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A statistical method to calculate blood contamination in the measurement of salivary hormones in healthy women

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ABSTRACT

Objectives: Previous studies have reported that salivary concentrations of certain hormones correlate with their respective serum levels. However, most of these studies did not control for potential blood contamination in saliva. In the present study we developed a statistical method to test the amount of blood contamination that needs to be avoided in saliva samples for the following hormones: cortisol, estradiol, progesterone, testosterone and oxytocin.

Design & methods: Saliva and serum samples were collected from 38 healthy, medication-free women (mean age = 33.8 ± 7.3 yr.; range = 19–45). Serum and salivary hormonal levels and the amount of transferrin in saliva samples were determined using enzyme immunoassays.

Results: Salivary transferrin levels did not correlate with salivary cortisol or estradiol (up to 3 mg/dl), but they were positively correlated with salivary testosterone, progesterone and oxytocin ($p < 0.05$). After controlling for blood contamination, only cortisol ($r = 0.65$, $P < 0.001$) and progesterone levels ($r = 0.57$, $P = 0.002$) displayed a positive correlation between saliva and serum. Our analyses suggest that transferrin levels higher than 0.80, 0.92 and 0.64 mg/dl should be avoided for testosterone, progesterone and oxytocin salivary analyses, respectively.

Conclusions: We recommend that salivary transferrin is measured in research involving salivary hormones in order to determine the level of blood contamination that might affect specific hormonal salivary concentrations.

1. Introduction

The use of saliva as a biological fluid to determine steroid hormone concentrations has grown immensely. Saliva measures provide researchers with a noninvasive, simple collection procedure with an added benefit of representing the unbound, biologically active hormone. Poor oral hygiene is an important factor to be considered, as it can cause inflamed bleeding gums, which will result in various degrees of blood contamination of saliva. Trauma or damage to the mouth, certain medications, and vigorous flossing or brushing may also increase blood contamination in the saliva [1]. Saliva steroid concentrations are approximately 1 to 10% of serum/plasma levels. Thus, the presence of even small amounts of blood may substantially interfere with saliva steroid assays. The issue of blood contamination pertaining to saliva collection has been well documented [1] but there continues to exist a lack of correction for blood contamination when it comes to salivary hormone analysis [2–5]. The effects of blood contamination on salivary hormone detection can vary from hormone to hormone [1,3,6] and, therefore, it is necessary to address the issue of sample contamination on a case-by-case (or hormone-by-hormone) basis.

The *primary* objective of this study was to develop a statistical method to quantify the threshold of blood contamination in saliva that interfere with salivary levels of cortisol, estradiol, testosterone, progesterone

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and oxytocin. The *secondary* objective was to correlate the serum and saliva levels of these hormones after controlling for salivary blood contamination.

2. Methods

2.1. Subjects

Thirty-eight healthy females between the ages of 18 to 45 years old were recruited at St. Joseph's Healthcare, Hamilton, Ontario. Twenty-eight were women with regular menstrual cycles and ten were breastfeeding postpartum women, which allowed us to study a wider range of oxytocin levels. All women were medication free. Informed consent was obtained from all study participants and the study was approved by the St. Joseph's Healthcare Research Ethics Board.

2.2. Serum and passive drool collection

Serum and saliva samples were collected from the women with regular menstrual cycles between days 19–22 of their cycle. Samples from the postpartum women were collected 30 min prior to breastfeeding. All samples were collected between 9 and 11 am to minimize diurnal changes.

Subjects were oral resting (no eating, chewing gum, smoking, drinking) for at least 30 min prior to collection. Subjects rinsed their mouths with water and spit it out, waited 10 min, and then spit (via passive drool) into a 15 ml sterile conical tube until 3 ml of saliva was collected. After collection, the saliva was centrifuged at 4 °C for 15 min at 3000 rpm. Seven aliquots were obtained and the samples were frozen at −80 °C until they were assayed.

Immediately following the saliva collection, subjects had 10 ml of whole blood taken in serum tubes. The blood was clotted at room temperature for 45 min, and then centrifuged at 4 °C for 15 min at 3000 rpm. Seven aliquots were obtained and the samples were frozen at −80 °C until assayed.

2.3. Serum assays

Serum was assayed for cortisol, progesterone, free testosterone and estradiol using commercially prepared solid phase enzyme-linked immunosorbent assay (ELISA) kits (ALPCO Diagnostics, Salem, NH). Serum oxytocin was assayed using an ELISA kit (Assay Designs, Inc., Ann Arbor, MI). All serum samples were assayed in duplicate as per manufacturers' protocol, with a fresh aliquot for each analyte. For all of the assays, the inter-assay variations were <11.4% and the intra-assay variations were <10.4%.

