The effects of saliva collection, handling and storage on salivary testosterone measurement

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A B S T R A C T
Several endocrine parameters commonly measured in plasma, such as steroid hormones, can be measured in the oral fluid. However, there are several technical aspects of saliva sampling and processing that can potentially bias the validity of salivary testosterone measurement. The aim of this study was to evaluate the effects caused by repeated sampling; 5 min centrifugation (at 2000, 6000 or 10,000g); the stimulation of saliva flow by a cotton swab soaked in 2% citric acid touching the tongue; different storage times and conditions as well as the impact of blood contamination on salivary testosterone concentration measured using a commercially available ELISA kit. Fresh, unprocessed, unstimulated saliva samples served as a control. Salivary testosterone concentrations were influenced neither by repeated sampling nor by stimulation of saliva flow. Testosterone levels determined in samples stored in various laboratory conditions for time periods up to 1 month did not differ in comparison with controls. For both genders, salivary testosterone levels were substantially reduced after centrifugation (men F = 29.1; women F = 56.17, p < 0.0001). Blood contamination decreased salivary testosterone levels in a dose-dependent manner (men F = 6.54, p < 0.01, F = 5.01, p < 0.05). Salivary testosterone can be considered a robust and stable marker. However, salivary testosterone levels were substantially reduced after centrifugation (men F = 29.1; women F = 56.17, p < 0.0001). Blood contamination decreased salivary testosterone levels in a dose-dependent manner (men F = 6.54, p < 0.01, F = 5.01, p < 0.05). Salivary testosterone can be considered a robust and stable marker. However, blood leakage can introduce bias into measurements of salivary testosterone using ELISA. Our observations should be considered in studies focusing on salivary testosterone.

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1. Introduction
Saliva is a diagnostic fluid that can be easily and noninvasively collected. Currently, saliva is used in endocrine research, especially in studies where blood samples are difficult to obtain, such as studies with large sample size and with children [1–3]. The noninvasive and simple collection techniques for saliva can dramatically reduce anxiety and discomfort, thereby simplifying collection of serial samples for monitoring chosen parameters over time. This makes saliva an attractive and widely used alternative to blood [4,5].

Some hormones commonly measured in plasma, such as steroids, can be measured in saliva [6]. Saliva is very sensitive to acute changes in blood hormone concentrations and to diurnal fluctuations. Therefore, researchers can investigate sequential and immediate changes in internal endocrine physiology by repeatedly collecting over the course of minutes, hours, days, or longer [7]. Whether repeated sampling itself affects salivary testosterone concentrations is, however, not clear.

The salivary testosterone assays are advocated for their noninvasive, easy sample collection method. Salivary testosterone is of great research value for it represents a filtered fraction of plasma testosterone and is independent of flow rate. It has been shown that salivary testosterone coming from circulation by passive diffusion correlates well with either free or non-SHBG bound testosterone [8,9]. There is a role for salivary testosterone assays in field studies, long-term studies, in athletes and studies in children [10,11]. While saliva testing has the promise of becoming a valuable and widely used tool in research, there are also some disadvantages to the method that must be kept in mind, including the cost of collecting and processing the samples and the reliability of the measure itself.

Previously published papers pointed towards several factors that potentially bias the validity of salivary testosterone measurement [12]. Most of the published studies focused on the effect of devices for sample collection on measured hormone concentrations. Although the mechanism of bias introduced by the sampling devices is not clear, the material used to absorb saliva is able to
falsey alter salivary concentrations of sex steroids [13–15]. There are, nevertheless, other pre-analytic factors that might affect the analysis of saliva. One of them is blood contamination. Blood and its components can leak from the oral mucosa as a result of micro-injuries such as burns or abrasions and due to gingivitis and periodontitis [16]. This might compromise quantitative estimates of hormones measured in saliva [12].

Stability of salivary testosterone was proved under various conditions [17]. Storage conditions and storage time can affect the analysis of biochemical variables due to temperature instability, bacterial growth and other reasons. Whether storage affects a particular assay depends on the type of molecule and its stability [1].

