ORIGINAL ARTICLE

Awakening responses and diurnal fluctuations of salivary cortisol, DHEA-S and alpha-amylase in healthy male subjects

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Submitted: 2011-05-05 Accepted: 2011-07-26 Published online: 2011-08-29

Key words: **awakening response; cortisol; DHEA-S; α-amylase; salivary biomarkers**

Neuroendocrinol Lett 2011; **32**(4):475–480 **PMID:** 21876512 NEL320411A16 © 2011 Neuroendocrinology Letters • **www.nel.edu**

Abstract

OBJECTIVES: Because the cortisol awakening response (CAR) has received increasing attention as a useful index of adrenocortical activity, the primary objective of this study was to investigate the presence of an awakening response for various salivary biomarkers of adrenocortical activity, including dehydroepiandrosterone-sulphate (DHEA-S), which acts as a cortisol antagonist, and α-amylase, which is a predictor of circulating catecholamine activity. Salivary biological indicators are considered to be valuable markers of hypothalamus-pituitary-adrenal (HPA) axis diurnal activity. **METHODS:** In an attempt to overcome problems associated with non-adherence to the requested sampling protocol, only young, healthy males with a physiological CAR value (defined as a 50% increase in salivary cortisol within 30 min after waking) were included in the study (67 out of 102 who initially enrolled met this criterion). **RESULTS:** Our results suggested that, as is already known for cortisol, DHEA-S and α-amylase have significant awakening responses. In addition, daily profile of salivary cortisol, α-amylase and DHEA-S fluctuations were analysed. Significant correlations were found between salivary cortisol, DHEA-S and α-amylase levels. The results showed that cortisol and DHEA-S concentrations were inversely correlated with α-amylase levels. **DISCUSSION:** This correlation confirmed the distinctiveness of the two regulatory systems: salivary cortisol and DHEA-S concentrations reflect the activity of the HPA axis, whereas α-amylase activity is more closely related to sympathetic activity. In addition, the present study emphasizes the potential value of saliva collection (which is both easy and stress-free) in monitoring changes of adrenal function, confirming that multiple sampling (especially within 1 h after awakening) is necessary to reliably characterise biomarker activity when investigating neuroendocrine changes under various conditions.

INTRODUCTION

Circadian, monthly and even seasonal fluctuations reflect hormone and autacoid secretions in the human body (Swaab *et al.* 1996). The hypothalamus-pituitary-adrenal (HPA) axis and the sympathetic-adrenomedullary system (SAM) are two major biological systems involved in homeostatic and allostatic adaptations to environmental and internal stimuli (Kopin 1995; McEwen 2002; de Kloet 2003; Goldstein & McEwen 2002; McEwen 2005). Dysregulation of physiological rhythms is believed to play a role in the initiation or progression of some diseases (Giubilei *et al.* 2001; Patacchioli *et al.* 2003; Ball 2006; Mendlewicz 2009; Westrich & Sprouse 2010; Weinrib *et al.* 2010).

Cortisol, the most important steroid product of the adrenal gland, is a well-known marker of HPA axis activity; cortisol secretion follows a circadian rhythm, with significantly higher concentrations in the morning and lower concentrations in the evening (Young *et al.* 2004; Ice *et al.* 2004). Furthermore, a typical peculiar cortisol awakening response (CAR) has been described that reflects changes in cortisol concentration that occur during the first hour after waking in the morning (Pruessner *et al.* 1997). The CAR is reportedly highly stable within individuals (Fries *et al.* 2009) and is higher during the week than on the weekend (Thorn *et al.* 2006; Kunz-Ebrecht *et al.* 2004). Both enhanced and reduced CARs are associated with various psychosocial factors (Kirschbaum *et al.* 1995; Chida & Steptoe 2009), including depression and anxiety disorders (Bhattacharyya *et al.* 2008, Vreeburg *et al.* 2009; 2010). Moreover, a reduced CAR has been described in subjects suffering from either post-traumatic stress disorder syndrome (Wessa *et al.* 2006) or fatigue-related symptoms (Nater *et al.* 2008).

