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Original Research Communication

Free Radical Scavenging Activity in the Nonenzymatic Fraction of Human Saliva: A Simple DPPH Assay Showing the Effect of Physical Exercise

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ABSTRACT

Free-radicals and other reactive oxygen species (ROS) have been implicated as being major damaging species in pathology and they have been widely investigated. Using 1,1'-diphenyl-2-picrylhydrazyl (DPPH), we estimated total free radical scavenging activity in the low-molecular-weight nonenzymatic fraction (LMNEF) of human whole saliva. The activity of the whole saliva and serum were measured in terms of the rate of decrease in the absorbance at 517 nm in a 40% ethanol DPPH solution (pH 7.4) at room temperature. The DPPH activity of saliva and serum showed a significant linear relationship. The mean DPPH activities of saliva from 257 subjects aged 4–72 was found to be 0.389±.190 μmol/ml and bore no relation to age or sex. The activity in saliva of 86 subjects aged 4–11 was significantly different before and after exhaustive aerobic dance exercise for 1 hr. Physical exercise markedly decreased free radical scavenging activity in whole saliva of children. On the basis of the above results, we concluded that DPPH is useful for evaluating the total antioxidant capacity of LMNEF of human saliva. Antiox. Redox Signal. 1, 537–546.

INTRODUCTION

REACTIVE OXYGEN SPECIES (ROS) react with the lipids of membranes and, through a series of reactions, generate alkoxyl or peroxyl radicals, all of which may cause damage to living cells. ROS have been implicated as etiological factors in a number of biological phenomena such as aging, mutagenesis, inflammation, and other pathological conditions (McCord, 1974; Petkau, 1980; Harman, 1984; Halliwell *et al.*, 1992). ROS was also reported that oxidative stress-damage is related to exhaustive exercise (Dillard *et al.*, 1978; Lovlin *et al.*, 1987; Loft *et al.*, 1994; Morales *et al.*, 1993; Sen, 1995; Ashton *et al.*, 1998). On the other hand, all living cells possess a number of protective mechanisms against an acute change caused by ROS. Because saliva is derived from blood, it would be expected to have a free radical scavenging activity comparable to that of blood. In fact, there are many reports showing the evidence of their similarity (Chapple *et al.*, 1997; Satoh *et al.*, 1997). For example, salivary peroxidase acts as a defense against oxidative damage (Carlsson, 1987), promotes healing of wounds (Bonder, 1991), and lessens the mutagenicity of carcinogens (Nishioka *et al.*, 1981).

It is difficult to measure ROS in saliva, because of their very short lifetime and relatively lower radical intensity. Many studies have addressed salivary defense effects against oxida-

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tive damages (Miller *et al.*, 1993; Nair *et al.*, 1995; Nagler *et al.*, 1997). Some attempts have been made with instruments such as electron spin resonance apparatus (Satoh *et al.*, 1997; Ashton *et al.*, 1998) and chemiluminometer (Chapple *et al.*, 1997). However, these methods need specialized equipment and require technical skill for the analysis. In the present study, we estimated the free radical scavenging activity of human whole saliva obtained from 257 subjects aged 4–72 by the reduction rate of 1,1-diphenyl-2-picrylhydrazyl (DPPH) (Blois, 1958), which has frequently been used to measure the antioxidative activity of natural foods such as tea catechin (Hatano *et al.*, 1989; Nanjo *et al.*, 1996; Yamaguchi *et al.*, 1998) and flavonoid (Ratty *et al.*, 1998). Furthermore, we studied the change in DPPH scavenging activity of whole saliva in 86 children before and after exhaustive aerobic dance exercise for 1 hr.

MATERIALS AND METHODS

Reagents

DPPH was obtained from Tokyo Kasei (Tokyo, Japan) and the chemical structure of DPPH is shown in Fig. 1. DL-α-Tocopherol, L-ascorbic acid, glutathione, uric acid, NaSCN, and KSCN were obtained from Wako Chemicals (Tokyo Japan).

Collection of saliva

The subjects were students and staff members in our university and members of some sport clubs in our community. They were healthy males and females, ranging in age from 4 to 72 years. Any individuals taking medication, including vitamins, were excluded from the study. The informed consent of each subject was obtained according to standard University guidelines. The salivary samples were

