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Importance of methodological details in the measurement of cortisol in human hair

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Objective. The measurement of cortisol in hair became a popular and frequently used methodology in human stress research. This methodological approach, depending on the length of hair analyzed, allows to reflect cortisol secretion over prolong time periods in a retrospective fashion. There is a big variability in the experimental approaches to cortisol extraction used in individual laboratories. Moreover, there are many methodological details which are not described in most of the published papers, although they may be influential. The aim of the present study was to identify and optimize selected methodological steps of hair cortisol extraction.

Methods. As the starting point served the methodology of Xiang et al. (2016). A hair pool was used to test the procedures. The main steps modified were pulverization, methanol extraction and centrifugation.

Results. In the presented procedure, we decreased the speed and duration of the pulverization, we increased the volume of methanol and increased the time and speed of centrifugation. The results showed obtaining lower variability and higher cortisol concentrations than those we obtained by the methodology of Xiang et al. (2016), which was optimized.

Conclusion. The presented methodology is relatively simple and is likely to provide reliable results with low variability of cortisol concentrations measured in the same sample.

Key words: cortisol extraction, human hair, method

Cortisol is the main glucocorticoid hormone in humans and it plays an important role in many physiological functions as well as in pharmacological treatments. A big attention is given to measurement of cortisol release during stress. There are also multiple clinical conditions, which require analysis of cortisol in biological fluids (Nunes et al. 2015). In the past, cortisol concentrations were measured mainly in blood serum or plasma. To avoid the stressfulness of venipuncture, determination of cortisol in saliva is recently often preferred (Jezova and Hlavacova 2008).

Since 2004 (Raul et al. 2004), the measurement of cortisol in hair became a popular and frequently used approach in human stress research. It is considered a suitable methodology particularly for evaluation of long term presence of chronic stress conditions. Hair grows approximately 1 cm per month (Wennig 2000). Therefore, concentration of cortisol measured in 1 cm long hair (starting from the scalp) reflects a cumulative value for cortisol secretion during the last month. The hair sampling does not require a specially educated staff and it can be done non-invasively at any time of the day. Moreover, the hair samples can easily by transported and stored at room temperature (Gow et al. 2010).

There are several commercially available kits for measurement of cortisol by radioimmunoassay or enzyme-linked immunosorbent assay (ELISA). Par-

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ticularly the analysis by ELISA kits is relatively simple and may be performed even by people without deep laboratory experience. This is however different with respect to the preparation of the sample and extraction of cortisol from the hair. The importance of laboratory expertise needed for proper hormone extraction is often neglected.

There is a big variability in the experimental approaches to cortisol extraction used in individual laboratories. In the last 10 years, there are more than 300 papers published on measurement of hair cortisol, which differ in the amount of hair taken into extraction, the way of hair cutting or pulverization, duration of extraction or way of extract evaporation. More importantly, there are many methodological details which are not described in most of the published papers, although they may be influential. These include details on washing method, timing and extend of sample preparation or concrete information on centrifugation conditions.

The aim of the present study was to identify and optimize selected methodological steps of hair cortisol extraction. As a starting point we have used one of the few papers providing detailed description of methodology by Xiang and colleagues (2016). These authors compared the suitability of three methodological steps and revealed that it is better to pulverize than cut the hair, to perform the extraction at room temperature or 50°C and evaporation of organic solvent by room air than by a stream of nitrogen.

Methods and results

The amounts of hair for extraction were 12.5, 25 and 50 mg. The present study was purely methodological, human hair pool was used without measurement of cortisol concentrations of concrete individuals. According to Xiang et al. (2016) the hair sample was washed 3 times with 5 ml aliquots of isopropyl alcohol (Serva, Germany), dried overnight and pulverized with ULTRA-TURRAX Tube Drive (IKA® Works, Inc., Wilmington, NC) at 4000 rounds per minute (rpm) for 6 min followed by 6000 rpm for 6 min. The BMT-20 tubes contained 40 steel balls. The hair powder was eluted with 1.5 ml of methanol (Sigma-Aldrich, USA) in glass tubes on a rotating shaker at room temperature overnight, centrifuged at 1000 rpm for 2 min and 1 ml of supernatant was transferred in a new glass tube. Methanol was evaporated at 50°C (Xiang et al. 2016) in a water bath. The residue was diluted in 250 µl of 0.01 M phosphate buffer saline (pH 7.4) and 50 µl per well was used for the analysis. Cortisol concentrations in hair extracts were measured by the Salivary Cortisol Enzyme Immunoassay (ELISA) kit (IBL International, Hamburg, Germany).