In recent years, salivary secretion of α-amylase has been proposed as an indicator of plasma catecholamine modifications under a variety of conditions (Chatterton *et al.* 1996; Walsh *et al.* 1999; Skosnik *et al.* 2000; Li & Gleeson 2004; Wolf *et al.* 2008). Moreover, significant diurnal fluctuations in salivary α-amylase have been reported, with low values reached within 60 min after awakening (Nater *et al.* 2007) and much higher values reached in the evening (Parkkila *et al.* 1995; Artino *et al.* 1998; Yamaguchi *et al.* 2006; Nater *et al.* 2007).

Dehydroepiandrosterone-sulphate (DHEA-S) is also secreted by the adrenal cortex and plays a pivotal role in the regulation of HPA activity, with effects that are opposite to cortisol at both peripheral and central levels (Majewska *et al.* 1990; Ebeling & Koivisto 1994; Ferrari *et al.* 2008). Currently, little is known about the daily fluctuations of DHEA-S, although circadian variation (with a trough concentration in the evening) has been reported in adults (Zhao *et al.* 2003) but not in the elderly (Del Ponte *et al.* 1990). To our knowledge, no available data describe a salivary DHEA-S awakening response.

Hormone salivary biomarkers have received increasing attention from researchers. However, the daily trajectories of salivary α-amylase and DHEA-S, as well as their relationship with cortisol, need to be further characterised in healthy populations before their roles in disease states are studied .

Failure to comply with the strict timing of saliva sampling can influence hormonal measurements and compromise the accuracy and reliability of the results (Hanrahan *et al.* 2006; Kudielka *et al.* 2009). Because CAR is considered a reliable parameter for detecting participants who are non-adherent to a study protocol (Steptoe *et al.* 2006; Thorn *et al.* 2006; O'Connor *et al.* 2009), only subjects who showed a significant CAR were included in this study.

The aim of our study was to investigate whether salivary α-amylase and DHEA-S awakening responses were present in young, healthy subjects who met the CAR criterion. The diurnal trajectories of salivary cortisol, α-amylase and DHEA-S as well as their interrelationships were also evaluated.

MATERIALS AND METHODS

Study population

This study was conducted over a period of 2 years, from February 2009 to January 2011. A total of 102 healthy male university (Rome and Iasi) employees and medical students were enrolled in the study. Participants with metabolic, cardiovascular or endocrine disease were excluded. None of the study participants had received anti-inflammatory or immunosuppressive therapy during the previous six months, and the use of vasoactive drugs that could influence cortisol secretion (e.g., antihypertensive agents, antidepressants or thyroid medications) was also contraindicated. During the 3 days before the "saliva sampling day", the subjects were asked to maintain a complete resting state without physical activity or significant psychological input. Moreover, the diet was similar and standardised among the subjects. The participants were taught how to collect saliva using the Salivette sampling device (Sarstedt, Italy) and asked to avoid food, coffee or alcohol consumption, teeth brushing, smoking or any physical exercise both for 60 min after waking and for 30 min prior to the evening saliva collection (Hanrahan *et al.* 2006; Hackney & Viru 2008).

For measuring salivary biomarker awakening responses and circadian fluctuations, saliva was collected upon waking (at 07:00 h) and 15, 30, 45 and 60 min thereafter; and again at 20:00 h. The participants were asked to store the samples in their home refrigerators and to deliver the samples to the laboratory the day after collection.

The study protocol was approved by the Institutional Ethics Committee, and all participants were fully informed of the procedures and gave written, informed consent to participate in the study.

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Salivary sampling protocol and cortisol, DHEA-S and α-amylase measurements

Saliva was collected using the Salivette sampling device (Sarstedt), which allows for quick and hygienic saliva recovery from a polyester swab by centrifugation at 3,000 rpm for 15 min (Gröschl *et al.* 2008). Salivary samples were immediately frozen at –20 °C until analysis.

For each sample, duplicate measurements were performed on 25 µl of saliva using commercially available immunoenzymatic Cortisol Saliva and DHEA-S Saliva kits (Diametra, Italy) for the direct measurement of salivary cortisol (with an inter-assay coefficient of variation of <10%, an intra-assay coefficient of variation of <7% and a minimum detectable concentration of 0.5 ng/ml) and DHEA-S (with an inter-assay coefficient of variation of <10%, an intra-assay coefficient of variation of <7% and a minimum detectable concentration of 25 pg/ml). Salivary α-amylase was measured by a commercially available assay kit (Diametra) that was specifically designed to quantify α-amylase activity by colourimetric assay (the inter-assay coefficient of variation was <1.5%, the intra-assay coefficient of variation was <1.5%, and the minimum detectable concentration of α-amylase that could be distinguished from the blank standard at a 95% confidence interval was 2.5 U/ml).

