Measurement of cortisol in human hair as a biomarker of systemic exposure

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Abstract

Purpose: Current methods for measuring long-term endogenous production of cortisol can be challenging due to the need to take multiple urine, saliva or serum samples. Hair grows approximately 1 centimeter per month, and hair analysis accurately reflects exposure to drug abuse and environmental toxins. Here we describe a new assay for measurement of cortisol in hair, and determined a reference range for non-obese subjects.

Methods: For measurement of cortisol in hair we modified an immunoassay originally developed for measuring cortisol in saliva. We compared hair samples obtained from various parts of the head, and assessed the effect of hair dying. We analyzed hair samples from non-obese subjects, in whom we also obtained urine, saliva and blood samples for cortisol measurements.

Results: The mean extraction recovery for hair cortisol standards of 100 ng/ml, 50 ng/ml and 2 ng/ml (n=6) was 87.9%, 88.9% and 87.4%, respectively. Hair cortisol levels were not affected by hair color or by dying hair samples after they were obtained. Cortisol levels were decreased in hair that was artificially colored before taking the sample. The coefficient of variation was high for cortisol levels in

hair from different sections of the head (30.5%), but was smaller when comparing between hair samples obtained from the vertex posterior (15.6%). The reference range for cortisol in hair was 17.7-153.2 pg/mg of hair (median 46.1 pg/mg). Hair cortisol levels correlated significantly with cortisol in 24-hour urine ($r=0.33$; $P=0.041$).

Conclusion: The correlation of hair cortisol with 24-hour urine cortisol supports its relevance as biomarker for long-term exposure.

Cortisol is a glucocorticoid hormone produced by the adrenal glands. In humans, cortisol levels are increased in severe stress, depression, and selected systemic diseases such as Cushing's syndrome. Proper assessment of endogenous cortisol secretion is required to adequately determine its relation to these clinical conditions. For that end, cortisol is currently measured in saliva, serum and urine. While each of these measurements has advantages and limitations, all these methods require multiple samples to provide an assessment of cortisol levels over prolonged periods of time. Furthermore, due to diurnal variations

samples either need to be taken at specific times of the day (saliva and serum), or require a laborious collection method (24 hr urine collection), making them unsuitable for population analysis.¹ Even 24-hr urine collection reflects the last day and not changes that can occur over weeks to months.

Hair analysis is increasingly used to reflect exposure to drugs of abuse and environmental toxins.² Incorporation of hormones in hair is thought to occur mainly via blood circulation during the formation of the hair shaft.³ Because of the balance existing between serum levels and hair concentration, measurement of endogenously produced hormonal levels in hair may reflect average hormone levels over months. Specifically, because hair grows approximately 1 centimeter per month (range 0.6-1.4 cm)⁴, a hair sample of 2-3 cm reflects average hormone levels over the previous 2-3 months. Hence, unlike other matrices, hair cortisol may represent long-term exposure to the hormone.

During the last few years, the Strasbourg group has reported measurement of cortisol in human hair.^{5,6} Until now, no studies have contrasted cortisol levels in hair with measurements of cortisol in saliva, serum and/or urine cortisol within the same individuals. The objective of the present study was to develop an assay for measuring cortisol in hair, to determine a reference range for cortisol in hair in a non-obese control population, and to correlate these novel measures with cortisol levels in serum, saliva and 24-hour urine.

Methods

A) Evaluation of ELISA assay for hair cortisol

The study was approved by the Research Ethics Board of the University of Western Ontario, and written informed consent was obtained from all subjects prior to participation.

Hair collection

Hair samples consisting of approximately 150 strands of hair or approximately 20 mg, were collected from the posterior vertex. The hair was cut with scissors as close to the scalp as possible. The hair was then taped

to a piece of paper using Scotch® Tape, the scalp end was clearly marked and the sample was stored in an envelope in room temperature up to 12 months before analysis.