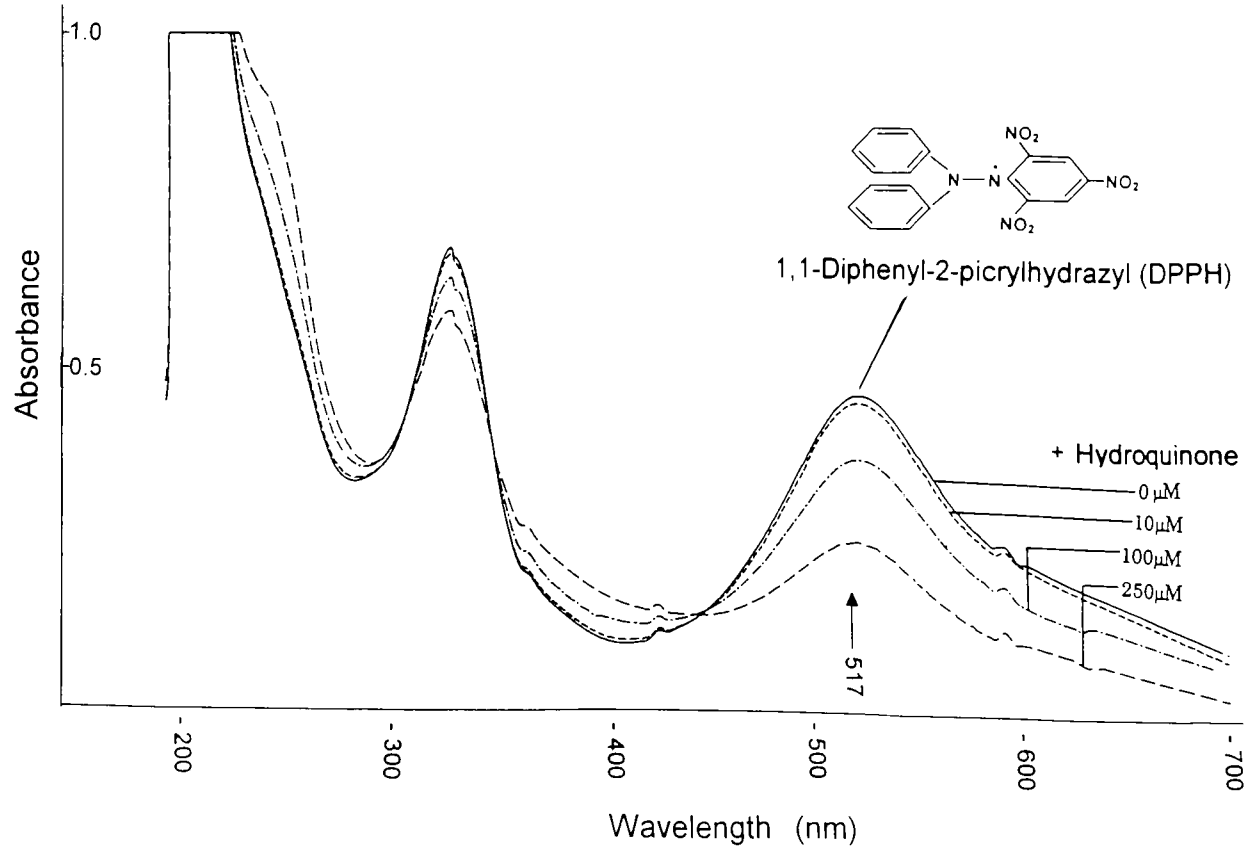

FIG. 1. Absorption spectra of DPPH in ethanol and the decrease in the absorbance at 517 nm by addition of a free radical scavenging agent, hydroquinone. DPPH (0.1 m*M*) was dissolved in ethanol. The upper right corner of the figure shows the chemical structure of DPPH.

taken at 10:00 AM to 12:00 noon or 3:00 to 5:00 PM to exclude the influence of the nutrition taken through a meal. These subjects were instructed not to eat, drink, smoke tobacco, chew gum, or engage in heavy physical exercise for 1 hr prior to collection. The subjects who performed strenuous aerobic dance exercise were 86 females, 4–11 years old. Salivary samples were gathered at 3:30 to 5:00 PM. After sufficient gargling with tap water, nonstimulated whole saliva (0.5–1.0 ml) was collected from each subject according to the standard guidelines for saliva collection (Atkinson *et al.*, 1993) and immediately applied to the experiment or stocked at −20°C.

Part of the collected saliva was applied to fractionation as follows: about 10 ml of saliva was pooled from several healthy volunteers and centrifuged at 10,000 rpm for 20 min. The resulting supernatant was boiled at 95°C for 10 min and then centrifuged at 10,000 rpm for 20 min again. Each pellet after centrifugation was resuspended up to the original volume.

