Short Communication

Determination of Appropriate Sampling Time for Job Stress Assessment: the Salivary Chromogranin A and Cortisol in Adult Females

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Objectives : This study was conducted to determine the appropriate sampling time of the salivary stress markers, chromogranin A (CgA) and cortisol as objective indices of job stress assessment in adult females.

Methods : The subjects were 20~39-year-old women (13 office workers, 11 sales-service workers, and 11 college students) who were eligible for the study and free of acute and chronic medical conditions. Salivary CgA and cortisol levels were determined by enzyme-linked immunosorbent assay (ELISA). Saliva samples were collected (2 ㎖ each) at 7:00, 8:00, 10:30, 12:00, 17:30, and 22:30 on a typical day. Salivary CgA and cortisol levels, according to sampling time, were compared among the three groups using general linear model. The full version of the Korean Occupational Stress Scale (KOSS), which includes socioeconomic characteristics, health behavior, work-related characteristics, and BMI, was used to access the subjects' job stress. Multiple regression analysis of the job stressors identified by the KOSS was performed on salivary CgA and cortisol levels.

Results : The salivary CgA level peaked at 7:00 (time of awakening), then decreased and were maintained at a low level throughout the day, and increased slightly at 17:30. The salivary cortisol level increased steeply within the 1st hour after awakening, followed by a gradual decrease by 12:00, and was then maintained at a low level throughout the day. The salivary cortisol levels of subjects who worked ≤5 days per week and graduated from the university were significantly lower at 8:00 (p=0.006). The salivary cortisol levels of non-smokers were significantly lower at 7:00 (p=0.040) and 8:00 (p=0.003) compared to smokers. There were no significant differences in salivary CgA and cortisol levels at 10:30 and 12:00 in general characteristics. The regression coefficients on salivary CgA level were significant with interpersonal conflict at 17:30 and job insecurity at 22:30. Regression coefficients on salivary cortisol level were significant with organizational system and total job stressors at 17:30.

Conclusions : We suggest that the appropriate sampling times for the salivary stress markers, CgA and cortisol, are at 7:00 (time of awakening), 8:00 (1 hour after awakening), 17:30 (early evening), and 22:30 (before sleep).

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Key words : Circadian rhythm, Saliva, Cortisol, Chromogranin A, Stress, Biomarker

INTRODUCTION

In modern society, job stress focuses on job features that pose a threat to the worker caused by excessive job demands, insufficient supplies to meet the workers´ needs, or the possibility of loss [1]. An increasing number of women work in service areas and the necessity for stress management has attracted considerable social attention. Therefore, intervention at both the organization and personal levels for stress management is required. To evaluate of intervention effects, valid measurement tools must be developed and infact, Structured question-naires and biomarkers have been introduced as measurement tools for stress. Representative structured questionnaires include the Korean Occupational Stress Scale (KOSS) [2] and the Job Content Questionnaire (JCQ) [3]. However, the questionnaires express the subjective status of stress. For this reason, biomarkers have been used for the objective measurement of stress. Salivary biomarkers have many advantages such as being stress-free and non-invasive, thus allowing for frequent and rapid sampling [4]. Catecholamine and cortisol levels serve as an objective index of stress because the sympathetic/adrenomedullary system and the hypothalamic-pituitary-adrenal axis respond to stress [5]. The surrogate of catecholamines detectable in saliva, chromogranin A (CgA), has been shown to be a useful index of psychological stress [6]. Salivary cortisol accurately reflects serum cortisol, the physiologically active component [7] and salivary cortisol is now a widely used index of stress [8]. Salivary cortisol and CgA exhibit a circadian rhythm [9] and racial/ethnic differences [10,11]. Salivary cortisol and CgA were measured 5-6 times in a single day in previous studies [10,12,13] because of the circadian rhythm. Disruption of the circadian rhythm might be mediated by chronic high stress [14-17], specially, physical workload increases the morning cortisol level and decreases the afternoon cortisol level [15]. A change in the circadian rhythm is essential in

evaluating the level of strss. For a large scale epidemiologic study regarding stress and the workplace, it is important to determine the appropriate sampling time through out the day forsalivary CgA and cortisol levels.

