REVIEW

SALIVARY CORTISOL IN PSYCHONEUROENDOCRINE RESEARCH: RECENT DEVELOPMENTS AND APPLICATIONS

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SUMMARY

The assessment of cortisol in saliva has proven a valid and reliable reflection of the respective unbound hormone in blood. To date, assessment of cortisol in saliva is a widely accepted and frequently employed method in psychoneuroendocrinology. Due to several advantages over blood cortisol analyses (e.g., stress-free sampling, laboratory independence, lower costs) saliva cortisol assessment can be the method of choice in basic research and clinical environments. The determination of cortisol in saliva can facilitate stress studies including newborns and infants and replace blood sampling for diagnostic endocrine tests like the dexamethasone suppression test. The present paper provides an up-to-date overview of recent methodological developments, novel applications as well as a discussion of possible future applications of salivary cortisol determination.

Keywords—Cortisol; Saliva; Stress; Exercise; Humans; Review.

INTRODUCTION

The assessment of cortisol in saliva has recently become a valuable alternative to blood-borne analysis. Due to the noninvasiveness and laboratory independence of sampling, salivary cortisol can be measured at almost unlimited frequency under a wide variety of clinical and field settings. Since cortisol is considered to enter saliva by passive diffusion or other means independent of an active transport mechanism, cortisol levels in saliva are unaffected by saliva flow rate. Moreover, the acinar cells lining the saliva glands prevent proteins and protein-bound molecules from entering saliva. Salivary cortisol is therefore an easy-to-assess measure of the unbound, 'free' hormone fraction. According to the free hormone concept (Mendel, 1989; Robbins & Rall, 1957), only unbound cortisol reaches the target tissue and elicits glucocorticoid effects. Earlier reviews of the literature outlined the history of salivary cortisol assessment, discussed the mode of entry of cortisol into saliva, provided normal values for a variety of subject populations, and stressed the possible advantages of salivary sampling over blood analyses.

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as well as the particular usefulness of this method in psychoneuroendocrinological research (Hellhammer et al., 1987; Kirschbaum & Hellhammer, 1989; Kirschbaum et al., 1992a; Riad–Fahmy et al., 1982; Vining & McGinley, 1984). The present paper adds an overview of new methods and applications of salivary cortisol assessment and summarizes recent data and findings obtained with this method. It is intended to provide additional evidence for the valuable contributions of cortisol measurement in saliva and to encourage a broader employment of this method in basic and clinical psychoendocrinological research.

**SAMPLING AND STORAGE OF SALIVA**

The ease of sampling is one of the most obvious advantages of cortisol assessment in saliva. In general, saliva samples can be obtained stress-free at almost any desired frequency in most subjects. Depending on the sensitivity and reliability of the read-out system involved, sample volume may vary between 0.05–2 ml saliva. While whole saliva sampled in wide disposable containers provides adequate material for analysis, a convenient sampling device called “Salivette” (Sarstedt Inc., Rommelsdorf, Germany) is used by an increasing number of investigators. The Salivette mainly consists of a small cotton swab, which fits inside a standard centrifugation tube. By gently chewing on the swab, subjects stimulate saliva flow to a rate that provides sufficient material within 30–60 sec. Not only does saliva sampling with the Salivette avoid emotive biases toward the specimen, it also facilitates pipetting of the sample since the debris is separated from the clear watery saliva supernatant.

While saliva sampling can be efficiently performed with the Salivette by conscious children and adults, different techniques are required if cortisol levels are to be assessed in infants or over prolonged periods, for example, during sleep. In newborns, saliva can be obtained by gently swabbing the baby’s mouth with a cotton dental roll (Gunnar et al., 1989) or by aspirating saliva with a small pipette or a disposable mucus extractor (Hiramatsu, 1981; Riad–Fahmy et al., 1982). Hänecke and Haeckel (1992) described an alternative approach. They used modified feeding bottles containing absorption material inside the sucker. Although a convenient method for saliva collection, the authors emphasize that varying amounts of saliva are obtained and thus a sensitive assay requiring only small volumes is needed. A note of caution was issued by Magnano et al. (1989), who assayed formula and breast milk for cortisol immunoreactivity. They found significant cortisol immunoreactivity in three different common infant milk formulas as well as in breast milk if samples were assayed directly. After defatting and extracting the samples, only little immunoreactivity was found for formula milk, but a higher level was found in one out of two breast milk samples. Saliva samples should, therefore, be collected a considerable time following feeding. In addition to a potential rise in cortisol levels (post-prandial rise) in infants (Spangler, 1991), milk contamination of infant saliva samples may yield false high cortisol results.

