Quantifying blood leakage into the oral mucosa and its effects on the measurement of cortisol, dehydroepiandrosterone, and testosterone in saliva

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Abstract

The impact of blood leakage due to microinjury to the oral cavity on the measurement of salivary hormones was examined. Saliva samples were collected before, immediately after, and then every 15 min for 1 h following vigorous tooth brushing. Blood in saliva was quantified by visual inspection of discoloration, HemaStix reagent strips to detect hemoglobin, and an immunoassay for transferrin. The presence of blood in saliva immediately after microinjury was confirmed by all methods. Hemoglobin and transferrin levels remained elevated over baseline for at least 30 min. Levels of salivary testosterone increased over baseline and remained elevated for 30 min in response to microinjury. Microinjury induced change in salivary testosterone was more closely associated with the change in transferrin than hemoglobin levels or discoloration ratings. On average, levels of salivary dehydroepiandrosterone (DHEA) did not increase in response to microinjury. However, individual differences in microinjury induced change in DHEA were associated with discoloration ratings. Salivary cortisol levels, on average, were neither responsive to microinjury, nor were individual differences in cortisol change associated with blood contamination measures. Neither diurnal nor gender-related differences in baseline hormone levels predicted the impact of blood leakage on quantitative salivary measurements. The findings suggest ecologically valid minor-to-moderate level microinjuries to the oral cavity have negligible effects on the measurement of salivary cortisol, but may be important to quantify and control when assessing other hormones especially testosterone.

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Keywords: Blood contamination in saliva; Cortisol; Testosterone; Dehydroepiandrosterone

Introduction

Monitoring steroid hormones in saliva rather than in urine, blood spot, or serum specimens has several well-documented advantages for biobehavioral researchers (see Kirschbaum et al., 1992; Malamud and Tabak, 1993). Yet, as the number of studies assessing salivary hormones has increased, so has our understanding that special circumstances involving the presence of exogenous cross-reacting substances in the mouth (i.e., infant formula, breast milk; Magnano et al., 1989), as well as characteristics of the process by which saliva flow is stimulated (i.e., citric acid containing powders, chewing gum; Schwartz et al., 1998) and collected (i.e., cotton-based absorbent material; Shirtcliff et al., 2001), have potential to influence assay validity. Ten years ago, the literature warned that when blood is present in the oral mucosa, quantitative estimates of hormones measured in saliva may be compromised (Malamud and Tabak, 1993). Given the rising popularity of the use of salivary biomarkers, it is somewhat surprising that little empirical research has specified the impact of this potential confound on measurement validity. In this study, we attempted to fill this important knowledge gap.

Blood and its components can leak into the oral mucosa as a result of microinjuries such as burns, abrasions, or cuts.
to the cheek, tongue, or gums. The probability of blood leakage into saliva is also higher for individuals suffering from poor oral health (i.e., open sores, periodontal disease, gingivitis) or certain infectious diseases (e.g., HIV; Arendorf et al., 1998), and those who engage in risky health behavior known to influence oral health negatively (e.g., chronic tobacco use; Reibel, 2003). Epidemiologic studies reveal that periodontal disease is more prevalent among minority (Gilbert et al., 2002), lower socioeconomic (Chavers et al., 2002), rural (Vargas et al., 2003), and third-world populations (e.g., Mengel et al., 1993). Blood leakage into saliva is also associated with normative developmental processes related to teething (common during infancy) and shedding teeth (common in middle childhood, early adolescence, and then again later in life). We anticipate the likelihood of blood contamination of saliva may covary with demographic variables frequently employed as mediators or moderators of individual differences in salivary hormone levels, or hormone–behavior relationships.

The concentration of most steroid hormones in saliva is typically several times lower than in serum (e.g., Kirschbaum et al., 1992). To be a meaningful index of systemic hormonal activity, quantitative estimates of steroid hormone levels in saliva (as opposed to assays that determine whether a substance, such as an antibody or drug, is present or not present) must be highly correlated with the levels measured in the general circulation. A high serum–saliva correlation depends on consistency in the process (i.e., active transport or passive diffusion, see Malamud and Tabak, 1993 for review) via which steroid hormones in the circulation move into the oral mucosa. At the lowest levels of blood leakage, the effect on salivary hormone levels is likely to be negligible. However, because of the higher concentration of steroid levels in serum than in saliva, after blood leakage reaches a certain threshold, the salivary levels should be artificially elevated.