Measurement of free radical scavenging activity of saliva

Saliva samples were homogenized in a Potter Elveljem homogenizer for 1 min in ice water to control for food debris and plaque material. A volume of 0.01–0.1 ml of sample was added to the DPPH solution (final volume was 1 ml), which contained 0.1 m*M* DPPH, 0.9% NaCl, and 25 m*M* HEPES buffer, pH 7.4, in 40% ethanol. When the volume of ethanol was less than 40%, DPPH was crystallized from the solution; and therefore the final ethanol concentration was kept at 40%. The solution stood for 10 min at room temperature in the dark and then centrifuged at 3,000 rpm for 10 min to remove precipitates of saliva. Then, the absorbance at 517 nm of the supernatant was measured with a spectrophotometer (Shimadzu UV 240). The free radical scavenging activity was calculated as (A_{517} control − A_{517} sample) ÷ A_{517} control. Samples having a higher value of activity than 0.5 (A_{517}) were diluted and were again measured repeatedly. The activity was presented as the concentration of DPPH scavenged per 1 ml of saliva.

Measurement of free radical scavenging activity of typical reagents

Ascorbic acid, glutathione, uric acid, KSCN, and NaSCN were dissolved in water at 11 concentrations ranging from 0.0001 m*M* to 10 m*M*. α-Tocopherol was dissolved in ethanol. Each sample was added to the DPPH solution. After the incubation for 10 min at room temperature, the absorbance at 517 nm in the sample solution was determined. The activity of each sample was expressed in terms of IC_{50} (the molar concentration required to reduce the DPPH radical level by 50%), which was calculated from log-dose curves.

Preparation of serum and its measurement of the free radical scavenging activity

About 2 ml of blood was gently withdrawn from an antecubital vein of 21 healthy human volunteers ranging in age from 20 to 26 years, and who were the same individuals used for saliva collections, by the use of 19-gauge needles; then the serum was separated by centrifugation at 4°C. Saliva samples from the same subjects were taken within 5 min after drawing of the blood. The serum and saliva were boiled at 95°C for 10 min and then were centrifuged at 10,000 rpm for 20 min. Each supernatant was measured for its free radical scavenging activity by the method described for the saliva sample.

RESULTS

Property of the DPPH method for measurement of the free radical scavenging activity in saliva

DPPH had an independent peak (λ_{max} at 517 nm) in the visual light spectrum in ethanol solution (Fig. 1). The peak height was reduced in the presence of hydroquinone, a typical free radical scavenging reagent, in a dose-dependent manner. The absorbance value at 517 nm of DPPH in 40% ethanol solution was almost constant in the range from pH 5 to 9 (Fig. 2), whereas after the addition of saliva (0.1 ml) it was constant in the pH range from pH 5 to 7.4. As the pH was increased gradually in pH value from pH 7.4 to 9, the absorbance of DPPH in

