

**Original****Efficacy of Stress Measurements Using Salivary Ultra-weak Chemiluminescence**Masamitsu Nakamura<sup>1,2)</sup>, Kazuo Umemura<sup>3)</sup>, Mitsuo Hiramatsu<sup>4)</sup>, Hiroko Oishi<sup>5)</sup> and Masato Maekawa<sup>6)</sup><sup>1)</sup>Graduate School of Medicine, Hamamatsu University School of Medicine<sup>2)</sup>Department of Laboratory, Hamamatsu Rosai Hospital<sup>3)</sup>Department of Pharmacology, Hamamatsu University School of Medicine<sup>4)</sup>Central Research Laboratory, Hamamatsu Photonics K.K.<sup>5)</sup>Department of Endocrinology, Hamamatsu Rosai Hospital<sup>6)</sup>Department of Laboratory Medicine, Hamamatsu University School of Medicine

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**Abstract**

The incidence of stress-induced psychiatric disorders has increased rapidly in recent years. Screening for stress markers enables early detection and treatment of conditions in which stress responses affect health.

Salivary ultra-weak chemiluminescence (UCL) can detect peroxidase and thiocyanate ions, which are secreted and increased in saliva under stress. Forty-four patients with diabetes mellitus who were admitted for diabetic education because of their high prevalence of depression and 14 healthy hospital staff members were included. Level variations of stress-related substances in saliva were measured by UCL.

Simultaneously, salivary levels of cortisol and chromogranin A (CgA) were measured, and the short form of the Profile of Mood States questionnaire was administered. Chemiluminescence intensity correlated with increases in depression-dejection subscale scores, and had a positive and negative correlation with CgA and cortisol levels, respectively. The hospital staff and patients differed significantly in chemiluminescence intensities, cortisol levels, and depression-dejection scores. In conclusion, UCL intensity was correlated with depression scores and levels of classical stress markers, with significant differences between hospital staff and patients. UCL intensity may thus allow the measurement of increases in psychological stress.

Furthermore, because saliva absorbed by filter paper can be used, samples can easily be collected and analyzed.

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**—Key words—**

ultra-weak chemiluminescence, Profile of Mood States, stress marker

**Introduction**

In recent years, the incidence of stress-induced psychiatric disorders, such as depression, has increased rapidly. At present, stress is mainly measured and assessed using psychological questionnaires, which need to be administered and evaluated by physicians trained in psychology or by psychologists. Meanwhile, although measurements of biomarkers such as cortisol, amylase, chromogranin A (CgA), and immunoglobulin A (IgA),<sup>1-6)</sup> in blood, urine, and saliva have been reported to be useful, these biomarkers have not been studied for long enough to be adequate and quantifiable markers. Stress markers that are easy to measure and highly specific seem to be needed. Salivary ultra-weak chemiluminescence (UCL) can detect peroxidase and thiocyanate ions, which are secreted and increased in saliva under stress.<sup>7-9)</sup> It has been reported that salivary UCL intensity significantly increased in patients with severe diseases, such as lung cancer,<sup>8)</sup> and in people under stress

caused by mental workload in the Kraepelin test.<sup>9</sup> Salivary UCL intensity can be measured in saliva absorbed by filter paper, which gives this marker an advantage in that saliva samples can be collected easily and repeatedly.<sup>7-9</sup>

This study targeted patients admitted to a hospital for diabetic education. According to a statistical survey conducted by the Japanese Ministry of Health, Labour and Welfare in 2011,<sup>10</sup> diabetes mellitus affects approximately 10.67 million of the Japanese adult population of approximately 95.34 million, and its prevalence reached 11.2% in 2011. While diabetes mellitus causes cardiovascular complications, the prevalence of depression is also reported to be as high as 14% in patients with diabetes mellitus.<sup>10</sup> Those with concomitant depression are unable to exercise glycemic self-control, which leads to a vicious cycle. When we previously administered the Profile of Mood States (POMS) questionnaire to patients admitted for diabetic education, psychological stress was observed in 11 of 16 surveyed patients (unpublished data). This observation indicates that patients with severe psychological stress are likely to be encountered in such a patient group. Thus, by targeting patients admitted for diabetic education, this study aimed to compare salivary UCL to a conventional psychological questionnaire as well as to measurements of salivary CgA and cortisol, to determine whether salivary UCL intensity could be a useful stress marker in the future.