Hair sample preparation

Samples were prepared using methods that have been previously reported by our lab (Yamada *et al*,⁷). A minimum of 10 mg of hair from the scalp end was used for each sample. The hair was weighed, cut into small pieces using small surgical scissors, put into a disposable glass scintillation vial and 1 ml of methanol was added. The scintillation vial was sealed and incubated overnight (~16 hr) at 52 °C while shaking (Gyromax® Amerex Instruments Inc.). After incubation, the supernatant was removed and put into disposable glass culture tubes. The supernatant was evaporated in a dry bath (Thermolyne® Dri-Bath) under nitrogen (Techne® Sample Concentrator) until completely dry. Once the methanol was removed, the sample was resuspended in 150-250 µL of phosphate buffered saline (PBS) at pH 8.0. Samples were vortexed for one minute followed by another 30 seconds until they were well mixed.

Hair cortisol analysis

The cortisol in the hair samples was measured using the Salivary ELISA Cortisol kit® (Alpco Diagnostics®, Windham, NH) as per the manufacturer's directions with the reagents provided. The cross reactivity of other steroids with the kit's antibodies was reported as follows: corticosterone 31%, progesterone <2%, deoxycortisol <2%, dexamethasone <2%, and estriol, estrone and testosterone were all <0.001%. The cross-reactivity of cortisone was tested using cortisone standards varying from 3.125 to 5000 ng/ml created in PBS in the presence or absence of 50 ng/ml of cortisol and run on the ELISA kit. The percent of the cortisone detected did not exceed 1%.

Hair cortisol ELISA evaluation

Intra-assay and interday variation. We used two hair samples, one with an average (about 60 pg/mg) and

one with a high cortisol level (about 600 pg/mg), to determine both the intra-assay and interday variation of hair cortisol measurement.

Effects of natural hair color on cortisol levels. To determine if hair matrix affects cortisol detection on the ELISA, hair from 4 brown, 4 blond, and 4 black haired volunteers was collected. Each subject's hair was finely chopped to create a homologous mixture. Six vials of hair for each subject were weighed out and incubated overnight as previously described. Following incubation, the six reconstituted samples from each individual were combined and the hair matrix solution was used as zero standard as well as spiked with cortisol to create standards of the following concentrations: 3.125, 6.25, 12.5, 25, 50 and 100 ng/ml. Measurements were performed in duplicate and the means of cortisol detected in the brown hair group, black hair group and blond hair group were compared to the cortisol detected in the PBS standards.

Hair cortisol levels in samples from different sections of the head. In 14 volunteers, we collected hair from the posterior vertex, anterior vertex, nape, temporal and frontal sections of the head. Four to five centimeters of hair from the scalp end was finely cut and analyzed for cortisol concentration.

Intra-individual variation. To determine the within-subject variation in hair cortisol levels in vertex posterior hair, two different hair samples in close proximity were collected simultaneously from the vertex posterior and analyzed.

Stability of cortisol in overnight incubation. To determine if cortisol is stable during overnight incubation, we created a standard curve in PBS pH8 (0, 2, 5, 10, 20, 40, 80 ng/ml) and incubated 500 µl of each standard overnight at 52 °C while shaking for 16 hours. This standard was run with the unincubated standard curve which was kept at -4 °C overnight.

Effect of hair dye on hair cortisol levels. To determine the effect of hair dying prior to hair sampling, we compared cortisol levels in untreated hair samples

with levels in hair samples obtained from subjects who had applied hair dye before the samples were taken. To evaluate the effect of hair dyeing after the hair samples had been taken, untreated, long hair strands from seven volunteers were collected divided in four equal groups. One group was not dyed, the other groups were dyed once, twice and thrice using Garnier BelleColor® as per manufacturer's instructions, such that each individual had hair strands treated zero, one, two and three times with dye. Twelve centimeters of hair from the scalp end was taken from all treatment groups in all the individuals to analyze on ELISA.

B) Correlation studies and determination of reference range

Participants

Non-obese (BMI < 30 mg/kg²) subjects were recruited via advertising in local media. Participants were excluded if there was insufficient hair for collection or they were using glucocorticoid drugs during the last 3 months.

Procedures

All participants were seen by a research nurse between 7:30 and 10 AM. Data on medical and surgical history, current intake of drugs, and use of alcohol and nicotine were obtained. Age, blood pressure, weight, height and waist circumference were recorded. All volunteers collected 24-hr urine for cortisol and creatinine. A saliva sample and a venous blood sample were taken for cortisol measurement. All samples for steroid measurements in serum and urine were frozen at -79 °C until analyzed.