As an alternative to urinary cortisol measurement, time-integrated assessment of unbound cortisol has been made available by introduction of a new sampling device called “Oral Diffusion Sink” (ODS; Wade & Haegele, 1991a). This device consists of a 2 × 15 mm radio-opaque polycarbonate resin shell with 12 perforations (ports). The ports are covered by a membrane limiting the diffusion of molecules from saliva into the device according to their molecular weight. The ODS is secured in the mouth of the subject by attaching it to a tooth with dental floss. Once the device is secured, the subjects tested
so far did not report discomfort wearing the ODS. Cortisol molecules are bound inside the ODS either by a specific antiserum (Wade & Haegel, 1991a) or β-cyclodextrin (Wade, 1992) which is less expensive and capable of binding several steroid hormones inside the same device. The binding capacity allows for sampling intervals of 1–8 h, thus salivary cortisol measurement over prolonged periods is possible, e.g., during sleep. The validity of the results obtained with the ODS was investigated in vitro as well as in vivo. Gehris and Kathol (1992) found high correlations between cortisol concentration measured with the ODS compared to cortisol levels in serum and saliva at baseline and following stimulation with ACTH1–24. Similarly, the ODS was reported to parallel results obtained from analysis of urinary free cortisol in 4-h samples (Shipley et al., 1992). The ODS device appears to be a useful expansion of available technology in salivary cortisol assessment. It may provide us with a substitute for urinary cortisol determination which can lead to erroneous results due to collection errors in unsupervised subjects. However, so far the device has only been evaluated in a relative small number of subjects. More tests in larger populations are therefore needed to assess the utility of the ODS device in different settings.

Saliva samples should be stored frozen whenever possible. However, under certain circumstances samples are stored at ambient temperature, e.g., during field studies in the natural environment. We (Kirschbaum & Hellhammer, 1989) and others (Leonard et al., 1991) have shown, that saliva samples can be stored at 20°C for up to 4 weeks without significant reduction in cortisol levels. Even longer periods of storage at room temperature up to 16 weeks can be achieved by adding 10 g/l citric acid to the samples or by the use of citric acid-treated “Salivette” devices. Caution is necessary when citric acid samples are analyzed, because in some immunoassays a low sample pH results in false high cortisol levels. Before sampling with acid-treated Salivettes in studied patients or volunteers, the investigator should carefully run parallel analyses on acid-free and acid-treated samples to show that their assay system is unaffected by the acid.

In conclusion, samples for salivary cortisol determination can be easily obtained and stored in nearly every possible environment. Remote monitoring of a patient with intermittent Cushing’s disease (Thomopoulos et al., 1992), frequent sampling in parachute jumpers (Deinzer et al., 1991), ambulatory sampling by subjects in their natural environment (Nicolson, 1992), and psychoendocrine investigations during space flights (scheduled project) show some possibilities brought about by easy sampling and storage of saliva.

ASSAYS FOR SALIVARY CORTISOL DETERMINATION

Sensitive radioimmunoassays (RIA) for reliable cortisol measurement in saliva have been established in the past, some of which were adaptations of commercially available serum kits (overview given in Kirschbaum & Hellhammer, 1989). These assays can be performed at reasonable costs in any laboratory allowed to use isotopes and equipped with a gamma-counter. Apart from potential health hazards and environmental problems associated with the radioactive waste produced, RIAs are perfectly suited for salivary cortisol measurement. Especially in psychology departments, in absence of medical or neuroscience laboratories, however, researchers in psychoneuroendocrinology often lack the appropriate facilities or permissions. Thus, they often have their saliva samples analyzed by a specialized laboratory. Recent advances in immunoassay developments possibly provide an alternative. Two nonisotopic immunoassays for salivary cortisol
determination have been described. Cooper et al. (1989) used horseradish peroxidase labelled cortisol tracers in their enzyme-linked immunosorbent assay (ELISA). This assay was reported to yield consistently a sensitivity of less than 1 pg and less than 2% intra- and inter-assay coefficients of variation. The latter results cast some doubt on the calculations of the assay performance, since even the use of pipette robots and high-tech coated assay tubes will hardly yield inter-assay variations below 4–5% in the normal range of salivary cortisol concentrations (1–25 nmol/l). A universal nonisotopic assay system for cortisol measurement in saliva was developed by Dressendorfer et al. (1990). Following the synthesis of a stable cortisol-biotin conjugate (Strasburger et al., 1990) salivary cortisol levels can be measured reliably using time-resolved fluorescence, luminescence, or enzyme-linked read-out systems. In the authors’ laboratory the time-resolved fluorescence immunoassay (Dressendorfer et al., 1992) was used on over 60,000 samples over the course of 3 years. The assay proved to be a highly reliable system with consistent results as indicated by the in-house quality controls.

For simultaneous and differential determination of cortisol and cortisone in saliva, a sensitive high-performance liquid chromatography (HPLC) method has been described recently (Wade & Haegel, 1991b). In addition to the two endogenous steroids, salivary levels of dexamethasone could possibly be detected on the same chromatogram. Thus, HPLC analysis of saliva samples could be the method of choice if smaller quantities of saliva samples are to be analyzed for these steroids, for example, in the DST (see above).