METHODS

Ⅰ. Subjects

Thirty-six female volunteers, 20-39 years of age, were recruited from 13 office workers (administrators and/or sales coordinator), 11 sales-service workers (department store sales-service), and 12 college students. One student was excluded due to nephritis; thus 35 subjects who were healthy and free from acute and chronic medical conditions, such as endocrine, eating, or psychiatric disorders, based on blood and liver function tests within 3 months, were enrolled in the study. All protocols described were approved by the Ethics Committee for the Protection of Persons in Biochemical Research at the Institute of Medical Science of Chung-Ang University in Seoul, Korea. We used the full version of the KOSS to evaluate the level of job stress in workers. The KOSS was developed over 2 years (2002-2004) and is considered to be unique and specific for occupational stressors in Korean employees [2]. A structured, self-reported questionnaire was used to assess the socioeconomic characteristics, health behavior, work-related characteristics, and BMI. Questionnaires and informed consents were received from all subjects.

Ⅱ. Salivary CgA and Cortisol

Saliva samples (2 ml) were collected 6 times a day in 15 ml sterilized conical tubes (Greiner, Frickenhausen, Hannover, Germany) at 7:00 (awakening), 8:00 (1st hour after awakening), 10:30, 12:00, 17:30, and 22:30 (before sleep). Stimulated saliva was obtained by chewing on a piece of tasteless Parafilm (0.29 g, Parafilm "M" ®; American National Can TM, Chicago, IL, USA) [18]. All subjects were asked to go to sleep between 22:30 and 23:00. When the subjects awakend, they provided the first saliva sample prior to brushing their teeth. Subjects were requested in advance not to eat or drink 1 hour before saliva collection. After the collections, all samples were kept in an icebox and immediately transferred to the laboratory, where they were stored at -80℃ until analysis. The intra- and inter-assay coefficients of variation for saliva CgA and cortisol were determined with researchers's saliva. The saliva samples were further subdivided into intra assay (within-day), which assesses precision during a single analytical run, and inter-assay (between-days), which measures precision with time and may involve different analysts, equipment, reagents, and laboratories [19]. For intra-assay precision - Three samples in triplicate were run (a total of nine assays). For inter-assay precision, the three runs on triplicate samples were run on three different days.

A. CgA concentration

The concentration of CgA in saliva (pmol/㎖) was determined using a YK070 Chromogranin A (human) electroimmu-noassay kit (Yanaihara Institute, Fujinomiya, Japan). The plate was read in the plate reader (Tecan Sunrise TW, Salzburg, Austria) at 490 nm.

The intra- and inter-assay coefficients of variation for saliva CgA were 6.2-11.7% and 7.4-23.3%, respectively.

B. Cortisol concentration

The concentration of cortisol in saliva (μg/d�报) was determined using an enzyme immunoassay kit (Salimetrics, State College, PA, USA). These were assayed in duplicate. The intra- and inter-assay coefficients of variation for saliva cortisol were 1.7-5.5% and 7.2-18.3%, respectively.

Table 1. The frequency distribution of general characteristics

Categorical variables	Office workers (N=13)		Sales-service workers (N=11)		Students (N=11)		p-value*
	Mean ±SD		Mean ±SD		Mean ±SD		
Age (yr)	33.2±4.0		26.0±3.0		20.0±2.0		<0.001
BMI (kg/m²)	20.3±1.9		20.6±1.5		20.0±1.4		0.739
	no.	%	no.	%	no.	%	
Working hrs per day							
≤8	2	15.4	0	0.0			0.308
>8	11	84.6	10	90.1			
Non-response	0	0.0	1	9.9			
Working days per wk							
≤5	13	100.0	3	27.3			<0.001
>5	0	0.0	7	63.6			
Non-response	0	0.0	1	9.1			
Working duration (yr)							
≤ 2	7	46.1	6	54.6			0.873
2.1 - 5.9	2	23.1	3	27.3			
≥ 6	4	30.8	2	18.2			
Education							
High school or College	2	15.4	9	81.9			0.002
University	11	84.5	2	18.1			
Marital status							
Single	5	38.5	8	72.7			0.102
Married	8	61.5	3	27.3			
Family Income (10,000 korean won)							
≤500	4	30.8	6	54.6			0.223
>500	9	69.2	5	45.4			
Alcohol intake (g/day)							
Non-36 g	13	100.0	6	54.5	5	45.5	0.005
36 g-	0	0.0	5	45.5	6	55.5	
Smoking amount (pack/yr)							
Nonsmoker	13	100.0	3	18.2	8	72.8	<0.001
Smoker (0-9)	0	0.0	8	81.8	3	17.2	
Exercise							
Yes+	4	30.8	1	9.1	6	54.6	0.083
No	9	69.2	10	90.9	5	45.5	

* p-value calculated by Chi-square test or Fisher's exact test, + Exercise yes: defined by subjects who exercise at least more once per week.