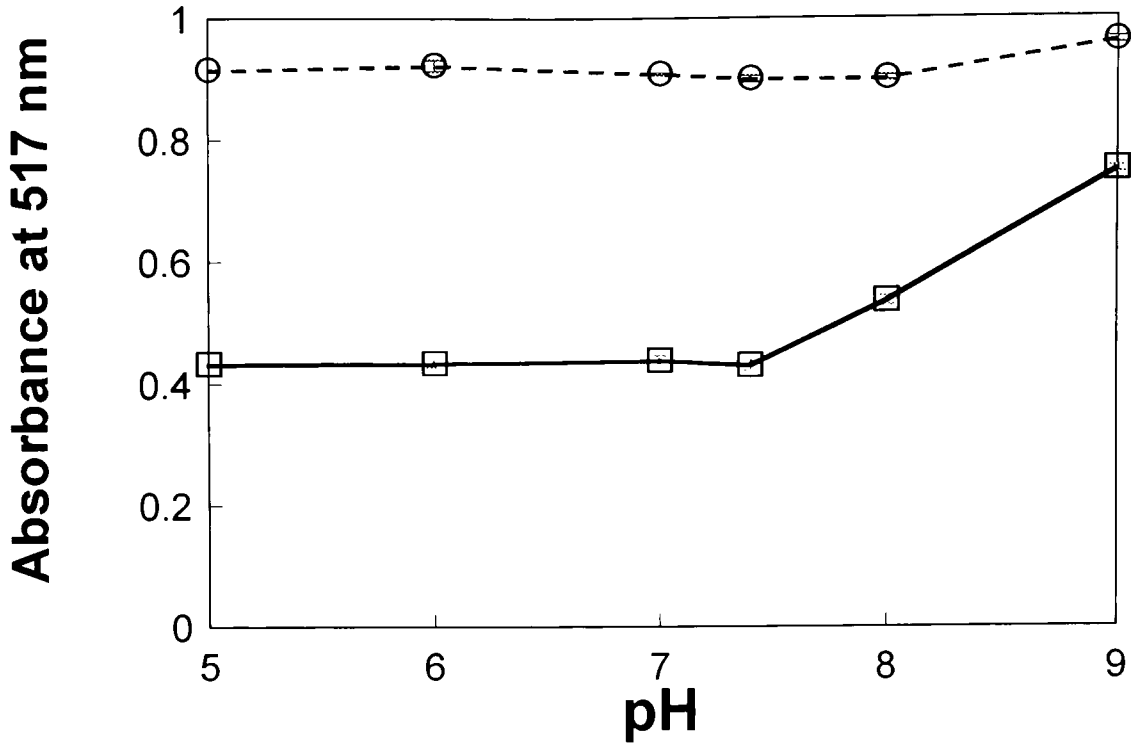

FIG. 2. Effect of pH on the absorbance at 517 nm of the DPPH solution with (□) and without (○) the addition of saliva. A saliva sample was taken from some subjects as described in the text. A volume of 0.1 ml of saliva was added to the DPPH solution (final volume was 1 ml), which consisted of 0.1 m*M* DPPH in ethanol, 0.9% NaCl, 25 m*M* HEPES buffer (pH 5–9; final volume [%] of ethanol: H_2O = 40:60). Each sample stood in the dark for 10 min at room temperature, and the absorbance of the supernatant was measured at 517 nm by a spectrophotometer following centrifugation at 3,000 rpm for 10 min. Data are the mean ± SD (n = 4).

the presence of saliva also was increased; therefore, by using the formula A_{517} of DPPH − A_{517} of (DPPH + saliva), the amount of DPPH scavenged, was correctly determined in the pH range from 5 to 7.4, which is the physiological pH of saliva. In this experiment, the pH of DPPH solution was set at pH 7.4.

Next, we studied the effects of temperature on DPPH. Figure 3a shows the absorbance at 517 nm of the DPPH solution versus the volume of saliva at 0°C, 23°C (room temperature), or 37°C. The absorbance at 517 nm was dependent on temperature. As the volume of saliva was increased, the absorbance at 517 nm was reduced in all three temperature groups. When no saliva was added to the DPPH solution, the absorbance at 517 nm decreased temperature-dependently: when that at 0°C was expressed as 100, that at 23°C was 83.7 and that at 37°C was 70.1. Therefore, the rate of decrease was calculated from the formula (A_{517} of control − A_{517} sample) ÷ A_{517} of control; plots of this rate as for volumes of saliva added are shown in Fig. 3b. Plotting of the decrease rate of A_{517} from 0 to 0.5 against volume of saliva showed a linear slope in both 0°C and 23°C, demonstrating that the activity of small amount of saliva sample (≦ 20 μl) can be correctly measured by the DPPH method at room temperature (20–25°C).

DPPH scavenging activity by nonprotein antioxidants

The DPPH scavenging activity in various fractions of whole saliva is shown in Fig. 4. The activity of pellet B obtained by centrifugation of the original saliva was thought to be mainly a plaque, showing that the activity was much weaker than that of the original saliva, A. D was a component derived from proteins because it was precipitated by heating of C. This fraction, however, had a very small activity. E was a heated supernatant of C and it was as al-