**COMPARISON OF CORTISOL LEVELS IN SALIVA AND SERUM**

Considerable doubts still seem to exist whether cortisol levels measured in saliva actually provide a valid and reliable correlate of serum or plasma cortisol concentrations. Although more than a dozen papers had been cited earlier (Kirschbaum & Hellhammer, 1989) indicating a high agreement between the two measures, additional studies compared salivary cortisol with serum/plasma cortisol concentrations in different populations. Supporting data obtained in healthy adults, high correlations between salivary and serum cortisol were reported in newborns (Gunnar et al., 1989), children and adolescents (Woodside et al., 1991), elderly subjects (Reid et al., 1992), as well as in psychiatric patients on an acute-admission ward (Harris et al., 1990). Simultaneous measurement in saliva and blood in volunteers following administration of exogenous cortisol (Tunn et al., 1992) and dexamethasone (Harris et al., 1990; McCracken & Poland, 1989; Woodside et al., 1991) also showed significant covariation. The correlation coefficients between cortisol in saliva and cortisol in serum ranged between $r = .71$ (patients on alpha-cholinergic medication) to $r = .96$ (healthy elderly subjects) in these studies, thus determining 50.4–86.4% of the total variance. Given that in most studies only total blood cortisol was measured (not solely the unbound fraction), a certain nonlinearity between total and unbound cortisol, and measurement errors in two separate analyses, these results clearly suggest that salivary cortisol assessment is closely correlated with cortisol levels in blood. However, one has to note that the absolute levels of cortisol found in saliva are significantly lower than in blood. This is at least in part due to an enhanced conversion of cortisol to cortisone by 11β-hydroxysteroid dehydrogenase activity in saliva. As summarized earlier (Kirschbaum & Hellhammer, 1989), the available literature clearly suggests that despite lower concentration in saliva, salivary cortisol is even closer correlated with the free cortisol fraction in serum compared to total serum cortisol.
CIRCADIAN RHYTHM OF CORTISOL AND CORTISOL RESPONSES DURING PREGNANCY

Among the multitude of altered physiological parameters in pregnant women are profound changes in adrenocortical activity. While an increase in total cortisol levels over the course of pregnancy has long been known, only recent studies investigated the circadian fluctuation and responses of salivary cortisol in pregnant women systematically. Converging data from different laboratories suggest that unstimulated salivary cortisol levels start to rise steadily at approximately the 25th week of gestation and stay elevated until delivery (Allolio et al., 1990; Meulenberg & Hofman, 1990a, 1990b; Scott et al., 1990). Although mean cortisol levels are elevated 1.5- to 2-fold, the circadian rhythm is preserved. While early morning concentrations in late pregnancy exceed values in nonpregnant women by far, this difference becomes less evident in samples obtained later in the afternoon. Meulenberg and Hofman (1990a) observed a shift in peak values for salivary cortisol with pregnant women lagging 90 min behind their controls. They concluded that this may reflect a delayed activation of the HPA axis in pregnancy. Shortly after parturition (5–7 days) salivary cortisol levels return to normal levels.

The reasons for elevated free cortisol levels in pregnancy is still unknown. It was hypothesized that the well-documented elevation of corticosteroid-binding globulin (CBG) and/or the increase in production of placental CRH is responsible for the increase in salivary cortisol. However, studies by Allolio et al. (1990) and Scott et al. (1990) concluded that neither CBG nor CRH levels alone could explain this phenomenon. They proposed a resetting of hypothalamic–pituitary sensitivity to cortisol feedback and a transient glucocorticoid resistance due to high progesterone concentrations. This hypothesis is supported by findings suggesting that in the third trimester pregnant women do not respond to exogenous administration of CRH with an increase in ACTH and salivary cortisol (Schulte et al., 1990). In conclusion, both circadian rhythm and responses to exogenous stimulation are altered in pregnant women and the differences to nonpregnant controls become evident after 20 weeks of gestation.

SALIVARY CORTISOL AND THE DEXAMETHASONE SUPPRESSION TEST

Since its introduction by Liddle (1960), the Dexamethasone Suppression Test (DST) continued to serve as a tool in the diagnosis of Cushing's syndrome. However, the reliability of the DST has been questioned in studies with women taking oral contraceptives. Due to an increased production of CBG induced by the exogenous steroids, healthy women on contraceptive medication failed to show adequate suppression of cortisol levels after ingestion of 1 mg dexamethasone in some studies (Grant et al., 1965; Treece et al., 1977). Since the unbound cortisol fraction remains unchanged under estrogen medication (Vining & McGinley, 1986), recent studies evaluated the usefulness of salivary cortisol instead of total blood cortisol DST assessment in the diagnosis of Cushing's syndrome (Allolio et al., 1986, 1992; Reinicke et al., 1992). The data obtained suggest that cortisol assessment in saliva can replace serum measures since they prevent false positive results. Investigating the effect of estrogen medication on the DST outcome in healthy females, Nickelsen et al. (1989) found six nonsuppressors (31%) according to their serum cortisol levels, but none when salivary cortisol levels were employed. Unfortunately, this study did not measure mood or personality to exclude subjects with high scores on depression ratings.
In contrast to its diagnostic value in the diagnosis of Cushing's syndrome, the usefulness of the DST as a biological marker for melancholic depression (Carroll et al., 1981) is discussed controversially. However, the test is nevertheless currently used as a diagnostic tool in many psychiatric institutions. Several investigators used matched serum and saliva samples to evaluate the performance of the DST with salivary cortisol as the read-out system. The efficacy of the salivary DST yielded conflicting results (Galar et al., 1991; Hanada et al., 1985; Poland & Rubin, 1982), which may in part be due to the small number of patients investigated. Moreover, different cut-off values for salivary cortisol were defined in these studies attempting to separate nonsuppressors from suppressors most efficiently. Two recent larger-scale studies with more than 400 patients, however, concluded that the diagnostic value of salivary cortisol DSTs equals that of blood-borne tests (Cook et al., 1986; Harris et al., 1990). To avoid the problem of random definition of the cut-off criterion, Harris et al. (1990) employed the technique of Receiver Operating Characteristic (ROC) curves, plotting specificity against sensitivity of the test. The comparison of the salivary and serum cortisol ROC curves showed almost identical performance of the DST in both specimen. In accordance with previous studies of their group, Copolov et al. (1989) again failed to obtain a high specificity of the DST in the differentiation between melancholic and nonmelancholic depressives. Determination of salivary dexamethasone levels did not increase the specificity of the test. Unfortunately, no matched serum samples were obtained in this study, thus a comparison of the saliva test performance with the serum DST is impossible. One interesting finding of their study is, however, that hospitalized patients showed significantly higher cortisol levels than outpatients and healthy controls. Therefore, the stress of hospitalization (separation from family, novel environment, etc.) must be regarded as a potential variable influencing the outcome of the DST.