Enzyme-linked immunosorbent assays (ELISA) are widely used for measuring analytes in saliva. They are simple, rapid, convenient, relatively inexpensive and requiring a lower sample volume [18]. Several studies have documented the internal validity (i.e., reliability, precision, accuracy, analytical recovery) of immunoassays designed to measure salivary testosterone [11,19]. On the other hand, there are several disadvantages that can affect the assay and lead to falsely high or low results. Despite advances in our knowledge and understanding of the mechanisms of interference in immunoassays, there is no single procedure that can rule out all interferences. It is important to recognize the potential for interference in immunoassay and to put procedures in place to identify them [20,21].

The aim of this study was to evaluate the effects of repeated salivary sampling, of using a stimulant to aid collection, of sample storage and processing, as well as of blood contamination on salivary testosterone concentration measured using ELISA.

2. Experimental and methods

2.1. Subjects

Ten volunteers (5 women and 5 men) were recruited for this study. All participants were university students between 19 and 21 years and apparently healthy. The participants signed an informed consent. All procedures were conducted in accordance with the Declaration of Helsinki. The study was approved by the Ethics Committee of the Institute of Molecular Biomedicine, Comenius University.

2.2. Sample collection and preparation

Saliva samples were collected using a standard protocol. Sampling was performed in the morning between 8:00 to 10:00 am in respect of the circadian rhythm of testosterone. All volunteers were asked to refrain from eating, drinking or oral hygiene procedures for at least 30 min prior to the collection. Probands were asked to spit the whole saliva. In effort to collect unstimulated saliva, subjects were recommended to drop down the head and let the saliva run naturally to the front of mouth, hold for a while and spit into a sterile polypropylene tube provided (Sarstedt, Nümbrecht, Germany). The goal for the whole saliva donation was about 2 ml. Probands were kindly reminded not to cough up mucus as saliva is collected, not phlegm.

In effort to analyze the effect of centrifugation immediately after the collection of the fresh saliva sample, aliquots (500 µl) of the whole saliva were centrifuged for 5 min at 2000, 6000 or 10,000g immediately after collection. The clear top-phase (100 µl) was used as a sample for ELISA assay. In order to test the effect of sample collections on testosterone levels in saliva, the volunteers were asked for repeated sampling in 5 min intervals (1 ml of unstimulated saliva each). Stimulation can be used to make sampling easier. Therefore, probands were instructed to stimulate saliva flow using a cotton swab soaked in 2% citric acid by touching the tongue several times. The goal for the stimulated saliva donation was about 2 ml taken to the sterile provided tube. No further processing of these samples was carried out. Repeatedly taken and stimulated samples were dispensed into ELISA plate immediately after collection and were measured in the same ELISA assay together with fresh unstimulated samples added to the plate after centrifugation. Sample of fresh unstimulated saliva served as a control. Control aliquots were not centrifuged and were dispensed into ELISA plate immediately after collection without any other processing before testosterone assay. All the samples measured in this ELISA assay (stimulated samples, repeatedly taken samples, centrifuged samples) were fresh, underwent no freeze-thaw cycle as the measurement was conducted in time of several minutes after collection.

In the next phase of the experiment the impact of storage on salivary testosterone measurements was assessed. Sample storage temperatures were chosen according to common temperatures available in the laboratory (room temperature, refrigerator, 4 °C; standard freezer, −20 °C; ultra-low temperature freezer, −80 °C). Immediately after the collection, whole unstimulated saliva was aliquoted. No other processing in term of centrifugation was carried out. Subset of aliquots was archived immediately after collection and then assayed after 1 day (24 h), 1 week (7 days) and 1 month (28 days) in various conditions mentioned above. On the day of testing, frozen samples were brought to room temperature and pipetted into testing plate without any centrifugation of other further processing. Samples of freshly collected unstimulated and unprocessed saliva served as a control.

Blood contamination of samples was simulated constructed by spiking venous blood into aliquots of saliva. On the day of collection, venous blood from each subject was spiked into one aliquot of that subject’s saliva, and serially diluted. Spiked samples represented a range from 0.01% to 10% of whole blood in saliva. All samples (except controls) appeared visibly contaminated with lower to higher degree of yellow, brown, or red hue. After obtaining the results proving the effect of blood contamination on testosterone measurements, the experiment was repeated with slightly modified design. Saliva samples were spiked with hemoglobin (Sigma, St. Louis, MO, USA) at concentrations corresponding to the hemoglobin content of the blood spiked samples.

