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Stress affects daily salivary cortisol profiles

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Abstract

Cortisol is a well-established biomarker of stress, and measurement of salivary cortisol (sCort) has attracted research interest because saliva collection is a non-invasive and subject-friendly procedure that does not require medical staff. Previous studies have shown inconsistent results in the association between stress and daily sCort profiles. This study aimed to compare sCort daily profiles between stressful and ordinary days in the same people. Twenty healthy participants collected saliva four times a day (awakening, at 10:00h, 12:00h, 16:00h) on an ordinary day when the participants had general duties and on a stressful day where there were known stressful events happening to the participants. The results showed that sCort levels on a stressful day were significantly higher than those on an ordinary day at two time points (10:00h and 12:00h, $p = 0.000$ and $p = 0.038$, respectively). In addition, on an ordinary day, the sCort level at awakening was significantly higher than at 10:00h ($p = 0.005$), at 12:00h ($p = 0.005$), and at 16:00h ($p = 0.000$). Meanwhile, the sCort value at 16:00h was the lowest value of the day with no difference between ordinary and stressful days. In summary, this study found increased sCort values on a stressful day compared with an ordinary day, and the sCort levels at different times of the day were also different.

Keywords**:** *cortisol*; *cortisol profiles*; *daily sCort profiles*; *saliva*; *salivary cortisol*; *stress*.

1. Introduction

Stress is a response of the body to stressors, and it presents in many forms and situations in everyday life. It can be psychological and/or physiological stress, internal (e.g., hunger, thirst, insomnia, illness) or external (e.g., heat, loud noise, social evaluation, a promotion, a marriage) (Chu, Marwaha, & Ayers, 2020; Kogler et al., 2015). An acute stress response is an important bodily response to tackle the presenting threat. However, when stress persists, chronic activation of the stress response, which hyperactivates the hypothalamic-pituitary-adrenal (HPA) axis, can be deleterious to the body. Chronic stimulation of the heart, persistent hypertension, and suppression of the immune system can increase the risk of many disorders, such as cardiovascular diseases, stroke, gastric ulcers, sleep dysregulation, and even psychiatric disorders (Mifsud & Reul, 2018; Yaribeygi, Panahi, Sahraei, Johnston, & Sahebkar, 2017). Thus, it is essential to investigate, prevent and intervene the effects of stress (Ketchesin, Stinnett, & Seasholtz, 2017; Yaribeygi et al., 2017).

Regarding stress research, the HPA axis is the most widely studied system having cortisol as its final product. Cortisol is a type of steroid hormone produced by the adrenal gland. Its secretion is controlled by the HPA axis, so cortisol was considered as a gold standard biomarker of stress (Ali & Nater, 2020; Chrousos, 2009). Serum cortisol contains total cortisol, of which 70–85 % is bound to cortisol binding globulin (CBG), 10-15 % is bound

to albumin, and less than 10 % is free cortisol which is biologically active (Kirschbaum & Hellhammer, 1989; Mifsud & Reul, 2018). Aside from serum, cortisol can be found in urine, hair, and saliva. Salivary cortisol (sCort) is free cortisol which comes from serum to saliva by passive diffusion due to its low molecular weight and lipophilic nature (Miller, 2008). Measuring sCort has gained popularity due to its non-invasive, convenient, and subject-friendly characteristics (Vining, McGinley, Maksvytis, & Ho, 1983). Furthermore, sCort levels are directly proportional to the free serum cortisol (Vining et al., 1983) and are not impacted by salivary flow rate (Kirschbaum & Hellhammer, 1989).

There have been many studies regarding the relationship between stress and daily sCort profiles over the past 40 years. As mentioned above, sCort is generally associated with stress; however, there are some subtle conflicting details. A previous study that analysed sCort levels at awakening, 14:00h, 15:00h, 16:00h, 17:00h and bedtime found that a relationship between the functions and demands of home and work environments might be important predictors of individual differences in daily sCort concentrations in adult mothers of toddlers. For instance, poorer relationship functioning was related to lower morning sCort levels and a flatter decline in sCort throughout the day. However, the time of collection was mostly in the afternoon, and there was no information on the participants' emotions (Adam & Gunnar, 2001). In another recent study, female subjects reporting stress at home had significantly lower daily sCort levels and a flatter daily slope when compared with subjects reporting no stress at home. However, stress at work was not associated with levels of sCort (Sjörs, Ljung, & Jonsdottir, 2014). In this study, subjects chose an ordinary day and a known stressful day to collect saliva. Daily profiles of sCort were analysed to elucidate the effect of stress on daily sCort profiles.

