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ORIGINAL ARTICLE

Salivary free radical-scavenging activity is affected by physical and mental activities

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OBJECTIVE AND DESIGN: Free radicals/reactive oxygen species (ROS) are related to inflammation, aging, and cancer. However, living systems have essential antioxidant mechanisms by which these harmful radicals can be scavenged, i.e., free radical-scavenging activity (FRSA). We measured the circadian rhythm of such activities by detecting salivary FRSA in healthy adults, and also examined how salivary FRSA is affected by physical and mental activities, which included (1) ingestion of beverage, (2) exercise, (3) comfortable/uncomfortable stimulation, and (4) smoking.

METHODS: FRSA was determined by using the DPPH (1,1′-diphenyl-2-picrylhydrazyl) method. Statistical analysis for experimentally obtained median values was carried out using the Wilcoxon signed rank test.

RESULTS: In circadian rhythm, FRSA was increased by food ingestion and relaxation. As to the individual activities, green tea and coffee ingestion increased FRSA, whereas swimming ($P < 0.05$) and dance lessons ($P < 0.01$) decreased it. Watching an amusing video program ($P < 0.001$) or stimulation by a pleasant aroma ($P < 0.01$) increased FRSA. In contrast, an unpleasant odor had no effect on FRSA. FRSA decreased immediately after smoking ($P < 0.05$), but increased thereafter ($P < 0.01$).

CONCLUSION: Salivary FRSA was affected not only by physical activities, but also by mental activities. It may be a parameter for reflecting the health status of individuals.
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Keywords: circadian rhythm; free radical-scavenging activity; comfortable/uncomfortable, ROS; saliva; smoking

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Introduction

Free radicals/reactive oxygen species (ROS) are involved in the pathogenesis of several diseases, such as chronic inflammatory diseases and cancer, as well as in aging (Halliwell *et al*, 1992). Living systems cannot avoid producing ROS, as free radicals are byproducts of oxygen consumption related to respiration (Davies, 1995). Therefore, the living body possesses several essential antioxidant systems, of which free radical-scavenging activity (FRSA) systems are very important for preventing oxidative stress. Oxidative stress is the condition in which a lack of balance exists between the levels of oxidant stimuli and the various antioxidants in biologic systems (Sies, 1997).

Currently, a large number of antioxidants are being investigated (Conklin, 2000; Borek, 2004). However, it remains controversial whether ingestion of foods and supplements with antioxidant actions directly contributes to the antioxidative status in biologic systems. Antioxidants in biological systems can be classified into three basic categories: (1) enzymes (e.g., superoxide dismutase, glutathione peroxidase, and catalase), (2) large molecules (e.g., albumin, ceruloplasmin, and ferritin), and (3) small molecules (e.g., ascorbic acid, α-tochopherol, β-carotene, plasma ubiquinol, uric acid, methionine, bilirubin, and glutathione). These antioxidants are either water-soluble or lipid-soluble molecules (Nagler *et al*, 2002). Individual antioxidant moieties play specific roles in combating oxidative stress, and measurement of single antioxidants may be beneficial (Sies, 1991). However, individual results may not be indicative of the overall effects of multiple antioxidants working in concert with one another. Therefore, an important index in oxidative stress studies may be the measurement of the total antioxidant potential of the biologic system. Therefore, the total antioxidant status should be a matter of focus.

However, total FRSA is not merely the sum of these individual mechanisms. FRSA may be associated with the antioxidant components previously reported as well as with unknown components, and may depend on the

synergistic effects of these components (Sanchez-Moreno, 2002; Serafini and Del Rio, 2004). Thus, the mechanism of FRSA *in vivo* remains to be clarified. Currently, the Total Radical Trapping Antioxidant Parameter method (Wayner *et al*, 1985), Oxygen Radical Absorbance Capacity (Delange and Glazer, 1989) method, scavenging activity of ABTS (radical cation 2,2-azinobis-(3-ethl-benzothiazoline 6- sulphonate) (Miller *et al*, 1993) and others are employed to measure total FRSA *in vivo*. These methods have both merits and demerits.