## Experimental

### 1. Subjects and methods

In this study, salivary UCL, the POMS questionnaire, and conventional measurements of salivary CgA and cortisol were analyzed and compared to determine the usefulness of salivary UCL for stress measurements. This study included 44 patients admitted for diabetic education (31 men and 13 women) and 14 hospital staff members without diabetes mellitus (seven males and seven females). Prior to this study, the experimental protocol was approved by the ethics committee of Hamamatsu Rosai Hospital, and informed consent for participation in the study was obtained from all study participants.

### 2. Sample collection

During the diabetic education sessions, the POMS questionnaire was administered, and saliva samples were collected. In these sessions which were held between 15:00 and 16:00 p.m. before the evening meal, the patients were examined after receiving instructions for diabetes treatment while seated for approximately 30 minutes, to avoid any effects of exercise. The hospital staff members were also examined before meal and under resting conditions. Study participants who were willing to repeatedly participate in this study underwent several examinations on different days.

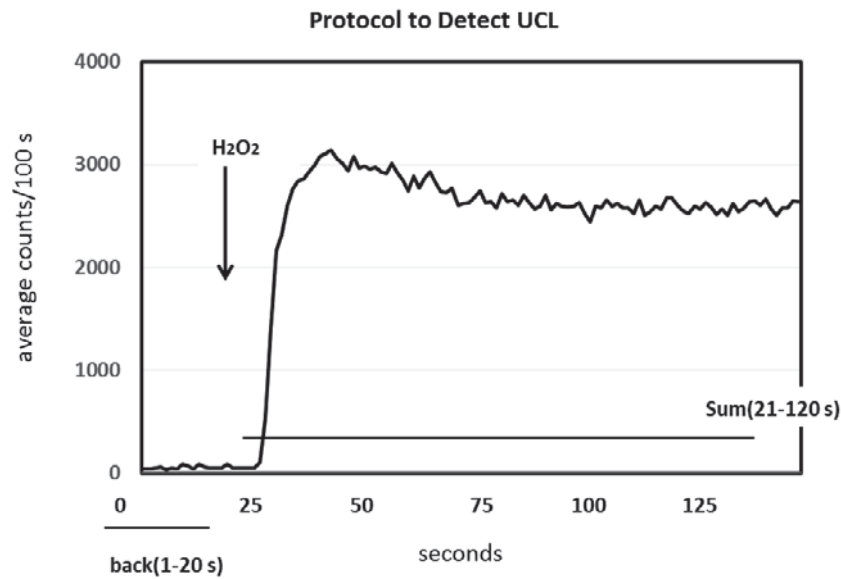
Salivary UCL and levels of salivary CgA and salivary cortisol were measured in the samples from the study participants. Filter paper was used for salivary UCL, and the Saliva Collection Aid (SCA; Salimetrics LLC, USA) was used for measurements of CgA and cortisol.

The filter paper (Nobuto Blood-Sampling Paper Strip type I) was placed in the mouth to absorb saliva for 60 seconds. Each filter strip that had absorbed saliva was placed in a zip-seal plastic bag, frozen, and stored at  $-80^{\circ}\text{C}$  until measurement.

### 3. Measurements

Salivary UCL intensity was measured with a Photon Counter C767 (Hamamatsu Photonics K.K., Hamamatsu, Japan).<sup>7</sup> The cryopreserved filter paper that had absorbed saliva was brought to room temperature under running water. After 1 mL of a gallate solution adjusted to a concentration of 3 mM (gallate 0.282 g+distilled water 500 mL) and the filter paper were placed in the measurement cell, the measurement started. When a 3% hydrogen peroxide solution was added 20 seconds later, chemiluminescence started. The mean intensity of chemiluminescence measured for 100 seconds was used for analysis. (Fig. 1)

Salivary CgA levels were measured by enzyme immunoassay using a microplate reader (SPECTRAmax<sup>®</sup> PLUS (384); Molecular Devices Inc., USA) and calibrated to protein equivalents (expressed as pmol/mg). Salivary cortisol levels were measured by solid-phase radioimmunoassay using a  $\gamma$  counter (Aloka ARC-950; Hitachi-Aloka Medical, Tokyo, Japan) and expressed as  $\mu\text{g}/\text{dL}$ .