2. Objectives

This study aims to compare sCort daily profiles between different emotional situations in a person, using stressful and ordinary days as representatives of different emotional situations.

3. Materials and methods

3.1 Participants and protocol

Twenty healthy volunteers, 18 years or older, were recruited in this study. All of them were in good health. None had underlying systemic disease, were on prescribed medications, pregnant, or smoked. Any individuals who were unable to collect saliva on all assigned days and times were excluded. The study was approved by the Human Research Ethics Committee of the Faculty of Dentistry and the Faculty of Pharmacy, Mahidol University. All participants gave written, informed consent.

For the first visit, participants were asked to answer part 1 of a questionnaire (personal information). They were shown how to collect and store their saliva. Each participant then received two sets of tubes for collecting saliva on two days. Each set contained four tubes for saliva collection at four time points (awakening, 10:00h, 12:00h, and 16:00 h). The participants were free to choose a typical, ordinary day and a stressful day defined as:

- *Ordinary day*: a common day not having too much pressure but not completely relaxing either (i.e., on a weekday doing a routine job or going to the university).

- *Stressful day*: a day with anticipatory stress or facing a difficult situation (i.e., an examination day, audit day with difficult supervisors, etc.).

The participants were free to choose whether to collect saliva for an ordinary day or a stressful day first. There was no restriction on how long these two days were apart from each other. On experimental days, participants answered part 2 of the questionnaire (withdrawal criteria) and recorded their heart rate. At each time point of the saliva collection, the participants also recorded their feelings at that moment with the visual analog scale (VAS), which is a scale of emotions ranging from 0 to 10 (no stress at all (0) to maximal stress (10)). Each individual was instructed to refrain from alcoholic beverage consumption for at least 12 hours and from eating or drinking an hour prior to salivary collection. However, plain water was allowed throughout the day. Participants were asked to collect saliva into a 2-mL tube at the assigned time via the passive drooling technique (i.e., pooling the saliva for four minutes in their mouth and not swallowing during saliva collection). The saliva sample should have filled at least 1/3 of the tube, or else the participants would be allowed more pooling time until they could collect sufficient saliva. The place of saliva collection could be anywhere in the participant's daily activities because they could keep the saliva at room temperature until they could find a freezer (-20^{o}C). The sample was stable at room temperature for up to 72h (Garde & Hansen, 2005).

Later the saliva sample was transferred to -80ºC until further analysis.

3.2 Measurement of sCort

For sCort analysis, the frozen saliva was thawed completely and centrifuged at 1500 × *g* for 15 minutes. The sCort levels were measured by an enzyme immunoassay kit (Salimetrics, State College, Pennsylvania, USA). In brief, antibodies to cortisol were bound at the bottom of a microtiter well. A 25 µL aliquot of saliva was added into a 96-well plate. A volume of 200 µL of the diluted cortisol-enzyme conjugate was added into the 96-well plate, followed by incubation at room temperature for one hour. Throughout this one-hour incubation, there was competition between the cortisol from the salivary sample and the cortisol–enzyme conjugate for antibody-binding sites. After the incubation period, wells were rinsed to remove the unbound materials. Next, 200 µL of 3,3',5,5'-tetramethylbenzidine substrate solution was added to each well that resulted in a reaction of substrate with the enzyme conjugate to produce a colored product. The degree of color in each reaction well was reported in units of optical density (OD) by a spectrophotometer at 450 nm (Varioskan Flash Multimode Reader, Thermo Fisher Scientific, Rockford, Illinois USA). The higher the amount of sCort, the lower the OD. All the protocols were followed according to the manufacturer's instructions. The minimum concentration of sCort that can be distinguished is from 0 was 0.007 µg/dL.