Table 2. Comparison of salivary CgA and cortisol levels with general characteristics among female workers

CgA (pmol/mg)		Time					
Categorical variables		7:00	8:00	10:30	12:00	17:30	22:30
Working days per wk	≤5 (no.=16)	104.1 ±97.4	105.2 ±94.0	61.9 ±91.0	84.0 ±130.9	92.2 ±116.6	87.4 ±80.2
	>5 (no.=7)	136.1 ±79.6	62.4 ±27.4	89.1 ±70.1	46.4 ±38.7	82.4 ±74.2	53.8 ±44.8
	p-value	0.350	0.593	0.160	0.789	0.712	0.462
Education	High school or College (no.=11)	110.8 ±81.6	92.7 ±87.4	77.9 ±80.5	58.3 ±47.3	78.8 ±69.5	78.9 ±74.1
	University (no.=13)	116.8 ±99.3	102.3 ±85.2	59.8 ±88.0	83.1 ±143.6	94.5 ±125.5	76.7 ±70.2
	p-value	0.794	0.750	0.505	0.339	0.709	0.977
Alcohol intake (gram/day)	Non-36 g (no.=19)	113.8 ±91.4	97.4 ±90.8	62.6 ±75.6	75.3 ±120.8	85.6 ±94.1	75.4 ±72.6
	36 g- (no.=5)	118.1 ±41.0	122.5 ±125.0	89.7 ±108.9	60.5 ±46.5	67.7 ±70.9	92.3 ±63.6
	p-value	0.915	0.972	0.804	0.594	0.935	0.500
Smoking amount (pack-yr)	Nonsmoker (no.=15)	118.6 ±97.2	82.8 ±53.7	59.7 ±85.4	70.1 ±123.5	85.6 ±103.8	65.5 ±64.5
	Smoker (0-9) (no.=9)	108.2 ±79.9	135.8 ±140.0	82.5 ±77.6	75.7 ±85.3	76.7 ±58.9	101.3 ±76.5
	p-value	0.835	0.698	0.297	0.270	0.606	0.128

Cortisol (μg/mℓ)		Time					
Categorical variables		7:00	8:00	10:30	12:00	17:30	22:30
Working days per wk	≤5 (no.=16)	0.47 ±0.21	0.45 ±0.17	0.29 ±0.16	0.21 ±0.09	0.14 ±0.09	0.15 ±0.14
	>5 (no.=7)	1.08 ±0.90	1.24 ±0.90	0.27 ±0.15	0.21 ±0.10	0.20 ±0.06	0.41 ±0.56
	p-value*	0.071	0.006	0.422	0.841	0.083	0.108
Education	High school or College (no.=11)	0.83 ±0.80	0.99 ±0.81	0.32 ±0.15	0.21 ±0.08	0.18 ±0.07	0.35 ±0.46
	University (no.=13)	0.49 ±0.19	0.47 ±0.19	0.27 ±0.16	0.21 ±0.10	0.15 ±0.10	0.11 ±0.09
	p-value*	0.622	0.077	0.369	0.931	0.239	0.030
Alcohol intake (gram/day)	Non-36 g (no.=19)	0.66 ±0.62	0.70 ±0.66	0.20 ±0.15	0.22 ±0.09	0.16 ±0.10	0.24 ±0.37
	36 g- (no.=5)	0.60 ±0.34	0.74 ±0.37	0.30 ±0.17	0.18 ±0.05	0.13 ±0.10	0.14 ±0.07
	p-value*	0.915	0.374	0.804	0.271	0.644	0.915
Smoking amount (pack-yr)	Nonsmoker (no.=15)	0.42 ±0.14	0.46 ±0.22	0.29 ±0.16	0.21 ±0.09	0.14 ±0.10	0.14 ±0.14
	Smoker (0-9) (no.=9)	1.01 ±0.81	1.13 ±0.81	0.28 ±0.13	0.21 ±0.09	0.18 ±0.08	0.36 ±0.50
	p-value*	0.040	0.003	0.835	0.858	0.144	0.069

* p-value calculated by Kruskal-Wallis test.