Does this mean that steroid hormone levels from samples with high levels of blood contamination could be easily identified as statistical outliers? We speculate that the presence of blood contamination is sufficient, but not necessary and sufficient, to cause statistical outliers in salivary hormone distributions. Many statistical outliers may be the result of blood contamination, but contaminated samples may also present salivary hormone levels within the normative range. The magnitude of the impact of blood leakage is certain to be influenced by individual differences in baseline hormone levels. That is, salivary hormone levels for subjects who naturally fall well below average might be moved to just above average if their saliva is contaminated and would thus not necessarily be outliers statistically.

To our surprise, studies rarely report they have screened samples for blood contamination, and if they do, the methods are typically very crude. Our computerized literature search revealed a few anecdotal reports that investigators excluded samples that appeared visually discolored, but no validation of this approach is typically provided. Others report using a filter-paper dipstick method originally designed to detect the presence of hemoglobin in urine (e.g., Mazur et al., 1997). The chemical reaction that is key to color development on the dipstick is driven by the activity of hemoglobin in urine. However, peroxidase, an enzyme naturally present in saliva in high concentration, is also a catalyst for this reaction. Thus, when used in saliva, the accuracy of the urine dipstick method is questionable. Recently, Shirtcliff et al. (2002) used a novel approach—an immunoassay for transferrin. Transferrin is a protein found in very high concentrations in serum (mg/dl), but only in trace amounts in saliva (under normal circumstances). Consistent with the logic outlined above, when samples that tested positive for high transferrin levels were excluded, the serum–saliva correlation for testosterone was substantially reduced. At present, there is no consensus about whether it is necessary to screen saliva samples for blood contamination. Nor is there agreement on the best method to quantify the presence of blood in saliva samples.

It appears this phenomenon has the potential to be a significant source of unsystematic error affecting our ability to detect interindividual differences and intraindividual change in salivary hormone levels as well as our ability to detect hormone–behavior relationships. In this study, we experimentally induced blood leakage into saliva by having participants brush their teeth, and quantified resulting blood contamination by three independent methods: visual analog scale of sample discoloration, Hemastix® dipstick for hemoglobin, and transferrin by immunoassay. Effects of microinjury were measured on the three most commonly measured salivary hormones in biobehavioral research—cortisol, testosterone, and dehydroepiandrosterone (DHEA). Diurnal and gender differences were used to designate conditions with systematic differences in baseline hormone levels.

We hypothesized that the agreement between methods in quantifying the effects of microinjury would be poor except at the highest levels of blood contamination (when samples were obviously discolored). More specifically, we expected that Hemastix® and transferrin methods would hold yield positive results even when visual examination of sample discoloration was negative, and the Hemastix® method may yield false positive results. Furthermore, we hypothesized that this moderate level microinjury would result in elevated levels of steroid hormones in saliva, but the injury would not influence each hormone the same. That is, the impact of microinjury would change the salivary levels of steroid hormones measured in the very low concentration per volume range (pg/ml range such as testosterone and DHEA) more so than those measured in higher concentration per volume range (µg/dl such as cortisol), and be most pronounced when baseline levels related to diurnal and gender-related differences were high. Finally, we evaluated which method of measuring blood contamination predicted the microinjury-related change in steroid hormone levels in saliva.
Methods

Participants

Subjects were 30 male and 32 female, healthy, university students between the ages of 18 and 40 years. Participants were excluded if they reported being in poor health, were currently under a physician’s care, were taking any over-the-counter or prescription medications (including birth control), had any symptoms of acute illness (fever, congestion, nasal drip), had experienced an injury, burn, or other trauma (including dental or orthodontic work) to their gums within the prior 48 h, or had chronic medical conditions. The Pennsylvania State University Institutional Review Board approved all procedures, and informed consent was obtained.