In summary, saliva samples could substitute for serum samples in the clinical evaluation of Cushing's disease and melancholic depression given that appropriate analytical techniques are employed. Combining the advantages of stress-free sampling, independence of estrogen levels and laboratory facilities, the salivary cortisol DST may be especially advantageous studying women under estrogen containing medication and/or outpatients.

**EFFECTS OF PSYCHOLOGICAL STRESS ON SALIVARY CORTISOL**

It is now widely accepted that psychological stress can increase the activity of the HPA axis. Especially in situations with high ego-involvement, low predictability, low controllability, and novelty, corticotropin releasing hormone (CRH) and ACTH are released with subsequent rise in cortisol levels. Table I summarizes recent studies on the effects of psychological stress on cortisol in saliva and shows that applications of this method range from investigations in newborns over studies among adults in the laboratory to field studies at the dentist's, during driving examinations or parachute jumping. In the following, two sets of studies will be elaborated in more detail, because (a) they demonstrate possible advantages of cortisol assessment in saliva in studies with children, or (b) they focused on elucidating sources of the large interindividual differences observed in the adrenocortical response.

**Studies on Adrenocortical Activity in Newborns and Infants**

While venipuncture has been proposed as a simple stress test of adrenocortical activity in infants (Mantagou et al., 1991), frequent blood sampling for hormone assessments in
children is difficult with regard to ethical considerations. Due to the noninvasiveness of saliva sampling, research activities this neglected area of psychoendocrinology received increasing attention. Several studies recently emerged on cortisol stress responses among newborns and infants. Gunnar and her group (1989) studied responses of 49 newborns to two discharge examinations of approximately 6 min duration each. While they observed a large cortisol response to the first examination (+11.6 nmol/l), the overall response to the second examination was blunted (+2.2 nmol/l). These results are supported in part by findings of Spangler and Scheubeck (1993), who observed similar stress responses in newborns. However they noted that only under simultaneous assessment of heart rates with a heart rate monitor attached to the child’s chest the subjects responded to both the first as well as to the second examination. Likewise, inoculation of infants by a pediatrician (Lewis & Thomas, 1990), brief maternal separation (Larson et al., 1991), neonatal behavior examination and heel stick (Magnano et al., 1992) were found to increase salivary cortisol levels. These results indicate that the human adrenocortex is highly responsive from a very early postnatal period with adult-like response patterns. Taking advantage of the opportunity to obtain serial samples from infants, future studies could now aim at a better understanding of the ontogenesis of psychoendocrine stress responses and possible implications for psychosomatic complaints in later life.

Studies on Interindividual Differences of Cortisol Responses

One of most prominent features in studies of adrenocortical responses under pharmacological, physical, or psychological stimulation is the large variation in both baseline levels and in response magnitude between subjects. While a similar time course of cortisol secretion, for example, after challenge with synthetic CRH, can be observed in most subjects, large interindividual variation exist with respect to the amount of cortisol released. A multitude of variables probably account for these differences, some of which have been unraveled in recent studies employing cortisol assessment in saliva.

A. Genetic factors. Possible contribution of genetic determination of baseline and stimulated cortisol levels were studied in a sample of monozygotic (MZ) and dizygotic (DZ) twins who (a) received a bolus injection of 100 μg h-CRH; (b) were exposed to a psychological stress task of public speaking and mental arithmetic ("Trier Mental Challenge Test," TSST; Kirschbaum & Hellhammer, 1993); and (c) performed bicycle ergometry until exhaustion. Analysis of intrapair correlation revealed a high resemblance of baseline cortisol levels prior to each stimulation, peak responses to CRH and to the TSST in MZ twins. No such resemblance was evident for cortisol release following physical stress (Kirschbaum et al., 1992b). These data suggest that baseline and response levels of cortisol are genetically determined. The impacts of such response tendency for psychosomatic and other complaints may be important for future studies.

