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Comparison of Total and Salivary Cortisol in a Low-Dose ACTH (Synacthen) Test: Influence of Three-Month Oral Contraceptives Administration to Healthy Women

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Summary

The objective of this study was to evaluate the influence of low-dose combined oral contraception (COC) on basal and stimulated (1 μg ACTH test) levels of serum and salivary cortisol (F), cortisone and on basal serum cortisol binding globulin (CBG), adrenocorticotropic hormone (ACTH), dehydroepiadrosterone (DHEA) and calculated free cortisol in healthy young women. Three-month administration of COC resulted in 1) significant increase of basal (454.0±125.0 to 860.9±179.7 nmol/l) and ACTH-stimulated serum cortisol in 30th min (652.3±60.5 to 1374.1±240.6 nmol/l); 2) no significant change of basal (15.4±7.3 to 18.9±8.5 nmol/l) and ACTH-stimulated salivary cortisol at the 30th min (32.4±8.8 to 32.9±9.0 nmol/l); 3) no significant change of basal serum cortisone (38,8±7.68 to 45.2±24.2 nmol/l) and ACTH-stimulated cortisone at the 30th (34.8±10.9 to 47.0±35.7 nmol/l); 4) significant increase of basal ACTH (17.2±9.0 to 38.2±29.4 ng/l), CBG (991.0±161.0 to 2332.0±428.0 nmol/l), and 5) no significant change of basal DHEA (24.6±15.7 to 22.6±11.7μmol/l) and calculated basal value for free cortisol (22.8±14.9 to 19.2±6.9 nmol/l). In conclusions, higher basal and ACTH-stimulated serum cortisol were found after three-month administration of COC, while basal and stimulated salivary cortisol were not significantly affected. Therefore, salivary cortisol can be used for assessment of adrenal function in women regularly using COC.

Key words

Low dose ACTH test • Salivary cortisol • Total serum cortisol • Free cortisol • Cortisol binding globulin • Oral contraceptives

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Introduction

Most of the cortisol (F) present in circulation is bound to proteins; only a small portion is found as free hormone, exerting hormonal activity. It is known that certain clinical conditions and drugs, for example estrogens as the component of hormonal contraception (COC), affect plasma concentration of cortisol binding globulin (CBG) and alter the total cortisol concentration. By contrast, the unbound fraction of cortisol is independent of CBG changes. Therefore, interpretation of the results of hypothalamic-pituitary-adrenal (HPA) axis based merely on the total serum cortisol level can be misleading (Christ-Crain *et al*. 2007, Gozansky *et al*. 2005).

As determination of free cortisol level in serum is not carried out routinely due to methodical reasons, alternative methods for adrenal function assessment were sought. One such possibility is to calculate the free cortisol index, a second is to determine salivary cortisol. Methodological details of salivary cortisol estimation have been published in recent papers (Šimůnková *et al*. 2007, Marcus-Perlman *et al*. 2006, Christ-Crain *et al*. 2007, Ho *et al* 2006.). The salivary cortisol measurement reflecting the free hormone fraction in serum appeared to be advantageous, which has been confirmed and successfully employed in diagnostics of HPA axis disorders (Gozansky *et al*. 2005, Contreras *et al*. 2004, Marcus-Perlman *et al*. 2006). This approach was frequently used as a screening test for the diagnosis of Cushing´s syndrome as well (Bjorntorp and Rosmond 1999, Reynolds *et al*. 2001, Vicennati and Pasquali 2000, Aardal and Holm 1995). Further advantages of salivary cortisol examination are non-invasive sample collection and its applicability in non-

standard conditions in outpatient clinics (Aardal and Holm 1995, Contreras *et al*. 2004).

Women using COC represent a large population group, including patients with autoimmune disorders and diabetes mellitus type I. The latter groups should have adrenal function examined regularly because of the higher risk of adrenal autoimmune function disturbances (Barker 2006, Betterle *et al*. 2002). In emergency situations and some other circumstances, it is necessary to test adrenal gland function as soon as possible without waiting for withdrawal of COC and consequent serum CBG and F correction influenced by COC use. For assessment of the adrenal reserve, a physiological stimulation has been used, which is ensured by administration of 1μg ACTH–low dose Synacthen test (Dickstein *et al*. 1991).

