Effect of Psychologic Stress on Peroxidase and Thiocyanate Levels in Human Saliva Detected by Ultraweak Chemiluminescence

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Saliva sampling has the advantage of being noninvasive and stress free. Based on a recent study, salivary ultraweak chemiluminescence (UCL) is a new biomarker of psychologic stress. However, it is not clear what causes changes in the UCL level and whether the change is biologically significant. We investigated the candidates for salivary UCL induced by psychological stressors and discuss the physiologic function of these candidates. Volunteers completed a questionnaire and then performed the Kraepelin test. Saliva was sampled just before, immediately after, and 30 min after the stress exposure. The UCL of saliva significantly increased just after stress exposure (1.56-fold) and returned to prestress levels after 30 min. The concentration of secretory immunoglobulin A also increased significantly and the change in both biomarkers was rapid. Similar significant changes were observed in salivary peroxidase activity and the concentration of thiocyanate (SCN^-). On the other hand, the levels of amylase activity did not significantly increase and the concentration of cortisol increased slowly. Moreover, in the reconstitution experiment, UCL was generated at the same level by a mixture of peroxidase and SCN^- at physiologic concentrations. In conclusion, we determined that the Kraepelin test as a mental arithmetic task elicited a significant response in the body and this response can be calculated using salivary UCL. Furthermore, SCN^- and peroxidase in the saliva play a key role in salivary UCL.

Key words —— saliva, ultraweak chemiluminescence, psychologic stress, peroxidase, thiocyanate

INTRODUCTION

Saliva contains various defense mechanisms to protect the body against infectious foreign agents in food and drink and against microorganisms. Numerous proteins such as secretory IgA (sIgA), lactoferrin, agglutin, and mucins also participate in the protection of oral tissues.[1–4] In addition, lysozyme and several peptides such as histatin, defensins, and LL-37 have been determined to have bactericidal activity.[5] Uric acid (UA) is the major component of the total antioxidant system, constituting 70% of total antioxidant capacity.[6, 7] Moreover, salivary peroxidase (Spx), thiocyanate ions (SCN^-), and hydrogen peroxidase (H_2O_2) constitute an antimicrobial system.[8–11]

Saliva has recently been used as a mental stress marker. The stress system coordinates the adaptive responses of the organism to stressors of any kind. The main components of the stress system are the corticotropin-releasing hormone (CRH) and locus ceruleus/norepinephrine (LC/NE) automatic systems. The CRH neurons and central catecholaminergic neurons of the LC/NE system innervate each other and are activated reciprocally. The hypothalamic/pituitary/adrenal (HPA) axis is controlled by several feedback loops that tend to normalize the time-integrated secretion of cortisol.[12] Therefore, cortisol has been used as an index for en-

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docrinologic stress.[13] In addition, the time-course changes in salivary amylase activity have been utilized as an index of psychologic stress using the Kraepelin test as a stress inducer.[14–16] The change in serum norepinephrine levels is delayed about 30 min in response to loading stress. In contrast, the secretion of salivary amylase is stimulated by direct innervation. This response is rapid, generally occurs in a few minutes, and is a markedly quicker response than that induced by hormonal regulation.[17,18] sIgA has also been measured because the duration of stress exposure may be an important factor in secretion rates.[19–21]

We previously demonstrated that the amount of salivary ultraweak chemiluminescence (UCL) based mainly on electronic transfer in the oxidation-reduction reaction changed before and after experimental examination under supervision that consisted of a serial aseptic manipulation for cell culture.[22] Therefore the level of salivary UCL may be a new biomarker to measure psychologic stress. However, it is not clear what causes changes in the UCL level and whether the change is biologically significant. Thus we studied: 1) the effects of the Kraepelin test as a psychologic stressor on salivary UCL in comparing amylase activity, levels of sIgA, and cortisol at the same time; and 2) the principles of the changes in UCL and whether the changes have biological significance.