B. Sex differences. In a series of studies, sex differences in cortisol responses to psychological stress have been studied in the authors’ laboratory. Using the TSST for reliable induction of significant cortisol release, consistent differences in the adrenocortical stress response were observed between healthy males and females in four independent studies (Kirschbaum et al., 1992c). Starting at similar baseline levels, males always released 1.5-2 fold more cortisol than females following psychological stress (Fig. 1). Even if the subjects only anticipated the stress without subsequent exposure, males had increased cortisol levels in contrast to females. These sex differences do not reflect an overall decreased responsiveness of the female adrenocor-
<table>
<thead>
<tr>
<th>Author(s), year</th>
<th>Subjects; age</th>
<th>Stressor; duration</th>
<th>Control group/session</th>
<th>Sampling time</th>
<th>Results</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benjamins et al. (1992)</td>
<td>Highly anxious dental patients ($n = 13$); 35 yr</td>
<td>Dental examination</td>
<td>Non-anxious subjects, male staff members ($n = 13$); 38 yr</td>
<td>Before examination (patients), 8:30–12:00 am; 8:30–12:00 am controls</td>
<td>14.7 nmol/l (patients) vs. 7.65 nmol/l (controls)</td>
<td>No sex differences</td>
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<td>Bohnen et al. (1990, 1991)</td>
<td>Healthy adults ($n = 24$); 41–69 yr</td>
<td>(a) Continuous cognitive tasks (4 h) b) Control session (4 h)</td>
<td>None</td>
<td>6 samples from 10:20 am to 3:30 pm</td>
<td>Higher cortisol levels in stress session (1.1 nmol/l); similar increase between 2:00 and 3:00 pm in both groups</td>
<td>Subjects were allowed to have a &quot;quick&quot; lunch</td>
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<tr>
<td>Cook et al. (1992)</td>
<td>Study 1: Healthy adults ($n = 5$) Study 2: Healthy adults ($n = 9$)</td>
<td>Study 1: Anticipation of parachute jumping Study 2: University exam</td>
<td>Control day, same subjects</td>
<td>Study 1: 5 samples on stress and control days Study 2: 8 samples on stress and control days</td>
<td>Study 1: 11.6 nmol/l 1 higher than on control day Study 2: higher levels on stress day (&lt; 4 nmol/l)</td>
<td>Use of assay system which gives false high free cortisol concentrations</td>
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<td>Croes et al. (1993)</td>
<td>a) Acutely depressed patients, untreated ($n = 11$); 46.2 yr b) Acutely depressed patients, treated ($n = 11$); 43.5 yr</td>
<td>Number completion and addition test (15 min)</td>
<td>Patients following traumatic surgery ($n = 11$); 45.5 yr</td>
<td>3 samples before, 3 samples after test at 5 min intervals</td>
<td>+1.4 nmol/l only in control subjects, uncontrollable stress; all other groups decreasing values</td>
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<td>Gunnar et al. (1989)</td>
<td>Newborns ($n = 49$); 29–82 h</td>
<td>Two discharge examinations; 6 min each</td>
<td>None</td>
<td>Before, +25 min</td>
<td>+11.6 nmol/l (day 1) +2.2 nmol/l (day 2)</td>
<td>Males also tended to respond on day 2</td>
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<td>Houtman &amp; Bakker (1991)</td>
<td>Healthy adults ($n = 45$); 23–31 yr</td>
<td>Two public lectures</td>
<td>None</td>
<td>Before, +15 min</td>
<td>No results on cortisol levels given</td>
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<td>Study</td>
<td>Participants</td>
<td>Intervention</td>
<td>Sample Size</td>
<td>Stressor Details</td>
<td>Stress Response</td>
<td>Notes</td>
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<td>Hubert &amp; de Jong–Meyer (1990)</td>
<td>General anxiety disorder patients (n = 12); 20–40 yr</td>
<td>a) “positive mood” film segments (9 min) b) “negative affect” film segments (9 min)</td>
<td>6 samples at 15 min intervals</td>
<td>Decreasing cortisol levels in both groups</td>
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<td>Hubert &amp; de Jong–Meyer (1992)</td>
<td>Healthy adults (n = 32); 18–40 yr</td>
<td>Suspense film (120 min)</td>
<td>Healthy adults, non-suspenseful film (n = 32)</td>
<td>10 samples at 20 min intervals</td>
<td>+1 nmol/l increase maximum in low anxious subjects, suspense film only; all other decreasing levels</td>
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<td>Hubert et al. (1993)</td>
<td>Healthy adults (n = 26); 19–32 yr</td>
<td>Humorous film (90 min)</td>
<td>Healthy adults, non-humorous film (n = 26)</td>
<td>9 samples at 20 min intervals</td>
<td>No increase over baseline levels; +1.5 nmol/l maximum difference between groups</td>
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<td>Hytten et al. (1990)</td>
<td>Study 1: Healthy adults (n = 44); 20.3 yr Study 2: Healthy adults (n = 21); 34.4 yr</td>
<td>Study 1: Smoke diving Study 2: Free fall lifeboat</td>
<td>Study 1: Healthy adults (n = 43); 27 yr Study 2: 34.4 yr</td>
<td>Study 1: Before, +10–+45 min (depending on performance speed) Study 2: After fall</td>
<td>Study 1: +3.4 in both groups Study 2: NS</td>
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<td>Kahn et al. (1992)</td>
<td>Undergraduate students (n = 73); 16.8 yr</td>
<td>Final exam</td>
<td>Control day, same subjects</td>
<td>6 samples on the stress day, 3 samples on the day prior to stress, 2 samples seven days following stress day</td>
<td>Higher cortisol levels prior to final exam</td>