2.3. Assay method

Salivary testosterone was measured using a commercially available ELISA kits (DRG Diagnostics, Marburg, Germany). Mean intra-assay and inter-assay coefficients of variation are 3.3% and 6.42%, respectively. The additional specific characteristics for every performed ELISA assay separately are summarized in Table 1. Presented data are calculated according our measurements, not provided by the manufacturer. Based on the information from the manufacturer, the range of the assay is 6.6–17335 pmol/L. Details of the recovery, specificity, sensitivity, reproducibility and linearity are available online in the ELISA kit manual (for more information see http://drg-international.com/ifu/slsv-3013.pdf).

2.4. Statistical analysis

Data were analyzed using the GraphPad Prism 5.0 software. Repeated measures analysis of variance was used to determine if different group means are equal. The test uses the F-distribution (probability distribution) function and information about the variances of each group (within) and between groups to help decide if variability between and within each group are significantly different. The within-sample is the average of the all the variances for each population (unexplained variation). The between-sample
The effect of storage time and temperature on salivary testosterone concentrations did not significantly change after centrifugation (for men $F = 2.22$, $p = ns$, Fig. 3A; for females $F = 2.47$, $p = ns$, Fig. 3B). This effect did not differ between the centrifugation forces used.

The effect of storage time and temperature on salivary testosterone levels was evaluated in whole saliva samples. Salivary testosterone concentrations did not significantly change after 1 day, 1 week or 1 month storage at room temperature (for men $F = 2.22$, $p = ns$, Fig. 3A; for women $F = 2.95$, $p = ns$, Fig. 3B). Similarly, storage at 4 °C for 1 day, 1 week or 1 month did not affect salivary testosterone levels (for men $F = 1.56$, $p = ns$, Fig. 3C; for women $F = 2.47$, $p = ns$, Fig. 3D). Freezing did not affect salivary testosterone levels. Neither storage at −20 °C (for men $F = 2.17$, $p = ns$, Fig. 3E; for women $F = 1.11$, $p = ns$, Fig. 3F) nor at −80 °C (for men $F = 1.05$, $p = ns$, Fig. 3G; for women $F = 1.40$, $p = ns$, Fig. 3H) for time periods up to 1 month caused any significant change to salivary testosterone levels measured using ELISA.

Blood contamination decreased salivary testosterone levels in a dose-dependent manner (for men $F = 6.54, p < 0.01$, Fig. 4A; for women $F = 5.01, p < 0.05$, Fig. 4B). Similarly, hemoglobin added to salivary samples decreased measured concentrations of salivary testosterone in a dose-dependent manner (for men $F = 14.61, p < 0.0001$, Fig. 4C; for women $F = 19.81, p < 0.0001$, Fig. 4D).

4. Discussion

The results of this study show that salivary testosterone is a robust and reliable marker, at least if measured by ELISA. Neither the tested collection procedures, nor the tested storage times and conditions affected the measured concentrations of testosterone in saliva. This is partially in line with previous studies [22]. However, a number of studies analyzing various substances used to stimulate saliva flow report a significant effect on salivary testosterone. Compared to unstimulated saliva, testosterone levels were higher after adding powdered drink-mix crystals placed in mouth and also after the chewing the gum [12,14,17]. In addition, previous research on the use saliva collection devices has shown bias introduced by the collection method likely due to stimulation of saliva production [7,15,23]. Stimulation of saliva flow by touching the tongue with a cotton swab treated with 2% citric acid in this study did not significantly affect the concentrations of salivary testosterone. It can be suggested that carefully applied stimulating agent such as using swabs with citric acid can be considered a reliable sample collection technique, if large volume of saliva is required and the probands do not have a sufficient spontaneous salivary flow. Swartz with colleagues pointed out a possible acid interference in samples with pH lower than 6, therefore, such treatment should be prevented or affected samples should be ruled out [24].