MATERIALS AND METHODS

Experimental Design and Saliva Collection —— The volunteers were 23 male (17 nonsmokers and 6 smokers) and 23 female (nonsmokers) students at the University of Shizuoka School of Pharmaceutical Science, mean age ± standard deviation (S.D.) 21.6 ± 1.3 (range 20–25) and 21.9 ± 2.3 (range 20–29) years, respectively. The aim of the experiment was explained and consent was obtained after confirmation that they fully understood the experiment. The experiment was the Kraepelin psychodiagnostic test (KN type, Employment Research Co., Tokyo, Japan)[23] as a psychologic stressor for the volunteers and was conducted between 14:00 and 16:00, 1 hr or more after lunch. The test consisted of two parts. In the first half, continuous additions must be performed for 15 min (120 figures from 3 to 9 were printed at random in each row on a sheet of paper, where 15 rows of figures were printed in the first and second half, and volunteers were instructed to change the row every minute). After a pause for 5 min, they must work again on the second half for 15 min.

Volunteers were assigned a Salivette (Salstedt, Germany) to collect saliva according to manual instructions. During collection, the position of the Salivette was free in the oral cavity and therefore the collected saliva was whole saliva composed of parotid, submandibular, and sublingual secretions. Volunteers were placed in a sitting position in a quiet environment for 5 min to ease their psychologic stress, and then saliva was sampled just before, immediately after, and 30 min after the beginning of the experiment. Volunteers could move freely for 30 min after the test. The samples were stored at −30 °C until measurement. This experimental design was approved by the Ethics Committee of the University of Shizuoka.

Salivary Flow Rate, and Protein and Cortisol Assays —— Saliva was defrosted on ice and centrifuged at 1000 *g* for 20 min. Only the liquid component volume of saliva was measured and the salivary flow rate was expressed in milliliters per minute. Amylase activity was determined using the quantitative kinetic determination kit (Wako Co., Osaka, Japan).[24] Hemoglobin and sIgA levels were measured using ELISA. After 1 μg of mouse monoclonal antibody to human hemoglobin (originally established) was coated on an ELISA plate (Falcon Co., Franklin Lakes, NJ, U.S.A), the plate was blocked with 200 μl of PBS solution containing 0.5% BSA and 2.5% skim milk for 2 hr at 4 °C. Samples and standards were applied to each well and incubated for 18 hr at 4 °. After washing three times with PBS-Tween 20, 100 μl of goat IgG anti-human hemoglobin (Bethyl Co., Montgomery, Texas, U.S.A) was added to each well and incubated for 1 hr at room temperature. Samples were washed out and 100 μl of biotinylated rabbit IgG anti-goat IgG was added and incubated (Zymed Laboratories, San Francisco, CA, U.S.A) for 1 hr at room temperature. Finally, 100 μl of streptavidin-peroxidase (Sigma, St. Louis, MO, U.S.A) was incubated for 30 min at room temperature and color-developed using 2,2′-Azino-bis(3-ethylbenzothiazoline 6-sulfonic Acid) (ABTS) and H_2O_2 reagents for 20 min at room temperature. Absorbance was recorded at 415 nm, and hemoglobin concentrations were calculated from the standard curve. IgA levels were measured using peroxidase conjugated-goat antibody to human IgA as previously described.[22]

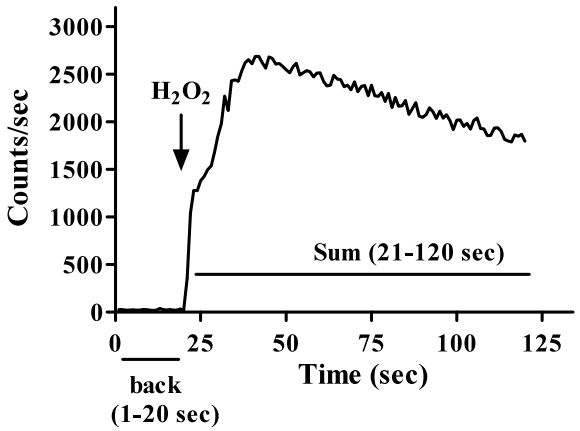

Fig. 1. Protocol to Detect UCL

After 3% (w/v) H_2O_2 solution was added to the saliva preparation, photon counts were conducted. The total number of counts for 100 sec was defined the level of UCL.