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<td>Kirschbaum et al. (1992e)</td>
<td>Study 1: Healthy adults (n = 30); 24 yr Study 2: Healthy adults (n = 37); 23.5 yr</td>
<td>“Trier Social Stress Test” (TSST; 20 min)</td>
<td>None</td>
<td>Study 1: +9 nmol/l Study 2: +6 nmol/l</td>
<td>No correlations with personality traits</td>
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<td>Kirschbaum et al. (1992c)</td>
<td>Healthy adults in three independent studies (n = 153); 22.6 yr</td>
<td>TSST; additionally in one study: h-CRH test; bicycle ergometry (all on different days)</td>
<td>None</td>
<td>Consistently higher stress responses in men (+7.1, +11.4, +10.9 nmol/l) compared to women (+4.3, +4.1, +6.2 nmol/l)</td>
<td>No sex differences in CRH test or bicycle ergometry</td>
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Cortisol levels over-estimated by far (e.g. 10 pm levels 10–15 nmol/l)
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</tr>
</thead>
<tbody>
<tr>
<td>Kirschbaum et al. (1992b)</td>
<td>Healthy monozygotic (MZ) twins ($n = 13$ pairs) and dizygotic (DZ) twins ($n = 11$) pairs; 22.4 yr</td>
<td>TSST; h-CRH test; bicycle ergometry (all on different days)</td>
<td>None</td>
<td>11 samples at 10 min intervals</td>
<td>Higher resemblance of baseline cortisol levels in MZ, trend towards higher resemblance in response to TSST</td>
<td>Higher resemblance of CRH stimulated cortisol levels in MZ; no differences after bicycle ergometry</td>
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<tr>
<td>Kirschbaum et al. (1993)</td>
<td>Chronic male smokers ($n = 10$); 24.7 yr</td>
<td>TSST; h-CRH; bicycle ergometry; saline injection (all on different days)</td>
<td>Male nonsmokers ($n = 10$)</td>
<td>8 samples at 10–30 min intervals</td>
<td>Higher stress responses to TSST in nonsmokers (+10.5 nmol/l) than smokers (+4.3 nmol/l); same trend in h-CRH test</td>
<td></td>
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<tr>
<td>Larson et al. (1991)</td>
<td>Infants ($n = 27$); 9.5 months</td>
<td>Separation stress; 30 min</td>
<td>Infants ($n = 24$), play with mother</td>
<td>Before, +30 min</td>
<td>+3.2 nmol/l (stress) −1.8 nmol/l (controls)</td>
<td>No sex differences</td>
</tr>
<tr>
<td>Lewis &amp; Thomas (1990)</td>
<td>Infants ($n = 69$); 2–6 months</td>
<td>Standard pediatric examination, inoculation; 10 min</td>
<td>None</td>
<td>Before, +15 min</td>
<td>+7.8 nmol/l</td>
<td>No sex differences; high baselines (20–26 nmol/l)</td>
</tr>
<tr>
<td>Magnano et al. (1992)</td>
<td>Preterm cocaine-exposed infants ($n = 11$)</td>
<td>a) Basal levels, b) neonatal behavior examine (20 min), c) Heel stick</td>
<td>Healthy preterm, noncocaine-exposed infants ($n = 35$)</td>
<td>a) +30 min b) +30 min</td>
<td>a) No baseline differences b) 23.7 nmol/l (non-cocaine) vs. 11.9 nmol/l (cocaine) c) 35.9 nmol/l (non-cocaine) vs. 22.9 nmol/l (cocaine)</td>
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<tr>
<td>Study</td>
<td>Group Description</td>
<td>Task Description</td>
<td>Control</td>
<td>Sampling Method</td>
<td>Salivary Cortisol Levels</td>
<td></td>
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</tbody>
</table>
| Müller et al. (1992)          | Healthy adults 
(n = 64)    | Attention test; mild electric shock (80 min); 4 groups (controllable stress +/-; achievement orientation +/-) | None    | 8 samples at 5-15 min intervals      | n.s. differences between groups; max increase 1.9 nmol/l |
| Nicolson (1992)               | a) Adults 
(n = 28); n.a.  | a) Minor written exam                                                             | Control day, same subjects | 10 samples at approx. 90 min intervals from 7:30 am to 10:30 p.m. | a) +2.2 nmol/l before test; b) +4.3 nmol/l before test; c) +4.5 nmol/l before and after test |
|                               | b) Adults 
(n = 13); n.a.  | b) Test of clinical skills                                                        |         |                                       | Experienced sampling method employed         |
|                               | c) Adults 
(n = 10); n.a.  | c) Driving examination                                                            |         |                                       |                                               |
| Rahe et al. (1990)            | Adults, military personnel 
(n = 21); 
31.5 yr; civilians 
(n = 31); 42 yr | Hostage in Iran for 444 days                                                       | 3 samples at 11 pm on consecutive nights | Absolute mean cortisol level: 5.5 nmol/l | Salivary cortisol the only physiological measure to correlate with psychiatric disturbance +8.8 nmol/l |
| Schreinicke et al. (1990)     | Healthy adults 
(n = 77); 43.2 yr | Choice reaction task at computer (30 min)                                        | None    | Before, +35 min                       | High cortisol levels (mean level at 10 am: 24.8 nmol/l); antiserum? |
| Spangler & Scheubeck (1993)   | Newborns 
(n = 42)         | Assessment of neonatal behavioral organization (twice; no duration given)         | None    | -90 min, immediately before and +15 min | +9.1 nmol/l and +9.6 nmol/l (first, second examination) |
| Tarui & Nakamura (1991)       | Fighter aircraft aircrew 
(n = 7); 26.9 yr | 7 repetitive missions (1 h each) over 15 h                                        | Control day, same subjects | 2-h intervals from 6:30 am to 8 pm | Higher cortisol levels on stress day compared to control day |