3.3 Statistical analysis

Data are presented as mean ± SD. Paired t-test was used to compare the parameters between ordinary and stressful days. Repeated-measures one-way ANOVA was used to compare the means of sCort levels and VAS scores at different times in each day and between ordinary and stressful days. Statistical analysis was set at $p < 0.05$ and performed using SPSS statistics program version 20 (IBM, New York, USA).

4. Results

The baseline characteristics of the participants are shown in Table 1. From a total of 20 participants, most were female and 15% were male. The age range of the participants was between 27 and 34 years old. Most participants were of medium built. Six were underweight (BMI < 18.5 kg/m^2) with only one overweight participant (BMI ≥ 25 kg/m^2). No participant was obese (BMI ≥ 30 kg/m^2). The BMI range of all participants was from 16.8 to 26.5 kg/m^2. The participants recorded the time of sleep the night before saliva collection. The results showed that the duration of sleep prior to a stressful day was less (approximately one hour less) than that prior to an ordinary day ($p = 0.018$). On an ordinary day, durations of participants' sleep had been from four hours to eight hours and 30 minutes. Meanwhile, the duration of sleep before a stressful day ranged from one hour to nine hours' sleep. Regarding the timing of the stressful event, there were 13 participants who had a stressful event in the morning, four participants who experienced stress in the afternoon, and three participants who reported having stressful events both in the morning and the afternoon.

Table 1 Characteristics of participants (mean ± SD)

	All	Male	Female
N	20	3	17
Age (years)	29.25 ± 1.70	28.67 ± 1.56	29.35 ± 1.80
BMI (kg/m^2)	20.60 ± 2.60	23.73 ± 0.40	20.05 ± 2.47
Duration of sleep the night before an ordinary day (h:mm)	6:34 ± 1:10	6:55 ± 1:38	6:30 ± 1:07
Duration of sleep the night before a stressful day (h:mm)	5:21 ± 1:41*	4:00 ± 2:36*	5:35 ± 1:27*

*$p < 0.05$ compared with ordinary day

The profiles of sCort levels on two emotional days are shown in Table 2 and Figure 1. They ranged from 0.02 to 1.07 µg/dL. On an ordinary day, sCort level sharply decreased from awakening to 10:00h, then remained stable before gradually falling towards the lowest level of the day at 16:00h. The sCort level at awakening was significantly higher than at 10:00h ($p = 0.005$), at 12:00h ($p = 0.005$), and at 16:00h ($p = 0.000$).

Table 2 sCort profiles on two different emotional days

sCort levels (μg/dL)		Awakening	10:00	12:00	16:00	Daily average
Ordinary day	Mean	0.31	0.16	0.16	0.11	0.18
	(SD)	(0.16)	(0.07)	(0.09)	(0.07)	(0.07)
	Range	0.03-0.69	0.04-0.36	0.07-0.37	0.02-0.31	0.08-0.38
Stressful day	Mean	0.32	0.39*	0.27*	0.11	0.27*
	(SD)	(0.25)	(0.25)	(0.23)	(0.05)	(0.15)
	Range	0.03-1.02	0.06-1.07	0.09-0.97	0.02-0.23	0.11-0.7

*$p < 0.05$ compared with ordinary day

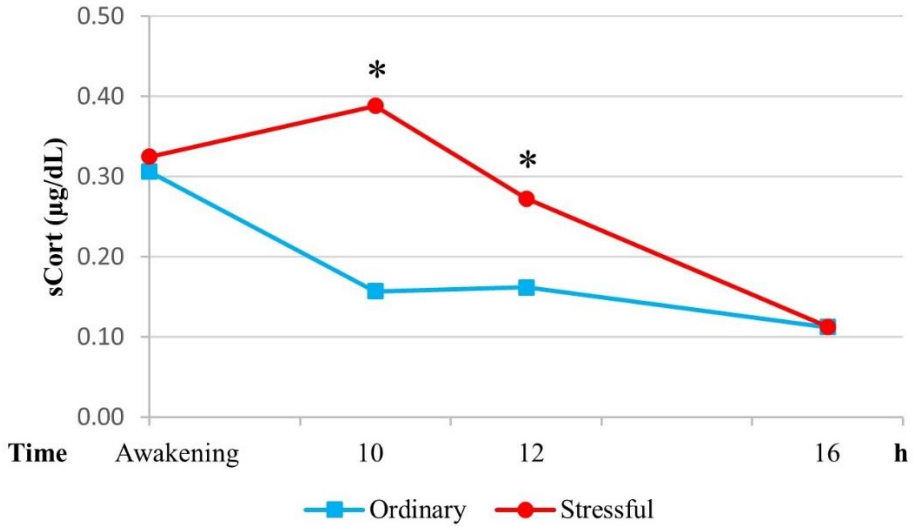

Figure 1 Daily profiles of sCort on two emotional days. **p < 0.05 compared with ordinary day.*