Salivary peroxidase activity was measured according to the 2,7-diaminofluorene (DAF) assay. The method relies upon the oxidation of DAF by the pseudo-peroxidase activity of hemoglobin.[25–27] Briefly, 50 μl of samples and standard were added to 96 wells plated in duplicate. Then, 150 μl of DAF 1 mM dissolved in 90% glacial acetic acid and urea 6 mM in Tris-phosphate buffer 100 mM (pH 7.0) was added to each well and incubated for 12 min at room temperature. Absorbance was recorded at 600 nm, and peroxidase activity was calculated from the standard curve. Determination of free salivary cortisol was done using an enzyme immunoassay kit (Salimetrics Co., State College, PA, U.S.A) with a lower limit of sensitivity of < 0.007 μg/dl.[28]

Detection of UCL —— Detection of salivary UCL was measured as described previously.[22] In brief, 200 μl of saliva was placed on filter paper (Advantec Co., Tokyo, Japan) in a plastic dish (Nunc Co., Tokyo, Japan) and 1 ml of gallic acid 3 mM was added. After mixing for 20 sec at room temperature, the dish was set in the UCL measuring counter C767 (Hamamatsu Photonics, Hamamatsu, Japan). After adding 1 ml of 3% (w/v) hydrogen peroxide (H_2O_2) to the saliva preparation, the reaction commenced. The UCL in the present study was defined as the total number of photons for 100 sec after the addition of H_2O_2 (see Fig. 1).

Determination of UA Concentration —— UA concentration was measured with a kit (Wako Co.). In this assay, UA was transformed by uricase into allantoin and H_2O_2, which, under the catalytic influence of peroxidase, oxidized the chromogen (4-aminophenazone/N-ethyl-methylaniline propanesulfonate sodium) to form a red compound of which the intensity of color was proportional to the amount of UA present in the sample; it was read at a wavelength of 540 nm.

Determination of SCN^- Concentration —— The concentration of SCN^- was measured spectrophotometrically as described by Aune and Thomas.[29] In brief, 100 μl of samples was added to a mixture of 800 μl of HCl 0.1 M and 100 μl of ferric chloride 0.1 M. After centrifugation at 1000 *g* for 1 min, absorbance of the supernatant due to $FeSCN^{2+}$ was measured at 450 nm.

Measurement of UCL with Varying Concentrations of Spx and SCN^- —— Artificial saliva for in vitro UCL assay was reconstituted with a solution of hemoglobin (1–30 μg/ml) as the Spx standard and sodium thiocyanate as the SCN^- standard (0.3–1.5 mM) in phosphate buffer 10 mM (pH 6.9), containing sodium chloride 150 mM (PBS). Detection of artificial salivary UCL was measured as described above.

Statistical Analysis —— GraphPad Prism was used for statistical calculations. One-way analysis of variance (ANOVA) procedures for repeated measures were utilized for evaluating levels of UCL; concentrations of total protein, sIgA, SCN^-, and uric acid; and activities of amylase and peroxidase, followed by the Tukey multiple-comparison test. Spearman's rank correlation test was used to estimate bivariate correlation coefficients. The level of statistical significance was set at $p < 0.05$.

RESULTS

Kraepelin Test Changed Biochemical Parameters in Whole Saliva

To examine the effect of the Kraepelin test on biochemical parameters in whole saliva, we collected the saliva just before, just after, and 30 min after the test and measured several parameters. The Kraepelin test induced transient changes in the IgA concentration (Table 1). The IgA concentration increased immediately after and decreased 30 min after the Kraepelin test. Amylase, hemoglobin, and cortisol concentrations also increased but were not significantly affected. Flow rates within each session were not different.

Kraepelin Test Changed the Salivary Antioxidant Component

The UCL of saliva was highest (1.56-fold) im-

mediately after the Kraepelin test (23.62 ± 21.67 × 10^4 counts/100 sec) and decreased to the same level 30 min later (15.15 ± 9.94 × 10^4 counts/100 sec), compared with those just before the test (15.08 ± 8.85 × 10^4 counts/100 sec) (Fig. 2).