As we desired a simple procedure that would be suitable for measuring the influence of complicate physical and mental activities in human life on FRSA, we chose saliva as a test solution, because saliva has an FRSA in addition to immunologic and enzymatic systems, and a lower protein content than serum. Earlier we modified the DPPH (1,1-diphenyl-2-picrylhydrazyl) method, which is usually used for measuring the FRSA of natural products, to improve it as a new method for measuring total FRSA in saliva samples mixed with a 40% alcoholic solution of DPPH (pH 7.4) (Atsumi *et al*, 1999). The DPPH method was based on the reduction rate of stable DPPH radical. This method is rapid, simple, with good cost-effectiveness and high reproducibility; and, therefore, it facilitates the measurement of fluctuations in total FRSA due to subtle changes in vital activities without damaging the human body.

The human body is always exposed to stress, producing ROS (Frei and Higdon, 2003). Recently, we found that the smell of lavender or rosemary kinds of aroma decreased cortisol which is a kind of stress hormone, but increased FRSA in human saliva (Atsumi and Tonosaki, 2007). We also reported a comfortable stimulation whereby watching TV increased FRSA in saliva (Atsumi *et al*, 2004). Therefore, we hypothesized that factors involved in changes in FRSA must include both physical and mental activities. To test this hypothesis we used our new DPPH method to measure FRSA under various conditions involving such activities described in Subjects and methods. A causal linkage between these activities and the FRSA was discussed to clarify factors involved in changes in FRSA.

Subjects and methods

Subjects

The subjects were 36 healthy volunteers who understood the objective of this study and consented verbally to cooperate in the experiment according to the university guidelines. Age, gender, and number of subjects in each experiment are described in each figure legend. The subjects were instructed to avoid drinking/eating, smoking, and intense exercise for 1 h prior to the experiment.

Collection of saliva samples

The collection of saliva samples was started 10 min after the subjects had brushed their teeth for about 1 min without toothpaste to avoid any effects of food and drink. For the study of the circadian rhythm, the subjects always gargled mouthwash in their mouth and throat for about 1 min for collection at time points other than after ingestion of food, as it was impossible for them to brush their teeth every time. For the collection of saliva samples, the subjects were instructed to bite a piece of cotton wool (Salivette; Sarstedt Inc. Numbrecht, Germany) every second for 1 min to avoid the effect of salivary flow rate. Immediately after collection, the saliva samples were centrifuged at 1200 ***g*** for 10 min to isolate cotton wool filtrate (0.5–1.5 ml). The filtrate was then snap-frozen at −40°C, thawed, and additionally centrifuged at 13500 ***g*** to remove contaminating mucin. We excluded samples having a volume of 0.5 ml or less and those of pH 6 or lower. In addition, blood-contaminated samples were recognized by transferrin detection made with a Salivary blood contamination Enzyme Immunoassay Kit (Salmetrics, State College, PA, USA) and were excluded.

Quantification of FRSA

The former DPPH method (Atsumi *et al*, 1999) was modified. Briefly, each saliva sample (20 μl) was added to 0.2 mM DPPH in 40% ethanol solution (pH 7.4) in wells of a 96-hole plate. The absorbance at 540 nm was measured after 10 min by using a Labsystems Multiskan^{R} (Bichromatic, Helsinki, Finland) to obtain the amount of decrease in the DPPH concentration. FRSA was presented as micromoles of DPPH radicals scavenged by 1 ml of saliva.

Design of the study

In the present study, we first measured the circadian rhythm by salivary FRSA for three healthy adult subjects in daily life. Secondly, we measured FRSA before and after individual physical or mental activities such as (1) ingestion of beverage, (2) exercise, (3) comfortable/uncomfortable stimulation, and (4) smoking.

Experiment on the circadian rhythm

In three subjects (A: a 26-year-old male; B: a 32-year-old female; and C: a 45-year-old female), saliva was collected at 30-min intervals starting from the hour of rising (5:30–8:00) until bedtime (23:00–1:00), and FRSA value was determined. During these hours, the subjects lived their normal daily life (e.g., going to the office, working, taking meals, and resting). Therefore, the contents of work and meals differed among the subjects.