**Fig. 1** Salivary UCL levels. After 3% (w/v)  $H_2O_2$  solution was added to the saliva preparation, photo counts were conducted. The total number of counts for 100 seconds was defined as the level of UCL.

**Table 1** Sample size according to the study parameters and average age

	All participants			Hospital staff			Patients		
	total	male	female	total	male	female	total	male	female
UCL	83	55	28	34	18	16	49	37	12
CgA	70	48	22	33	17	16	37	31	6
Cortisol	70	50	20	33	18	15	37	32	5
POMS	82	55	27	33	18	15	49	37	12
Average Age	55 ± 15	55 ± 14	55 ± 16	42 ± 13	47 ± 11	36 ± 13	57 ± 14	57 ± 14	59 ± 13

## Results

Table 1 shows the number of saliva samples collected from the study participants according to the study parameters (UCL, CgA, and cortisol), the number of times when the POMS questionnaire was administered, and the mean age of the study participants.

For measurements of salivary UCL intensity, 83 saliva samples (55 from male and 28 from female participants) were collected overall, and the mean age at the time of this study was  $55 \pm 15$  years ( $55 \pm 14$  vs.  $55 \pm 16$  for male/female participants). From the patients admitted for diabetic education, 49 saliva samples (37 vs. 12) were collected, and the mean age at the time of this study was  $57 \pm 14$  years ( $57 \pm 14$  vs.  $59 \pm 13$ ). From the hospital staff members, 34 saliva samples (18 from male and 16 from female participants) were collected, and the mean age at the time of this study was  $42 \pm 13$  years ( $47 \pm 11$  vs.  $36 \pm 13$ ). The survey and measurement results of individual participants are shown in detail in the supplementary tables.

The salivary UCL intensity measured in 83 saliva samples widely varied from 149 to 20,000 average counts/100 seconds.

### 1. POMS questionnaire

For the POMS questionnaire, responses were converted to numerical scores. In this study, the vigor POMS subscale scores (healthy range:  $\geq 8$  for men and  $\geq 9$  for women) decreased in 32 of the 58 study participants (25 patients admitted for diabetic education and seven hospital staff members). Meanwhile, the POMS scores increased on the tension-anxiety subscale (healthy range:  $\geq 11$  for men and  $\geq 12$  for women) in 11 participants (eight patients and three hospital staff members), on the anger-hostility subscale (healthy range:  $\leq 9$  for both men and women) in nine participants (nine patients), on the fatigue subscale (healthy range:  $\leq 12$  for men

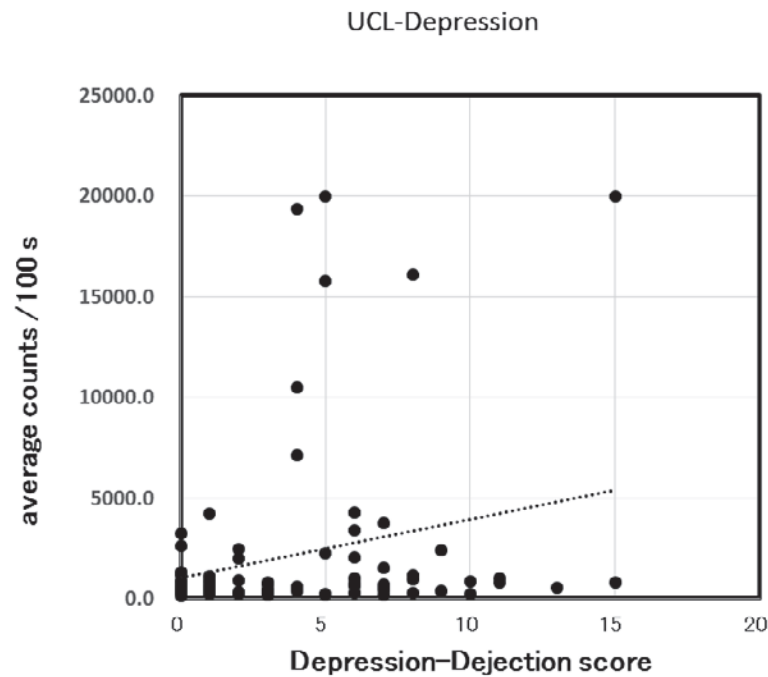


Fig. 2 POMS questionnaire. There was a significant correlation between UCL intensity and the depression-dejection POMS subscale scores ( $p < 0.05$   $r = 0.24$ ; 82 saliva samples).