C. Protein concentration

The concentration of protein (μg/mℓ) in saliva was determined using a BCA Protein assay kit (Pierce Biotechnology Inc., Rockford, IL, USA). The measured value of CgA-like immunoreactivity must be corrected on the basis of the protein concentration in the saliva sample.

D. Statistical analysis

Data analysis was performed using STATA ver. 10.0 (StataCorp LP College Station, TX, USA). A chi-square test or Fisher's exact test was used to compare the subjects' general characteristics. A general linear model was used for salivary CgA and cortisol to obtain adjusted (least squares means) estimates. The model was adjusted for age and smoking amount. A comparison of salivary CgA and cortisol levels among three groups were performed according to sampling time. A comparison of salivary CgA and cortisol levels with general characteristics at various time points among female workers was performed with the Kruskal-Wallis test. CgA and cortisol levels were natural logarithmi-cally-transformed before analysis to correct a strong positive skew in the distributions. Multiple regression analysis of the job stressors included in the KOSS was performed to evaluate the effects of confounding variables (age, exercise, smoking amount, education, and working days per week, and alcohol intake) on salivary CgA and cortisol levels. The criterion of significance was set at p<0.05.

RESULTS

Ⅰ. General Characteristics

The general characteristics of subjects are described in Table 1. There was no significant difference in working hours per day, working duration per year, marital status, and family income between the office workers and the sales-service workers. There was a significant difference in working days per week (p<0.001) and education (p<0.001) between office workers and sales-service workers. Smoking (p<0.001) and alcohol intake (p=0.005) in students and sales-service workers were higher than office workers. Comparison of salivary CgA and cortisol levels according to sampling times on general characteristics had a significant difference, as shown in Table 2. There were no significant differences in salivary CgA with respect to working days per week, education, alcohol intake, and smoking amount at all sampling times. The salivary cortisol levels of subjects who worked ≤5 days per week and graduated from the university were lower in the morning and evening and shown to be significantly different at 8:00 (p=0.006) and 22:30 (p=0.03), respectively. The cortisol level of non-smokers were significantly lower at 7:00 (p=0.040) and 8:00 (p=0.003) than smokers. There were no significant difference in salivary CgA and cortisol levels at 10:30 and 12:00 in general characteristics.