Experiment on a single stimulus

Saliva was collected before and after each of the following four activities (after which the FRSA was measured): (1) ingestion of green tea or coffee: green tea and coffee were bought from a vending machine, and the subjects drank 100 ml of either in one gulp. Saliva was then collected at 10-min intervals until 120 min after drinking (subjects A, B, and C, as described for the circadian rhythm); (2) swimming and dance lessons: a swimming lesson was given for 50 min. The contents of exercise differed among individuals (17 subjects: eight males, nine females, aged 6–16 years, mean age: 11.9 years). A dance lesson (same exercise) was taken for 1 h (37 subjects: young girls, aged 4–7 years, mean

age: 5.8 years); (3) comfortable/uncomfortable stimuli: (a) viewing a video program [general amusement program, 30 min (26 subjects: 17 males, 9 females, aged 19 to 30 years, mean age: 23.5 years)], and (b) the subjects smelled a pleasant/unpleasant odor for 10 min: the pleasant odor was a commercially available lavender essence oil (Charis Essential Oil, Krisu Seijo, Japan, No. 14D3T), which is used for aroma therapy, and was diluted with propylene glycol at a ratio of 1:25. The unpleasant odor was a standard malodorous substance, isovaleric acid (Daiichi Pharmaceutical Co., Ltd. Osaka, Japan, special grade) which was diluted with propylene glycol at a ratio of 1:50 (18 subjects: 11 males, 7 females, aged 20–29 years, mean age: 21.8 years); and (4) smoking: the subjects smoked a cigarette for about 5 min (14 subjects: 11 males, 3 females, mean age: 24.0 years).

Statistical analysis

In experiments on a single stimulus, there was no normality in the salivary FRSA value. Therefore, median values rather than averages were used for the tests. Wilcoxon signed-rank sum test was used for analysis of the significance of differences between the control (before stimulation) group and each stimulation group.

Results

Circadian rhythm by FRSA

Figure 1 shows the circadian rhythm of salivary FRSA in subjects A, B, and C. Furthermore, activities at the hour indicated by individual arrows are described in the legend. The arrows were classified into three types: arrow ① represents an increase in FRSA related to taking meals; arrow ② represents an increase in FRSA related to ingestion of drinks/snacks (sweets, tea, and fruits) other than meals; and arrow ③ represents an increase in FRSA related to other activities. In all subjects, FRSA increased after meals (arrow ①). It also increased after ingestion of other drinks/foods (arrow ②). The rate of increase differed among the subjects and with respect to the meal content. After ingestion, the FRSA gradually decreased. During these hours, the subjects worked, suggesting that fatigue reduced FRSA. Among the activities other than ingestion (arrow ③), FRSA increased with mental relaxation such as watching TV and smoking. However, several factors were complexly involved in the above increases, and in many cases, it was difficult to evaluate factors involved in changes in FRSA based on the circadian rhythm of FRSA alone. On the other hand, the minimum FRSA value was almost constant in the individual subjects; the minimum value was approximately 40 μmol ml^{-1} in subject A, approximately 10 μmol ml^{-1} in subject B, and approximately 20 μmol ml^{-1} in subject C.

Effect of ingestion of beverage

As shown in Figure 1, ingestion of beverage increased FRSA; therefore, we examined the influence of taking green tea or coffee itself. Figure 2 shows the changes

