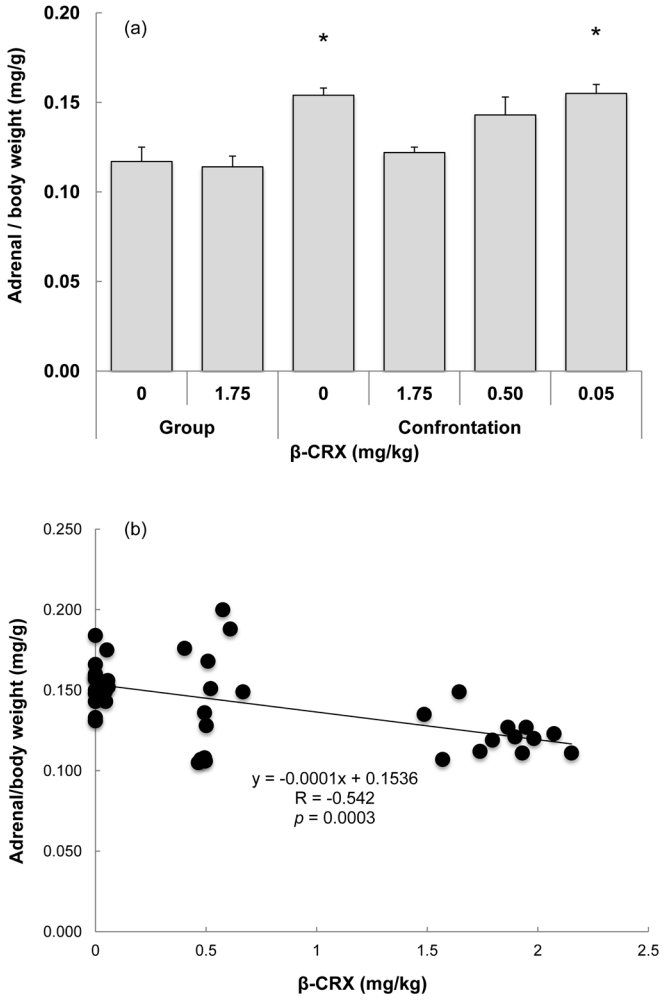

Fig. 3. Effect of β-CRX on the Adrenal Gland

Mice ingested β-CRX in drinking water that was consumed *ad libitum* for 7 d (single housing for 6 d and confrontational housing for 1 d). The weights of adrenal glands were measured one day after confrontational housing. Control mice similarly ingested β-CRX in drinking water for 7 d under group housing. The stress-reducing effect of β-CRX was compared among the six groups as follows: Group 1 was group housing mice that drank water; Group 2 was group housing mice that drank 5-times diluted β-CRX (5.3 μg/mL), corresponding to about 1.75 mg/kg; Group 3 was confrontational housing mice that drank water; Group 4 was confrontational housing mice that drank 5-times diluted β-CRX, corresponding to about 1.75 mg/kg; Group 5 was confrontational housing mice that drank 16-times diluted β-CRX (1.65 μg/mL), corresponding to about 0.5 mg/kg; and group 6 was confrontational housing mice that drank 160-times diluted β-CRX (0.17 μg/mL), corresponding to about 0.05 mg/kg. Adrenal weights were normalized by body weight. The adrenal weight in each group (a), and the effect of β-CRX intake in mice under confrontational housing (b) are shown. Each bar represents the mean ± S.E.M. ($n = 6$–12, $^*p < 0.05$).

mouse model. There was no difference in body weight or intake volume of juice between groups. The mean body weight of mice was 32.8 ± 0.3 g. The average intake volume of juice was 10.2 ± 0.3 mL. The weight of adrenal glands increased to about 5 mg in mice after confrontational housing from about 4 mg before confrontation. However, adrenal hypertrophy was significantly suppressed after the ingestion of β-CRX (Fig. 3a). A close correlation was observed between actual β-CRX consumption and normalized adrenal weight of confrontational mice (Fig. 3b).

DISCUSSION

This study was carried out to examine the anti-stress effect of β-CRX on humans. We preliminarily found that the ingestion of β-CRX reduced post-stress sAA in females during pharmacy practice.[25] Similarly, in this study with approximately twice the number of participants, post-practice sAA was significantly higher than pre-practice sAA in the placebo group. In contrast, the increase in post-practice sAA was suppressed in the β-CRX group. These results clarified that the ingestion of β-CRX reduced post-stress sAA in females. The level of sAA is low in the morning and rises according to activities during the day, but hardly changes under stress-free conditions.[27–30] In addition, lack of sleep increases the level of sAA in the morning.[31] As the mean sleeping time of the participants in this study exceeded 6 h in both β-CRX and placebo groups, there was no change in the levels of pre-stress sAA in these groups. On the other hand, the difference of post-stress sAA in β-CRX and placebo groups was considered to reflect difference in the stress response between these groups. Although there was a significant individual difference in sAA, the participants of higher pre-stress sAA showed higher post-stress sAA. The difference between β-CRX and placebo groups was clearly observed in the participants of higher pre-stress sAA. Lower sAA implies lower physiological and psychological stress,[32] indicating that the ingestion of β-CRX reduced stress. While these participants were sensitive to tension caused by new training at the pharmacy, excessive tension was suppressed in the β-CRX group. To reduce tension is important in people who are sensitive to stress, because excessive tension contributes to psychological stress.

On the other hand, as in a previous study,[25] there was no significant difference in the STAI value and subjective stress between the β-CRX and placebo groups in this study. Since participants in this study also reported that they enjoyed the pharmacy practice, it was considered that their state anxiety was not too high, even in the placebo group. It may be better to examine the effect of β-CRX in participants under another stressful condition in a future study. However, the participants displaying higher subjective stress also showed high values of anxiety. As the measurement of sAA based on physiological response is a sensitive biosensor,[33] it may give stress estimates more accurately than the STAI value and subjective stress based on psychological questions.

The anti-stress effect of β-CRX in females was observed at about 0.06 mg/kg (3.3 mg/50–60 kg/d), but it is necessary to examine if a lower amount of β-CRX can reduce stress. The stress response in mice showed that β-CRX can reduce stress dose-dependently. This result suggests that its stress-reducing effect may be observed in humans even at less than 3.3 mg/d. The correlation between blood β-CRX and sAA levels may need to be considered in the future. As this and previous studies are the result of young participants in their twenties, it is also necessary to examine middle-aged and elderly people to

assess if the anti-stress effect is age-dependent.

In conclusion, we examined whether the ingestion of *β*-CRX is able to reduce the stress response in female students. Post-stress sAA levels were significantly higher in the placebo group than in pre-stress sAA, but were suppressed in the *β*-CRX group. The ingestion of *β*-CRX may be helpful for reducing stress.

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Conflict of Interest The authors declare no conflict of interest.

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