Measurement of cortisol in hair, saliva, serum and urine

Hair cortisol was measured as described above. Saliva cortisol was measured using the same salivary ELISA kit (Alpco®) as per the manufacturer's instructions. Cortisol in serum was measured by Chemiluminescent Immunoassay (Bayer Centaur Analyzer) using trilevel serum quality control material for monitoring per-

FIGURE 1. Effect of natural hair colour on the detection of cortisol in hair samples. Cortisol standards were created in basic PBS and in hair solutions generated from blond, brown and black hair samples during overnight hair incubation. The Y-axis represents *exogenous* cortisol-conjugate bound as a percent of the amount of cortisol-conjugate measured in the zero standard. Therefore, as a result of ELISA's competitive binding process, the amount of cortisol in a sample is inversely proportional to the amount of *exogenous* cortisol-conjugate measured.

formance. Urine cortisol was first extracted with methylene chloride, then constituted and tested using the same method as for serum cortisol. The creatinine level in the urine was used to ensure there was a complete 24-hr urine collection. For cortisol measurements in saliva, the sensitivity is 1 ng/mL; the coefficient of variation (CV) is 10.3 % at 6.6 ng/mL, and 6.5% at 52.4 ng/mL (manufacturer information). For mean serum cortisol concentrations of 753, 549 and 59 nmol/L, the CV was 6.34, 7.08, and 11.52%, respectively. For a mean urine cortisol of 39 nmol/L, the CV was 12%. The LOD for urine and serum cortisol was 5 nmol/L and 5 nmol/day, respectively.

Data Analysis

All data are presented as median (range) unless indicated otherwise. All statistics were run using the Sigma Stat program (Version 3.1). Comparison between groups was done using unpaired Student t-test or the Mann-Whitney U test as appropriate based on data-distribution. Correlation coefficients between different parameters were calculated with the Spearman's

rank correlation. Differences in hair cortisol levels from various parts of the head as well as changes in hair cortisol levels with sequential dye treatment were evaluated using Kruskal-Wallis One Way Analysis of Variance on Ranks.

Results

A) Evaluation of ELISA Assay for Hair Cortisol

Extraction recovery and precision

The mean extraction recovery of 80 ng/ml and 2 ng/ml cortisol standards (n=5) from PBS was 87.4% and 83.6% respectively. When hair samples were spiked with 100 ng/ml, 50 ng/ml and 2 ng/ml (n=6) cortisol standards, incubated and extracted, the mean extraction recoveries were 87.9%, 88.9% and 87.4%, respectively.

According to the manufacturer, the lowest level of detection for cortisol 1.14 ng/ml. The precision of the ELISA assay was determined using two hair samples with an average (about 60 pg/mg) and a high cortisol level (about 600 pg/mg). The intra-assay CVs for these samples were 7.2% and 6.0%, respectively. Inter-day precision was slightly more variable, with a CV of 10.6% for the sample of average concentration and 7.6% for the sample of high cortisol content.

Effect natural hair color on hair cortisol levels

Cortisol levels in the standards created from different hair color matrices did not differ significantly from the levels in PBS standards (Figure 1), indicating that measurement of hair cortisol levels is not affected by variation in natural hair color.

Hair cortisol levels in hair collected from different sections of the head, and intra-individual variation in two samples from the vertex posterior

Hair cortisol levels in samples obtained from posterior vertex, nape, temporal, anterior vertex, and frontal sections of the head in 14 individuals were 72.9 (32.4-120), 63.4 (31.2-1010.4), 61.6 (30.0-257.3), 50.3 (34.0-133.0) and 86.5 (14.6-183.3) pg/mg, respectively. One-way ANOVA analysis did not reveal

FIGURE 2. Boxplots of hair cortisol concentrations in untreated hair and in hair that had been dyed before taking the hair samples.

any differences in mean hair cortisol levels obtained from various parts of the head. However, the intra-individual variation was high with a mean coefficient of variation of 30.5%.

Our standard hair collection procedure, as developed within the Motherisk program, is to take hair samples from the vertex posterior. Therefore we compared intra-individual variation in hair cortisol levels obtained simultaneously from the vertex posterior. The mean CV for the 28 sample pairs was 15.6%

Effect of hair dying on hair cortisol levels.

