

Response of Cortisol Metabolites in the Insulin Tolerance Test and Synacthen Tests

K. SIMUNKOVA^{1,2}, M. DUSKOVA², M. KOSAK¹, M. KRSEK¹, V. HANA¹, M. HILL²,
H. JANDIKOVA², H. POSPISILOVA², M. SRAMKOVA², E. BIFULCO³, L. STARKA²

¹Third Department of Medicine, General University Hospital and First Faculty of Medicine, Charles University, Prague, Czech Republic, ²Institute of Endocrinology, Prague, Czech Republic, ³Department of Clinical Science, University of Bergen, Bergen, Norway

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Summary

Determination of response of cortisol and its metabolites to different stimuli may be important for adrenal gland disorders. To date, only one metabolite, cortisone, has been followed in stimulation tests of the adrenal gland. We aimed to describe a response of cortisol metabolites to the standard short Synacthen test (HDST), insulin tolerance test (ITT), low dose Synacthen test (LDST) and medium dose Synacthen test (MDST). Sixty healthy subjects were investigated: 30 men and 30 women. Plasma for measurements of cortisol and its metabolites was obtained before and 30th and 60th min after Synacthen and insulin administration. The cut-off 500 nmol/l of cortisol was reached after stimulation in all of tests, the maximal stimulation level was reached in 60th min in all of the tests except for LDST. The response of cortisol and its metabolites at 30th and 60th min strongly correlated in all of the tests except for LDST. Cortisol and its metabolites increased after stimulation; in contrast, cortisone and its metabolites decreased. We showed that the response of the cortisol metabolites during the Synacthen tests and ITT well correlated, and the MDST showed similar response compared to HDST. The decrease in cortisone metabolites may correspond to the regeneration of cortisol from cortisone in response to stimulation test.

Key words

Synacthen test • Cortisol • Cortisone • Insulin tolerance test

Corresponding author

K. Simunkova, Third department of Medicine, General University Hospital and First Faculty of Medicine, Charles University, U Nemocnice 1, 128 08 Prague 2, Czech Republic. E-mail: ksimunkova@gmail.com

Introduction

Cortisol is the key hormone responsible for maintaining the homeostasis in the body and for responses to every stress event. Adrenal glands are releasing cortisol under specific rhythm almost every hour with the higher amplitude of peaks in the morning and the lowest in the evening (Lightman 2014). Cortisol precursor levels are essential for the diagnostic procedure in stimulation test of adrenal disorders and response of cortisol precursors has been described (Willenberg *et al.* 2002). So far, only cortisone has been followed in stimulation test of adrenal gland, response of other cortisol and cortisone metabolites have not yet been described.

The critical and crucial step in cortisol metabolism is cortisol regeneration from cortisone or its conversion to cortisone by 11 β -hydroxysteroid dehydrogenase 1 (11 β -HSD1). That conversion enables the recycling of cortisol and cortisone in peripheral tissues (Hughes *et al.* 2012, Nixon *et al.* 2012). 11 β -hydroxysteroid dehydrogenase 2 (11 β -HSD2) inactivates cortisol in the kidney and protects mineralocorticoid receptors against cortisol excess (Dotsch *et al.* 2001). Cortisol and cortisone are then converted to dihydrocortisol (DHF) and dihydrocortisone, which are further reduced to tetrahydrocortisol (THF) and tetrahydrocortisone (THE). This process constitutes the main pathway. DHF may be reduced by 5 α -reductase or 5 β -reductase to two isoforms THF and allo-THF. The minor pathway involves hydroxylation by 6 β -hydroxylase to 6 β -hydroxycortisol (Larsen *et al.*

2002). Urinary cortisol and its metabolites as well as cortisol/cortisone ratio are used to evaluate the activity of cortisol metabolising hormones in several disorders and may be used to predict cardiovascular risk in various diseases (Gomes-Santos *et al.* 2014).

The insulin tolerance test (ITT) has been traditionally regarded as the gold standard for evaluation of the hypothalamic-pituitary-adrenal (HPA) axis. However, the ITT has several limitations and has to be avoided in several conditions due to artificially caused hypoglycemia. Several alternative tests for the HPA axis assessment have been proposed over the years (e.g. glucagon or metyrapon test) but only the Synacthen test has demonstrated good sensitivity and specificity compared to the ITT in many studies (Hurel *et al.* 1996, Rasmuson *et al.* 1996, Abdu *et al.* 1999). The standard short Synacthen test is cheaper, safer and less unpleasant for patient than the ITT and assesses directly the capacity of the adrenal cortex and indirectly of HPA. We focused on the response of metabolites of cortisol to the ITT and Synacthen tests involving three different doses. The main aim was to compare the maximal response of cortisol and its metabolites to the stimulation tests and to determine whether the low dose Synacthen test (LDST, 1 µg dose) results in the same level of cortisol metabolites as the standard short Synacthen test (HDST, 250 µg) and the ITT. The second aim was to investigate the response of cortisol and its metabolites to a medium dose Synacthen test (MDST, 10 µg Synacthen test) and to compare the response with the responses to the other tests.