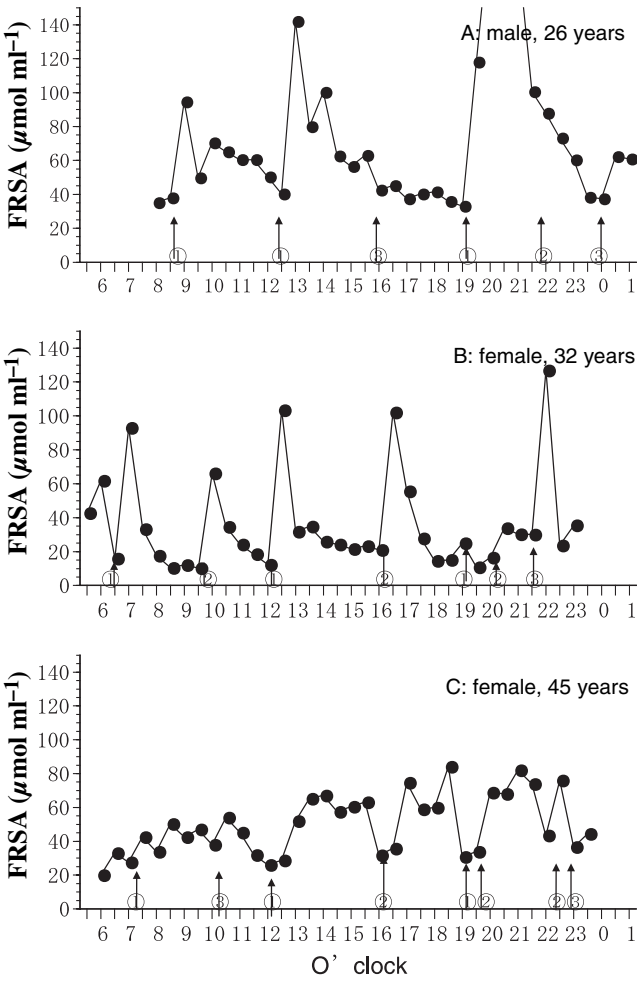

Figure 1 Circadianrhythms in salivary FRSA of three subjects (**A**, **B**, and **C**). The saliva was obtained from three volunteer subjects (A: male, 26 years old; **B**: female, 32 years old; **C**: female, 45 years) starting from their getting up to going to bed at 30-min intervals during the day. The behaviors of subjects were as follows. Subject **A**: 8:30, breakfast; 12:30, lunch; 14:00, smoking; 19:00, dinner; 22:00, smoking while watching TV; 0.00, bathing. Subject **B**: 6:30, breakfast; 9:30, tea; 12:00, lunch; 16:00, snack and tea; 19:00, dinner; 20:00, fruits and tea; 21:30, watching TV with dozing. Subject **C**: 7:00, breakfast; 10:00, meeting; 12:00, lunch; 16:00, tea and snack; 19:00, dinner; 19:30, cake and tea; 22:00, bathing. The salivary FRSA values were determined by the DPPH method described in the text

in FRSA values before and after ingestion of green tea (a) or coffee (b). The pre-drinking values for subjects (control) markedly differed from each other, resembling each minimum value shown in Figure 1. However, as indicated in the time–FRSA curves, these were similar behavior to that of the FRSA enhanced by ingestion of tea/coffee. The time at which FRSA reached its peak after ingestion was different in individual subjects; namely, that for subject A was 10 min after taking either beverage, whereas that for subject C was 30 min. For subject B the time was 10 min for green tea and 30 min for coffee. Thereafter, the FRSA for each subject gradually decreased. There was no marked difference in FRSA between green tea and coffee values.

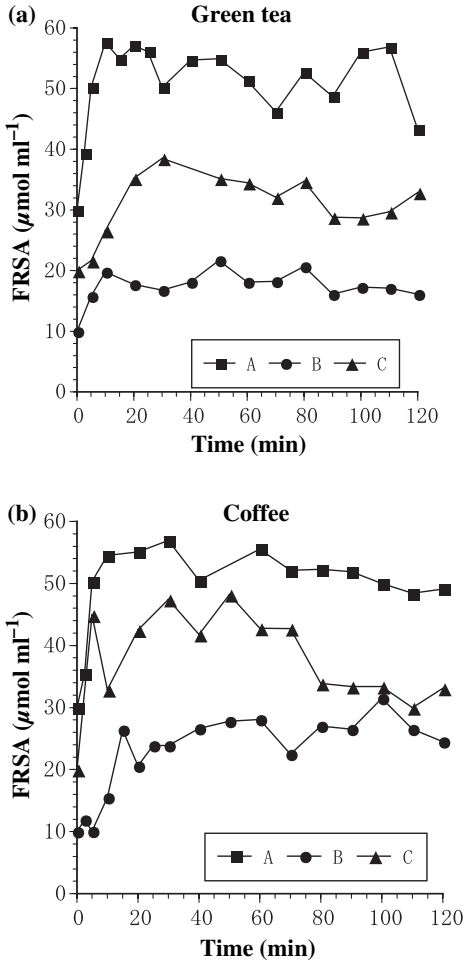

Figure 2 Changesin the salivary FRSA after drinking green tea or coffee. The subjects (A, B, and C: as in Figure 1) drank a cup of green tea (**a**) or coffee (**b**) (100 ml) obtained from a vending machine. The saliva was collected before drinking and then at 10-min intervals for 120 min afterwards. FRSA in the saliva was determined by the DPPH method