Design and procedure

This study employed a 2 (time of day) × 2 (sex) × 2 (treatment condition) × 6 (sample collection time) mixed-model factorial design. Time of day, sex, and treatment condition were between subject variables, sample collection time was a repeated measure. Salivary transferrin, hemoglobin, discoloration ratings, DHEA, testosterone, and cortisol were dependent measures.

Two conditions in the design introduced systematic differences in the baseline levels of DHEA, testosterone, and cortisol. First, due to known diurnal differences in hormone production, conditions with higher (AM) vs. lower (PM) baseline levels were created for DHEA, cortisol, and testosterone. Samples were collected from participants either in the AM between 9 and 11 or in the PM between 12:30 and 5. Also, sex was included in the model because, on average, DHEA levels are higher in females than in males (Granger et al., 1999a), and testosterone levels are higher in males than females (Granger et al., 1999b).

In the microinjury group (n = 42), participants first donated a baseline saliva sample. Next, participants were instructed to manually brush their teeth for 2 min using an American Dental Association (ADA) approved, medium bristle brush without toothpaste. The pace was controlled at one stroke per 1–2 s and medium pressure was advised. After 30 s of brushing, participants were instructed to allow saliva generated to pool in their mouths. Immediately after brushing, and also every 15 min for 1 h, saliva samples were again collected. To index the size of the effect of blood leakage relative to the expected diurnal decline of these hormones, a second group (N = 20; no-treatment control) provided saliva samples only without brushing teeth.

Sample collection and preparation

At baseline, participants were asked to donate 3–5 ml of saliva into a collection vial. Following tooth brushing, 1–2 ml of saliva was collected by passive drool. Saliva samples were divided into 200- to 500-μl aliquots and stored at -40°C until assay. All saliva samples were subject to a single freeze thaw cycle to break down mucopolysaccharides that can interfere with pipetting (Worthman et al., 1990). On the day of assay, samples were centrifuged (1500 × g, 15 min) to remove particulate matter, and clear samples were transferred into appropriate test wells.

Hormone determinations

Saliva samples were assayed (without separation or extraction) for DHEA, cortisol, and testosterone using commercially available immunoassay protocols (Salimetrics, State College, PA). All samples were assayed for salivary cortisol using a high-sensitivity enzyme immunoassay (EIA) with a lower limit of sensitivity of <0.007 μg/dl, and average intra- and inter-assay coefficients of variation 4.13% and 8.89%, respectively. Saliva samples were assayed for testosterone using an EIA with a lower limit of sensitivity of 1.5 pg/ml, and average intra- and inter-assay coefficients of variation less than 10% and 15%, respectively. Samples were assayed for salivary DHEA using an EIA with a lower detection limit of 10.0 pg/ml, and average intra- and inter-assay coefficients of variation less than 10% and 15%, respectively. The units of measurement for salivary DHEA and testosterone are picograms per milliliter (pg/ml) and micrograms per deciliter (μg/dl) for cortisol.

To minimize error, all samples were tested in duplicate. Duplicates that varied by more than 20% were subject to repeat testing, and the average of the duplicates for each sample was used in the analyses (Chard, 1990). With the exception of repeats, all samples from each individual subject were tested in the same assay run.

Measuring blood contamination in saliva

Given a lack of consensus as to how blood contamination of saliva should be measured, we employed a multimeter approach. Three independent methods were applied ranging with respect to ease of use, time efficiency, expense, and accuracy.

Discoloration ratings

Each saliva sample was evaluated by two independent observers using a five-point visual analog scale designed to quantify sample discoloration. The “Blood Contamination in Saliva Scale” (BCSS) had the following response options: 1, “saliva appears clear, no visible color”; 2, “saliva has a hint of color, a little brown or yellow tint is barely visible”; 3, “saliva has a clearly visible yellow or brown tint”; 4, “yellow or brown coloring is more than just a tint, color is obvious but not very deep”; and 5, “saliva is very colored, deep, rich, dark yellow or brown is very apparent”. There was very high agreement between observer ratings (r = 0.95), and values were averaged across observers to represent the scores used in the analyses. This
very straightforward method requires minimal training and can easily be used in the field when samples are collected.