Therefore, we decided to obtain more detailed data on the influence of low-dose hormonal contraception (COC) on the basal and stimulated (1 μg ACTH test) levels of serum and salivary cortisol (F) and serum cortisone in healthy young women. In addition to serum and salivary cortisol, basal serum CBG, adrenocorticotropic hormone (ACTH), calculated free serum F, serum cortisone and DHEA were measured in order to detect possible changes in the HPA axis and peripheral tissue metabolism of glucocorticoids.

Subjects and Methods

Subjects

Eleven healthy, 22-30 year-old women with normal weight (BMI within 20-25 kg/m^2) were examined in the follicular phase of the menstrual cycle without using hormonal contraceptives (baseline period). None of them used any other medication affecting adrenal function for at least three months before testing. The same women were investigated after three-month administration of oral contraceptives (COC period). The contraceptives used by these subjects were monophasic, with either 30 μg ethinylestradiol + 2 mg dienogestodum, 20 μg ethinylestradiol + 75 μg gestodenum, or 35 μg ethinylestradiol + 250 μg norgestimatum. The protocol of the study was approved by the Ethical Committee of the Institute of Endocrinology, and the subject signed the informed consent.

The low-dose (1 μg) ACTH (Synacthen) test

The tests were carried out in the Institute of Endocrinology, Prague, in the Laboratory for Function Tests, always starting at 9.00 a.m., after an overnight fast. Sixty minutes before the test the subjects were not allowed to smoke, drink liquids or brush their teeth. After a 30-min rest in bed with a cannula introduced into the cubital vein, samples of blood and saliva were taken, and then 1μg of ACTH was administered intravenously (Time 0). The samples of blood and saliva were taken at the 20th 30th, 40th and 60th minutes after ACTH administration, while patients were in a supine position. Basal concentration of ACTH, CBG, calculated value of free cortisol, DHEA were measured, basal and ACTH-stimulated serum, salivary cortisol and serum cortisone were measured. Thirty minutes after sample collection, blood was centrifuged at 3000 rpm for 15 min. and serum was collected into plastic tubes. Saliva was sampled by spitting into plastic tubes; both serum and saliva samples were frozen at –20 °C and stored at this temperature until analysis. No saliva sample was contaminated with blood. The above-described low-dose ACTH test was performed twice in every woman, at the baseline period and after 3 months use of COC (COC period).

Solution preparation

The entire content of an ampoule with ACTH (250 μg/ml) (Synacthen, Novartis Pharma GmbH, Nurnberg, Germany) was added to 249 ml of sterile 0.9 % NaCl (saline) solution. One milliliter of this solution was administered intravenously during ACTH test, corresponding to 1 μg of ACTH. The dose was prepared at most 10 min before administration.

A high performance liquid chromatography (HPLC) system (Dionex Softron, Germering, Germany) was used for separation of cortisol from cortisone and for their quantification. It consisted of a HPLC pump system P 680 equipped with automatic flow rate control, automated sample injector ASI-100, thermostated column compartment TCC-100 and photodiode array detector PDA-100 with wavelength range 190-600 nm. A speed-vacuum centrifuge (HETO, Melsungen, Germany) was used for solvent evaporation.

Serum cortisol and cortisone concentrations were determined by a method using high HPLC with UV/VIS detection.

Serum sample (200 μl) was diluted with releasing buffer (200 μl) and 4-androsten-3, 17-dione-11β-ol (40 ng per sample) was added as an internal standard. Buffered sample solution was extracted with diethylether (2 ml) for 1 min (2000 rpm) and frozen. The organic phase containing released steroids was decanted and evaporated under the stream of nitrogen.

To avoid possible column contamination from free fatty acids, the dry residue from the extract was dissolved in 80 % methanol (1 ml, v/v) and n-hexane (1 ml). The mixture was extracted again for 1 min (2000 rpm). N-hexane-containing phase was removed and discarded. The residual polar phase was evaporated at 55 °C and the dry residue was dissolved in 15 % acetonitrile (50 µl, v/v) and mixed vigorously to rinse the tube walls appropriately. The samples were then centrifuged (2000 *g*, 3 min, 22 °C) and decanted solution transferred into vials.

Simultaneously, blank samples and quality control samples were processed in the same way to avoid possible contamination and to determine procedural losses of individual metabolites.

HPLC separation

Standard mixtures of cortisone and cortisol in the following concentrations were used for calibration by an external standard method: cortisone (25, 50, 100, 250, 500, 625 and 750 pg/µl), cortisol (100, 250, 500, 750, 1500, 2250 and 3000 pg/µl). HPLC separation was carried out with reverse phase Macherey-Nagel EC 250/4 nucleosil 100-5 C_{18} column (250 x 4 mm) with particle size of 5 µm (Macherey-Nagel, Dueren, Germany). The following protocol was used.