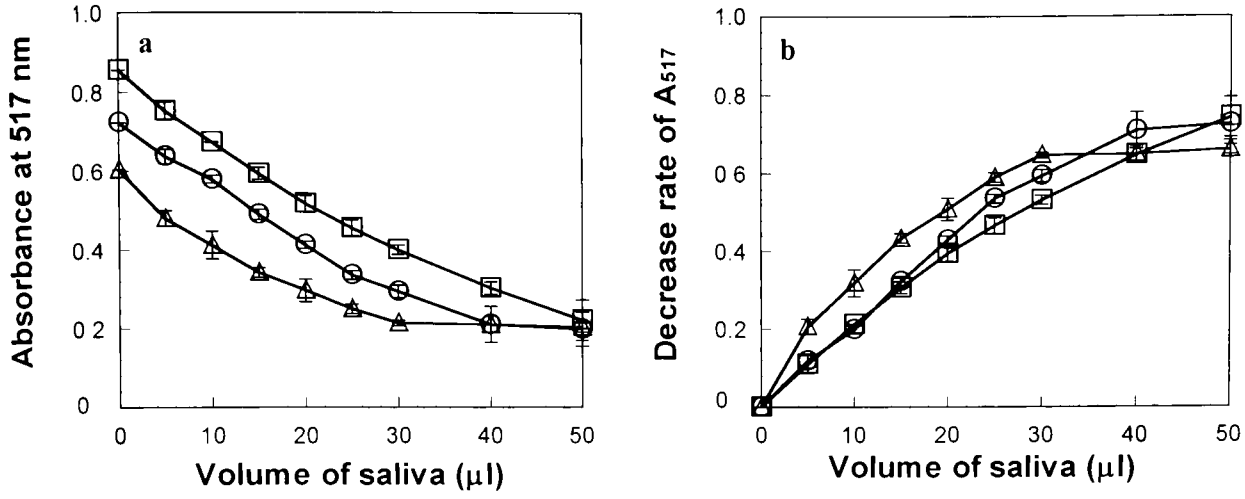

FIG. 3. Dose–response of absorbance at 517 nm to saliva volume (a) and the rate of decrease in A_{517nm} (b) after addition of saliva to the DPPH solution. Saliva collection and measurement of free radical scavenging activity of the collected saliva were the same as described in the legend of Fig. 2. The absorbance at 517 nm of the supernatant in the centrifuged DPPH solution was measured. The rate of decrease in A_{517} was calculated as (A_{517} control − A_{517} sample) ÷ A_{517} control. This experiment was performed at 0°C (□), 23°C (room temperature) (○), and 37°C (△). Data are the mean ± SD (n = 4). At 0°C and 23°C, linearity was achieved from the start point to the rate of decrease of 0.5. Data are the mean ± SD (n = 4).

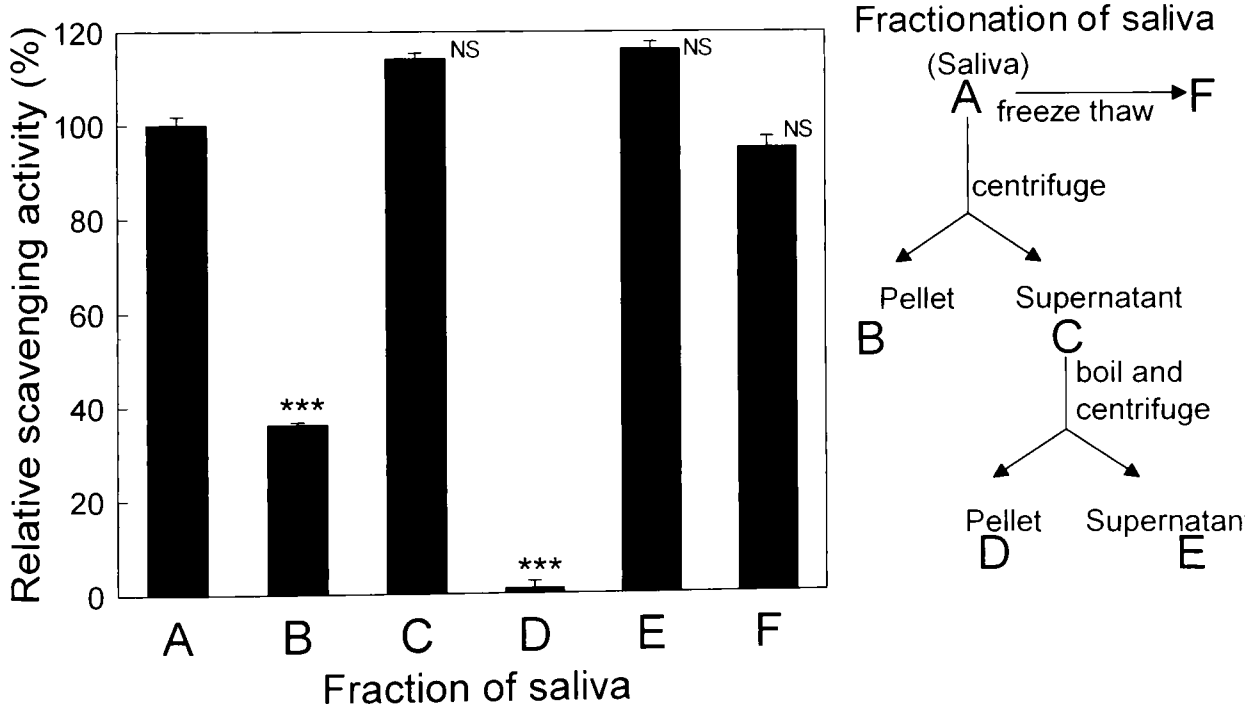

FIG. 4. Free radical scavenging activity of various fractions of saliva estimated by the DPPH method. Approximately 10 ml of nonstimulated whole saliva was pooled from several healthy volunteers and soon fractionated by the scheme illustrated at the right side of the figure. **A.** Original saliva; **B.** pellet of **A** obtained by centrifugation (10,000 rpm, 20 min); **C.** supernatant of **B**; **D.** precipitate formed by boiling of **C** (95°C, 10 min) and collected by centrifugation (10,000 rpm, 20 min); **E.** supernatant of **D**; **F.** **A** kept frozen at −80°C for 2 days and then thawed at room temperature. Each pellet or precipitation was resuspended up to the original volume. The measurement of the free radical scavenging activity was conducted as described in Materials and Methods. The data are expressed as relative activity with A as 100. Data are the mean ± SD (n = 4). Symbols indicate a significant difference from **A**: *** $p < 0.001$; NS, not significant, Student's t-test.