**Hemastix® reagent strip**

Each saliva sample was tested with a Hemastix® reagent strip, a commercially available method used to screen for blood in urine. Ten microliters of saliva is pipetted onto filter paper (similar to pH paper). Reagents on the paper undergo a color change, and the color is visually compared to a scalar measure of blood contamination: “Negative”, “Trace”, “+”, “++”, “+++”, and “++++”. These outcomes were coded 0 “negative” through 5 “++++” for analytic purposes. The procedure is simple, fast, and inexpensive (US$0.35/test) but requires some basic technical skills and equipment.

**Salivary transferrin**

All samples were assayed for blood contamination using an enzyme immunoassay kit for transferrin (Salimetrics). Transferrin is a large protein prevalent in blood at very high concentrations. Its large size prevents transferrin from being passively or actively transported from the general circulation into saliva. Under normal conditions, transferrin is present in saliva in the low <0.50 mg/dl range. When blood is spiked into saliva, there is a linear increase in salivary transferrin levels (Schwartz and Granger, 2004). There is no detectable cross-reactivity of the antibody used in this assay with testosterone, cortisol, or DHEA. Saliva samples were assayed for transferrin using an EIA with a lower limit of sensitivity of 0.08 mg/dl, and average intra- and inter-assay coefficients of variation less than 10% and 15%, respectively. This procedure (US$1.50/test) is run using a 96-well microtiter plate format and requires precision multichannel pipettes, an optical density reader, and technically trained laboratory personnel.

**Analytical strategy**

Examination of the blood contamination measures and hormone data revealed that the distributions were positively skewed. Therefore, log transformations were used to establish approximately normal distributions before analyses. All analyses used the log-transformed hormone values; however, nontransformed data are reported in the tables, figures, and text to facilitate interpretation. The main analyses evaluated the impact of microinjury by computing mixed-model ANOVAs following the omnibus 2 (time of day) × 2 (sex) × 2 (treatment condition) × 6 (sample collection time) factorial design. Time of day, sex, and treatment condition were between subject variables, sample collection time was a repeated measure. The first series of analyses used transferrin, hemoglobin, and discoloration ratings as separate dependent variables. The second set tested main and interactive effects of microinjury on DHEA, testosterone, and cortisol. Secondary analyses used multiple regression to determine which of the blood contamination screening methods best predicted the effects of microinjury on the change in hormone levels.

**Results**

**Did microinjury induce blood contamination of saliva?**

The effect of microinjury on the presence of blood in saliva was determined using a series of mixed-model ANOVAs with sample collection time (six collection times) as a repeated measure and microinjury condition (microinjury vs. passive drool) as a blocking factor, with either discoloration ratings, Hemastix®, or transferrin as the dependent variable. To decompose the source of interactions, planned contrasts (t tests) compared change in blood contamination measures from baseline to each time point after tooth brushing between the microinjury and control groups.

**Discoloration ratings**

There were significant effects of sample collection time \[ F(5,275) = 28.04, \ P < 0.001 \], microinjury condition \[ F(1,55) = 4.46, \ P < 0.05 \], and sample collection time by microinjury condition interaction \[ F(5, 275) = 9.78, \ P < 0.001 \]. As can be seen in Fig. 1A, in comparison to the control condition, the microinjury group displayed an increase in discoloration ratings from baseline to each time point immediately after brushing \[ (t(51.67) = 4.79, \ P < 0.001) \]. Interestingly, at no other time point after microinjury were discoloration ratings different than the passive drool condition.

**Hemastix® reagent strips**

There were significant effects of sample collection time \[ F(5, 265) = 8.95, \ P < 0.001 \] and sample collection time by microinjury condition interaction \[ F(5, 265) = 8.12, \ P < 0.001 \]. In comparison to the passive drool condition, the microinjury group showed elevations in Hemastix® ratings immediately after microinjury \( (t(56) = 5.87, \ P < 0.001) \), as well as 15 min \( (t(54) = 2.99, \ P < 0.01) \), 30 min \( (t(52.96) = 3.28, \ P < 0.01) \), and 45 min later \( (t(55) = 2.03, \ P < 0.05) \). Thus, in contrast to discoloration ratings, in the microinjury group, Hemastix® ratings peaked immediately after microinjury but remained elevated over baseline even 45 min after tooth brushing (Fig. 1B).