In contrast, there was a considerable increase of sCort level from awakening to 10:00h on a stressful day. However, sCort level dropped continuously throughout the course of day until 16:00h. The sCort value at 16:00h was significantly lower than all other time points (at awakening, 10:00h, and 12:00h with $p = 0.006$, $p = 0.000$, and $p = 0.033$, respectively). On a stressful day, sCort levels at 10:00h and 12:00h were significantly higher compared with those on an ordinary day ($p = 0.000$ at 10:00h and $p = 0.038$ at 12:00h). In addition, on a stressful day, daily average of sCort was higher than that of an ordinary day ($p = 0.014$).

Average VAS scores are shown in Table 3 and Figure 2. On an ordinary day, VAS score at awakening was higher than at 10:00h ($p = 0.005$). On a stressful day, VAS score at 10:00h was higher than that at 16:00h ($p = 0.035$) on the same day as well as at the same time compared to an ordinary day ($p = 0.000$). In addition, the daily average VAS score was also higher on a stressful compared to an ordinary day ($p = 0.022$).

Table 3 VAS profiles on two different emotional days

VAS score		Awakening	10:00	12:00	16:00	Daily average
Ordinary day	Mean	5.58	4.7	5.1	5.1	5.12
	(SD)	(1.17)	(1.3)	(1.02)	(1.17)	(0.97)
	Range	3-8	2-6	3-7	2-7	3-6.5
Stressful day	Mean	5.94	6.55*	5.45	4.94	5.72*
	(SD)	(1.2)	(1.57)	(1.61)	(2.04)	(0.96)
	Range	4-8	3-9	2-8	0-8	3.5-7.25

*$p < 0.05$ compared with ordinary day

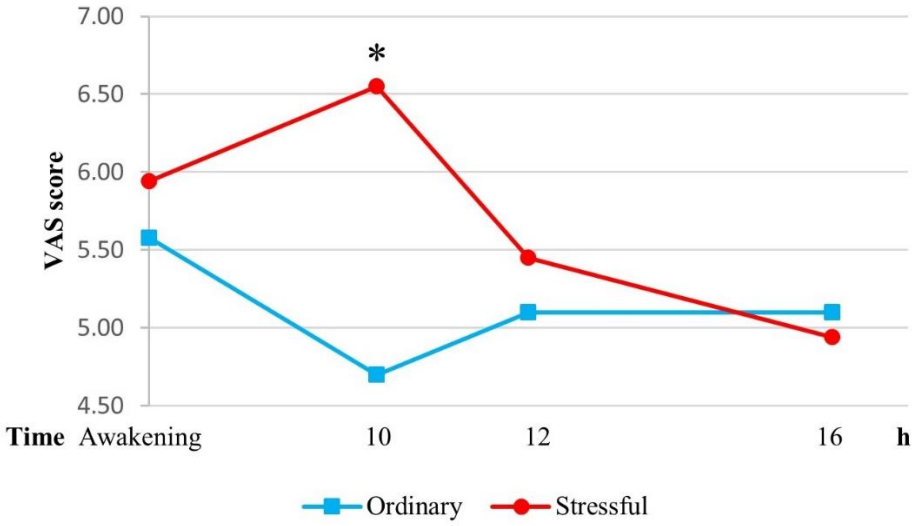

Figure 2 VAS scores on two emotional days. *$p < 0.05$ compared with ordinary day

5. Discussion

The current study found the profiles of daily sCort levels to be high in the morning and low in the afternoon in healthy volunteers on an ordinary day. Similarly, most previous studies indicated that sCort had a highest level in the morning followed by a gradual decline throughout the day (Bedini et al., 2017; Kobayashi et al., 2017; Sjörs et al., 2014; Van Lenten & Doane, 2016). The peak level was recorded in the first 30-45 minutes post-waking (Stalder, Hucklebridge, Evans, & Clow, 2009; Van Lenten & Doane, 2016) and the lowest level was found in the evening or immediately before bedtime (Van Lenten & Doane, 2016). Our results and other studies also showed that there were significant differences of sCort levels among various times in a day (Adam, Hawkley, Kudielka, & Cacioppo, 2006; Van Lenten & Doane, 2016).