Subjects and Methods

Subjects

Sixty healthy subjects, 30 men and 30 women (age 38±10 years (mean ± SD), BMI 24.5±2.7 kg/m², p=0.08) were examined by the ITT, HDST (250 µg), LDST (1 µg) and MDST (10 µg). The study was approved by the local Ethical Committee.

The healthy subjects did not have a medical history of steroid treatment. Women included in the study were not pregnant, were in the follicular phase of the menstrual cycle and did not use any contraceptives or their use was discontinued at least 6 weeks before the investigation. The healthy subjects signed an informed consent before entering the study, which was approved by the Ethical Committee of the Institute of Endocrinology and General University Hospital.

Performance of dynamic tests of the HPA axis

Each healthy subject underwent all 4 tests. Adrenal function was evaluated by measurement of basal and stimulated values of following the steroids cortisol, cortisone, tetrahydrocortisone (THE), tetrahydrocortisol (THF), allo THF and 6β-hydroxycortisol (6β-OHF) in plasma during the ITT, LDST, HDST, MDST.

The insulin dose was calculated according to the body weight and a dose of 0.1-0.2 IU/kg; of rapid acting insulin was administered intravenously and hypoglycemia <2.2 nmol/l was reached.

Preparation of the dose of 1 µg Synacthen for LDST

The content of an ampoule with 250 µg/1 ml ACTH (Synacthen, tetracosactide 250 µg, Novartis Pharma GmbH, Nuernberg, Germany) were added to 249 ml sterile normal saline solution. Each subject received intravenously 1 ml of the solution containing 1 µg Synacthen. Individual doses were prepared 10 min before administration to the subject.

Preparation of the dose of 10 µg Synacthen for MDST

The content of the ampoule with 250 µg/1 ml ACTH (Synacthen, tetracosactid 250 µg, Novartis Pharma GmbH, Nuernberg, Germany) was added to 249 ml sterile normal saline solution. Each subject received 10 ml of the solution intravenously containing 10 µg Synacthen. Individual doses were prepared 10 min before administration to the subject.

All of the tests were carried out in a specialized Laboratory for Functional Tests in the Institute of Endocrinology, after overnight fasting between 8-9 a.m. in recumbence position of a subject. After 30 min of rest in bed with a cannula introduced into the cubital vein, blood samples were collected for the determination of basal plasma hormones (Time 0), and then the dose of Synacthen or insulin was administered intravenously. The next blood samples were taken at 30th, 60th and 90th min after 250 µg, 10 µg Synacthen or insulin administration and 30th, 60th min after the administration of 1 µg of Synacthen. Thirty minutes after withdrawal of blood samples, they were centrifuged at 3000 rpm for 15 min, and obtained plasma was frozen in plastic tubes and stored at -20 °C until analyzed.

A peak plasma cortisol response of greater 500 nmol/l (Stewart 2001) in each of the test was considered to be normal physiological response.

Methods for steroid determination

Cortisol, cortisone, tetrahydrocortisol, allo-tetrahydrocortisol, tetrahydrocortisone and 6beta-OH-cortisol were measured by liquid chromatography-tandem mass spectrometry (LC-MS/MS). Sample processing was completely robotized (Hamilton Robotics, Inc., Reno, NV, USA). Internal standards (cortisol-d4, THE-d5 and THF-d3) were added to 100 µl of human EDTA plasma, which was then subjected to liquid-liquid extraction with 480 µl of ethylacetate-heptane (80:20; v:v). The supernatant (280 µl) was subsequently washed with sodium hydroxide buffer (0.1 M), evaporated and reconstituted in 100 µl of 0.01 % aqueous solution of formic acid:methanol (50:50, v:v). The samples were analyzed on a Waters Acquity UPLC system connected to a Waters Xevo TQ-S tandem mass spectrometer. The compounds were separated on a C-18 BEH phenyl column from Waters (100 x 2.1 mm column, 1.7 mm particle size, 60 °C), which was developed by gradient elution over 5.5 min, using an aqueous solution of formic acid and acetonitrile as mobile phases. Multiple reaction monitoring (MRM) transitions were monitored for formic adducts of cortisol (409.18→333.19), cortisone (405.22→301.24), tetra-hydrocortisol (411.20→335.20), allo-tetrahydrocortisol (411.20→335.20), tetrahydrocortisone (409.18→333.19) and 6beta-OH-cortisol (424.29→347.20), which were detected in negative mode. The range of measurement was 1-200 nM for cortisone, TH cortisone and TH cortisol; 1-1000 nM for cortisol; 6-200 nM for allo TH cortisol and 2-20 nM for 6beta-OH-cortisol.