Ⅱ. Circadian Rhythm of Salivary CgA and Cortisol

Figure 1 shows the levels of CgA and cortisol in saliva at various time points. The level of CgA peaked at the time of awakening and then slowly decreased by 12:00. Thereafter, it remained at a low level throughout the day, and increased slightly in the early evening. In sales-
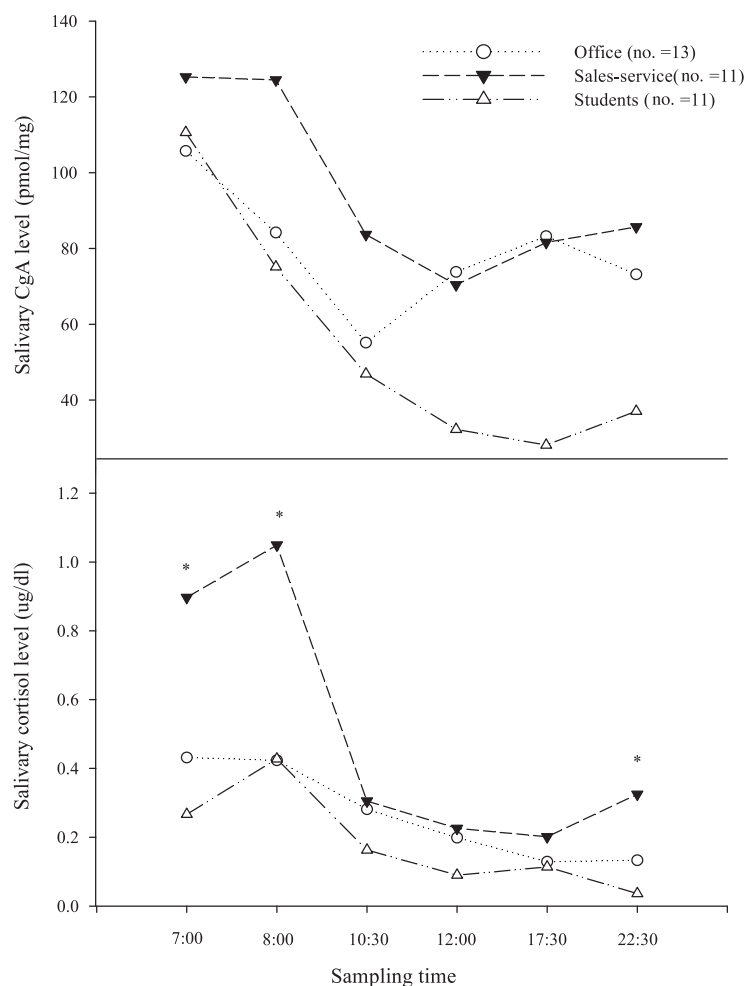
Figure 1. Chromogranin A and cortisol levels in saliva of three groups sampled at various time points. At sampling time, 7:00 means the time of awakening and 22:30 means the time before sleeping. Values were expressed as the mean. In comparison of salivary CgA and cortisol levels among three groups, general linear model was used for salivary CgA and cortisol to obtain adjusted (least squares means) estimates and model was adjusted for age and smoking amount. There were significant differences at 7:00 (p=0.045) and 8:00 (p=0.010) and 22:30 (p=0.007) between sales-service workers and students in salivary cortisol.

service workers, there were higher levels at various times than other groups, except at 12:00. In contrast, students showed lower levels than other groups. With respect to the cortisol level, there was a steep increase within the 1st hour after awakening, followed by a gradual decrease throughout the day. Before the time of sleep, the cortisol level was significantly lower than the 1st hour after awakening and the morning time. In office workers, the salivary cortisol levels had flatter slopes among the other workers. The circadian rhythm of sales-service workers had higher cortisol levels at awakening time and 1 hour after that than the other groups. At the time before sleep, the sales-service workers' levels was slightly

increased. The circadian rhythm of students showed a similar pattern to the mean level of the other two groups in cortisol levels. In comparison of salivary CgA and cortisol levels among working types, there were significant difference at 7:00 (p=0.045) and 8:00 (p=0.010) and 22:30 (p=0.007) in cortisol.

III. Multiple Regression Analysis to Adjust Confounding Variables Between Salivary CgA and Cortisol Levels and KOSS Job Stressors in Workers.

The coefficient and standard error between salivary CgA and cortisol levels and KOSS job stressors were estimated by multiple

regression model adjusted for age, smoking, and working days per week (Table 3). Regression coefficients on salivary CgA level were significant with interpersonal conflict at 17:30 and job insecurity at 22:30 (p=0.025). Regression coefficients on the salivary cortisol level were significant with organizational system (p=0.011) and total job stressors (p=0.015) at 17:30.

DISCUSSION

In this study, we determined the appropriate sampling time of salivary CgA and cortisol in female adults in their normal life as a function of circadian rhythm effects. We propose that stress biomarkers (salivary CgA and cortisol) for service area workers should be measured at 7:00 (time of awakening), 8:00 (1 hour after awakening), 17:30 (early evening), and 22:30 (immediately before sleep) to objectively assess the level of stress in the workplace. This is the first study to investigate circadian rhythm changes in salivary CgA and cortisol in Korea. The measurement of CgA and cortisol six times a day by saliva sampling was based on previous research [9,10,12,13].