The mean cortisol level in hair prior to dyeing was 51.0 (32.0-146.7). In hair samples that were dyed once, twice, or thrice *in vitro*, after the samples had been obtained, the hair cortisol levels were 67.9 (48.6-150.7), 64.4 (42.2-144.9) and 79.6 (47.2-156.1) pg/mg, respectively ($P = 0.4$; Kruskal-Wallis test).

We compared cortisol levels in untreated hair samples with levels in hair samples that had been dyed before taking the hair samples (Figure 2), and found lower hair cortisol levels in hair that had been dyed ($P < 0.05$, Mann-Whitney U).

TABLE 1. Clinical and laboratory characteristics of participants

Variable	Subjects
Sex (males/females)	19/20
Age (yr)	39 (20-76)
Weight (kg)	70 (45-92)
Waist (cm)	82 (60-106)
BMI (kg/m ²)	24 (18-29)
Systolic BP (mmHg)	108 (92-138)
Diastolic BP (mmHg)	70 (56-82)
PSS Score	12 (3-31)
Salivary cortisol (ng/ml)	10 (2-13)
Serum cortisol (nmol/L)	222 (25-545)
Urinary cortisol (nmol/24hr)	38 (2-213)
Hair cortisol (pg/mg)	46 (27-200)

Data are presented as median (range).

BP = blood pressure, PSS = Perceived Stress Scale

B) Correlation of cortisol in hair with cortisol in saliva, serum, and urine

In total, 39 non-obese subjects, 19 male and 20 female, participated (Table 1).

There was a correlation between hair cortisol levels and 24-hr urine cortisol ($r=0.33$; $P=0.041$) and between serum and salivary cortisol ($r=0.58$; $P=0.0016$) (Figure 3).

Hair cortisol reference range

Among the 39 non-obese participants the hair cortisol levels were not normally distributed ($P < 0.001$, Kolmogorov-Smirnov test), but after log transformation the distribution was rendered normal ($P=0.19$). Therefore, we used the log transformed data to determine the mean $\pm$ 2SD of the log values, after which an antilog transformation was used to define the 95th percentile reference range. Thus, the reference range for cortisol levels in hair of healthy non-obese individuals was 17.7-153.2 pg/mg of hair with a median of 46.1 pg/mg.

FIGURE 3. Correlations between cortisol levels in serum and saliva (A) and between hair cortisol and cortisol in saliva (B), 24-hr urine (C) and serum (D).

Discussion

In this study we describe a novel assay for measurement of cortisol levels in human hair obtained from the head. Although preliminary studies have described measurement of cortisol levels in hair^{5,6}, the present study is the first to compare these levels with cortisol levels in other, commonly used matrices.

We found a correlation between cortisol in hair and in urine, and between cortisol in saliva and in serum. Neither hair cortisol nor urine cortisol was significantly correlated with either serum or saliva cortisol. The strong correlation between saliva and serum cortisol is well-supported in the literature as two biomarkers of short term measurements.^{8,9} In comparison, the correlation between hair cortisol and urinary cortisol is much weaker, most probably because of the difference in the time frames that are reflected by the measurements: saliva and serum levels both reflect acute levels, urine represents cortisol secretion during

one day, whereas hair cortisol levels represent levels during 1-2 months.

The use of a commercial salivary ELISA for measuring hair cortisol resulted in a good precision and extraction recovery, and hair cortisol levels were not affected by the overnight incubation for the extraction procedure and are stable up to 6 months in the freezer (-20°C or -80°C). Hair cortisol could also be reliably tested without cross-reactivity to cortisone.

Hair matrix effects are a well-known confounder in the interpretation of hair testing results.^{10,11} Matrix effects are typically related to hair colour, but can be associated with differences in hair quality, surface and texture. We did not find any effect of hair colour on hair cortisol levels. These reassuring results are in keeping with the study by Raul *et al.*⁶, who did not find a correlation between hair cortisol levels and melanin concentrations.