Accuracies were in the range between 94 and 105 %, and imprecision was <10 % for Cortisol, cortisone, tetrahydrocortisone and 6beta-OH-cortisol and <12 % tetrahydrocortisol, allo-tetrahydrocortisol.

Statistical analysis

Respecting the skewed distribution and non-constant variance in most dependent variables, these were transformed by power transformations to data symmetry and homoscedasticity prior further processing (Meloun *et al.* 2000). The homogeneity and distribution of the transformed data and residuals was checked by residual analysis as described elsewhere (Meloun *et al.* 2002, 2004). The repeated measurements ANOVA model was used for the evaluation of the relationships between steroid levels, gender, type of test and time after

stimulation. The model consisted of subject factor explaining inter-individual variability, between-subject factor Gender (men vs. women), within-subject factors Test (four types of test were investigated in each subject such as HDST, ITT, LDST, and MDST) and Time (times after stimulation 0, 30 and 60 min), and all corresponding interaction between the factors except of the subject factor. For instance, significant Test × Time interaction indicates that the type of test significantly influences the course of the time profile (there are significantly different shapes of time profiles between individual tests). F represents the Fisher's statistic and p designates statistical significance for the factors and interaction. The symbols with error bars represent re-transformed means with their 95 % confidence intervals (triangles, diamonds, squares, and circles symbolize HDST, ITT, LDST, and MDST test, respectively. The full and empty symbols represent men and women, respectively. The 95 % confidence intervals are computed using the least significant difference multiple comparisons ($p < 0.05$). The confidence intervals, which do not overlap each other, denote significant difference between the respective subgroup means. Statistical software Statgraphics Centurion, version XV from Statpoint Inc. (Herndon, Virginia, USA) was used for the statistical analysis.

Results

We simultaneously evaluated plasma cortisol, cortisone, THF, THE, all THF and 6β-OHF in the ITT, LDST, HDST and MDST in the same subjects. The results are described in Table 1 and expressed as the means and SD. The cut-off 500 nmol/l of cortisol was exceeded at 30th min in the HDST, LDST, and MDST, however, in the ITT it was reached with some delay at 60th min. The peaks of all of the hormones were observed at 60th min in the ITT, HSDT, MDST, apart from LDST (Figs 1-6). Levels of cortisol and its metabolites decreased at 60th min in the LDST. We found significant correlation of cortisol and its metabolites levels at 30th min in the LDST, HDST and MDST.

The ratio of plasma cortisol/cortisone increased in all of the test with a higher degree of interindividual variation; the peak were observed at 60th min in all of the tests excluding the LDST (Fig. 7).

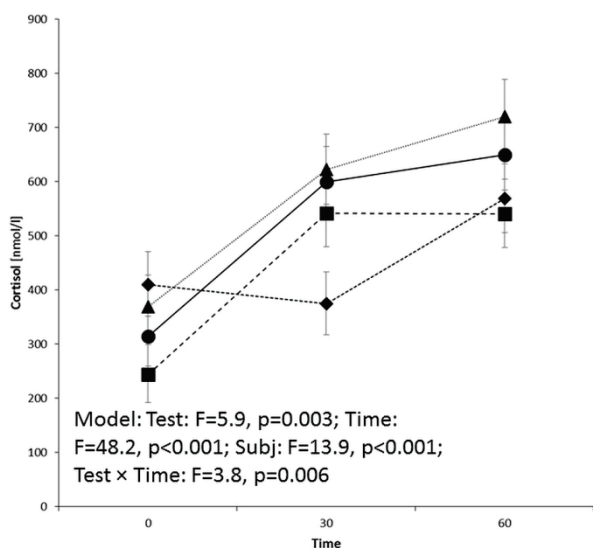


Fig. 1. Different profiles of plasma cortisol for all tests as evaluated using repeated measures ANOVA model. The circles represent MDST, triangles HDST, squares LDST, diamond ITT with error bars represent group means with their 95 % confidence intervals. F and p represent the correlation coefficients of the ANOVA model, F-ratio and level of statistical significance.