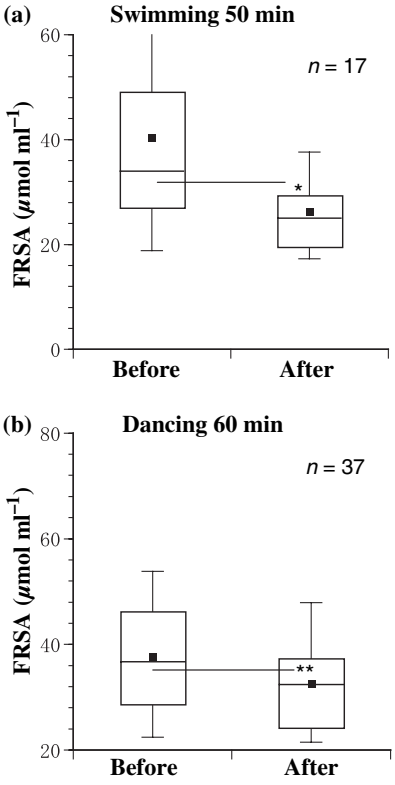

Figure 3 Changesin the salivary FRSA after swimming (**a**) or dancing (**b**). Saliva was obtained before and after the exercise from subjects as follows: (**a**) 17 subjects (boys and girls, mean age: 11.9 years) took a swimming lesson for 50 min and (**b**) 36 subjects (young females, mean age: 5.8 years) took a dance exercise for 1 h. Salivary FRSA was determined by the DPPH method. In the box-whisker plot, the median values are represented by the central line, and the mean values, by the square symbol, in the box. The horizontal lines in the regions below and above the box represent the 25% rank value and the 75% rank value, respectively, and the vertical lines in the regions above and below correspond to the range from 10% to 90%. The statistical difference between the median value before and after was determined by Wilcoxon signed-ranks test. Symbols represent significance of the difference between median values before and after exercise: $*P < 0.05$, $**P < 0.01$

Effect of physical exercise

As also shown in Figure 1, the FRSA gradually decreased after ingestion, possibly due to fatigue. Therefore, we measured the FRSA before and after intense exercise (Figure 3). For swimming (a), the median FRSA value before exercise was 34.0 μmol ml^{-1}; and it was significantly reduced to 25.0 μmol ml^{-1} after exercise ($P < 0.05$). Similarly, the median FRSA value before dancing (b) was 36.7 μmol ml^{-1}, and this value was significantly reduced to 32.4 μmol ml^{-1} after this activity ($P < 0.01$). These results suggest that the fatigue caused by physical exercise decreases the FRSA.

Effect of comfortable/uncomfortable stimuli

The median FRSA value before watching the video program was 39.9 μmol ml^{-1}, and it significantly ($P < 0.001$) increased to the level of 48.6 μmol ml^{-1} immediately after the subjects had finished watching the program (Figure 4). After surveying the subjects for their opinion regarding the video program, 21 out of 26 subjects reported that the program was 'interesting.' Therefore, the video program was considered to be a comfortable stimulus, and such stimuli may increase the FRSA.

We next examined the FRSA response to a pleasant odor (lavender) and to an unpleasant one (isovaleric acid). The median FRSA value before lavender stimulation was 35.9 μmol ml^{-1}, and it significantly increased to the level of 39.9 μmol ml^{-1} by 10 min after the stimulation ($P < 0.01$, Figure 5a). In contrast, the median FRSA values before and after isovaleric acid stimulation was 37.6 and 36.6 μmol ml^{-1}, respectively; there was no significant difference (Figure 5b). These results suggest that pleasant odors increase the FRSA, whereas unpleasant odors do not.