As the UCL is based mainly on electronic transfer in an oxidation-reduction reaction, we analyzed the Spx activities, and SCN^-, and UA concentrations in saliva (Fig. 3). The Spx and SCN^- concentrations increased significantly immediately after administration of the Kraepelin test. These increases were followed by a decrease after 30 min. The UA concentration also increased with the Kraepelin test, but this change was not significant.

To determine the effects of Spx, SCN^-, and UA on salivary UCL, we compared the concentrations of these antioxidant components compared with the UCL level (Fig. 4). All of the antioxidant components correlated with the UCL immediately after the test.

Generation of UCL by Reconstitution of Spx and SCN^- at Physiologic Concentrations in Saliva

To examine the effects of antioxidant components on salivary UCL, we prepared sequential concentrations of Spx, SCN^-, and UA with phosphate buffered saline (PBS) and measured the UCL (Fig. 5). The UCL of the solutions prepared with

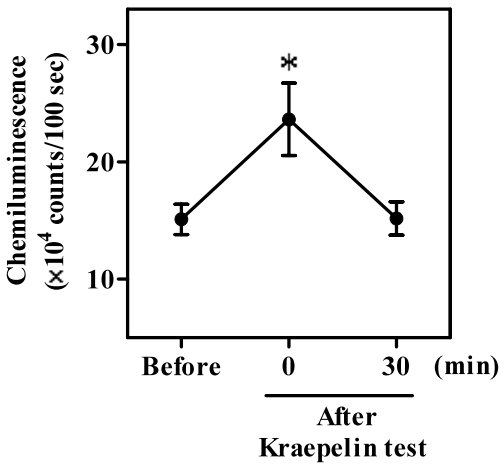

Fig. 2. Weak Chemiluminescence of Saliva Before and After the Kraepelin Arithmetic Test (n = 46)

The overall test of differences among the times was statistically significant ($p < 0.01$ by one-way analysis of variance); after 0 min the values were significantly different from before as determined using the Tukey multiple-comparison procedure ($^*p < 0.05$).

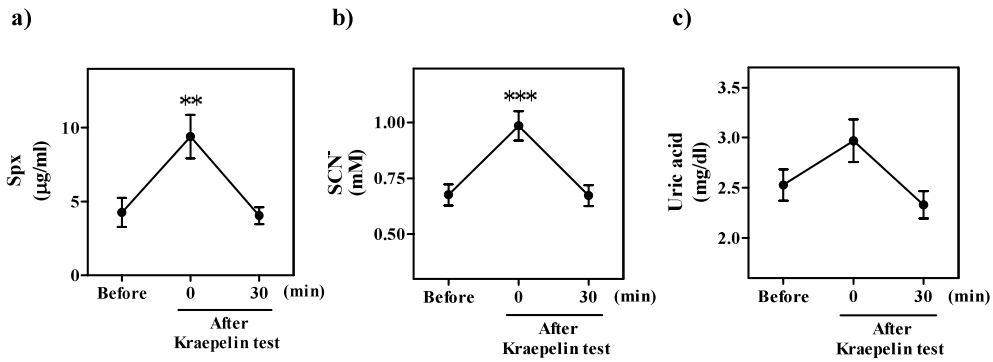

Fig. 3. Salivary Antioxidant Component of a) Spx (n = 40), b) SCN^- (n = 46), and c) Uric Acid (n = 46) Concentrations Before and After the Kraepelin Arithmetic Test

The overall test of differences among the times was statistically significant ($p < 0.001$ by one-way analysis of variance); after 0 min the values were significantly different from before as determined using the Tukey multiple-comparison procedure ($^{**}p < 0.01$, $^{***}p < 0.001$).

Table 1. Biochemical Parameters in Whole Saliva of Healthy Volunteers Before and After the Kraepelin Test

	Before	Time after Kraepelin test (min)	
	Kraepelin test	0	30
Amylase (×10^4U/ml)	17.69 ± 10.93	21.54 ± 15.59	17.99 ± 10.31
IgA (μg/ml)[a)]	7.48 ± 4.06	12.09 ± 6.33***	9.12 ± 6.05
Hemoglobin (μg/ml)	0.21 ± 0.24	0.45 ± 0.35	0.19 ± 0.20
Cortisol (μg/dl)	0.75 ± 0.30	1.50 ± 1.10	1.56 ± 1.14
Flow rate (ml/min)	1.63 ± 0.64	1.62 ± 0.79	1.74 ± 0.92

Date are expressed as mean ± S.D. (n = 46) except for cortisol (n = 6). *a)* One-way analysis of variance, $p < 0.05$. Tukey test vs. baseline, $^{***}p < 0.001$.