Data analysis and statistics

Statistical analyses were performed using Statistica 6 (StatSoft, Inc.). Unless otherwise stated, data are presented as mean±SD. ANOVAs (with repeated measures) were computed to reveal the TIME effect on salivary cortisol, DHEA-S and α-amylase for the first 5 samples (taken at 07:00, 07:15, 07:30, 07:45 and 08:00 h) and for the samples taken 20:00 h. To test for significance with more than two levels, the Bonferroni *post-hoc* multiple comparison test was performed. For all of the evaluated substances, we also separately computed Pearson's correlation coefficients of concentration against time for the samples taken at all 6 time points. For the significance of *r*, the parameters of the interpolated regression line equations were also calculated. A subsequent *t*-test to check for parallelism between regression lines was performed on pairs of *b* parameters derived from the equations.

One-way ANOVA power analyses were calculated for a total sample size of 67 using Statistica 6 (StatSoft, Inc.); the chosen sample size was of sufficient power (α=0.05). Specifically, we found a cortisol root mean square standardised effect (RMSSE) of 0.65 (*f*=0.60 with a power of 1.0); for α-amylase, the RMSSE was 0.29 (*f*=0.26 with a power of 0.99); for DHEA-S, the RMSSE was 0.35 (*f*=0.32 with a power of 0.99).

RESULTS

Characteristics of the study population

Of the 102 subjects who enrolled in the study, 35 failed to meet the CAR inclusion criterion (which was defined as a 50% increase in salivary cortisol within the first 30 min of awakening) and were excluded (Clow *et al.* 2004).

The subjects' socio-demographic characteristics are summarised in Table 1. The study cohort was composed of 67 young, healthy males (mean age 28±7 years) with at least a college education. The majority of the participants were non-smokers (94%), and 9 participants (13%) were married.

Tab. 1. Socio-demographic characteristics of the 67 participants that were selected according to their CAR values (see Materials and Methods).

Age (years)	28±7
BMI (kg/m^2)	24.5±2.5
Current smokers (%)	4 (5.9)
Educational level (years)	14±3
Married (%)	9 (13.4)
Sleep duration on workday (h)	7.44±0.7

Data are expressed as mean±SD or number of subjects (% of total).

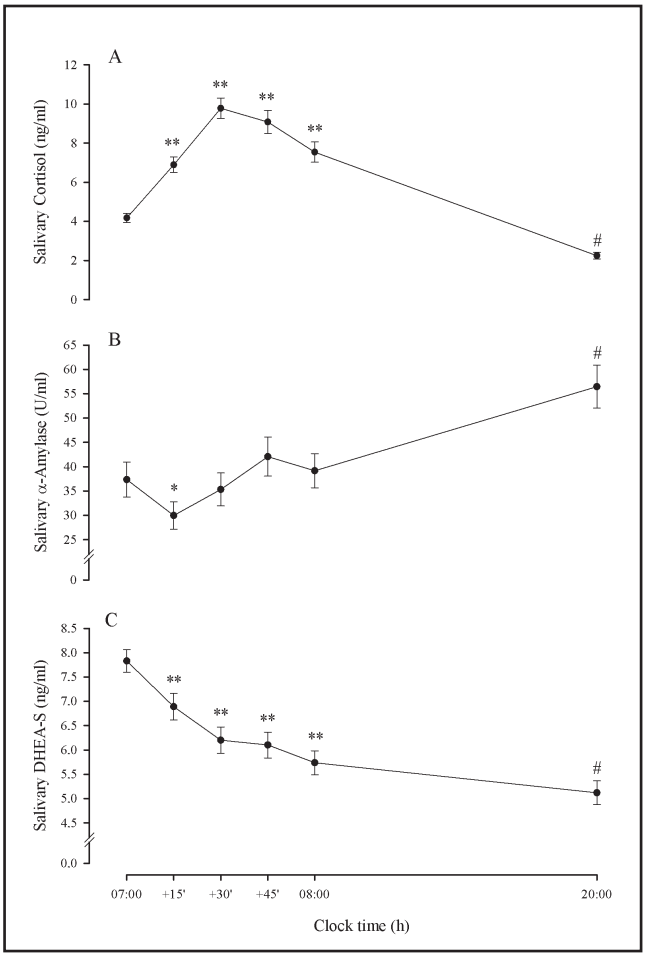

Fig. 1. Daily profile of salivary Cortisol (A), ct-Amylase (B) and DHEA-S (C) concentrations in young healthy subjects. Data are presented as mean ± SEM. * and **: $p<0.05$ and $p<0.001$, respectively vs 07:00; #$p<0.001$ vs 08:00.