Elution gradient: 0.0-2.1 min constant mobile phase acetonitrile-water (15:85), 2.1-12.0 min, linear gradient from methanol-acetonitrile-water (40:9:51) to methanol-acetonitrile-water (49:11.3:39.7), 12.0-15.0 min, constant mobile phase methanol (100 %), 15.0-19.0, constant mobile phase acetonitrile-water (15:85).

The temperature in the column compartment was 35 °C and the flow rate of the mobile phase was kept constant at 0.8 ml/min. Under these conditions the retention times of authentic cortisone and cortisol standards were 12.13 min and 12.84 min, respectively.

The PDA-100 detector response was recorded in UV mode at 239 nm for cortisone and 243 nm for cortisol at the appropriate retention times. Cortisone and cortisol amounts were determined according to a calibration curve and final values were corrected for procedural and extraction losses according to yields of internal standard.

Salivary cortisol was determined using the method published by Bičíková *et al*. (1988). The method consisted of non-extraction solid phase radioimmuno-assay using coated tubes with rabbit polyclonal antiserum to cortisol-3-O(carboxymethyloxime) bovine serum albumin conjugate, and homologous [^{125}I]tyrosine methylester derivative as a tracer. Frozen saliva was thawed and centrifuged at 3000 rpm for 10 min to clear off the debris and mucosa residues. The material was then pipetted by automatic analyzer (Stratec, Immunotech, Marseille, France). Intra-assay coefficients of variation (CVs) were 7.4 %, and inter-assay CVs were 10.2 % for salivary cortisol.

Unconjugated DHEA was determined by radioimmunoassay kit from Immunotech (Marseille, France) after extraction with dichloromethane. Intra- and inter-assay CVs 7.2 % and CVs 11.9 %.

Plasma-ACTH was measured using commercial Immunoradiometric assay (IRMA) kits (Immunotech, France). Minimum detection limit for ACTH was 1.2 pg/mL. Intra- and inter-assay CVs were 9.1 % and 9.6 %.

CBG was measured by radioimmunoassay kit (MG 130 61, Immuno-Biological Laboratories, Hamburg, Germany). The analytical parameters corresponded to those given by the manufacturer.

Value of free (unbound) cortisol was calculated according to Coolen´s equation (Coolens *et al*. 1987).

Statistics

Differences in the basal levels of substances before and after treatment by COC were tested using Wilcoxon's robust paired test. The effects of COC and time in ACTH tests were evaluated using ANOVA model consisting of the independent factors of treatment (with COC, without COC), Time (0 min, 20, 30, 40, 60 min) and the subject (each of the examined women) and the Treatment × Time interaction followed by the least significant difference multiple comparisons. Due to non-Gaussian data distribution and heteroscedasticity in some data and residuals, the variables concerned were transformed using power transformation before the ANOVA testing. The non-homogeneities after data transformation were identified using studentized residuals. If the absolute value of the studentized residual was greater than 3, the experimental point was regarded as outlier and excluded from the analysis. The number of outliers never exceeded 5 % of the total number of experimental points.

Results

Serum cortisol

The mean basal concentration of total serum cortisol at baseline period was 454.0±125.0 nmol/l (mean ± SD) and it increased to 860.9±179.7 nmol/l; at COC

