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Analysis of hair cortisol levels in captive chimpanzees: Effect of various methods on cortisol stability and variability

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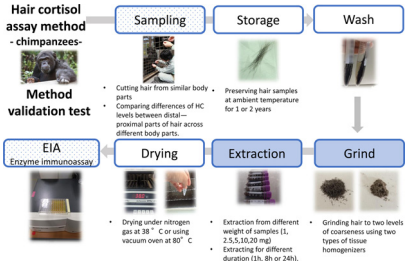
GRAPHICAL ABSTRACT

Summary of the experimental process. The items colored blue affected the results of the hair cortisol analysis in the present study. The stippled items were found to affect the results obtained in our previous study [1].



ABSTRACT

Hair cortisol has been reported to be a useful measure of long-term hypothalamic–pituitary–adrenal (HPA) axis activation in several species. It serves as a practical tool for long-term stress assessment, but it is important to understand the methodological factors that can affects hair cortisol assays to avoid methodological artifacts. To that end, we tested several procedures for measuring cortisol levels in hair collected from captive chimpanzees. The results showed that reproducibility was high, and we found no differences in cortisol levels among the various storage, drying, and sampling methods. However, the fineness of homogenized hair, sample weight, and

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extraction time affected absolute hair cortisol concentration. Although hair cortisol levels were stable over time, factors that may influence measurement results should be kept constant throughout a study.

- We modified and validated a methodology involving enzyme immunoassays to reliably measure the hair cortisol levels of captive chimpanzees.
- The results revealed that the fineness of homogenized hair, sample weight, and extraction time caused variations in absolute hair cortisol concentrations in chimpanzees. In contrast, storage, drying, and sampling from similar body parts did not affect the results.

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Method details

Samples were collected from chimpanzees by cutting arm hair (approximately 200 mg) with scissors. Samples were washed with 5 ml isopropanol by shaking the tubes three times for 2 min each. After drying, the samples were stored at ambient temperature until analysis. Then, about 150 mg of the sample was ground into a fine powder for 4 min at 6500 rpm with a Precellys 24 tissue homogenizer (Bertin Technologies, Orléans, France). The powdered samples were weighed (minimum 5 mg) and placed in 2-ml tubes, and 1-ml methanol was added. Cortisol was extracted by shaking the tubes for 24 h at ambient temperature. Following extraction, the samples were centrifuged, and 0.6 ml of supernatant was aliquoted into different tubes and evaporated by vacuum oven at 80 °C. Samples were reconstituted using phosphate buffer, and cortisol concentrations were measured using a commercially available enzyme immunoassay (EIA) kit (Salivary cortisol, Salimetrics LLC, Philadelphia, PA, USA). These methods to quantify hair cortisol levels were based on our previous study, which was modified according to the method-validation results presented below [1].

Method validation

We investigated the effects of various sampling, grinding, and drying methods and storage and extraction times on the analysis of hair cortisol. In addition, we checked the minimum number of samples necessary to estimate the average hair cortisol level during a year. Samples were collected from 72 captive chimpanzees (38 males and 42 females) living in the Kumamoto Sanctuary (KS), the Primate Research Institute (PRI), and the Great Ape Research Institute. (For information about care and husbandry of these chimpanzees, see Refs. [2–4]). Following the procedure described in the Methods section, except when we tested the grinding process, we used a Precelly 24 tissue homogenizer for grinding hair. According to the instructions, the minimum concentration of cortisol that can be measured by this kit is 0.07 ng/ml. Intra-assay variability was 5.12%, and the inter-assay variability values for high and low controls were 4.88 and 7.17%, respectively (mean of nine plates). The results from all of the validation tests are shown in Table 1 and Supplementary Material.

Statistical analysis

Pearson's correlation was used to test the correlation between samples. Paired *t*-tests or a one-way analysis of variance (ANOVA) was used to assess differences between samples. Tukey's HSD tests were performed as a post hoc test following the ANOVA to examine pairwise differences. All statistical tests were conducted using R 3.1.0. [5]. Data on hair cortisol concentrations were log-transformed prior to statistical analysis.

(a) Effects of sampling location

Our previous study [1] reported variation in hair cortisol levels across different body regions. To reduce this variation, we suggested using a consistent body region for multiple samplings.