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

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ABSTRACT

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 salivary 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, saliva processing and blood leakage can introduce bias into measurements of salivary testoterone using ELISA. Our observations should be considered in studies focusing on salivary testosterone.

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

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falsely 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 micr-oinjuries 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 saliva 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 10,000*g* 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 3.31% 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/slv-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