Salivary cortisol in low dose (1 µg) ACTH test in healthy women : comparison with serum cortisol

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Summary

To date, a single report has appeared on the use of salivary cortisol for adrenal function testing with low dose (1 μg) ACTH, although 1 μg has become preferred as a more physiological stimulus than common 250 μg ACTH test. This study was aimed to obtain physiological data on changes of free salivary cortisol after 1μg ACTH stimulation. This approach was compared with the common method based on the changes of total serum cortisol. Intravenous, low dose ACTH test was performed in fifteen 22-40 years old healthy women with normal body weight, not using hormonal contraceptives, in follicular phase of menstrual cycle. Blood and saliva for determination of cortisol were collected before ACTH administration and 30 and 60 min after ACTH administration. Basal concentration (mean±SE) of salivary cortisol 15.9±1.96 nmol/l increased after 1 μg ACTH to 29.1±2.01 nmol/l in the 30^{th}, and to 27.4±2.15 nmol/l in 60^{th} min, respectively. The differences between basal and stimulated values were highly significant ($p<0.0001$). The values of salivary cortisol displayed very little inter-individual variability ($p<0.04$) in contrast to total serum cortisol ($p<0.0001$) A comparison of areas under the curve (AUC) related to initial values indicated significantly higher AUC for salivary cortisol than for total serum cortisol (1.89±0.881 vs. 1.22±0.19, $p< 0.01$). Correlation analysis of serum and salivary cortisol levels showed borderline relationship between basal levels ($r=0.5183$, $p=0.0525$); correlations after stimulation were insignificant.

Low dose ACTH administration appeared as a sufficient stimulus for increase of salivary cortisol to a range considered as normal adrenal function reserve.

Introduction

Determination of free cortisol in serum is not part of routine tests due to its methodological and economical expensiveness (Gozansky et al., 2005). Therefore, for a routine evaluation of adrenal function, total serum cortisol is currently being used. The main disadvantage of this method is that the cortisol values are significantly influenced by the binding proteins (Hamrahiam et al.,2004, Le Roux et al., 2003, Ho et al. 2006, Gozansky et al., 2005). Their levels may be altered considerably under various clinical states: for instance they are increased when using estrogen preparations or in pregnancy, while liver or kidney disorders or catabolic post-operation states generally decrease CBG concentration (Ho et al., 2006, Landon et al., 1984, Meulenberg et al., 1987). Attempts were therefore made to obtain information of free cortisol indirectly, by using indexes calculated from total cortisol and CBG, or directly by analyzing urine or saliva (Le Roux et al., 2003).

The salivary cortisol, which very well reflects the free hormone fraction in serum, appeared to be advantageous (Gozansky et al., 2005, Contreras et al., 2004). The follow up of free salivary cortisol has been successfully applied in diagnostics of disorders of hypothalamo-pituitary-adrenal (HPA) axis under various situations, as for instance in metabolic syndrome (Bjorntorp et al., 1999, Reanolds et al., 2001, Vicennati et al., 2000, Aardal et al., 1995). The authors of these studies emphasize further advantages of salivary cortisol in evaluation of HPA function: mainly the non-invasiveness of sample collection and its applicability in non-standard conditions outside an outpatient clinic (Aardal et al., 1995, Contreras et al., 2004).

Free cortisol represents hormone immediately available for use in tissues. Its response to a secretory stimulus is, in contrast to basal conditions, considerably influenced by sex. In addition it depends on the phase of the menstrual cycle (Kirschbaum et al., 1999). In the case of total cortisol, these situations do not influence considerably the magnitude of the response.

Therefore we have focused in our study on a well-defined group of healthy young women, all of them in a follicular phase of the cycle. Stimulation with 250μg ACTH is now considered pharmacological by character and therefore low dose (1μg) variant has begun to be used, especially for detection of subclinical forms of disorders of adrenal function or HPA axis (Dickstein et al., 2003, Dickstein et al., 1991, Laureti et al., 2002). To the best of our knowledge, the values of salivary cortisol after 1 μg (ACTH) stimulus have been published in only one study (Marcus-Perlman et al. 2006) .