Daily profiles of salivary cortisol, DHEA-S and α-amylase

Figure 1 shows the mean (±SEM) concentrations of salivary cortisol, α-amylase and DHEA-S measured during the sampling day in the study population. Salivary cortisol levels (Figure 1A) upon waking and 15, 30, 45 and 60 minutes thereafter reflected a typical CAR course. Using a repeated-measures ANOVA, we detected a significant change in cortisol concentration over TIME ($F_{5,330}$=79.76; p<0.001). Salivary cortisol concentrations increased from 4.18±0.23 ng/ml at waking to 6.89±0.40 and 9.78±0.53 ng/ml after 15 and 30 min, respectively, which represented significant increases of 65 (p<0.001) and 134% (p<0.001), respectively; the levels then declined within 45 min of waking. Moreover, significant diurnal cortisol fluctuations were measured in the study population (Figure 1A), with evening and morning cortisol levels of 2.25±0.16 and 5.74±0.25 ng/ml, respectively (p<0.001).

As shown in Figure 1B, secreted salivary α-amylase levels decreased in the first 15 min after waking (from 37.4±3.56 to 29.9±2.79 U/ml; p<0.05). After this initial decrease, α-amylase secretion then increased, reaching 35.3±3.37, 42.0±4.01 and 39.2±3.52 U/ml at 30, 45 and 60 min after waking, respectively. Using the repeated measures ANOVA test, TIME was found to have a significant effect ($F_{5,330}$=12.83; p<0.001). We also found a significant diurnal increase in α-amylase activity (Figure 1B); the evening (20:00 h) and morning (08:00 h) levels were 56.5±4.42 and 39.2±3.52 U/ml, respectively (p<0.001).

After waking, salivary DHEA-S levels (Figure 1C) decreased steadily and significantly (p<0.001), from 7.83±0.23 ng/ml at awaking to 6.4±0.22, 5.92±0.21, 5.86±0.21 and 5.74±0.25 ng/ml after 15, 30, 45 and 60 min, respectively. Applying repeated measures ANOVA revealed a significant effect of TIME ($F_{5,330}$=58.4; p<0.001). DHEA-S levels further decreased during the day (Figure 1C), from 5.74±0.25 ng/ml at 08:00 h to 5.12±0.25 ng/ml at 20:00 h (p<0.001).

Correlations among fluctuations in salivary cortisol, DHEA-S and α-amylase

To evaluate the daily trend of each salivary biomarker, the Pearson coefficient (for salivary biomarker concentration against time) was determined. When the resulting r value was significant, an equation that described the interpolated regression line was derived. The following parameters were obtained for the various biomarkers: cortisol r=–0.41 (p<0.001) with the equation y=10.494–0.0066x; α-amylase r= 0.24 (p<0.01) with the equation y=23.864+0.2754x; DHEA-S r=–0.26 (p<0.01) with the equation y=7.6039–0.0022x (scatterplots are not shown). All t-tests for pairs of b parameters yielded significance (cortisol vs. α-amylase t [gdl800] = 85.96 [p<0.01]; cortisol vs. DHEA-S t [gdl800] = 540.13 [p<0.01]; α-amylase vs. DHEA-S t [gdl800] = 86.60 [p<0.01]). These results showed that while cortisol and DHEA-S concentrations were decreasing throughout the day, α-amylase concentration was increasing. In contrast, cortisol and DHEA-S decreased in parallel, though at significantly different rates.

DISCUSSION

The present study demonstrated that, similar to cortisol, salivary α-amylase and DHEA-S produce awakening responses in healthy subjects (Pruessner *et al.* 1997; Young *et al.* 2004; Ice *et al.* 2004). Moreover, we found significant relationships among these salivary biomarkers.