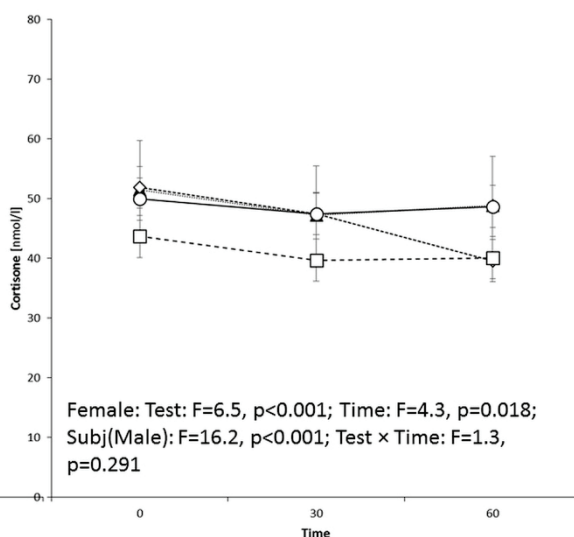
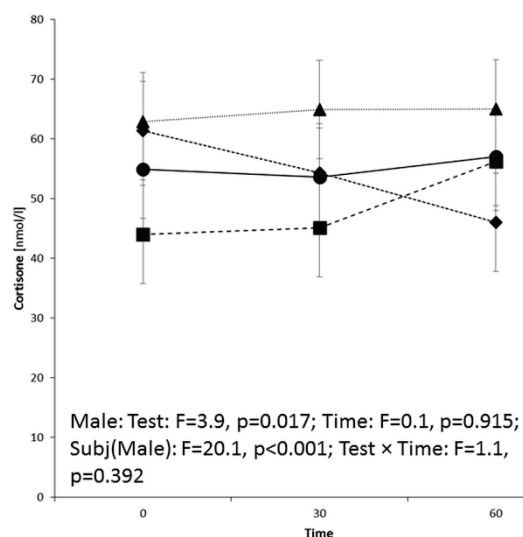


Fig. 2. Different profiles of plasma cortisone for all tests are evaluated using repeated measures ANOVA model. The empty symbols represent female subject and full symbols male subjects, circles represent MDST, triangles HDST, squares LDST, diamond ITT with error bars represent group means with their 95 % confidence intervals. F and p represent the correlation coefficients of the ANOVA model, F-ratio and level of statistical significance.

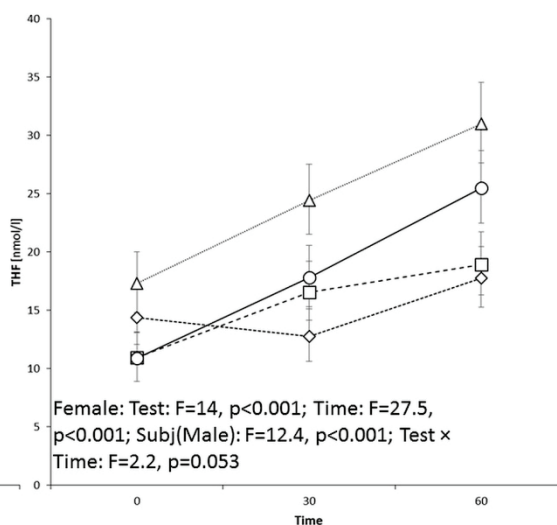
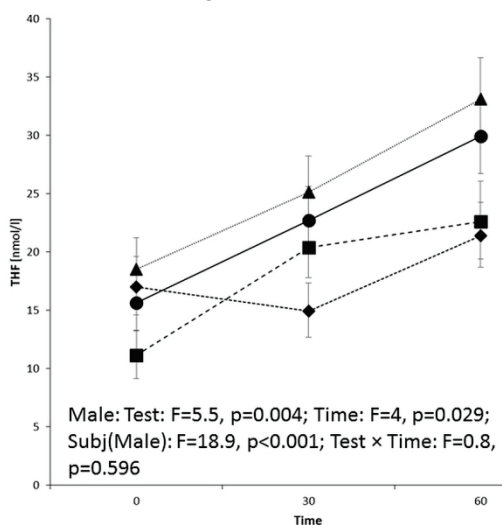


Fig. 3. Different profiles of plasma THF for all tests are evaluated using repeated measures ANOVA model. The empty symbols represent female subject and full symbols male subjects, circles represent MDST, triangles HDST, squares LDST, diamond ITT with error bars represent group means with their 95 % confidence intervals. F and p represent the correlation coefficients of the ANOVA model, F-ratio and level of statistical significance.