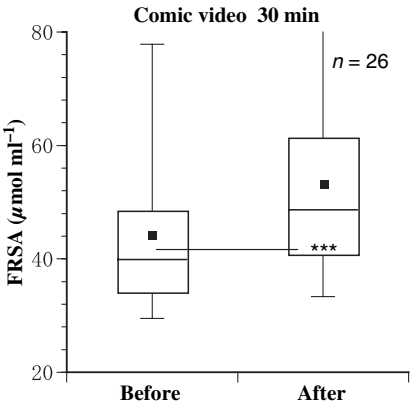

Figure 4 Changesin the salivary FRSA after watching a video. The saliva was collected from 26 subjects before and after watching a comic video for 30 min. FRSA in the saliva was measured by the DPPH method described in the text. The presentation in the box-whisker-plot is the same as in Figure 3. ***$P < 0.001$

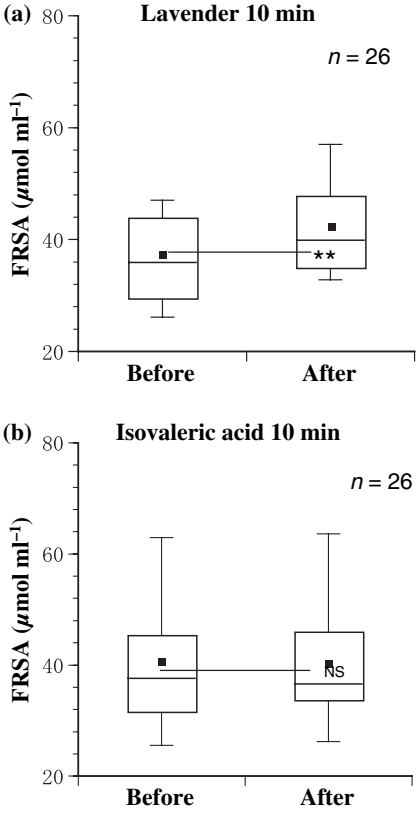

Figure 5 Changesin the salivary FRSA after stimulation with a pleasant or unpleasant odor. The subjects ($n = 26$) smelled the headspace in a wash bottle containing 5 ml of 25-fold diluted lavender essential oil (**a**) or 50-fold diluted isovaleric acid (**b**) for 10 min. Saliva was collected immediately before and after stimulation. FRSA was measured by the DPPH method. The presentation in the box-whisker plot is the same as in Figure 3. **$P < 0.01$, NS, not significant

Effect of smoking

The median FRSA values before smoking and immediately after smoking were 39.7 and 36.5 μmol ml^{-1}, respectively, which indicates a significant decrease

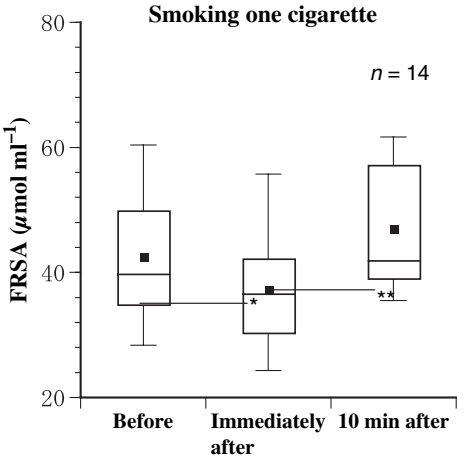

Figure 6 Changesin the salivary FRSA after smoking. The subjects ($n = 14$) smoked one cigarette for about 5 min. Saliva was collected from the subjects before, immediately after, and 10 min after smoking. Salivary FRSA was determined by the DPPH method described in the text. The presentation in the box-whisker plot is the same as in Figure 3. *$P < 0.05$, **$P < 0.01$

($P < 0.05$, Figure 6). Interestingly, the value after smoking then rose to 41.9 μmol ml^{-1} after a time lag of 10 min. There was a significant difference between the value immediately after and that at 10 min after smoking ($P < 0.01$, Figure 6).

Discussion

In the present study, there were marked individual differences in the salivary FRSA value when we evaluated the circadian rhythm (Figure 1), suggesting that many factors influence FRSA. Even though the FRSA was quite different among subjects, the biorhythm for each subject appeared to show a similar behavior after a meal or snack and during relaxation.