The level of CgA peaked at 7:00 and then dramatically decreased toward 10:30. With respect to the cortisol level, there was a steep increase within the first hour after awakening, followed by a gradual decrease by 12:00. These findings were similar with the results of Den et al. [9]. Thereafter, salivary CgA increased at 17:30 and cortisol remained flatter throughout the day. Den et al. [9] reported salivary CgA increased and cortisol gradually decreased after 17:30. These differences could reflect the different subjects used. Therefore, this result must be interpreted with physical workload and work factors [15]. Den et al. [9] studied male college students, whereas the present study was performed in female college students as the control group, and sales-service and office workers. In female students, the CgA level was increased at 22:30 and the

Table 3. Regression coefficients of job stressors of Korean Occupational Stress Scale on salivary CgA and cortisol levels among 24 female workers

CgA (pmol/mg)	Sampling time					
Job stressor	7:00	8:00	10:30	12:00	17:30	22:30
Physical environment	0.011(0.007)	-0.002(0.010)	0.004(0.015)	-0.001(0.016)	0.002(0.014)	-0.010(0.010)
p-value	0.141	0.815	0.801	0.961	0.909	0.328
Job demand	0.015(0.009)	0.005(0.012)	-0.012(0.019)	-0.019(0.020)	-0.011(0.018)	-0.012(0.012)
p-value	0.101	0.651	0.543	0.353	0.535	0.332
Insufficient job control	-0.007(0.013)	-0.018(0.016)	0.004(0.026)	-0.009(0.028)	0.013(0.024)	0.009(0.018)
p-value	0.591	0.269	0.872	0.735	0.608	0.609
Interpersonal conflict	0.006(0.009)	-0.005(0.012)	0.028(0.018)	0.019(0.020)	0.038(0.016)	0.019(0.012)
p-value	0.494	0.668	0.140	0.359	0.025	0.138
Job insecurity	-0.012(0.008)	-0.011(0.009)	-0.006(0.016)	-0.003(0.019)	-0.008(0.017)	-0.026(0.011)
p-value	0.127	0.261	0.718	0.888	0.641	0.025
Organizational system	-0.006(0.008)	-0.010(0.009)	0.003(0.016)	0.004(0.019)	0.009(0.016)	0.001(0.012)
p-value	0.424	0.303	0.845	0.847	0.575	0.930
Lack of reward	-0.005(0.009)	-0.013(0.010)	0.002(0.018)	-0.010(0.021)	0.020(0.019)	0.003(0.013)
p-value	0.579	0.236	0.933	0.626	0.290	0.851
Organizational climate	0.005(0.011)	-0.013(0.014)	0.021(0.023)	0.014(0.024)	0.010(0.022)	-0.007(0.015)
p-value	0.681	0.382	0.369	0.568	0.656	0.634
Total job stressors	0.002(0.014)	-0.023(0.016)	0.008(0.029)	-0.004(0.034)	0.020(0.030)	-0.010(0.021)
p-value	0.904	0.166	0.791	0.914	0.501	0.630

Cortisol (ug/ml)	Sampling time					
Job stressor	7:00	8:00	10:30	12:00	17:30	22:30
Physical environment	-0.019(0.011)	0.003(0.010)	-0.005(0.006)	-0.002(0.005)	-0.003(0.002)	0.004(0.014)
p-value	0.118	0.787	0.366	0.681	0.179	0.810
Job demand	0.015(0.012)	-0.002(0.010)	0.004(0.006)	0.002(0.006)	0.002(0.002)	0.001(0.015)
p-value	0.238	0.823	0.564	0.695	0.471	0.924
Insufficient job control	-0.021(0.017)	0.006(0.015)	-0.008(0.008)	-0.0008(0.008)	-0.005(0.003)	-0.010(0.020)
p-value	0.242	0.670	0.368	0.304	0.121	0.615
Interpersonal conflict	-0.019(0.011)	0.016(0.009)	-0.001(0.006)	-0.004(0.005)	-0.003(0.002)	0.005(0.014)
p-value	0.094	0.096	0.921	0.390	0.159	0.703
Job insecurity	0.006(0.012)	0.006(0.009)	0.003(0.005)	0.003(0.005)	-0.003(0.002)	0.006(0.013)
p-value	0.604	0.496	0.555	0.594	0.176	0.666
Organizational system	-0.012(0.011)	0.008(0.009)	-0.001(0.006)	-0.004(0.005)	-0.005(0.002)	-0.005(0.014)
p-value	0.312	0.374	0.836	0.397	0.011	0.743
Lack of reward	-0.012(0.014)	0.017(0.011)	-0.001(0.007)	-0.004(0.006)	-0.004(0.002)	-0.002(0.017)
p-value	0.427	0.136	0.948	0.567	0.085	0.887
Organizational climate	-0.006(0.016)	0.010(0.013)	-0.002(0.008)	-0.006(0.007)	-0.002(0.030)	0.019(0.022)
p-value	0.724	0.439	0.763	0.444	0.621	0.386
Total job stressors	-0.027(0.022)	0.022(0.017)	-0.002(0.011)	-0.007(0.010)	-0.009(0.003)	-0.003(0.026)
p-value	0.228	0.224	0.873	0.471	0.015	0.906