The aim of this study was to gain normal physiological data about salivary cortisol after stimulation of adrenal gland with low dose ACTH test in population of young women in follicular phase of menstrual cycle a nd to compare the data of salivary cortisol after stimulation compare with data in serum cortisol. The comparison with stimmulated serum cortisol provide to other infomation about salivary cortisol.

Subjects and Methods

Subjects

Fifteen healthy, 22-40 years-old healthy women with normal weight (BMI within 20-25 kg/m^2), not using hormonal contraceptives, in a follicular phase of the menstrual cycle were investigated. None used hormonal contraceptives or other medication for at least three months. The study was approved by the Ethical Committee of the Institute of Endocrinology.

Testing procedure

The low-dose (1μg) ACTH test

The test was carried in the Institute of Endocrinology, Prague, in a specialized Laboratory for Functional Tests, always in the morning at 9.00 a.m., after overnight fasting. Sixty min before the test the subjects were not allowed to smoke, drink liquids and brush their teeth. After a 30-

min rest in bed with a cannula introduced into cubital vein, blood and saliva were collected, and then 1μg ACTH was intravenously administered (Time 0). The next withdrawals of blood and saliva were performed at 30^{th} and 60^{th} min after ACTH administration in supine position of patients. Thirty min after withdrawal blood was centrifuged at 3000 rpm for 15 min, and the serum was left frozen in plastic tubes and stored at -20 ^{o}C until analyzed. Saliva were collected by spitting into plastic tubes, the material was frozen at –20 ^{o}C and stored at this temperature. No saliva sample was contaminated with blood.

Solution preparation

The contents of ampule, 250μg/1ml ACTH (Synacthen, Novartis Pharma GmbH, Nurnberg, Germany) was added to 249 ml of sterile physiological solution. Each subject received 1 ml of the solution i.v., corresponding to 1μg ACTH. The dose was prepared 10 min before application.

Cortisol determination

Total serum cortisol was determined by original methodology (Bicikova et al., 1988). It was determined by non-extraction solid phase radioimmunoassay 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 were thawed and centrifuged at 3000 rpm for 10 min to clear off the debris and mucosa residues. The material could then be pipetted by automatic analyzer (Stratec, Immunotech, Marseille, France). Salivary cortisol was determined by the same method as serum cortisol, but using 30 μl of saliva instead of 1.5 μl of serum. Intra-assay coefficients of variation (c.v.) were 5.2% and 7.4, inter-assay c. v. 9.8% and 10.2 % for serum and salivary cortisol, respectively.

Statistical data treatment

Two linear models were used to compare the methods. The first, ANOVA model, was set up to evaluate the dissimilarity of inter-individual differences in the method based on the salivary cortisol and the common approach based on cortisol levels in the serum. This model consisted from Time and Subject as the factors. The F- and p-value of the later factor express its contribution for explanation of the total variability in cortisol levels. The second, general linear model (GLM), was built to compare the dissimilarity between time profiles of the salivary and blood cortisol after ACTH-stimulation. The Bonferroni multiple comparisons following the testing by GLM were used to compare the distinctiveness of the responses on ACTH stimulation in the methods based on the salivary- and blood cortisol. To approximate Gaussian distribution and constant variance, the original data were transformed by a power transformation. Wilcoxon test was used for a comparison of areas under the curve (AUC)between the methods and Spearman correlations were employed to evaluate the relationships between the basal and stimulated levels. Statistical software Statgraphics Plus version 5.1 was used for the analysis.

Results

1) a) The basal levels of total cortisol 486±122.7 nmol/l (mean ± SD), increased after administration of 1 µg ACTH to 607.2±92.4 nmol/l in the 30^{th} min, and to 610.9±131.27 nmol/l in 60^{th} min. The difference between basal value and values in 30^{th} and 60^{th} min were significant with $p<0.01$ and with $p<0.05$, respectively.

b) Basal concentration of salivary cortisol (mean ± SD) 15.9±7.6 nmol/l increased after 1µg ACTH to 29.1±7.8 nmol/l in the 30^{th}, and to 27.4±8.3 nmol/l in 60th min, respectively. The

differences between basal and stimulated values were highly significant with $p<0.00001$ and with $p<0.0001$, respectively..