most as reactive as A or C. E was LMNEF. Additionally, F was a freeze-thawed saliva sample and its activity was equivalent to that of A. The results described above (Fig. 4) show that the scavenging activity of saliva assessed by the DPPH method did not change even if saliva was physically treated by either heating or by freeze-thawing. This demonstrated that the activity of the LMNEF in whole saliva was able to be measured correctly by heating the sample without laborious preparations using DPPH.

The scavenging activity of some typical nonprotein antioxidants, commonly contained in whole human saliva, were determined by this method. Plots of the A_{517} of DPPH as a function of increasing concentrations of the antioxidants such as α-tocopherol, ascorbic acid, glutathione, uric acid, NaSCN, or KSCN are shown in Fig. 5. The IC_{50} was estimated from the curves shown in Fig. 5. IC_{50} (unit; μmol/ml) increased as follows: α-tocopherol (0.0168) < ascorbic acid (0.0194) < glutathione (0.0734) < uric acid (0.514) < NaSCN (1.360) < KSCN (1.500). Antioxidants such as α-tocopherol, ascorbic acid, or glutathione showed a stronger scavenging effect, compared to inorganic antioxidants such NaSCN or KSCN.

Relationship between DPPH scavenging activity of saliva and serum

To study whether the free-radical scavenging activity of human whole saliva correlates with that of serum from human blood, we collected both saliva and serum from 21 subjects at the same time and the activity in each samples was determined by the DPPH method (Fig. 6). The linear regression curve with the positive correlation ($r = 0.770$, $p = 0.001$, single-regression Student's t-test) was obtained between saliva and serum, demonstrating that the DPPH scavenging activity of saliva significantly correlated with that of serum.

Free readical scavenging activity of human whole saliva by DPPH method and its effect of age or sex

Table 1 shows the free radical scavenging effect of saliva obtained from 257 subjects aged 4–72, as classified by age or sex. Average of the activity obtained from all subjects was 0.389 ± 0.190 μmol/ml; the activity for females was 0.369 ± 0.174 μmol/ml and that for males was 0.424 ± 0.222 μmol/ml. The activity appears to be enhanced with increasing age, and, in particular, to be stronger for males than for fe-

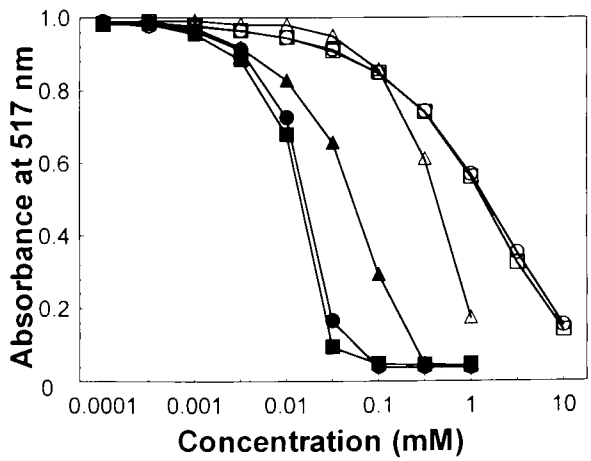

FIG. 5. Dose–response curve for scavenging of DPPH by various antioxidants: α-tocopherol (■), ascorbic acid (●), glutathione (▲), uric acid (△), NaSCN (□), and KSCN (○). Each was diluted to 11 appropriate concentrations (0.0001–10 m*M*). The free radical scavenging activity of tested solutions was measured as described in Materials and Methods.

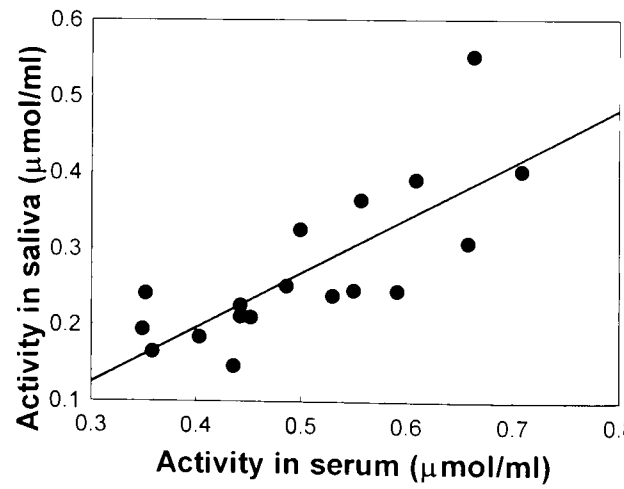

FIG. 6. Comparison of the free-radical activity in saliva and in serum from 21 subjects. Serum and saliva were collected at the same time from healthy male and female subjects (20–26 years old), and their free radical scavenging activity was measured as described in Materials and Methods. The regression line for the activity in saliva versus the activity in serum is shown ($r = 0.770$, $p > 0.001$; Student's t-test).