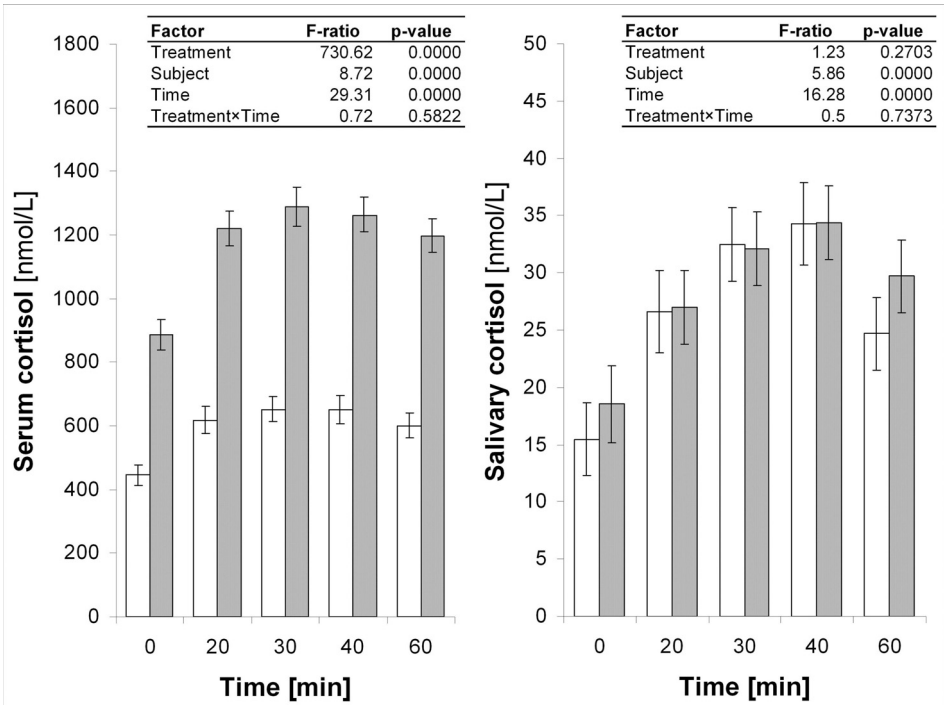

Fig. 1. Serum and salivary levels before and after the injection of 1 μg ACTH in normal women (shaded bars) and after 3 month of using oral contracetion (open bars). The empty and dotted bars with error bars represent retransformed means with their 95 % confidence intervals for the stages with and without treatment with COC, respectively, in individual time points of the ACTH test. The group means the confidence intervals of which do not overlap are significantly different ($p<0.05$, least significant difference multiple comparisons). For details see "Statistical data analysis".

Table 1. Basal levels cortisol and related substances before and after treatment and their differences.

Substance		Without COC		With COC		Difference (with - without) COC		Significance of the mean difference*
	n	mean	SD	mean	SD	mean	SD	
Transcortin [nmol/l]	11	991.0	161.2	2331.8	428.3	1340.7	354.9	**p<0.004**
Free cortisol [nmol/l]	11	22.78	14.85	19.20	6.956	-3.572	13.91	NS
Salivary cortisol [nmol/l]	11	15.48	7.655	18.99	8.913	3.516	10.85	NS
Total cortisol [nmol/l]	11	454.6	131.1	860.9	188.4	406.3	159.5	**p<0.004**
Cortisone [nmol/l]	11	38.84	7.679	45.15	24.23	6.309	22.84	NS
DHEA [micromol/l]	11	24.6	15.7	22.6	11.7	9.1	23.8	NS
ACTH [nmol/l]	11	17.20	9.540	38.24	30.88	21.03	30.66	**p<0.03**

*Wilcoxon's robust paired

period ($p<0.0001$). After 1 μg ACTH stimulus, the basal serum F increased at the 20^{th}, 30^{th}, 40^{th} min to 599.9±68.2; 652.3±60.5;632.3±67.5; 603.7±52.2 nmol/l at baseline period, while at COC period it rised to 1231.4±232.7, 1374.1±240.6, 1270.4±197.6, 1204.6±186.5 nmo/l at the 20^{th},30^{th}, 40^{th}, 60^{th} min ($p<0.0001$) (Fig. 1, Table 1).

The basal calculated value of free cortisol in blood was 22.8±14.9 nmol/l at baseline period; while at COC period it decreased to 19.0±9.6 nmol/l. However, this difference was not statistically significant.

Salivary cortisol

The mean basal salivary F was 15.4±7.3 nmol/l at baseline period at COC period was 18.9±8.5 nmol/l; p=NS. The ACTH-stimulated levels of salivary cortisol were at the 20^{th} 30^{th} and 40^{th} min with concentration of 25.2±6.8, 32.4±8.8 and 32.1±10.6 nmol/l at baseline

period, respectively, and 27.0±9.4, 32.9±9.0 and 34.3±7.4 nmol/l at COC period; p=NS. In the 60th min of ACTH test, the concentration of salivary cortisol was 24.6±3.4 and 29.7± 9.6 nmol/l at baseline period and COC period respectively; p=NS.

Other hormones

The basal plasma concentration of ACTH was 17.2± 9.0 ng/l at baseline period and increased significantly to 38.2±29.4 ng/l at COC period (p<0.03).

Baseline basal and ACTH-stimulated levels of cortisone in blood did not differ significantly from the data of COC period (basal 38.8±7.7, at 20th min. 33.6±8.1, at 30th min. 34.8±10.9, at 40th min 37.1±6.9, at 60th min 38.1±11.2 nmol/l in baseline period vs basal 45.2±24.2, at 20th min 49.1±30.1, at 30th min. 47.0±35.7, at 40th min 46.8±35.9, at 60th min 47.9±35.6 nmol/l in the COC period (NS).