Table 2 Hospital staff crowd and Patients

	Hospital staff	Patients	P-value
UCL (average counts/100 s)	$490.5 \pm 270.9$	$3,330.9 \pm 5,445.2$	$p < 0.05$
CgA (pmol/ml)	$4.2 \pm 3.6$	$5.5 \pm 3.2$	ns
Cortisol (IU/L)	$0.3 \pm 0.3$	$0.2 \pm 0.1$	$p < 0.05$
Depression-Dejection (score)	$3.1 \pm 3.6$	$4.5 \pm 3.8$	$0.05 < p < 0.1$

and  $\leq 13$  for women) in eight participants (seven patients and one hospital staff member), and on the confusion subscale (healthy range:  $\leq 9$  for both men and women) in 12 participants (nine patients and three hospital staff members). Depression (dejection) (healthy range of the POMS subscale score:  $\leq 8$  for men and  $\leq 9$  for women) was observed in 10 participants (eight patients and two hospital staff members). In some surveyed patients whose profiles indicated that they were treated for depression, suffered from insomnia, or were under excessive stress from working at night for a long period, the POMS scores increased on the depression-dejection and tension-anxiety subscales and decreased on the vigor subscale. When we examined whether salivary UCL intensity measured in 82 saliva samples was correlated with increases and decreases in the depression-dejection POMS subscale scores, a correlation was observed between intensity and scores ( $p < 0.05$ ,  $r = 0.24$ ). (Fig. 2)

## 2. Comparison between hospital staff and patients

The comparison between the hospital staff and the patient groups revealed significant differences in salivary UCL intensity ( $p < 0.05$ ), cortisol levels ( $p < 0.05$ ), and depression-dejection POMS subscale scores ( $0.05 < p < 0.1$ ). (Table 2)

## 3. Chromogranin A

CgA levels varied from 0.5 to 14.6 pmol/mg of protein. Although no correlation was observed with the depression-dejection POMS subscale scores, CgA levels were correlated with salivary UCL intensity measured in 70 saliva samples ( $p < 0.05$ ,  $r = 0.23$ ). A Spearman rank correlation analysis also showed a positive correlation ( $p < 0.01$ ,  $r_s = 0.388$ ). (Fig. 3)

## 4. Cortisol

Cortisol levels ranged from 0.06 to 1.20  $\mu\text{g}/\text{dL}$ . When compared between the hospital staff and the patient

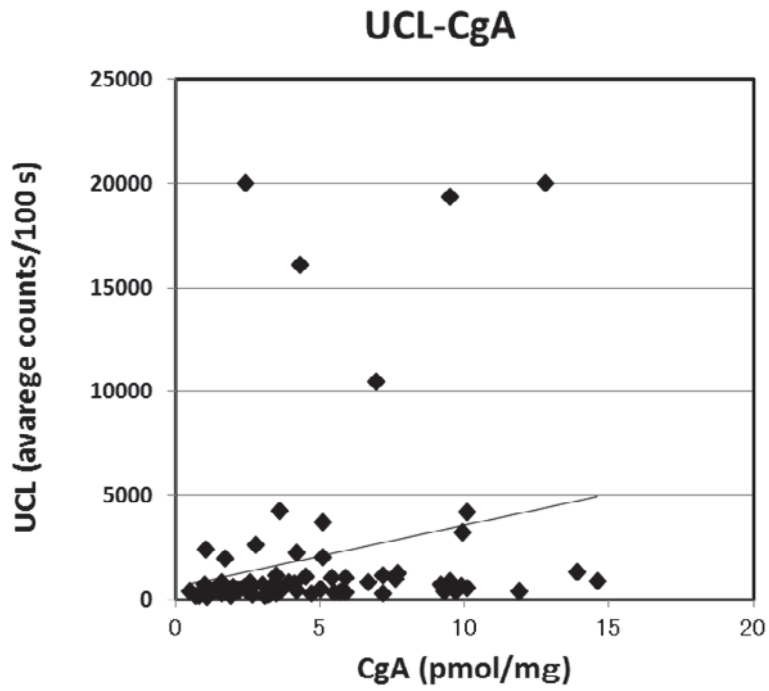


Fig. 3 Chromogranin A. CgA levels were correlated with salivary UCL intensity measured in 70 saliva samples ( $p < 0.05$ ,  $r = 0.23$ ).