TABLE 1. FREE RADICAL SCAVENGING ACTIVITY IN HUMAN WHOLE SALIVA MEASURED BY THE DPPH METHOD AND CLASSIFICATION ACCORDING TO AGE OR SEX

Age (years)	*Sex*	*Number of subjects*	*Mean SD (μmol/ml)*
4–9	Female	77	0.377 ± 0.139
	Male	10	0.278 ± 0.143
10–19	Female	53	0.324 ± 0.189
	Male	13	0.322 ± 0.162
20–29	Female	15	0.333 ± 0.143
	Male	18	0.388 ± 0.158
30–39	Female	13	0.340 ± 0.162
	Male	10	0.463 ± 0.231
40–49	Female	10	0.485 ± 0.211
	Male	10	0.567 ± 0.213
50–59	Female	12	0.434 ± 0.281
	Male	10	0.561 ± 0.300
60–69	Female	4	0.434 ± 0.281
70–	Female	2	0.422 ± 0.059
Mean	Female	186	0.369 ± 0.174
	Male	71	0.424 ± 0.222
Total		257	0.389 ± 0.190

Saliva samples were collected from 257 subjects. Each subject thoroughly rinsed his or her mouth with tap water and then spit nonstimulated whole saliva, 0.5–1 ml, into a tube. The free radical scavenging activity of saliva samples was then measured. The activity is expressed as the molar concentration of DPPH scavenged by 1 ml of saliva. There was no significant difference in terms of age or sex.

TABLE 2. EFFECT OF PHYSICAL EXERCISE OF AEROBIC DANCE FOR 1 HR ON THE FREE RADICAL SCAVENGING ACTIVITY IN WHOLE SALIVA OF HUMAN CHILDREN MEASURED BY THE DPPH METHOD

Age (years)	*Number of subjects*	*Mean SD (μmol/ml)*	
		Before	*After*
4	7	0.357 ± 0.081	0.346 ± 0.077
5	8	0.360 ± 0.099	0.318 ± 0.121
6	9	0.413 ± 0.165	0.332 ± 0.127
7	14	0.429 ± 0.221	0.335 ± 0.104
8	17	0.400 ± 0.189	0.326 ± 0.109
9	14	0.383 ± 0.146	0.353 ± 0.148
10	6	0.397 ± 0.106	0.353 ± 0.266
11	11	0.447 ± 0.191	0.366 ± 0.114
Total	86	0.402 ± 0.184	0.340 ± 0.131***

Saliva samples were obtained from 86 young females aged 4–11 years before and after exhaustive aerobic dance exercise for 1 hr. *** shows significant difference at level of 0.1% from the values before on the same line (Student's *t*-test).

males. However, there was no significant difference between the activity and age or sex due to high standard deviation.

Free radical scavenging activity of the whole saliva of children before or after exhaustive dance exercise

The radical scavenging activity in saliva collected from 86 young females (4–11 years old) before and after the exhaustive exercise of aerobic dance for 1 hr was determined (Table 2). The average value before the exercise was 0.402 ± 0.184 μmol/ml, whereas that after was 0.340 ± 0.131 μmol/ml. Exercise significantly ($p < 0.001$) lowered free radical scavenging activity in whole saliva of children.

DISCUSSION

Saliva is known to be an important defense against the oxygen toxicity induced by ROS in the oral environment. For example, salivary peroxidase detoxifies hydrogen peroxide (H_2O_2) in the presence of the thiocyanate by converting into hypothiocyanite, dioxygen, and water (Carlsson, 1987). Salivary peroxidase (Mansson-Rahemtulla *et al.*, 1988; Al-Essa *et al.*, 1994; Ueta *et al.*, 1991) and lactoperoxidase (Mandel *et al.*, 1983) were reported to have defective activities against caries formation of teeth or tumor formation of salivary glands. But these studies mostly focused on the defense effects by enzymes. On the other hand, the low-molecular-weight nonenzymatic fraction (LMNEF) of saliva plays an important role because vitamins, hormones, or minerals contained in saliva act as radical scavenger. To evaluate the activity of the free radical scavenging effect of whole saliva, we focused on the LMNEF of saliva in this study.