Generally, saliva samples are freed from mucopolysaccharides and other residuals by three freeze–thaw cycles followed by centrifugation [25–27]. Centrifugation is helpful in reducing viscosity, leads to considerable loss of protein content and produces a clear, easily pipettable supernatant [28]. If the specific steroid binding proteins are present in saliva, the preparation of sample before assay may greatly influence the results of steroid hormones determinations [29]. According to the reported results of this study centrifugation of the saliva sample decreases salivary testosterone levels in comparison to unprocessed saliva. However, centrifugation seems to be the useful step in sample preparation, caution must be taken as centrifugation even at low relative centrifugation force caused a decrease of measured salivary testosterone concentrations by 47%. Similarly to our observation, other studies indicate loss of steroid hormones when saliva is centrifuged. It is possible that the pelleted debris of saliva contains most of the binding
proteins bring measured metabolite with them [30]. The processing of saliva after saliva collection can, thus, be seen as a factor contributing to the variability of reported salivary testosterone concentrations between published studies. In several relevant studies, the issue of saliva processing and handling immediately after collection is overlooked and even not reported in the Methods. This study does not answer the question whether saliva should or should not be centrifuged before ELISA, but shows that it can be at least recommended to report in the Methods whether whole unprocessed saliva sample or the supernatant after centrifugation of saliva was used for testosterone measurements to enable comparisons between studies.

The caution is also needed in the interpretation of data from salivary testosterone measurements. It was believed that salivary testosterone comes from circulation by passive diffusion representing free, unbound, bioactive fraction. However, processes in salivary glands are not fully understood yet. It has been pointed out salivary concentrations of unconjugated steroids may differ and do not have to correspond necessarily with free levels in circulation because of salivary gland metabolism, particularly metabolism of the measured analyte [31–35]. Also the presence of specific binding globulins in saliva should be considered. If they are indeed present in the saliva, the question of whether salivary hormone concentrations reflect the plasma free level has to be re-opened [29,30].

Samples of saliva collected in clinical studies are commonly archived and analyzed later. Potential changes in salivary testosterone levels were evaluated when samples were stored up to 1 month at room temperature, 4 °C, −20 °C or −80 °C. No effects of storage time and temperature on salivary testosterone concentrations were found in our experiment. Our findings support the hypothesis that testosterone can be reliably measured in saliva samples archived for a longer time period. Similar results were published in previous studies suggesting that steroid hormones (namely testosterone and cortisol) and their binding globulins are stable over time and to variations in storage conditions [36,37]. Stability of steroid hormones stored over four decades at −20 °C suggests that stored samples from longitudinal studies may be used to investigate links between prenatal testosterone and long-term offspring outcomes [37]. Other studies showed that testosterone levels in saliva are stable only when samples are frozen at −80 °C [12]. Storage at 4 °C or −20 °C was associated with lower concentrations of testosterone probably caused by degradation and coinciding with bacterial accumulation [7,17]. Differences to the present study might explain the divergent results – including sample collection, time of observation and the analytical method. The findings of the present study are in line with some previous observations. It was shown that samples can remain unrefrigerated for several days without distorting salivary testosterone assay scores [17]. These characteristics make salivary collections for testosterone measurement easier also out of the laboratory. It might be possible to mail instruction and collection tubes to subjects and have them mail samples back. It is essential to remark that testosterone scores among female subjects were elevated when saliva was mailed or sat at room temperature for 2 weeks. It is not clear whether female salivary testosterone measurements are more affected because testosterone levels are so low to begin with or whether other confounding substances are present in female saliva [17].

A high serum–saliva correlation depends on consistency in the process via which steroid hormones from circulation diffuse into the oral mucosa. When the integrity of the physical barrier maintaining this consistency is compromised, salivary testosterone levels should be falsely changed. Published literature warns that when blood is present in saliva, hormonal concentrations are biased [22]. Concentrations of testosterone in plasma are much higher than in saliva. Theoretically, salivary testosterone should, thus, be higher in samples with blood such as from patients with periodontitis or due to microinjuries after brushing the teeth. To test the effect of blood contamination peripheral venous blood was added to saliva. Sample spiking ranged from 0.001% to 10% of whole blood in saliva. Previously published papers showed that salivary testosterone levels measured using a high-sensitivity enzyme immunoassay are elevated after microinjury of the oral mucosa [2,16,38]. Levels of salivary testosterone increased in response to microinjury. This change was more closely associated with the change in transferrin than hemoglobin levels [38]. In contrast to these studies, the results of the present study show the measured salivary testosterone concentrations are lower in association with the percentage of blood spiked into saliva. Despite reported inconsistency in findings, it can be concluded that the quantitative measurements of testosterone in saliva are sensitive to the effect of blood contamination. Regardless, what is not yet clear is whether hormones themselves or nonspecific blood components leaking into saliva cause the effects we observed. Kivlighan with colleagues (2004) suggested 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 [38]. We hypothesized that our observation might be explained by potential interference of hemoglobin with ELISA assay system. To test this, saliva samples contaminated with hemoglobin were prepared. Hemoglobin was added to saliva samples in amounts that adhere to the concentrations present in whole blood contaminated saliva from the previous experiment. Hemoglobin contamination falsely reduced testosterone concentrations measured in saliva samples. These results indicate that hemoglobin in blood may interfere with the colorimetric absorbance measurement in ELISA. Thus it seems to be worthwhile to screen samples to be assayed and samples with visible blood contamination should be excluded to prevent the undesired bias and unsystematic variance.