We found that dying of hair *after* it was removed from the head did not result in an increase in hair cortisol levels. In contrast, we found that cortisol levels

in hair samples obtained from individuals who had previously applied cosmetic colouring were lower than levels in hair samples that had not been treated. Several studies have reported that hair treatments can decrease hair levels of different drugs by more than 30%, e.g. Yegles *et al.*¹² demonstrated that bleaching resulted in decreases in hair levels of cocaine (24.6%), codeine (57.5%), morphine (67.4%), and diazepam (39.7%). Dyes, bleach and permanent waves have been shown to reduce cotinine and nicotine levels in hair.¹³ Several factors may help explain this disparity in the effect of hair dying on cortisol levels. First, the laboratory experiment examines the effect of hair dyeing after the hair collection in the same individuals; the clinical comparison is in hair samples dyed before sampling in two separate groups of individuals. Secondly, while the laboratory experiment is controlled and the treatment of all the hair samples is the same, in the clinical group different cosmetic treatments and products were used at variable times. Thirdly, it is unknown how hair dying affects the shaft of the hair. If hair dyeing increases hair mass, the concentration of cortisol may decrease, while removal of oil or other components may decrease the total hair mass, thereby increasing the cortisol concentration. Further studies need to determine if hair cortisol levels are affected by the time period between hair dying and hair sampling, or by application of other *in vivo* cosmetic products including shampoos and conditioners.

Although we did not find a significant difference between hair cortisol levels obtained from different sections of the head, the mean coefficient of intra-individual variation was large (30.5%). When comparing hair cortisol levels in two adjacent hair samples obtained from the vertex posterior, we found a notably lower CV of 15.6%. To date, very sparse information exists on drug concentrations in hair collected from different scalp regions, showing intra-individual variation as high as 24%.¹⁴ This along with our study supports the current practice to obtain hair samples from the vertex posterior. Presently, all published hair collection is done from this region.^{15,16}

The calculated reference range of cortisol levels in hair in the normal non-obese population is tenfold (17.7-153.2 pg/mg), indicating less variation than in

the hair cortisol range of 5.2-91 (mean 18, median 15.4) determined by Raul *et al.*⁶ Raul's population had a very large age range, from 2 to 90 year old, and other clinical details were not provided. Importantly, the Raul study used LC-MS, while we used an ELISA assay, and the extraction methods were different. In future work the two methods should be compared using the same samples in a larger study population.

There are several limitations associated with measurement of endogenous hormones in hair. It is restricted to individuals who have sufficient hair at the posterior vertex and do not have cultural/religious objections to taking a hair sample. Hair cortisol levels are not able to determine brief cortisol responses, and cannot be used to determine day-to-day variation. It is not known whether hair cortisol levels vary with hair growth rate, which could be important as the activity of hair follicles is intermittent, consisting of active phase (anagen), transitional (catagen) and resting phase (telogen).¹⁷ Further, hair growth rate decreases with age, in various diseases (e.g. hyper- and hypothyroidism) and varies among ethnic groups.¹⁸ For saliva samples the saliva flow rate has only a minor effect on saliva cortisol levels.¹⁹ By analogy, it is conceivable that for hair cortisol levels the hair growth rate may have limited importance. Finally, levels of cortisol in hair may be affected by local synthesis or metabolism in the hair follicles²⁰, although the relative contribution of locally produced hormones may be limited. Currently it is not known if hair levels of cortisol vary throughout the various seasons, or if hair growth rate follows a diurnal rhythm.

Measurement of hormone levels in hair has several advantages. Its collection is non-invasive and can be performed by non-health care workers at any time of the day. Samples can be stored at room temperature and be sent by mail, making it potentially useful in population studies. Further, levels reflect average hormone levels over the last two months, as opposed to blood, saliva and urine samples, which reflect acute or daily cortisol levels. Another important characteristic of measurement of cortisol in hair is that the levels are not affected by acute stress. Hair cortisol may be important in the diagnosis of cyclical Cushing's syndrome, and in depression, in which daily cortisol ex-

cretion in urine is increased in a higher number of days, but not all days.²¹

Recently, hair cortisol measurement has received substantial experimental support. In male rhesus macaque monkeys, hair cortisol levels, measured by ELISA, were increased upon stress of relocation, and returned to pre-stressor levels after the animals had adapted to their new environment.²² Further, we found a positive correlation between hair cortisol levels and chronic stress, as assessed by the Perceived Stress Questionnaire, in pregnant women.²³

In summary, the current study describes a novel assay for measurement of cortisol in hair, and establishes a reference range for non-obese healthy subjects.

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