2) A comparison of AUC related to initial values showed significantly higher AUC for salivary cortisol than AUC for total serum cortisol: 1.89±0.88 vs. 1.22±0.19, $p< 0.01$ (Wilcoxon´s test).

3) The individual results showed high inter-individual variability in the case of serum cortisol ($p<0.0001$), while only borderline inter-individual differences were found when analyzing the salivary cortisol ($p<0.04$).

4) A correlation analysis of serum and salivary cortisol levels showed only a marginal relationship between the basal levels ($r=0.5183$, $p=0.0525$), while correlations at all the times after stimulation were insignificant.

5) Although the Matrix ×Time interaction indicating different response to ACTH stimulation in serum and saliva was insignificant, Bonferroni multiple comparisons found more significant differences between basal and stimulated cortisol levels in saliva compared to serum (Fig. 1). For a better lucidity, the salivary cortisol is displayed as ten times the amount.

Discussion

A comparison of changes of total serum cortisol with results obtained by measuring salivary cortisol response to 1 µg ACTH stimulus pointed to some differences: The results showed high interindividual variability in the case of serum cortisol ($p<0.0001$), while only borderline interindividual differences were found when analyzing the salivary cortisol ($p<0.04$). It means that the between-subject variance is substantially lower in salivary cortisol compared to serum cortisol as documented by different F-and p-values in ACTH test in serum and saliva (Fig. 1.).

On the other hand, as expected, in both parameters, the differences between basal and

stimulated values are highly significant ($p<0.0001$). The poststimulatory response of salivary cortisol in comparison with total serum cortisol was considerably higher, as demonstrated by a significant difference between AUC for salivary and serum cortisol and figure 1. This finding is in agreement with some recent studies, describing more pronounced increase of free cortisol in comparison of the total one, as a consequence of CBG changes. The assumed changes of CBG concentrations and its saturation could explain not only the high variability of post-stimulatory values of total serum cortisol, but also the fact that stimulated values of serum cortisol did not correlate with salivary concentrations in individual times during the test (Gozansky et al., 2005, Contreras et al., 2004, Wong et al., 2004). In our study we did not follow CBG levels.

Of interest may be the finding that a maximum response of cortisol in saliva as well as in serum after l µg stimulus occurred in all the subjects at the same time, i.e. in the 30^{th} or 60^{th} min. It confirms earlier findings that actual levels of salivary cortisol react almost immediately to changes of the free fraction of circulating hormone (Landon et al., 1984). According to Kirschbaum, the poststimulatory values of free salivary cortisol are markedly influenced by sex; in women also by a phase of the menstrual cycle, probably as a result of different adrenal sensitivity (Kirschbaum et al., 1999). Therefore, it is difficult to closely compare our results from healthy women with normal weight with recent data of Marcus-Perlman et al., who used in their study of the effect of 1µg ACTH stimulus on salivary cortisol a mixed control group (males and females) (Marcus-Perlaman et al.,2006). Also, in their group the body weight and the phase of menstrual cycle in women were not specified.

From our results as well as from the data in the literature it is obvious that the measurement of salivary cortisol is less dependent on other factors than the total serum cortisol is, and therefore reflects more accurately the adrenal function reserve (Contreras et al.,2004). In the

case of diagnostic use, the salivary cortisol appears to be a better marker of adrenal function because the individual response to the stimulus does not vary significantly in healthy subjects contrasting the wide range of responses seen in the serum total hormone.

The results about salivary cortisol in low dose ACTH test in young women shown in this study, can be use as physiological references in diagnostic decision of exlusion andrenal insufficiency.