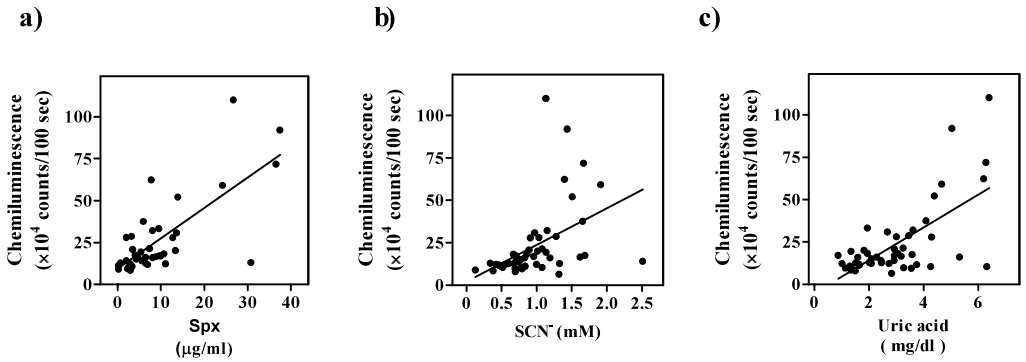

Fig. 4. Correlation between Ultraweak Chemiluminescence of Saliva and Salivary Peroxidase (Spx) a), SCN^- b), and Uric Acid c)
The univariate association with the fitted line is shown between chemiluminescence and a) Spx ($r = 0.618$, $p < 0.0001$), b) SCN^- ($r = 0.615$, $p < 0.0001$), and c) uric acid ($r = 0.490$, $p = 0.0004$).

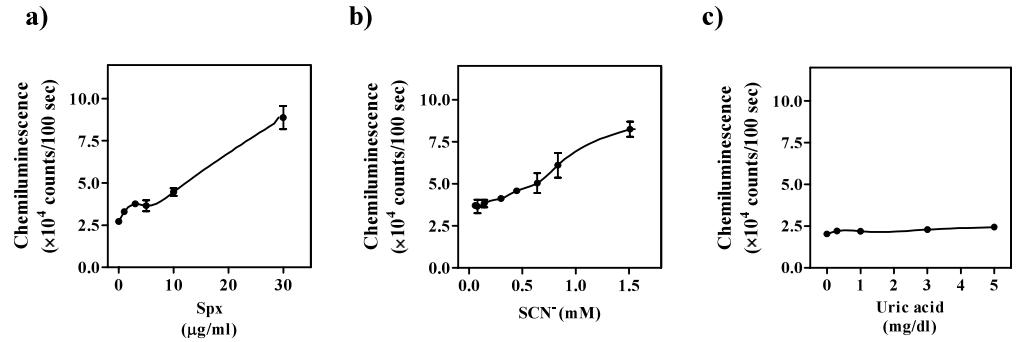

Fig. 5. Effects of Salivary Antioxidant Component of a) Spx, b) SCN^-, and c) Uric Acid in PBS 10 mM (pH 7.4) on Weak Chemiluminescence
Artificial saliva was reconstituted with a solution of hemoglobin (1–30 μg/ml) as the Spx standard, sodium thiocyanate (0.3–1.5 mM), and uric acid (0.3–5 mg/dl) in phosphate buffer 10 mM (pH 6.9), containing sodium chloride 150 mM (PBS).

Spx and SCN^-increased dose dependently. The concentrations of Spx and SCN^- in human whole saliva were 1–36 μg/ml and 0.13–2.51 mM, respectively; therefore the concentrations of Spx and SCN^- used here were in the physiologic range. However, the generation of UCL by each of these components was at a lower level by itself than that by innate whole saliva. On the other hand, UA did not affect the generation of UCL at any concentration.