Table 1
The high variability of cortisol concentrations in human hair measured in parallel samples before the present modification of the methodology

Sample	Amount of hair (mg)	Cortisol (ng/100 ml)	Cortisol (pg/mg of hair)
1	12.5	36 7	10.47 2.04
2	25	69 23	10.45 3.48
3	50	79 55	6.44 4.48
4	12.5	62 28	16.49 7.45
5	25	54 56	7.97 8.27
6	50	89 167	7.01 13.18

Figure 1. Examples of methanol extract of human hair following centrifugation. The second, clear sample was performed using present methodology.

We used the above mentioned methodology (Xiang et al. 2016) on a test hair extraction and we obtained values of cortisol concentrations in appropriate range of the calibration curve. However, the duplicate tubes from the same hair extracts were very variable (Table 1).

Moreover, it happened repeatedly that the methanol extract of hair was cloudy (Figure 1). Next, it was uneasy to pipette the supernatant into the new tube without indirectly disturbing the sediment. Thus, an idea raised that the centrifugation efficacy was not sufficient. We therefore increased the volume of methanol for extraction from 1.5 to 2.0 ml and following centrifugation at 2000 rpm for 10 min we transferred 1.5 ml of supernatant into an Eppendorf

Table 2
Cortisol concentrations measured in human hair pool by two methodologies of hair cortisol extraction

Cortisol	Methodology according to Xiang et al. (2016) A	Present methodology B
(pg/mg of hair)	4.1	6.0
	2.5	5.2
	4.8	4.9
	3.1	5.8
	5.1	6.3
	4.8	5.3
	3.6	5.5
	3.8	5.3
	3.9	5.9
	4.5	4.8
Mean	4.01	5.49 ($p<0.001$)
SD	0.77	0.48
SD²	0.59	0.23

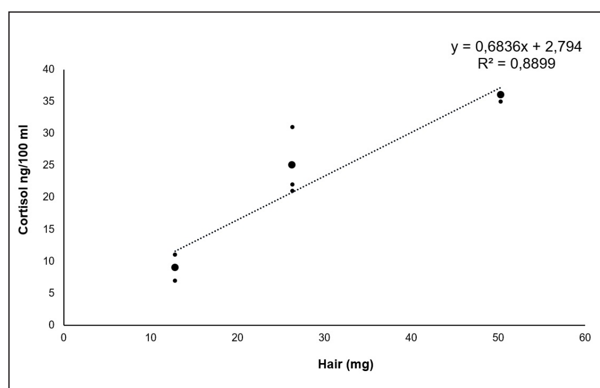

Figure 2. The relationship between the amount of hair pool analyzed and cortisol concentrations obtained.

tube and centrifuged again at 10 000 rpm for 10 min. The variability of duplicate samples from the same hair extract was lower, though not ideal.

The next step was to test different speeds and durations of pulverization of hair and their combinations. The results were statistically evaluated using the independent Student t-test. Differences were considered significant at a $p<0.05$. The linear regression analysis was used to study the linear relationship between different amounts of hair pool and cortisol concentration.

The modified methodology of hair cortisol extraction. Wash the hair sample 3 times with 5 ml aliquots for 2 min of isopropyl alcohol, dry overnight and pulverize with ULTRA-TURRAX Tube Drive (IKA® Works, Inc., Wilmington, NC) at 4 000 rpm for 6 min. The BMT-20 tubes contained 40 steel balls. Elute the hair powder (50 mg) with 2.0 ml of methanol on a rotating shaker at room temperature overnight, centrifuge at 2 000 rpm for 10 min, transfer 1.5 ml of supernatant into an Eppendorf tube and centrifuge again at 10 000 rpm for 10 min. Transfer 1.0 ml of methanol to a glass tube, evaporate at 50 °C (water bath) with room air overnight and dilute with 250 µl of 0.01 M phosphate buffer saline (pH 7.4). The sample is ready for cortisol analysis by ELISA as described above.

To compare the above described methodology with the method of Xiang et al. (2016), we performed the washing step with a 200 mg of hair pool and the pulverization at 4 000 rpm for 6 min. Thereafter, 50 mg of the powder was transferred to a glass tube for further extraction with 2 ml methanol (sample B). We continued to pulverize the rest of the hair powder by 6 000 rpm for 6 min (Xiang et al. 2016) and 50 mg of the powder was transferred to a glass tube for further extraction with 1.5 ml methanol (sample A). The elution as well as first centrifugation was 1 000 rpm for 2 min (sample A) or 2 000 rpm for 10 min (sample B). Sample B was centrifuged again in an Eppendorf tube at 10 000 rpm for 10 min. The evaporation step was performed under the same conditions.