When relying upon saliva sampling in an outpatient setting, compliance with the sampling schedule is essential. Compliance was ensured in the present study by excluding subjects suspected of non-adherence based on a flat CAR (Steptoe *et al.* 2006; Thorn *et al.* 2006; O'Connor *et al.* 2009).

A salivary α-amylase awakening response was observed in the study population; specifically, a pronounced decrease in α-amylase concentration was measured 15 min after waking. Nater and co-workers (Nater *et al.* 2007) have previously reported sharp drops in salivary α-amylase activity 30 and 60 min after waking. This slight discrepancy might be due to the different time schedules of salivary sampling and/or the strict criterion applied in the present study, i.e., evaluating α-amylase and DHEA-S only in those subjects who had demonstrated a sufficient CAR (Clow *et al.* 2004; Kudielka *et al.* 2003; Thorn *et al.* 2006; O'Connor *et al.* 2009). We also found a DHEA-S awakening response in our study population, with a significant decrease in DHEA-S levels within the first hour after waking.

As expected, we found significant diurnal fluctuations in salivary cortisol levels, with the highest concentrations measured in the morning and the lowest concentrations measured in the evening. Concomitantly, in the same subjects, a significant increase in α-amylase was measured in the evening compared to the morning. Our results are in agreement with other studies that have reported a DHEA-S concentration trough during the day (Zhao *et al.* 2003; Ahn *et al.* 2007).

The results of this study showed that salivary cortisol and α-amylase have opposite diurnal fluctuation patterns: as evening approaches, cortisol concentrations decrease, and α-amylase activity rises (Parkkila *et al.* 1995; Chatterton *et al.* 1996; Artino *et al.* 1998; Yamaguchi *et al.* 2006; Nater *et al.* 2007), confirming that these two systems are distinct (Wolf *et al.* 2008). On the whole, salivary cortisol and DHEA-S levels reflect the activity of the HPA axis, whereas α-amylase is considered to be a marker of sympathetic activity (Ebeling & Koivisto 1994; Wolf *et al.* 2008). Although Cortisol and DHEA-S are adrenocortical secretory products the responses are inverted probably because of their physiological opposite activities (Majewska *et al.* 1990; Ebeling & Koivisto 1994; Ferrari *et al.* 2008).

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No correlation among salivary biomarkers was reported by Yamaguchi and co-workers (Yamaguchi *et al.* 2009), who evaluated changes in α-amylase, DHEA-S and cortisol levels following the inhalation of plant essential oil fragrances by healthy young women; however, the diurnal course of the response was not analysed. Moreover, no correlation was found between cortisol and salivary α-amylase responses to various stress paradigms (Chatterton *et al.* 1996; Nater *et al.* 2005; Filaire *et al.* 2009). In contrast, we found a significant relationship between the diurnal curves of salivary cortisol, α-amylase and DHEA-S. However, this discrepancy may have been due to our study measuring these salivary biomarkers under basal conditions.

Previous studies from our group and others have shown that the measurement of salivary cortisol, α-amylase and DHEA-S is becoming more widely accepted for monitoring changes in HPA and SAM activity under stress-related conditions (Kirschbaum & Hellhammer 1989; Kirschbaum & Hellhammer 1994; Patacchioli *et al.* 2003; Patacchioli *et al.* 2006; Simeoni *et al.* 2011). Changes in salivary cortisol, α-amylase and DHEA-S levels, as well as their diurnal fluctuations, are thought to have implications for health (Ahn *et al.* 2007; Wolf *et al.* 2008; Chida & Steptoe 2009). Therefore, choosing both an appropriate time of day for sample collection and an appropriate sampling schedule are important when investigating neuroendocrine changes under different conditions (Hackney & Viru 2008). The present study confirmed that multiple sampling, especially within 1 h of waking, is necessary to reliably measure salivary biomarker levels.

In summary, the results of this study indicate that both α-amylase and DHEA-S have distinct and significant awakening responses in a homogenous group of young, healthy males stringently selected for their adherence to the protocol. Future studies will be necessary to elucidate the presence and/or modification of α-amylase and DHEA-S awakening responses in other populations.

AKNOWLEDGEMENTS

This study was partially supported by a grant from MIUR to F.R.P. and P.R.

The authors wish to thank Diametra (Italy) for generously supplying the salivary assay kits.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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