The daily sCort profile on a stressful day displayed a different pattern compared with that of an ordinary day as it did not show the usual drop after awakening. Instead, sCort levels significantly increased during the day before dropping down at the end of the day. The daily average sCort level on a stressful day was also higher than that of an ordinary day. This result was also confirmed in a previous study which showed that workers had higher sCort levels on a shift involving more stressful duties than on a shift having less stressful duties (Bedini et al., 2017). In contrast, this result was inconsistent with another study which demonstrated that participants reporting stress at home had a significantly smaller total sCort output (Sjörs et al., 2014), and it also contradicted another study that showed participants in a high stress group secreted less sCort throughout the day compared to a low stress group (O'Connor et al., 2009). These findings may be attributed to the hyporeactivity of the HPA axis in the case of chronic stress (Hellhammer, Wüst, & Kudielka, 2009). These conflicts suggest the complexity of sCort levels and stress (i.e. it's not just a simple correlation of high stress and high cortisol). Chronic stress could also blunt awakening cortisol response and cause a flatter slope of daily cortisol, which was found to be related to a higher production of inflammatory markers (Herriot, Wrosch, Hamm, & Pruessner, 2020; Knight et al., 2021).

sCort levels on a stressful day were higher than those on an ordinary day only at two time points (10:00h and 12:00h). These likely point to a temporary nature of stress as reported by the participants since they did not experience the stress all day. Only four of the participants experienced stressful events both in the morning and afternoon. The stressful events reported in this study were psychological (academic presentations, an examination, or a challenging duty under the supervision of a very difficult supervisor). Previous studies showed that sCort levels could be elevated in these situations (Merz & Wolf, 2015; Preuss, Schoofs, Schlotz, & Wolf, 2010). Prolonged elevated levels of sCort can lead to serious diseases such as metabolic disorders, immune system disorders, psychiatric disorders (Bozovic, Racic, & Ivkovic, 2013) as well as

increased morbidity and mortality (Watson et al., 2015). Thus, management of psychological stress might reduce the possibility of long-term adverse effects. Indeed, a recent study found that stress management helped reduce total sCort levels in chronically stressed midlife and older adults (Urizar et al., 2021).

VAS scores were used to determine subjective feelings of stress, and daily VAS profiles corresponded with those of sCort levels on both ordinary and stressful days, which confirmed the presence of stress. However, VAS scores appeared to decline faster than sCort levels. Future studies are needed to confirm the period of stress and its correlation with the duration of increased sCort levels or VAS scores. Another factor that was different between ordinary and stressful days was the mean duration of sleep. However, even though the mean duration of sleep prior to a stressful day was significantly less than that of an ordinary day (Table 1), the awakening levels of sCort were not different. A previous study has shown that a shorter average objective sleep duration was associated with lower levels of awakening sCort and flatter slope of the sCort profile (Van Lenten & Doane, 2016).

There are several limitations in this study. First, the sample size was rather small with a narrow age range and a low number of male participants. Second, other parameters of body response to stress should also be employed to better report the relationship between the timing of stress and the initial response of the body to stress, such as blood pressure, pulse rate, or other biomarkers. Third, the frequency of assessing these parameters is also important. In the current study, only four time points throughout the day were selected. Ideally, an hourly assessment may give a clearer profile of sCort levels to stress response. These issues should be explored in future studies.

6. Conclusion

The daily profile of sCort on a stressful day was significantly different from that of an ordinary day showing higher average sCort levels overall as well as at 10:00h and 12:00h. The levels of sCort were also different at different times of the day both on stressful and ordinary days. VAS scores also confirmed the presence of stress in the participants as their profiles were in agreement with those of sCort profiles.

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