We next examined the interaction of Spx and SCN^- in terms of UCL generation. There was no effect when SCN^- was in the range of 0–0.5 mM; however, the level of UCL increased beginning at SCN^- 0.75 mM for all concentrations of Spx (Fig. 6). In this system, the value of UCL was about 40×10^4 counts/100 sec for 10 μg/ml of Spx and 1 mM of SCN^-, and this was at a similar level with the original whole saliva.

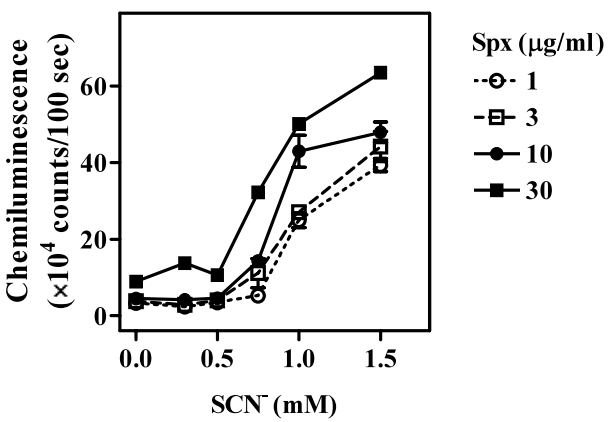

Fig. 6. Effects of Mixture with Spx and SCN^- on Chemiluminescence
Each point is the average of three experiments. Vertical bars represent S.D.

DISCUSSION

Several studies have demonstrated the role of salivary UCL,[30–32] although little research has

been published that assesses salivary UCL as an index of psychologic stress. We previously reported that the change in salivary UCL before and after an experimental examination might be a new sensitive biomarker of acute psychologic stress. In the present study, we investigated whether a mild psychologic stressor such as the Kraepelin test influenced the level of salivary UCL to compare with sIgA and cortisol concentrations and amylase activity. Previous reports demonstrated that the response of salivary amylase activity and cortisol level significantly increased from a state of rest to a psychologic stress state.[14, 33] As shown in Table 1, amylase activity immediately after stress was 38% higher than the levels prestress; however, no significant difference between before and immediately after stress was found using one-way ANOVA (p = 0.010, Wilcoxon test). In addition, cortisol levels did not change significantly and did not decrease from immediately after to 30 min after the Kraepelin test in our study. This means cortisol still reflected the influence of the test 30 min later and the highest level was not at a point immediately after the test. sIgA concentrations have also been utilized as an index of psychologic stress.[20, 34] Significant increases in concentrations of sIgA caused by the Kraepelin test were demonstrated. The UCL of saliva increased significantly immediately after the Kraepelin test, and this increase was similar to the change in sIgA (Fig. 2). However, during the restoration, the level of UCL decreased immediately from immediately after the Kraepelin test (23.62 ± 21.67 × 10^4 counts/100 sec) to 30 min later (15.15 ± 9.94 × 10^4 counts/100 sec) and this was the same as immediately before the test (15.08 ± 8.85 × 10^4 counts/100 sec), and sIgA did not return completely to the same level from immediately after (12.09 ± 6.33 μg/ml) to 30 min later (9.12 ± 6.05 μg/ml) compared with immadiately before the test (7.48 ± 4.06 μg/ml) (Fig. 2 and Table 1). From these results, it is suggested that salivary UCL reflects the influence of stress more sensitively than amylase activeity and more quickly than cortisol and expresses a recovery state from stress more immediately than sIgA.