**Transferrin**

There were significant effects of sample collection time \[ F(5, 285) = 12.98, \ P < 0.001 \] and sample collection time by microinjury condition interaction \[ F(5, 285) = 8.85, \ P < 0.001 \]. Contrasts revealed that in comparison to the passive drool condition, the microinjury group had elevations in transferrin over baseline immediately after brushing \( (t(43.73) = 5.92, \ P < 0.001) \), as well as 15 min \( (t(57) = 4.25, \ P < 0.001) \), 30 min \( (t(57) = 3.56, \ P < 0.01) \), and 45 min later \( (t(57) = 2.09, \ P < 0.05) \). Therefore, similar to discolor-
Injury and Hemastix R methods, in the microinjury group, transferrin rose immediately after brushing. Transferrin reached peak levels 15 min post-brushing, and similar to the Hemastix R method, the levels remained elevated over baseline even 45 min post-microinjury (Fig. 1C).

Intercorrelations between measures of blood contamination

There were stronger associations between the three measures of blood contamination in the microinjury group than in the control group. The average intercorrelation between discoloration and transferrin levels for the microinjury group (r = 0.264) was higher than those observed for the control group (r = 0.172). A similar pattern was observed for the relationship between discoloration ratings and Hemastix R analysis (microinjury: r = 0.392; control: r = 0.060). Lastly, the relationship between Hemastix R and transferrin levels was the strongest, but also followed the same trend with correlations in the microinjury group (r = 0.540) much higher than those observed in the control condition (r = 0.336).

Did microinjury influence salivary hormone levels?

The effect of microinjury on salivary hormones was determined using the general mixed-model ANOVA approach described above. Separate ANOVAs were computed for cortisol, DHEA, and testosterone with sample collection time (six sample collection times) included as a repeated measure and microinjury condition (microinjury vs. passive drool) a blocking factor. Means and standard errors are presented in Table 1.

Cortisol

There was a significant main effect of sample collection time \[ F(5, 295) = 65.10, P < 0.001 \], revealing that, on average, cortisol levels decreased over the sample collection period. There were no differences between the microinjury and control conditions. See Fig. 2A and Table 1 for means and standard errors.

Dehydroepiandrosterone (DHEA)

There was a significant main effect of sample collection time \[ F(5, 300) = 16.12, P < 0.001 \]. On average, DHEA levels decreased over the sample collection period, and similar to cortisol, there were no differences between the microinjury and control conditions. See Fig. 2B and Table 1 for means and standard errors.

Table 1

<table>
<thead>
<tr>
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<th>Microinjury (N = 42)</th>
<th>Control (N = 20)</th>
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<tr>
<td><strong>Cortisol (pg/dl)</strong></td>
<td></td>
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<tr>
<td>Baseline</td>
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<tr>
<td>Immediately post</td>
<td>0.32</td>
<td>0.40</td>
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<td>0.27</td>
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<tr>
<td>45 min post</td>
<td>0.21</td>
<td>0.24</td>
</tr>
<tr>
<td>1 h post</td>
<td>0.19</td>
<td>0.21</td>
</tr>
<tr>
<td><strong>DHEA (pg/ml)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>214.28</td>
<td>232.26</td>
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<tr>
<td>Immediately post</td>
<td>198.17</td>
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<td>45 min post</td>
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<td>1 h post</td>
<td>166.95</td>
<td>155.40</td>
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<tr>
<td><strong>Testosterone (pg/ml)</strong></td>
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<td></td>
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<tr>
<td>Baseline</td>
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<tr>
<td>Immediately post</td>
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<tr>
<td>1 h post</td>
<td>138.27</td>
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Fig. 1. (A) Sample discoloration ratings of blood contamination are elevated over baseline and control conditions only immediately after microinjury. (B) Hemastix R ratings of blood contamination are elevated over baseline and control conditions immediately after and until 45 min after microinjury. (C) Salivary transferrin levels (mg/dl) elevate immediately after, peak 15 min after, and remain elevated until 45 min after microinjury. *P < 0.05, **P < 0.01, ***P < 0.001.
There was a significant main effect of sample collection time \( F(5, 300) = 8.41, P < 0.001 \) and sample collection time by microinjury condition interaction \( F(5, 300) = 4.06, P < 0.01 \). To decompose the source of the \( 2 \times 6 \) interaction, a series of planned \( t \) tests compared the microinjury and control groups with respect to the change in testosterone levels from baseline to each time point after microinjury. As can be seen in Fig. 2C, microinjury caused elevations in testosterone 15 min \((t(60) = 3.11, P < 0.01)\), 30 min \((t(60) = 3.27, P < 0.01)\), and 45 min post-microinjury \((t(60) = 2.27, P < 0.05)\) (see Fig. 2C).