The results showed significantly higher cortisol concentrations ($t=4.93$; $p<0.001$) in the sample B analyzed by the presently proposed methodology (Table 2). Moreover, the variability of cortisol concentrations measured in the same sample in 10 parallel wells as shown by the values of standard deviation (SD) and squared SD was much lower in sample B.

To evaluate the relationship between the amount of hair pool analyzed and cortisol concentrations obtained, we performed the extraction using the presented methodology from 12.8, 26.3 and 50.3 mg. The cortisol assay was performed in triplicates. The results showed a clear linear regression (Figure 2). The coefficient of variation was 0.89.

Discussion

The main result of the present studies is the optimization of the methodology for extraction of cortisol from human hair. The important modifications were made in the pulverization and centrifugation steps (Figure 3).

The extraction of cortisol from hair starts with organic solvent. The majority of previously described procedures involves isopropyl alcohol. In many ar-

ticles, this step is not even mentioned (e.g. Karlen et al. 2011; Caparros-Gonzalez et al. 2017; Pochigaeva et al. 2017). Very variable duration of drying the hair after the washing was used in previous reports. In our hands, the optimal way is to dry the hair for longer time periods, the best is overnight.

There is a big diversity in the step of the hair preparation for extraction. The simplest technology is to mince the hair by scissors (e.g. Smy et al. 2016). To obtain extra fine powder, there is a possibility to pulverize the hair by using a ball mill device (e.g. Laudenslager et al. 2011; Skoluda et al. 2012; Krumbholz et al. 2018), which is rather expensive and not available for each laboratory. We used a middle way, namely the pulverization with a ball grinder. Interestingly, there was not a linear correlation between the length of grinding and the speed of the mixing with reliable results of cortisol analysis. In opposite, the best results were obtained with the lowest speed and the shortest duration of pulverization tested.

A rather uniform step described in the published studies is the extraction of cortisol by methanol. The individual methodologies differ only in the extraction temperature. A very convenient process is to perform the extraction at room temperature, which was used also in the present procedure.

The critical step is the centrifugation of the extracts. The details of the centrifugation conditions are usually not described in the published papers. We observed that the results of final cortisol analysis can be significantly influenced by insufficient centrifugation. Some authors used only slow rotation, e.g. 100 rpm (Albar et al. 2013). The present results are in favor of prolonged (2 times 10 min) and rapid (2 000 and 10 000 rpm) centrifugation. Such high speed centrifugation has been described only rarely (Kirschbaum et al. 2009; Meyer et al. 2014). It is possible, although we have no direct evidence that after very intensive pulverization, the smallest parts of hair powder may occasionally be transferred into the wells for ELISA analysis. Thus, an insufficient centrifugation along with very intensive pulverization may be the cause of the variability in parallel samples. It cannot be excluded that the variability observed in the mass density of hair (Kiazymov et al. 2009) may contribute to the difficulties mentioned.

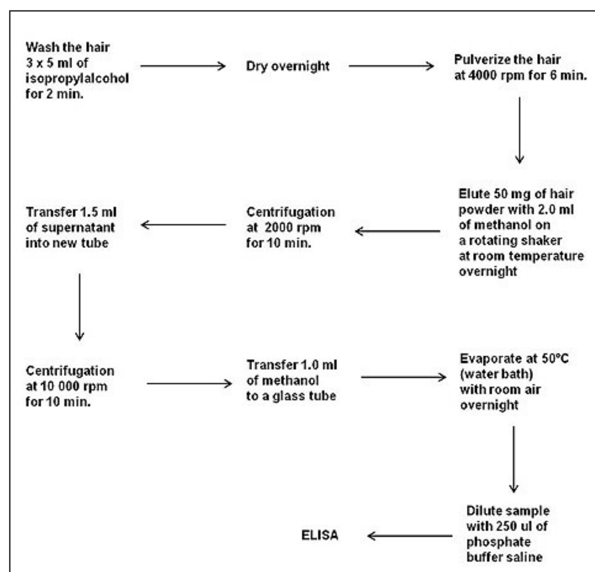

Figure 3. The simplified/schematic steps of the final modified method used for cortisol extraction from human hair.

Evaporation of methanol before cortisol analysis used to be performed by variable approaches often with necessity of the source of nitrogen stream or speed-vac. Present data are in favor of a simple evaporation with room air as suggested by Xiang et al. (2016), but at a higher temperature (50 °C) using a water bath.

In conclusion, we have optimized several methodological steps in the process of the hair cortisol extraction. The presented methodology is relatively simple and is likely to provide reliable results with low variability of cortisol concentrations measured in the same sample.

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