† No data on controls given in the results section. In the discussion the authors mention eight medical team members from whom serum cortisol levels were obtained; none of them had elevated cortisol levels (no data presented).

NS = not significant.
Fig. 1: Sex differences to psychological stress. In a series of studies (A–C), healthy male and female adults were exposed to a brief psychological stress of 5 min public speaking and 5 min mental arithmetic in front of an audience. Under these conditions, women (○) showed consistently lower cortisol responses than men (●). In contrast to the cortisol response to psychological stress, both sexes showed similar cortisol responses after CRH infusion and bicycle ergometry (data not shown). [Reprinted with permission from Kirschbaum C, Wüst S, Hellhammer DH (1992c) Consistent sex differences in cortisol responses to psychological stress. Psychosom Med 54:648–657. © American Psychosomatic Society.]
tex, since both sexes had similar cortisol increases after CRH or bicycle ergometry stimulation. Moreover, a sex-specific effect of short-term social support on stress-induced cortisol levels has been observed indicating decreased cortisol responses when males received social support from their girlfriends; the opposite was true for females (Kirschbaum et al., unpublished data). These findings are corroborated by a study among newborns (Gunnar et al., 1989). In contrast to females, male infants tended to respond repeatedly to a brief discharge examination with increased cortisol levels. However, no sex differences in cortisol responses to psychological stress were observed in other studies (Benjamins et al., 1992; Larson et al., 1991; Lewis & Thomas, 1990) while numerous publications lack data on putative adrenocortical sex differences.

C. Smoking. Nicotine is a potent stimulator of the HPA axis through induction of CRH release after binding to cholinergic receptors in the hypothalamus (Weidenfeld et al., 1989). Repeated exposure to nicotine could therefore lead to chronically elevated ACTH and/or cortisol with reduced responsiveness of the axis. Salivary cortisol levels seem to be elevated during the day in response to short-term effects of smoking (Kirschbaum et al., 1992d). Furthermore, habitual male smokers were found to show blunted cortisol responses to psychological stress with the same trend of decreased adrenocortical responses after exogenous CRH injection (Kirschbaum et al., 1993). Habitual smoking should therefore be considered a potential intervening variable which could account for some of the interindividual variation observed in HPA responses to pharmacological, physical, or psychological stimulation.

SALIVARY CORTISOL RESPONSES FOLLOWING PHYSICAL EXERCISE

Physical exercise-induced adrenocortical stimulation can be easily detected in saliva. Given adequate work load of >70% VO_{2max} (Mason et al., 1973), cortisol levels rise in a temporal pattern which closely reflects the duration of physical stress. For instance, in a short-term work load of 10–15 min duration, subjects show increasing salivary cortisol levels throughout the stress period with peak concentrations approximately 20–30 min after cessation of stress (Fig. 2). Similar response kinetics have been observed comparing salivary cortisol to total serum cortisol levels (O’Connor & Corrigan, 1987) as well as under more severe conditions, e.g. in marathon runners. Obtaining 22 samples throughout a day, Cook and co-workers (1992) were able to show that salivary cortisol levels rose continuously over more than 3 h reaching a peak mean concentration of approximately 100 nmol/l (!) shortly after completion of the run. Other studies, however, failed to observe significant changes in salivary cortisol levels following physical exercise (Table II). This was either due to insufficient workload (Kirschbaum et al., 1993) and/or inadequate timing of sampling. For instance, 9-min submaximal exercise and a subsequent 30 s anaerobic test was reported to not be associated with cortisol secretion (Tharp & Barnes, 1990). The same holds for two investigation on the effects of 2–3 min paced swimming (O’Connor et al., 1991a, 1991b). Although these exercises most probably exceeded the 70% VO_{2max} criterion for induction of cortisol secretion, those studies failed to observe any significant elevation simply because they obtained only one postexercise saliva sample immediately after the physical strain. If possible, saliva samples should be obtained at 10–15 min intervals during and, at least, for 30 min postexercise.
<table>
<thead>
<tr>
<th>Author(s), year</th>
<th>Subjects; age</th>
<th>Stressor; duration</th>
<th>Control group/session</th>
<th>Sampling time</th>
<th>Results</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ben-Aryeh et al. (1990)</td>
<td>Healthy adults $(n = 34)$</td>
<td>Test 1: Submaximal ergometric test (9 min) Test 2: Wingate anaerobic test (30 s)</td>
<td>None</td>
<td>90 min before and immediately after exercise</td>
<td>No increase in cortisol levels</td>
<td>Second sample obtained too early to detect a rise in cortisol</td>
</tr>
<tr>
<td>Cook et al. (1992)</td>
<td>Healthy adults $(n = 18)$</td>
<td>Marathon run</td>
<td>Control day</td>
<td>22 samples from 8 am to 10 pm</td>
<td>Increase $\geq 70$ nmol/l $+9.3$ nmol/l</td>
<td></td>
</tr>
<tr>
<td>Kirschbaum et al. (1992a)</td>
<td>Healthy adults $(n = 48)$</td>
<td>Bicycle ergometry until exhaustion (12–14 min)</td>
<td>None</td>
<td>11 samples at 10 min intervals</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kirschbaum et al. (1993)</td>
<td>Healthy adults $(n = 20)$</td>
<td>Bicycle ergometry until exhaustion or if heart rate exceeded 190 bpm</td>
<td>Saline injection, same subjects</td>
<td>8 samples at 10–30 min intervals</td>
<td>No increase in cortisol levels</td>
<td>Workload criterion (heart rate &lt;190 bpm) ambiguous</td>
</tr>
<tr>
<td>Study</td>
<td>Participants</td>
<td>Protocol</td>
<td>Outcome</td>
<td>Notes</td>
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<tr>
<td>O'Connor et al. (1989)</td>
<td>Healthy adults ($n = 14$); n.a.</td>
<td>Overtraining in competition swimmers</td>
<td>Healthy adults, physically active, noncompetitive</td>
<td>3 samples in total; one in September, January, and February each; obtained at 2–4 pm</td>
<td>No effect of overtraining, higher levels in competitors on two occasions</td>
<td></td>
</tr>
<tr>
<td>O'Connor et al. (1991)</td>
<td>Healthy adults ($n = 40$); 19.3 yr</td>
<td>Paced swim at 90% velocity (2–3 min)</td>
<td>None</td>
<td>-15 min, +1 min</td>
<td>Lower post exercise levels (n.s.); higher cortisol levels in females n.s. differences in cortisol levels; no sex differences</td>
<td></td>
</tr>
<tr>
<td>O'Connor et al. (1991)</td>
<td>Healthy adults ($n = 40$); 19.3 yr</td>
<td>3 days of increased training; paced swim at 90% velocity (2–3 min)</td>
<td>Swim test before increased training</td>
<td>-15 min, +1 min</td>
<td>See above</td>
<td></td>
</tr>
<tr>
<td>Tharp &amp; Barnes (1990)</td>
<td>Healthy adults ($n = 21$)</td>
<td>Swim training of different intensity levels</td>
<td>None</td>
<td>Before and after training (2 h)</td>
<td>Increase only after heavy-intensity training (+6.3 nmol/l)</td>
<td>Second sample taken too early to observe exercise-induced cortisol rise</td>
</tr>
</tbody>
</table>
Fig. 2: Example for extreme cortisol responses to strenous physical exercise. Marathon runners obtained saliva samples before, during, and after marathon running at 30-min intervals ($n = 18$, ●). Note the large differences between peak cortisol concentration at the end of the run compared to time-matched samples on a control day (■). [Reprinted with permission from Cook et al. (1992).]