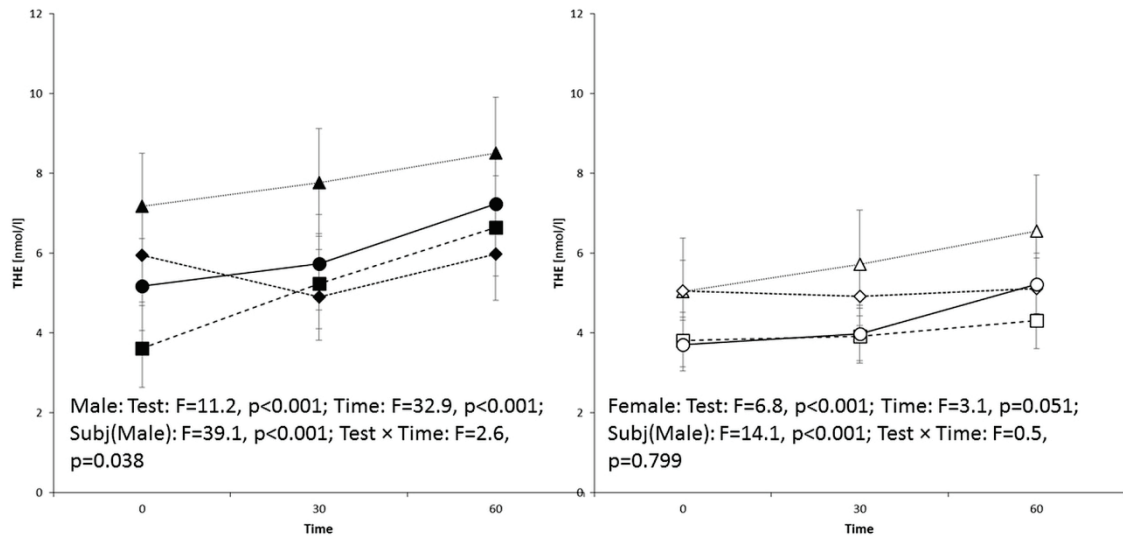


Fig. 4. Different profiles of plasma THE for all tests are evaluated using repeated measures ANOVA model. The empty symbols represent female subject and full symbols male subjects, circles represent MDST, triangles HDST, squares LDST, diamond ITT with error bars represent group means with their 95 % confidence intervals. F and p represent the correlation coefficients of the ANOVA model, F-ratio and level of statistical significance.

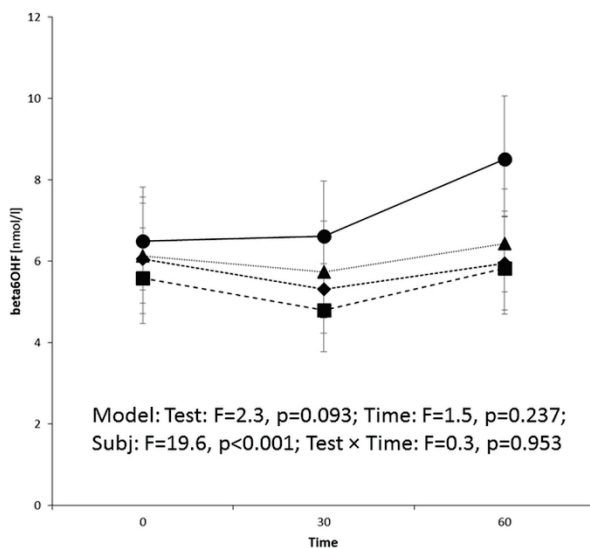


Fig. 5. Different profiles of plasma 6β-OHF for all tests as evaluated using repeated measures ANOVA model. The circles represent MDST, triangles HDST, squares LDST, diamond ITT with error bars represent group means with their 95 % confidence intervals. F and p represent the correlation coefficients of the ANOVA model, F-ratio and level of statistical significance.