In our system, major UCLs are the volume of photons generated after applying H_2O_2 as an oxidant. With regard to this oxidation-reduction reaction, it is known that SCN^- can be oxidized to $OSCN^-$ by Spx. Therefore we measured the SCN^- concentration and Spx activity in whole saliva. The concentration of UA, which is a major component of the total antioxidant system and constitutes 70% of the total antioxidant capacity, was also calculated. The Spx and SCN^- concentrations significantly increased immediately after the administration of stress and decreased 30 min later, reaching the prestress levels, which was similar to UCL. On the other hand, the UA concentration also increased with the Kraepelin test but not significantly (Fig. 3). In addition, all of the components, especially Spx and SCN^-, showed a significant positive correlation with UCL (Fig. 4). In the measurement of UCL with the addition of Spx, SCN^-, and UA to PBS instead of whole saliva, Spx and SCN^- increased UCL dose dependently at physiologic concentrations but UA did not change UCL at any concentration (Fig. 5). Spx and SCN^- alone showed low levels of UCL in comparison with original whole saliva; however, UCL generated by Spx and SCN^- together showed similar levels in the physiologic range (Fig. 6). These results indicate that Spx and SCN^- in saliva can play a key role in H_2O_2-induced UCL, and especially when the SCN^- concentration becomes greater than 0.75 mM, the amount of UCL increases greatly. This SCN^- concentration is similar to levels before and 30 min after stress (Fig. 3). Spx catalyzes the oxidation of SCN^- by H_2O_2 to the antimicrobial component, hypothiocyanate ($OSCN^-$) with the emission of UCL.

$$H_2O_2 + SCN^- \xrightarrow{\text{Spx}} OSCN^- + H_2O + UCL\uparrow$$

Because H_2O_2 is constantly generated in the mouth by aerobic bacteria and H_2O_2 as such is toxic to mucosal and gingival cells, saliva provides Spx to consume H_2O_2 by peroxidation. Therefore the amount of UCL means the abilities to consume H_2O_2 as an antioxidant component and produce $OSCN^-$.

The function of $OSCN^-$ in the oral cavity has been discussed in relation to antimicrobial activity.[35] Lactoperoxidase also contributes to the antimicrobial defense in secretory fluids.[36, 37] The hypothesis that salivary SCN^- can protect the stomach from OH radicals formed by ascorbic acid/H_2O_2/Fe(II) systems under acidic conditions was reported.[38] Thus the change in SCN^- concentration may have a significant effect not only on the oral cavity, but also on other sites in the body. We suggest that salivary UCL may be used to evaluate the strength of the innate immune system like the antibacterial system and that this innate immune system is sensitive to acute psychologic stress, such as results from the Kraepelin test.

We calculated total peroxidase concentration using hemoglobin as a standard. This assay are based on pseudoperoxidase activity of hemoglobin using DAF as a hydrogen donor for peroxidase.[25–27,39] The content of hemoglobin in saliva was 0.2 μg/ml on average, as detected by ELISA (Table 1). More than 1 μg/ml of hemoglobin increased UCL levels (Fig. 5). These results demonstrate that hemoglobin in the saliva at physiologic concentrations is not correlated with UCL levels. Peroxidase activity is mainly composed of Spx and myeloperoxidase in the body.[40] In addition, the peroxidase/H_2O_2 system is regulated by NO, chloride, pH, lysozyme, and other factors in addition to SCN^-.[41–45] From these observations, we propose that the changes in these components must balance each other, and therefore salivary UCL levels may reflect a more comprehensive ability for antioxidation and antimicrobial activity than mere concentrations of enzyme and SCN^-.

The occurrence of much higher concentrations of the enzymatic antioxidant parameters in the parotid saliva compared with whole and submadibular/sublingual saliva was reported.[7] In this study, we used only whole saliva, and parotid saliva may induce much higher and more sensitive changes in the amount of UCL. In addition, we considered the influence on the living body of a type of a stressor. We used the Kraepelin test as an acute stressor only once, but it is more important to examine the effects of events occurring over a long term or repeatedly as in several studies on cortisol, sIgA, and interleukin-6.[13,19,21,46] Schommer *et al*. suggested that although HPA responses quickly habituate, the sympathetic nervous system showed rather uniform activation patterns with repeated exposure to psychosocial challenge.[18] Whether we can evaluate the effects of chronic stress experience from measurements based on our UCL system should be investigated. Furthermore, the levels of some psychologic stress markers are influenced by the circadian rhythm, aging, and sex difference.[21,47,48] It is a future project to examine the effects of these factors on salivary UCL levels.

In conclusion, we determined that the Kraepelin test as a mental arithmetic task elicited a significant response in the body and this response can be calculated by salivary UCL. Furthermore, SCN^- and peroxidase in the saliva play a key role in H_2O_2-induced salivary UCL.

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