**Testosterone**

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Did sex-related or diurnal differences in baseline hormone levels influence the effect of microinjury on salivary hormones?

To determine if individual differences in baseline hormone levels influenced the impact of microinjury on the measurement of salivary cortisol, DHEA, or testosterone, sampling time of day (AM vs. PM) and sex were added to the omnibus ANOVA model above as well as subject variables. As expected, there was a main effect of sex for testosterone \((F = 47.13, P < 0.001)\), but not for DHEA or cortisol. Men \((M = 213.18 \text{ pg/ml})\) had higher testosterone levels than women \((M = 107.46 \text{ pg/ml})\). There were no significant interactions between either sex or sampling time of day with microinjury condition, revealing that these baseline differences did not effect the potential for microinjury to influence the measurement of salivary cortisol, DHEA, or testosterone.

Do individual differences in microinjury-induced blood contamination predict salivary hormone levels?

In a series of regression analyses, we evaluated whether individual differences in the levels of blood contamination predict corresponding individual differences in the change in salivary cortisol, DHEA, and testosterone in response to microinjury. Using separate regression analyses for each hormone, we first entered discoloration ratings (entry criterion \( P < 0.05 \)), then in a second step allowed the Hemastix® and transferrin measurements to compete (stepwise entry criterion, \( P < 0.05 \)) to predict variance in hormone levels above and beyond that explained by sample discoloration alone. In other words, we required the more involved assay methods to predict change in hormones levels “better” than what could be achieved by simply visually inspecting the sample.

These analyses used only the data from the microinjury group. However, when significant relationships were observed, parallel analyses were conducted in the control group to confirm the effect was specific to microinjury. Dependent variables were the respective changes in hormone levels over baseline at each of the five time points post-microinjury. Predictor variables were the corresponding changes in each of the three blood contamination measures compared to baseline at each time point post-microinjury.

**Cortisol**

The regressions revealed that individual differences in blood contamination levels never predicted individual differences in the change in salivary cortisol from baseline after microinjury. Consistent with the analyses of group differences above, individual differences in the measurement of salivary cortisol are unaffected by levels of blood contamination of saliva caused by mild-to-moderate microinjury to the oral mucosa.

**Dehydroepiandrosterone (DHEA)**

Discoloration ratings predicted individual differences in the change in DHEA levels from baseline to immediately post \((\beta = 0.475, P < 0.01)\), 15 min post \((\beta = 0.407, P < 0.05)\), and 30 min post \((\beta = 0.362, P < 0.05)\) microinjury. There were also trends \((P < 0.10)\) for visual ratings to predict change in DHEA at 45 min and 1 h post-microinjury. As expected, these associations were not present in the passive drool control condition. Neither Hemastix® nor
transferrin levels explained variance in individual differences in the change in DHEA in response to microinjury beyond that contributed by discoloration ratings.