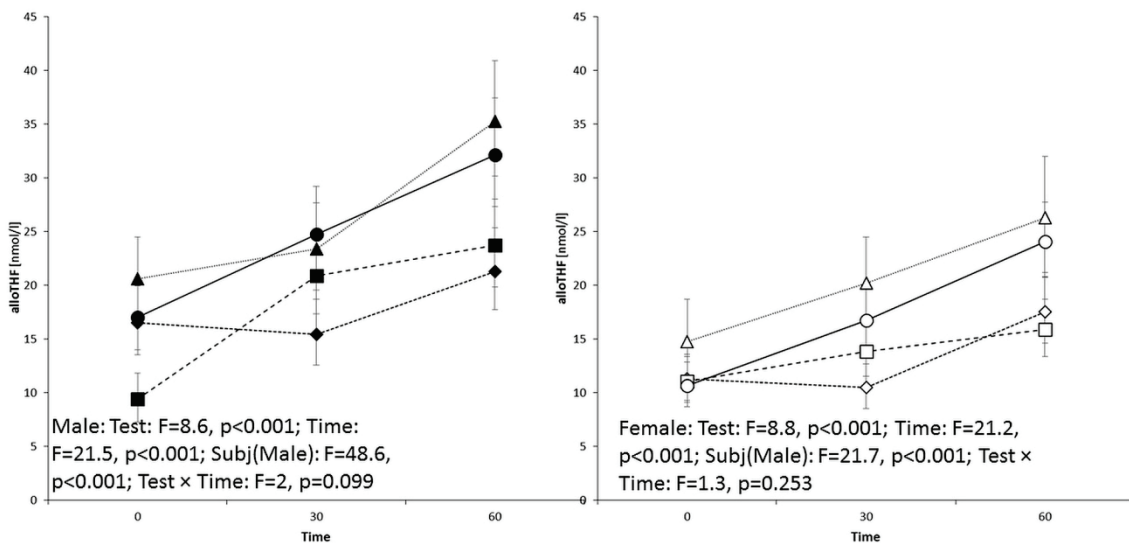


Fig. 6. Different profiles of plasma allo THF for all tests as evaluated using repeated measures ANOVA model. The circles represent MDST, triangles HDST, squares LDST, diamond ITT with error bars represent group means with their 95 % confidence intervals. F and p represent the correlation coefficients of the ANOVA model, F-ratio and level of statistical significance.

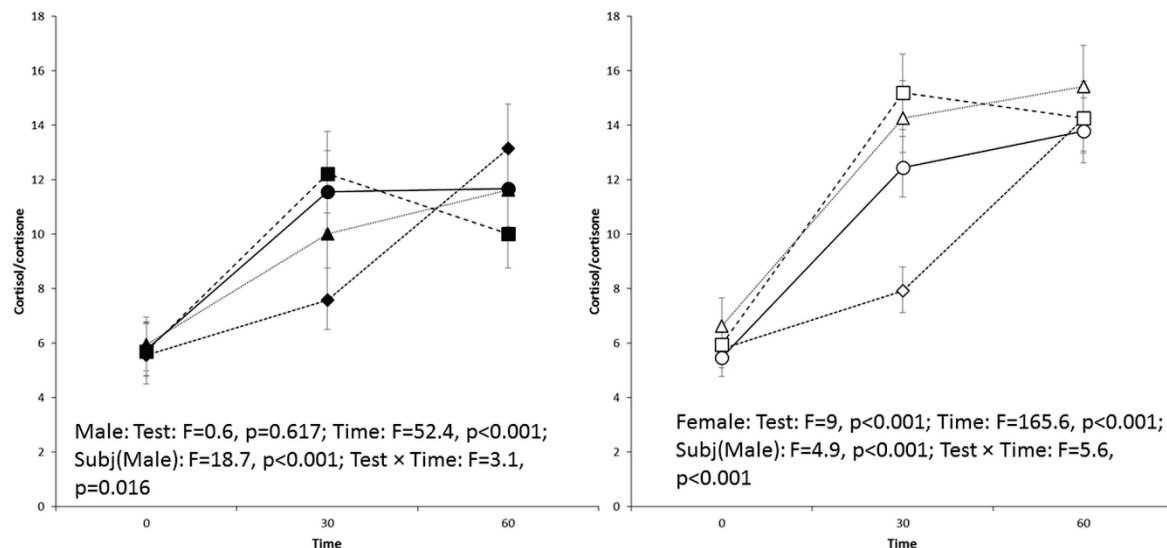


Fig. 7. Different profiles of ratio of cortisol/cortisone for all tests as evaluated using repeated measures ANOVA model. The empty symbols represent female subject and full symbols male subjects, circles represent MDST, triangles HDST, squares LDST, diamond ITT with error bars represent group means with their 95 % confidence intervals. F and p represent the correlation coefficients of the ANOVA model, F-ratio and level of statistical significance.