To optimize the detection of DPPH activity, the effects of pH (Fig. 2), temperature (Fig. 3), or the ratio of ethanol to water in test solutions were examined and the measurement conditions were optimized. The DPPH scavenging activity of whole saliva was not found to be influenced by heating or freeze-thawing (Fig. 4), indicating that LMNEF of saliva represents a main source for scavenging the DPPH radicals in whole saliva. Antioxidants such as glutathione or uric acid have been previously reported to have a strong antioxidant activity

during exercise-induced oxidative stress (Inoue, 1993; Morales *et al.*, 1993; Sen, 1995). In general, glutathione or uric acid is contained in the low-molecular-weight fraction in saliva, and the activity of these compounds was efficiently measured in our study (Fig. 5).

Our results summarized in Table 2 show a significant correlation between fatigue and the DPPH scavenging activity of saliva. There are a number of studies concerning the relation between exercise and free-radical generation, but, in some instances, contradictory findings have been previously reported. For example, Lovlin *et al.* (1987), Davies *et al.* (1995), and Alessio (1993) reported that physical exercise was capable of inducing oxidative stress, whereas Viinikka *et al.* (1984) demonstrated no change in plasma peroxide levels during a long moderate exercise period. One of the reasons for this confusion is thought to be related to the numerous indirect indices used to assess free-radical damage (Viinikka *et al.*, 1984; Lovlin *et al.*, 1987; Morales *et al.*, 1993; Alessio, 1993; Inoue *et al.*, 1993; Davies *et al.*, 1995; Loft *et al.*, 1994). Our results support that exhaustive exercise depletes antioxidant defenses in human saliva.

Previously, some attempts have been made to assess the free radical scavenging effects of saliva. Nagler *et al.* (1997) measured the salicylate in saliva that was converted into catechol metabolites in the presence of metal ions such as iron, copper, or manganese. Nair *et al.* (1995) reported the formation of *o*- and *m*-tyrosine from phenylalanine as a marker of hydroxyl radical generation. Bodis and Haregewoin (1993, 1994) measured nitric oxide in human saliva. Satoh *et al.* (1997) measured ascorbate derivatives in saliva or blood by electron spin resonance (ESR). These methods measured specific oxidants or antioxidants in the saliva and did not provide much information regarding total radical-scavenging activity. Miller *et al.* (1993) and Moore *et al.* (1994) previously measured the total antioxidant activity in saliva by using 2,2′-azinobis-(3-ethylbenzo-thiazoline-6-sulfonic acid) (ABTS) radical cation. Chapple *et al.* (1997) estimated the total antioxidant activity of saliva using chemiluminescence with trolox. The current DPPH-based assay represents a more simple approach compared to both ABTS-based and chemiluminescent methods. Yamaguchi *et al.* (1998) have previously evaluated the DPPH-radical scavenging activity of colored foods by high-performance liquid chromatography (HPLC). HPLC was used due to the interference of absorbance between DPPH and food pigments. Also, reduction of the signal of the DPPH radical has been shown to be comparable with scavenging of the superoxide anion radical (Hatano *et al.*, 1989), but it did not always reflect the scavenging of the hydroxyl radical. Furthermore, we previously found that the activity of DPPH was affected with the steric hindrance of chemical structure of antioxidants with bulky groups such as butylated hydroxy toluene (unpublished data). Thus, the total activity of the radical scavenging effect of saliva might not be completely estimated by the DPPH method. However, a good relationship between the DPPH scavenging activity of serum and of saliva indicates that the DPPH method could be useful to evaluate effects on exhaustive-induced antioxidant depletion in saliva.

In the DPPH-based assay described here, there is some room for improvement of the process for whole saliva collection. To eliminate the effect of nutrition from a meal, one must collect saliva early in the morning. Also the longer the time required for the collection of saliva, the higher is its activity in our experiment. Because the secretion time of aged subjects tended to be longer than that of younger subjects, aged subjects may have shown higher values of the activity in our experiment. The application of a filter paper and/or sample sheet to the oral environment may be applicable to resolve this problem. Also, to clarify the activity of saliva, there are some probe-material factors in sampling subjects, such as smoking addition or oral diseases, in particular, marginal periodontitis. These limitations were noted while employing the described DPPH-based assay.

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ABBREVIATIONS

ABTS, 2,2′-azinobis-(3-ethylbenzo-thiazoline-6-sulfonic acid; DPPH, 1,1′-diphenyl-2-picrylhydrazyl; ESR, electron spin resonance; H_2O_2, hydrogen peroxide; HPLC, high-performance liquid chromatography; LMNEF, low-molecular-weight nonenzymatic fraction; ROS, reactive oxygen species.

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