EFFECTS OF RELAXATION ON SALIVARY CORTISOL LEVELS

In contrast to results obtained in stress studies which clearly indicate increasing cortisol levels following appropriate stimulation (see above), only few data are available on possible effects of “anti-stress” behavior, that is, relaxation or meditation techniques. Approximately a dozen studies investigated this issue measuring cortisol levels in blood with varying results (Ahuja et al., 1981; Cooper et al., 1985; Holden–Lund, 1988; Jevning et al., 1978; Manyande et al., 1992; McGrady et al., 1987; McGrady & Higgins, 1989; Michaels et al., 1979; Sudsuan et al., 1991; Werner et al., 1986). Most of these studies either found no significant differences in serum cortisol levels or lacked appropriate control groups. One reason for the negative results obtained could have been the impact of blood sampling on the dependent measure. Assessment of cortisol in saliva could be advantageous in this respect since it circumvents the possible stress effects of blood sampling. Unfortunately, only three studies investigated the effects of relaxation/meditation on salivary cortisol levels. As was observed in studies using serum cortisol measures, one study failed to find any significant effect (Jin, 1992), a second did not employ any control group (Jin, 1989) while the third found minimal differences using a questionable control group and inadequate statistical methods (Field et al., 1992). Thus, the question whether there are ways to deliberately lower cortisol levels remains unanswered. The
use of salivary cortisol should facilitate the necessary studies since larger populations
(including control groups) can be studied with a nonreactive sampling regimen.

FUTURE APPLICATIONS

One of the predominant advantages of cortisol assessment in saliva is the possibility
that the studied volunteers and patients can obtain saliva samples independent of labora-
tory or medical personnel at almost any desired frequency. It may therefore turn out to
be the method of choice to collect samples on a daily or less frequent basis over prolonged
periods from populations who are thought to undergo fluctuations in adrenocortical
activity associated with concurrent or future diseases/disorders. For example, relapses
of certain autoimmune disorders and recurrent infectious diseases have been associated
with acute stress in retrospective analyses. Since in both groups of diseases cortisol
appears to play a modulating role, it would be challenging to investigate such patients
in longitudinal study designs to proof or disproof a correlation of stress, cortisol, and
disease relapse. First attempts to reveal patterns of disorder-associated cortisol changes
have been recently presented in single-case studies (Degenhard & Petermann, 1992;
Thomopoulos et al., 1992). A similar application would be to investigate chronically
stressed subjects. The use of daily cortisol profiles in combination with point assessment
of adrenocortical responses, subjective mood ratings, and external health status assess-
ment could yield valuable information on the time course of chronic stress effects. Cross-
sectional and longitudinal studies could be easily intertwined. It seems to us that the use
of noninvasive cortisol measurement in saliva can provide the basic researcher as well
as the clinician a valuable tool which is especially suited for psychoneuroendocrinological
investigations.

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