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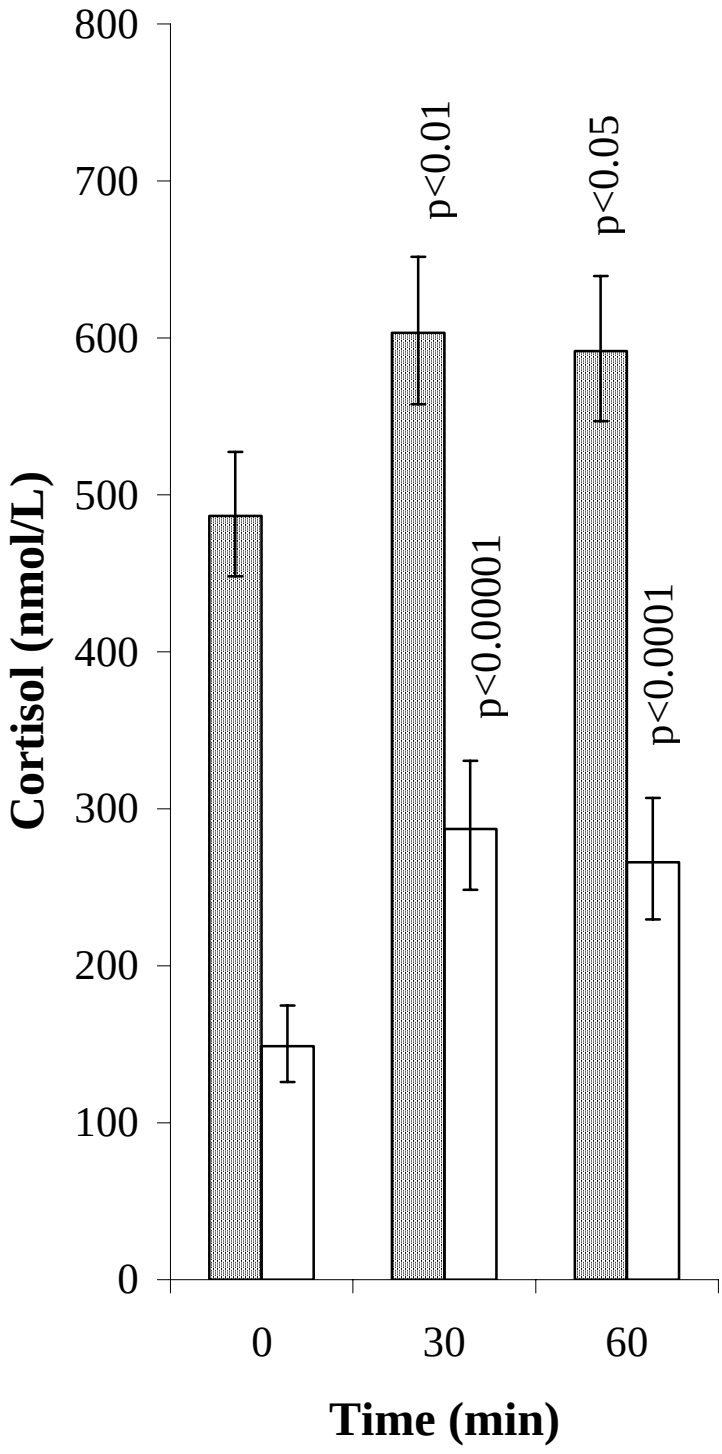
Legend to figure

Figure 1. Evaluation of ACTH tests for serum and salivary cortisol. The shaded and empty bars with error bars represent retransformed mean values and their 95% confidence intervals in individual stages of the ACTH test for serum and salivary cortisol, respectively. For a better lucidity, the salivary cortisol is displayed as ten times the amount. The F-values and significances for in the GLM consisting of the factors Matrix, Subject, and Time were as follows: Matrix: $F=6208$, $p<0.0001$; Subject: $F=4.19$, $p<0.0001$; Time: $F=29.7$, $p<0.0001$; Matrix×Time interaction: $F=1.22$, NS. The model was highly significant ($F=300$, $p<0.0001$) explaining 98.8% of the variability in cortisol levels. The p-values above the error bars demonstrate the significance of the difference between basal- and stimulated values as found using Bonferroni mulltiple comparisons following the GLM. When using the ANOVA model consisting of Subject and Time as factors separately for serum and saliva, the F-values and significances were as follows: Serum...Subject: $F=4.19$, $p<0.0003$; Time: $F=12.3$, $p<0.0001$; Saliva...Subject: $F=2.06$, $p<0.03$, Time: $F=22.1$, $p<0.0001$.


800
700
600
500
400
300
200
100
0
Cortisol (nmol/L)
p<0.01
p<0.05
p<0.00001
p<0.0001
0
30
60
Time (min)