Fig. 2. The effect of centrifugation on measured salivary testosterone concentrations in males (A) and females (B). Centrifugation of saliva decreased measured testosterone concentrations. Significant differences according to Tukey post hoc paired test are shown as ***p < 0.05, **p < 0.01, *p < 0.001.
Methods most widely used to measure levels of steroid hormones either in plasma or saliva sample include radioimmunoassays (RIAs) and ELISAs. Steroid RIAs are cumbersome, time-consuming, costly, and require relatively large sample volumes. ELISAs for steroid estimation made salivary assays more practical. They are convenient, simple, rapid, and relatively inexpensive, requiring a lower sample volume. As with all antibody-based assays, they often overestimate results the measurements due to a lack of specificity of the antibody. Cross-reactivity and exogenous interferences due to pre-analytical variation, matrix and equipment reaction also affect the results of immunoassays. Interfering substances may lead to falsely elevated or falsely low concentrations. The detection of interference may require the use of an alternate assay or additional measurements [39]. Salivary levels of testosterone (pg/mL) represent only a small fraction of the testosterone in the circulation [10, 40, 41]. Consequently, levels of testosterone in saliva may often be too low to be reliably measured by immunoassays [39]. Notable deficiencies in validation of immunoassays generated with commercial kits involve assay sensitivity and specificity [39]. Assay sensitivity is usually poorly defined, leading kit users to

Fig. 3. The effect of storage conditions on measured salivary testosterone concentrations. No significant difference was found after one day (1D), one week (1W) or one month (1M) storage at room temperature (RT), at 4 °C, at –20 °C or –80 °C in men and women.
believe that they can measure very low levels of an analyte. Similarly, validation of assay specificity is often limited to showing cross-reactivity with possible interfering compounds [39]. Despite these concerns, based on published data a well validated immunoassay can be suitable for majority of applications [42]. However, limitations of ELISAs and RIAs have led to increased usage of mass spectrometry methods [42]. High resolution fused silica columns enabled routine use of gas chromatography–mass spectrometry (GC–MS) [43]. Later, high performance liquid chromatography (HPLC)–MS was developed [44]. In the past decade, HPLC coupled with tandem mass spectrometry (LC–MS/MS) has improved the measurement of steroid hormones [45,46]. Although mass spectroscopy assays are accurate, reliable, can be carried out with high throughput and have made an important contribution in the endocrine testing, there are still many challenges they have to overcome [42,46–48]. The challenge is to have smaller diagnostic laboratories carry out steroid hormones measurement using these assays. Decrease the cost and complexity of instrumentation can help achieve this. There is also need to obtain valid ultrasensitive assay for measuring certain steroids, particularly estrogens. MS technologies face one seems to be a stable and robust marker that is not very sensitive to sample storage conditions. On the other hand, visible blood contamination in saliva can affect the measured concentrations of testosterone, using our standardized ELISA method.

It is essential to remark the main limitation of our study. Considering the low samples size, our results cannot be much generalized. Further research on larger sample size is essential to prove these findings. However the small study observed a striking effect and in larger sample the effect might be different, we believe that the scientific community should learn of the effect of different conditions on testosterone measurement in order to avoid erroneous results in the experiments. These findings might be helpful for scientists working with salivary testosterone as a biomarker in psychoneuroendocrine research.

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