**Testosterone**

Discoloration ratings and transferrin levels each contributed unique variance to the prediction of microinjury-related change in testosterone levels. Immediately after brushing, the change in transferrin levels was associated with a change in testosterone level from baseline ($\beta = 0.757, P < 0.001$). At 15 and 30 min post-microinjury, the change in discoloration ratings ($\beta = 0.347, P < 0.01; \beta = 0.257, P < 0.05$) and transferrin levels ($\beta = 0.663, P < 0.001; \beta = 0.690, P < 0.001$) was associated with the change in testosterone from baseline. The change in transferrin levels over baseline at 45 min to 1 h post-microinjury was also associated with a corresponding change in testosterone ($\beta = 0.698, P < 0.001; \beta = 0.742, P < 0.001$). Parallel regression analyses computed for the control group revealed, as expected, that discoloration ratings were not associated with testosterone levels at any time point. Surprisingly, within the control condition, the change in transferrin levels was associated with change in testosterone over baseline at two of the five assessment intervals (15 min, $\beta = 0.629, P < 0.05$; and 1 h, $\beta = 0.672, P < 0.01$). This observation raises the possibility that even under normal conditions, some of the variance in salivary testosterone might be attributable to blood leakage. In summary, mild-to-moderate microinjury to the oral mucosa did influence the measurement of salivary testosterone. The magnitude of this effect was associated with individual differences in blood contamination as measured by transferrin at all time points assessed and by discoloration ratings at some time points but not others. Although Hemastix® differentiates blood contaminated from noncontaminated saliva, individual differences in testosterone caused by microinjury were not associated with blood contamination levels estimated using the Hemastix® method.

**Discussion**

Despite previous speculation, the effects of ecologically valid, minor-to-moderate microinjury to the oral cavity on the measurement of salivary cortisol seem negligible. Given that cortisol is the most commonly measured salivary biomarker in biobehavioral research, this news should be welcomed. The less welcome news is that quantitative measurements of testosterone in saliva are sensitive to the effects of blood contamination. Importantly, the confounding effects of blood leakage cannot be adequately screened or controlled by visual inspection of sample discoloration or using the Hemastix® approach. Investigators should implement strategies (such as the measurement of salivary transferrin) to avoid this potential “invisible” source of unsystematic variation across repeated samplings from the same individual as well as between individuals. The news is less clear for DHEA. Group average measurements of salivary DHEA were largely unaffected by microinjury. However, researchers interested in individual differences should take note. Visual inspections of sample discoloration did accurately predict individual differences in the change of DHEA after microinjury. Thus, it does seem worthwhile to screen samples to be assayed for DHEA at the point of collection or in the laboratory using the BCSS. These findings have several noteworthy implications for the design of the next generation of studies as well as for investigators who have archived saliva samples for testing in the future.

Clearly, the presence of blood in the oral mucosa has the potential to introduce unsystematic variance in the levels of hormones measured in saliva. Regardless, what is not yet clear is whether hormones themselves or nonspecific blood components leaking into saliva cause the effects we observed. Our findings suggest that the nature of this effect is different depending on which hormone is studied, but does not appear to be linked to baseline, diurnal, or gender differences. The following recommendations seem well justified. Minimally, participants should be screened for events in their recent history that could cause blood leakage into saliva by asking questions related to teething, shedding teeth, open sores, and injury. Sampling saliva within 45 min of microinjury to the oral mucosa should be avoided. Samples should be systematically inspected at the point of collection, and if visibly contaminated with blood, they should be excluded from analyses. Given the presence of blood in saliva is not always visible, when measuring certain hormones (e.g., testosterone), the immunoassay of salivary transferrin may be a useful screening tool. The use of Hemastix® reagent strips to screen or control potential effects of blood contamination on the measurement of salivary biomarkers has very questionable validity.

In conclusion, there is consensus that salivary measures provide a window through which students of biobehavioral health may observe the interacting effects of biological, contextual, historical, and behavioral factors. As researchers become interested in incorporating salivary measures into future studies, care must be taken to insure accurate levels of hormones independent from interfering substances. This issue is not trivial, as the current generation of studies is constructing multivariate models of the variance in hormone levels, and variability in hormone levels induced by a variety of environmental circumstances. The success of that endeavor will of course depend on variance in saliva hormone measurements that reflect true individual differences and not the systematic effects of extraneous factors.

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References