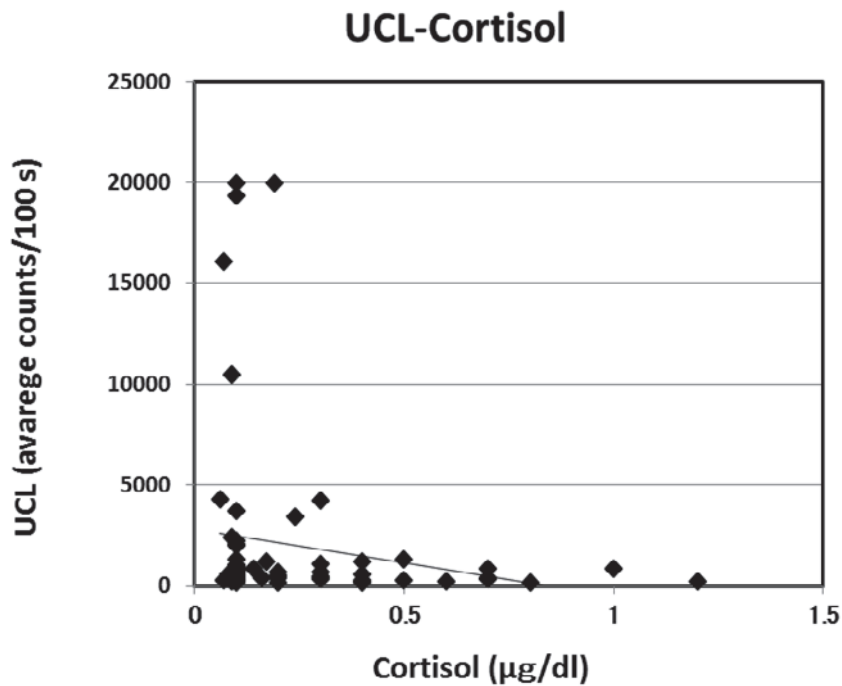


Fig. 4 Cortisol levels. There was a negative correlation between UCL intensity and cortisol levels in 70 saliva samples ( $p < 0.05$ ).

groups, the levels were correlated with the depression-dejection POMS subscale scores ( $p < 0.05$ ). The levels also showed a significant difference between the groups ( $p < 0.05$ ). When we examined whether the cortisol levels were correlated with salivary UCL intensity measured in 70 saliva samples, a negative correlation was observed ( $p < 0.05$ ). A Spearman rank correlation analysis also showed a negative correlation ( $p < 0.01$ ,  $r_s = -0.278$ ). (Fig. 4)

## Discussion

In this study, we examined whether salivary UCL intensity is an effective stress marker. The study included patients admitted to a hospital for diabetic education. Simultaneously, the POMS questionnaire, which is most commonly used for assessing psychological stress, was administered to the patients. In some surveyed patients whose profiles indicated that they were treated for depression, suffered insomnia, or were under excessive stress from working at night for a long period, the POMS scores increased on the depression-dejection and tension-anxiety subscales, and decreased on the vigor subscale. The salivary UCL intensity measured in 82 saliva samples was correlated with increases in the POMS scores, particularly on the depression-dejection subscale. These findings suggest UCL intensity as a candidate for a reliable stress marker.

Salivary level of CgA, which is a classical stress marker, was simultaneously measured. A Spearman rank correlation analysis of the measurement and salivary UCL intensity revealed a positive correlation between salivary CgA levels and UCL intensity. Salivary CgA levels increase under stress and mental load, such as tension and fear, in the early stages. These findings thus suggest that UCL intensity can detect stress as well as the classical stress marker CgA.