Table 1. The basal and stimulated cortisol and its metabolites in ITT, HDST, MDST and LDST.

Variable nmol/l	ITT		HDST		MDST		LDST	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
cortisol 0	350.40	144.10	403.10	181.30	287.60	181.30	259.10	67.60
cortisol 30	379.40	111.00	650.20	157.40	594.20	157.40	530.10	134.80
cortisol 60	578.20	142.90	739.80	122.90	661.90	122.90	486.40	148.60
cortisone 0	55.40	15.80	55.60	12.90	51.80	12.90	44.10	12.10
cortisone 30	50.00	16.40	53.70	19.00	49.70	19.00	40.40	7.40
cortisone 60	42.00	10.60	54.80	17.10	51.70	17.10	45.10	11.90
THF 0	16.00	6.30	18.50	8.20	13.00	8.20	12.20	4.00
THF30	14.20	6.00	25.30	7.90	20.00	7.90	19.60	6.40
THF60	20.00	8.00	32.10	5.90	27.70	5.90	21.80	6.70
THE0	5.60	2.30	5.90	1.90	4.40	1.90	4.20	1.60
THE30	5.10	2.30	6.60	2.10	4.70	2.10	5.00	1.90
THE60	6.50	2.40	7.30	2.00	6.10	2.00	5.70	2.20
6βOHF 0	6.90	3.80	6.20	2.10	6.40	2.00	5.00	1.90
6βOHF 30	5.70	2.70	6.20	2.40	6.50	2.30	5.30	2.10
6βOHF 60	7.60	2.90	7.50	2.50	7.90	2.40	6.00	2.60
allo THF 0	14.10	6.90	17.60	6.90	14.70	6.90	12.60	4.70
allo THF30	13.70	8.50	21.70	4.50	21.50	4.50	19.80	8.80
allo THF60	22.40	10.80	30.40	9.40	29.40	9.40	23.20	11.50
cortisole/cortisone 0	6.30	14.10	7.20	1.40	5.60	5.60	5.90	5.90
cortisole/cortisone 30	7.60	8.30	12.10	8.30	12.00	18.20	13.10	13.10
cortisole/cortisone 60	13.60	7.20	13.50	7.20	12.80	12.40	10.80	108.00

The stimulated and baseline levels of cortisol and 6 β -OHF did not differ significantly between female and male subjects, which contrasted with the results for THF, THE, cortisone and cortisol/cortisone ratio. Levels of cortisone and THF, THE, and allo THF were significantly higher in males, whereas the cortisol/cortisone ratios were higher in females.

The levels of cortisol, cortisone, and THF as well as cortisol/cortisone ratio were significantly lower at 0th and 30th min in the LDST compared to in the other tests, at 60th min cortisol reached significantly higher levels in the HDST compare the levels in the ITT. The levels of 6 β -OHF were significantly lower at 60th min in the ITT and LDST compared to in the MDST. The levels of the THE were significantly lower at 0th and 30th min in the LDST and MDST compared to the other tests; at 60th min, the THE levels were significantly higher in the HDST compared to in the ITT.

Discussion

In our study we observed a 2-fold increase in cortisol and cortisone after insulin and Synacthen stimulation, and the highest levels were reached in the HDST at 60th min. The cut-off of 500 nmol/l of cortisol was reached in all of the tests, which confirmed the feasibility of the use of the MDST. Additionally, the cortisol metabolites followed the pattern of the cortisol response in all tests.

Several alternative tests for the assessment of the HPA axis have been proposed over the years, but only the Synacthen test has demonstrated good sensitivity and specificity compared with the ITT in many studies (Hurel *et al.* 1996, Rasmuson *et al.* 1996, Abdu *et al.* 1999). Several controversies remain a matter of debate when using the Synacthen test to evaluate the HPA axis. The most discussed issue is the dose of Synacthen. The use of 1 μ g, 12.5 μ g and 25 μ g instead of standard 250 μ g of Synacthen has been suggested (Contreras *et al.* 2004, Dorin *et al.* 2003, Agha *et al.* 2006, Abdu *et al.* 1999). The LDST test showed a similar maximal response of cortisol in 30th min, which corresponded to cortisol response in HDST and ITT (Nye *et al.* 1999). With respect to the timing of blood tests after stimulation, the time of occurrence of maximal stimulation levels and of achievement of cut-off for cortisol have been published in more than 100 studies, yet clear consensus is still lacking (Deutschbein *et al.* 2009, Daidoh *et al.* 1995). Moreover, the maximal level of cortisol may vary and may also