Baseline serum CBG concentration in blood was 991.0±161.0 nmol/l and increased to 2332.0±428.0 nmol/l at COC period (p<0.004).

The serum concentrations of unconjugated DHEA did not show any significant difference between baseline and COC data. The respective obtained values were 24.6±15.7 μmol/l and 22.6±11.7 μmol/l (NS).

Discussion

Endogenous and synthetic estrogens (COC) cause an increase in CBG concentration, which results in an elevation of the total cortisol level. Therefore, it is considered appropriate to include free cortisol assessment in the interpretation of the results of the HPA axis examination (Gozansky *et al*. 2005, Meulenberg *et al*. 1987).

In our study, we found that the increase in the levels of CBG concentration at COC period. Therefore it is not surprising that basal and stimulated levels of serum cortisol after 3-months administration of COC increased, which is in agreement with other authors (Marcus-Perlman *et al*. 2006, Gozansky *et al*. 2005). Unlike serum cortisol, the baseline concentration of basal and ACTH-stimulated salivary cortisol did not differ from COC period. Our results concerning salivary cortisol are similar to data reported by Marcus-Perlman *et al*. (2006), although the study groups were different. In that study a group of healthy females and males was compared with other group of hyperestrogenic females using COC and hormone replacement therapy. The cohort of our study was defined more accurately, as the same women were examined before and after treatment with COC, and all of them used only low dose formulation, which is also important.

We found significantly higher levels of ACTH at COC period. Some experimental studies have shown that both basal as well as stimulated ACTH and corticosterone levels are higher in rats during the ovulation period. Studies with humans did not provide such definite conclusions (Kirschbaum *et al*. 1993) Two studies with humans (Stewart *et al*. 1993, Genazzani *et al*. 1975) showed cyclic changes only in non-stimulated ACTH during the menstrual cycle. The Kirschbaum´s study only proved changes in salivary cortisol in response to psychosocial stress and ACTH stimulation during menstrual cycle but serum ACTH concentration was unchanged. Jacobs *et al*. (1989) described, reduced ACTH plasma levels in oral triphasic contraceptive users, which were and significantly lower following the CRH infusion compared to controls. In Jacobs study, the group of women taking a triphasic oral contraceptive were examined and the results were compared with the control group of women. However, in our study, the same group of women was examined

Increased levels of ACTH may result from the change in the activity of the HPA axis after the administration of COC. However, this is in contrast to unchanged basal salivary cortisol and calculated value of free cortisol after the administration of COC. We did not investigat the ACTH-stimulated calculated value of cortisol. The ACTH-stimulated calculated cortisol did not correlate with salivary cortisol because of not account all of the proteins to which cortisol can potentially bind (Christ-Crain *et al*. 2007, Ho *et al*. 2006). Moreover, we did not observe an increased DHEA level as would be expected at higher levels of ACTH. More detailed investigation of the influence of COC administration on the HPA axis activity is highly needed.

Theoretically, the increased levels of ACTH, which we observed, could have resulted from COC induced changes of tissue metabolism of glucocorticoids in periphery. 11β-hydroxysteroid dehydrogenase type 1 is an enzyme allowing for conversion of cortisone to cortisol. Experimental studies have shown that estrogens reduce the activity of this enzyme (Low *et al*. 1993, Tomlinson *et al*. 2004). We did not observe any changes in basal or ACTH- stimulated serum cortisone levels at COC period as compared with baseline data. The changes of glucocorticoid metabolism may be observed only at

the tissue level without changed serum cortisone. Further investigation of glucocorticoid metabolism at tissue level in COC administration will be challenge.

To conclude, we proved that the assessment of free cortisol is a relevant approach to the evaluation of HPA axis function. We confirmed that salivary cortisol levels do not change after COC administration, and therefore its determination is more favorable for evaluation of adrenal function disorders by low-dose ACTH test, particularly in mild forms of adrenal insufficiency. Assessment of free salivary cortisol is especially valuable in cases with altered CBG concentration either due to either COC administration or some pathological conditions (obesity, catabolic states, renal and liver diseases, etc.).

Since COC administration is a widely used, including in groups of women with increased risk of adrenal gland disturbances, assessment of free salivary cortisol can provide a valuable method for earlier diagnosis of such serious disorders.

Conflict of Interest

There is no conflict of interest.

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