The coefficient and standard error estimated by multiple regression model adjusted by age, smoking, and working day per week.

cortisol level was decreased at 22:30. This finding is in agreement with the same previous study [9]. In sales-service workers, the CgA level was increased at 17:30 and the cortisol level was increased at 22:30. Salivary CgA has been reported to rapidly and sensitively respond to psychosomatic stressors [6]. Salivary cortisol levels at 7:00, 8:30, and 22:30 were significantly higher and salivary CgA and cortisol levels after 12:00 had a higher pattern of in office and sales-service workers, which might be associated with job strain. In stressed women, cortisol secretion was increased due to concerns about the negative consequences of not being able to manage all the workplace duties and responsibilities, and chronic stress induced a significantly larger increase in the cortisol levels compared to unstressed subjects

[14]. Lack of control and poor social support in the workplace contribute to staff burnout [20]. In office workers, the salivary CgA and cortisol levels were lower than sales-service workers (Figure 1). It is likely that an excessive number of duties and tasks of sales-service workers are related to working for an extended time period in an uncomfortable position and a lack of control of the work pace and time schedule.

In the morning and evening, the salivary cortisol level, were low in those who worked ≤5 days per week, had a higher education, and were non-smokers. Toda et al. [21] reported that the level of salivary cortisol may be affected by current circumstances and that level of CgA may be affected by changes in the comprehensive lifestyle, such as regular physical activity. The reports are similar with our results.

Regression coefficients of the effect of interpersonal conflict and job insecurity on salivary CgA levels were significant at 17:30 and, 22:30, and the effect of the organizational system and total job stressors on the salivary cortisol level was significant at 17:30. These findings could reflect day time stress. Interpersonal conflict was correlated more closely with significantly higher levels of CgA in the early evening before the closing time. The elevated evening cortisol associated with chronic high strain was consistent with the findings in other studies on long-term strain [22].

The inter-assay variation of CgA was higher than cortisol. The salivary cortisol appears to be useful as a stress biomarker.

Kang et al. [15] collected salivary cortisol

two times a day (in the morning and afternoon) to assess job stressors for male manufacturing workers. However, this study was not conducted for service area workers. Our results showed that there were no significant differences in salivary CgA and cortisol levels at 10:30 and 12:00 on general characteristics for service area workers. In previous studies, salivary cortisol levels were measured 5-6 times in a single day [10,12,13]. Job strain was associated with elevated free cortisol concentrations early in the work day [23] and elevated evening cortisol was associated with chronic high strain [22]. Evidence that stress may be a mediating factor in the disruption of the cortisol circadian rhythm has been derived from studies on animals [24]. After an exhausting day of work, cortisol levels in chronically stressed subjects were observed to be lower than those of non-stressed individuals [14].

However, individual variations should be considered because of the limited sample size and given that the KOSS is not ideal to reflect emotional labor. The use of a questionnaire designed for emotional stress, such as the JCQ ver. 2.0, will be considered in the search for an association with salivary stress biomarkers with a larger sample size in a future.

In conclusion, the salivary CgA level decreased after awakening and the salivary cortisol level, were elevated within at the 1st hour after awakening (8:00), followed by a gradual decrease throughout the day. The salivary CgA and cortisol levels were maintained at a lower level without significant change in the daytime and evening. This result further supports the hypothesis that optimal sampling time is a crucial condition for salivary biomarkers in job stress assessment. We therefore suggest that the proper sampling time for salivary CgA and cortisol for female workers is 7:00, 8:00, 17:30, and 22:30. Further studies are needed to reduce the sampling frequency for epidemiologic studies.

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