It is well known that green tea and coffee contain polyphenol molecules and these exhibit antioxidant activity (Frei and Higdon, 2003; Rietveld and Wiseman, 2003). The FRSA of green tea and coffee samples employed in this experiment was 36.5 and 33.0 mmol ml^{-1}, respectively (data not shown in Results). As shown in Figure 2, immediately after the intake of a cup of tea or coffee, the salivary FRSA increased rapidly by about twofold for pre-drinking value and reached the salivary maximum FRSA values of 30 μmol ml^{-1} (subject B), 50 μmol ml^{-1} (subject C), and 60 μmol ml^{-1} (subject A), suggesting that green tea/coffee polyphenol had been secreted into the saliva. No increase was observed when sugar was added in the coffee (data not shown). Thus, intake of a substance having antioxidant activity may play a crucial role in the biologic capacity of FRSA in the human body.

We next investigated the effect of exercise-related fatigue. The two kinds of exercise examined, swimming and dancing, were associated with a significant decreased in the FRSA (Figure 3). The reduction in FRSA may have been possibly related to the production of ROS caused by the physical exercise. Physical exercise

was previously reported to lead to the acute production of free radicals and other reactive species in animals and humans (Dillard *et al*, 1978; Banerjee *et al*, 2003; Holecek *et al*, 2004; Lamprecht *et al*, 2004) despite the health-promoting effects of physical activity.

In addition to the physical factors such as ingestion and fatigue, we investigated whether or not mental factors would affect the FRSA. Many studies have reported mental stress-related changes in physiologic actions such as immune, autonomic, and endocrine activities in human and rats (Bosch *et al*, 2003; Ishihara *et al*, 2003; Oishi *et al*, 2003; Bakke *et al*, 2004; Isowa *et al*, 2004). However, there have been only a few reports on the effect of mental activities in response to pleasant stimuli such as laughter (Berk *et al*, 2001), music (Hirokawa and Ohira, 2003) and humor (Christie and Moore, 2005) compared with the greater number regarding unpleasant stimuli. In most cases blood has been used as samples for biologic study. Therefore, the reason for few studies on the effect of pleasant stimuli is that the extracting of a blood sample is in itself considered to be an unpleasant stress. Using saliva samples, we found that stimulation with a comical video program (Figure 4) or with a pleasant odor, lavender, significantly increased FRSA (Figure 5a). In contrast, the unpleasant odor tested, isovaleric acid (wet sock-like odor), did not (Figure 5b). These results suggest that pleasant stimulation of the human body leads to increased FRSA regardless of the organ system stimulated. Berk *et al* (2001) reported that mirthful laughter positively modulated neuroimmune parameters such as natural killer activity and so on, but no earlier study has mentioned the relationship between mental activities perceived as pleasant and oxidative stress or FRSA. Atanackovic *et al* (2002) indicated that anger increased the blood levels of ROS and cortisol and decreased the number of natural killer cells. Conversely, the results of this study suggest that pleasant stimuli enhance the FRSA, possibly by preventing oxidative stress.

In the present study, the FRSA value decreased immediately after smoking (Figure 6), but significantly increased 10 min after smoking. This finding suggests that some stimulating substance contained in a cigarette, e.g., smoke free radicals or ROS, may reduce FRSA immediately after smoking, as formerly reported (Van Schooten *et al*, 2002; Nishizawa *et al*, 2005). However, the increase seen 10 min after smoking may be related to the relaxing effect of smoking, which is not immediate but corresponds to the time when the FRSA increased. Thus smoking may be thought to provide two stimuli: a physical stimulus that produces ROS and a subsequent mental stimulus that results in relaxation.

Furthermore, we found that the response of FRSA to mental activity appeared more promptly after stimulation than that to the physical action of ingestion or fatigue. Therefore, this method is useful for estimating mental effects, especially those perceived as pleasurable, on oxidative stress. As FRSA values are easily changed by physical and/or mental activity, the saliva obtained at the first time upon getting up is favorable as a health parameter. As salivary FRSA measured by the DPPH method is simple to obtain, it may be an important parameter for monitoring changes in the health status.

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