differ between secondary and primary adrenal insufficiency (Tordjman *et al.* 2000, Rasmuson *et al.* 1996). Studies comparing the ITT and the Synacthen tests showed good correlation, but the time at which maximal cortisol response was detected strongly depended on the Synacthen dose (Gonzalbez *et al.* 2000). In our study, the level of cortisol also depended on the administered dose of Synacthen and continued to increase, reaching the maximum at 60th min in the HDST and MDST. In contrast, the peak of cortisol in the LDST was reached at 30th min, and at 60th min, the levels of cortisol did not correlate with those in either the HDST or MDST. Early termination of the LDST test can contribute to a higher number of false positive results and 11 % of patients may be misdiagnosed (Karaca *et al.* 2011). This may be avoided thought the use of 10 μ g of Synacthen. Furthermore, aside from methodological issues, errors in the pre-analytic phase may also contribute to the variability in the response of cortisol to Synacthen. In particular, the stability of Synacthen after it is reconstituted and the method of reconstitution may play a role (Hana *et al.* 2015). Therefore, we introduced a 10 μ g dose of Synacthen. The stability of 1 μ g Synacthen for up to 30 days in storage has been recently demonstrated (Ananthanraman *et al.* 2015). We observed an excellent correlation between the results in the HDST and ITT at 30th and 60th min after Synacthen administration, and the maximum cortisol response that was observed at 60th min in ITT and HDST as well as MDST. That shows applicability of MDST test. The supraphysiological dose of 250 μ g of Synacthen causes prolonged production of cortisol to 90 min after the Synacthen administration (Dickstein *et al.* 1991). We also observed an ongoing increase of cortisol at 60th min in the MDST test. This result indicates that medium dose of 10 μ g of Synacthen is still a supraphysiological dose. Further comparative studies among ITT, HDST and MDST may be of importance.

The cortisol metabolites THF and allo THF followed the same pattern as the response of cortisol. In contrast, the cortisone metabolites either decreased at 30th min and recovered at 60th min or remained unchanged. The ratio of cortisol/cortisone increased and reached twice its basal level, which is in agreement with previous findings (Vogesel *et al.* 2001). Those findings correspond to the shift to the conversion of cortisone to cortisol, which increase the levels of biologically active hormone.

Stimulation of the HPA axis induced remarkable

increases in the cortisol/cortisone ratio and also significant increases in the levels of THF, allo THF and 6 β -OHF, which are steroids known as neuro-active compounds. It is possible that stimulation of adrenal cortisol secretion also directly influences the activity of 11 β -HSD. 11 β -HSD1 is a ubiquitous enzyme that predominantly acts as an oxoreductase and converts cortisone to cortisol. Hepatocytes and adipocytes are the major source of that conversion, many factors, such as hormones of adipose tissue regulate the activity of this enzyme (Morgan *et al.* 2014). Recent studies have shown the important role of 11 β -HSD1 in the development of insulin resistance (Morgan *et al.* 2009). The activity of 11 β -HSD1 may be altered by several conditions, e.g. obesity, and may determine the degree of cortisol regeneration. This is important particularly in critically ill patients and for glucocorticoid replacement treatment (Boonen and van den Berghe 2014). Another explanation is that rapid cortisol secretion supplies the circulation with an amount of the active steroid, that the steroid metabolising enzymes are unable adequately metabolise in time because the half-life of cortisol is 40 min (Kraan *et al.* 1997). Thus, a limitation of the study may be the short period of follow up after Synacthen stimulation.

Gender also plays an important role in the

regulation of 11 β -HSD1 activity. Estradiol has been suggested to inhibit hepatic 11 β -HSD1-mediated reduction of cortisone, but after adjusting for BMI, the estradiol effect disappeared; thus, other factors may play an important role in 11 β -HSD1 regulation. Further studies in mice confirmed that the activity of liver 11 β -HSD1 was lower in female mice and depended on body mass index (Mattson *et al.* 2012, Low *et al.* 1994). This may explain the gender difference in cortisone levels in our study because BMI was similar in both groups.

In conclusion, we observed a similar response of cortisol and its metabolites after 3 different doses of Synacthen test and in the ITT. These results suggest that a test with 10 μ g of Synacthen may be an option to replace the ITT and the HDST test, but further confirmation is essential.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

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