Cortisol levels tend to increase under intensive and persistent physical stress or chronic stress,<sup>11)</sup> but have been reported to be normal or low under acute stress.<sup>12)13)</sup> This study revealed a negative correlation between salivary cortisol levels and UCL intensity. When we had previously measured UCL intensity before and after mental workload in the Kraepelin test,<sup>9)</sup> the acute stress caused increases in UCL intensity levels. UCL levels are considered to increase during acute stress. Previous results and this study thus suggest that salivary UCL intensity increases with acute stress.

In this study, measurements obtained from patients with marked gingivitis varied, maybe due to bleeding in the oral cavity. Therefore, before saliva samples are collected for salivary UCL, gargling is necessary to reduce the influence of bleeding and other factors in the oral cavity. However, measurements of salivary UCL intensity widely varied from 149 to 200,000 average counts/100 seconds in this study, and we were unable to explain why they increased to such a high level.

The collection of saliva samples from patients with diabetes mellitus is often difficult in patients whose salivary secretion is low. Saliva samples for UCL can however be easily collected, even in patients with low salivary secretion, because salivary UCL intensity can be measured in saliva absorbed by filter paper.

In conclusion, we found that salivary UCL intensity is correlated with depression-dejection POMS subscale scores; it is also positively correlated with salivary CgA levels and negatively correlated with salivary cortisol levels. These findings suggest that UCL intensity can be used as an acute stress marker. Furthermore, because salivary UCL intensity can be measured in saliva absorbed by filter paper, saliva samples can be collected easily.

## Abbreviations

ultra-weak chemiluminescence (UCL); chromogranin A (CgA); Profile of Mood States (POMS); Saliva Collection Aid (SCA)

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## 唾液微弱発光測定法を用いたストレス調査の有効性について

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### —キーワード—

微弱発光, POMS, ストレスマーカー

【目的】近年、ストレスによるうつ病等の精神疾患が急増している。ストレス反応が健康に影響している状態を早期発見、早期対応するためストレスマーカーによるスクリーニング調査の必要性が求められている。唾液微弱発光測定 (Salivary Ultra-weak Chemiluminescence : UCL) はストレス時に唾液中に分泌増加するペルオキシターゼ (Peroxidase), チオシアン酸イオン (SCN<sup>-</sup>) を捕らえる事ができる。濾紙に唾液を湿らすだけで測定ができるため検体採取方法が簡便で、繰り返し採取できる利点がある。今後、ストレスマーカーとして活用できる可能性がある。

【対象】浜松労災病院にて糖尿病教育指導のため入院した 44 名の糖尿病患者と同病院職員で、糖尿病の発症のない 14 名を対象にした。

【方法】唾液微弱発光法を用いて、唾液中のストレス物質の増減を測定した。同時に、唾液中の cortisol、クロモグラニン A の測定及び気分プロフィール検査 (POMS 短縮版) も行った。

【結果】全調査症例の UCL の発光量と POMS 検査の気分尺度のうつのスコアの増減を比較したところ、UCL とうつのスコアに相関性を ( $p < 0.05$ ,  $r = 0.24$ ) 認めた。また、cortisol にも相関性を認めた。 ( $p < 0.05$ )

病院職員群と糖尿病患者群に分類して比較したところ、UCL, cortisol に有意差 ( $p < 0.05$ )、うつスコアにも有意差 ( $0.05 < p < 0.1$ ) を認めた。UCL の測定値と唾液 cortisol ( $p < 0.05$ ,  $r = 0.23$ )、cortisol ( $p < 0.05$ ) とともに、相関性を認めた。唾液検体採取において、唾液微弱発光測定法は、唾液を濾紙に湿らせる事で、測定が可能で、唾液の分泌の少ない糖尿病患者でも容易に採取測定が可能であった。

【結論】唾液微弱発光法は、POMS のうつスコアに相関性を認めた。糖尿病患者群においても弱い相関性を認めた。これにより、唾液微弱発光法唾液微弱発光測定法は、ストレスの増減を測定できる可能性がある。また、唾液測定試料の濾紙で採取、使用する事が可能で、容易に採取測定する事ができた。

利益相反：利益相反基準に該当無し

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