2.4. Saliva assays

Saliva was assayed for cortisol, progesterone, testosterone and estradiol using commercially prepared enzyme immunoassay (EIA) kits (Salimetrics, LLC, State College, PA). Salivary oxytocin was assayed using the same kit manufacturer as for serum, with the following modifications: 1 ml of saliva was thawed, centrifuged and dried by vacuum centrifugation, then reconstituted in 250 μl of assay buffer, of which 100 μl/well was used in the ELISA protocol [7]. The inter-assay variations were <10.7% and the intra-assay variations were <9.5%.

2.5. Salivary blood contamination testing

Saliva samples were also tested for blood contamination using an EIA kit (Salimetrics, LLC, State College, PA). This kit quantitatively measures the amount of transferrin in the saliva samples. Transferrin is present in large quantities in blood but there should only be trace amounts found in saliva. The inter-assay variation was 7.6%, the intra-assay variation was 9.5% and the sensitivity was 0.08 mg/dl.

2.6. Statistical analyses

In order to determine the contamination level that we must avoid for each analyte we correlated the salivary hormone levels with their respective transferrin levels using Pearson's or Spearman correlation coefficients using the following steps: For each hormone, we first rank ordered the 38 transferrin values, from higher to lower. Then, we performed a series of Spearman/Pearson's correlations where we correlated the salivary transferrin with the salivary hormone levels, starting with all 38 values, then repeating it with 37 values (excluding the higher transferrin level on the list), then with 36 values, 35 values, and so on, until we were left with ten pair of values (within the lower transferrin levels). Then, we plotted these correlation coefficients, which are represented by the circles in Fig. 1. Then, we used linear or logarithmic regressions to calculate the exact amount of contamination we must avoid for each salivary hormone. Circles in red (Fig. 1) indicate slopes with inclination significantly different from zero ($p \leq 0.05$), suggesting a positive correlation between hormonal level and blood contamination in saliva.

Next, we used Pearson's or Spearman correlation coefficients to calculate the correlation between salivary and serum hormone levels after excluding the data points associated with blood contamination (Fig. 2). We used GraphPad Prism software version 5.04 in all analyses.

3. Results

3.1. Saliva

For the salivary blood contamination analyses, the levels of contamination were low (0.08 to 0.50 mg/dl) for 23 subjects, medium (0.51 to 1.00 mg/dl) for 5 subjects and high (1.01 to 2.96 mg/dl) for 10 subjects. Overall, the salivary transferrin results indicated that levels up to 3 mg/dl did not correlate with salivary cortisol or estradiol (Fig. 1A and B), which suggests little if any interference of blood contamination with salivary levels of cortisol and estradiol. However, higher salivary transferrin levels were positively correlated with salivary testosterone, progesterone and oxytocin (all $p < 0.05$; Fig. 1C, D and E). We found that salivary transferrin levels higher than 0.80, 0.92 and 0.64 mg/dl should be avoided for testosterone, progesterone and oxytocin, respectively.

3.2. Serum

Correlations between the saliva samples and the respective serum samples controlled for transferrin contamination are shown in Fig. 2. Salivary cortisol and estradiol were not influenced by blood contaminations up to 3 mg/dl. After controlling for blood contaminations, only cortisol ($r = 0.65$, $p < 0.001$; Fig. 2A) and progesterone levels ($r = 0.57$, $p = 0.002$; Fig. 2D) displayed a positive correlation between salivary and serum samples in this cohort of young healthy women.

4. Discussion

The objective of this study was to quantify the effects of blood contamination in saliva samples of steroid hormones, as well as measure their saliva X serum correlation after controlling for salivary blood contamination. In the present study, we found that salivary cortisol levels were not influenced by blood contaminations up to 3 mg/dl. Our results are in agreement with a number of previous studies. A recent study of numerous saliva samples with unique combinations of differing yet known cortisol and hemoglobin concentrations reported that salivary contamination with blood undetectable to the eye did not affect salivary cortisol readings obtained via immunoassay [8]. In another study that looked at salivary hormone concentrations over the menstrual cycle, blood contamination in unstimulated whole saliva was not correlated with salivary cortisol concentrations [9]. Furthermore, in a study

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