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Pilot Study Open Access

Embedded Dental Cortisol Content: A Pilot Study

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Abstract

Objective: This study tests the feasibility and validity of analyzing cortisol levels within human teeth and the possibility of any potential gender differences.

Methods: As a precursor, to confirm the general presence and existence of cortisol within teeth, six healthy supernumerary teeth were initially extracted and examined following the ELISA method. After confirming that cortisol was in fact present within human teeth, we began the main experiment. In order to examine any potential gender differences, twenty-one extracted wisdom teeth were collected from subjects (11 male and 10 female) within the age range of 27-29 yrs.

Results: The results showed irrefutable proof of the existence of cortisol within the dentin of the teeth. Observations revealed that gender had no significant impact upon a subject's dental cortisol levels ($p>0.05$), suggesting that cortisol in teeth can be used as an unbiased and reliable means of studying stress.

Conclusions: This is the first report on extraction and analysis of cortisol levels in hard tissues, such as teeth, leading to the discovery of a novel biomarker that can be used when studying chronic stress. Practical application of this study can be used postmortem to assess cortisol levels in patients suffering from prolonged disorders, including patients having undergone chemotherapy treatments, when hair cortisol analysis proved to be problematic.

Keywords: Cortisol; Chronic stress; Tooth; Sex difference

Introduction

Cortisol is one of the most commonly used stress biomarkers and is produced by the zona fasciculate of the adrenal cortex. Some of the primary functions of cortisol in the blood stream include; increasing blood sugar, suppressing the immune system, and stimulating fat, protein and carbohydrate metabolisms [1-3]. Experiencing chronic stress seems to be inevitable in daily modern life, which adversely affects both physical and mental health. It has been proven that reacting to chronic stress negatively affects the autonomic nervous system and thus creates further stress, which is often associated with many physical and mental health problems. By studying cortisol levels within human teeth we may be able to address symptoms of several prolonged stress conditions. This study presents a potential breakthrough in cortisol measurement methods in hard tissues such as teeth.

Animal studies have suggested that increased levels of cortisol produce a glucocorticoid deficient state and can lead to a lower resistance to infection, intense susceptibility to autoimmune inflammatory diseases, and pain [4,5]. Psychological and dental studies have routinely exploited salivary cortisol as a reliable means of measuring hypothalamus-pituitary-adrenal axis (HPAA) adaptation to stress [6-8]. Historically speaking, saliva has been used for studying the psychobiological mechanisms of acute stress [8]. However, cortisol levels in saliva are influenced by numerous factors and can be inaccurate. If the research focuses on free cortisol and its effects on target tissues, salivary cortisol should be the measurement method of choice. However, as described above, a linear dose-response relation – in terms of target cell effects – should not be expected. The same phenomena in which cortisol infuses from blood into hair [3,5,8] is theorized to occur in the same way from saliva into teeth. Researchers who decide to assess salivary cortisol must consider that variables such as hormones (gender, menstrual cycle, oral contraceptives) or medical conditions could affect cortisol binding and HPAA, respectively. In addition, the expected range of cortisol measurements should be taken into consideration as levels are elevated once CBG binding is saturated. Moreover, fluctuations in day to day sampling of saliva exist and do not provide us with enough reliable data to be compared with teeth cortisol levels and correlations.

Hair, fingernails and wool are also known to keep a record of biological markers such as hormones and drug use [9-11]. Cortisol diffusion rates are directly related to high lipid solubility and low levels of protein binding. This suggests a preferential deposition of unbound cortisol when compared to a protein-bound hormone [12]. Thus, cortisol steroid levels measured in saliva and in hair could only reflect

the free or unbound fraction of cortisol present in the blood stream, non-bound cortisol, resulting in a less accurate measurement. This raises the question of whether or not it is possible for a hard matrix, such as a tooth, to be penetrated by the cortisol carried in the blood.

As the stress cycle continues, the progression of cortisol accumulation over time can result in receptor desensitization and tissue damage [13]. This damage can be reflected in a chronic dysregulation of the HPA axis. Therefore, finding a relatively stable indicator of cortisol with less variance, such as dental cortisol, would help scientists explain a variety of stress conditions. Despite the complexity of cortisol accumulation in hard matrices, the aim of the present study was first and foremost the evaluation of the existence of cortisol in dental tissues, and to test if gender differences affect said cortisol levels. In addition, this and future follow up studies have the potential to address several issues in relation to the feasibility of dental cortisol as a firm biomarker in both genders.

Materials and methods

Subjects

Before beginning the formal experiment, six healthy supernumerary teeth (5 men and one woman) were extracted and used to test for the presence and existence of cortisol within teeth. In the main experiment, twenty-one extracted wisdom teeth were collected from subjects (11 male and 10 female) within the age range of 27-29 yrs. Immediately after extraction, each tooth sample was preserved in a 10 ml isotonic sodium chloride, normal saline-0.9% NaCl solution capped in a 20 ml light-proof glass bottle (GC Fuji PLUS, Radiopaque reinforced, glass ionomer luting cement 0086, GC Corporation, Tokyo, Japan) and kept in a refrigerator (4°C) prior to cortisol analysis.

The study procedure was approved by the Ethics and Human Welfare Committee of Hallym University, Republic of Korea. All donors were informed of the purpose of the study and signed waivers to allow their teeth to be used in this study.

Teeth preparation for cortisol analysis

Each tooth was washed with isopropanol prior to drying and fine grinding for cortisol analysis. Using a ball mill or any other cutter for fine grinding requires washing the residue from the grinder after each sample is processed. Doing so would increase the dilution of the wash part of the cortisol since alcohol is used to wash the residue. In diluting the wash part measurements of cortisol could be corrupted and inaccurate. Hence, we have decided to mill the teeth by hand, insuring no dilution. Teeth were broken down into smaller fragments, then the teeth were dried and milled by-hand with a mortar and pestle.

Cortisol assay

One gram of each ground tooth sample (1 g) was washed twice with 2 ml of iso-propanol in 5 ml polypropylene tubes. The samples were centrifuged at 1000 g at room temperature, for 5 min using a Brushless D.C. Motor Centrifuge (Vision Scientific Co., Ltd., Bucheon, Gyunggi province, Republic of Korea). Samples were mixed on a rotor mechanical shaker at room temperature for 3 minutes following each wash and 1 ml of suspension from each wash was collected in a separate tube to determine the level of overall cortisol lost during the washing procedure.

The tooth samples were then allowed to dry for 7 days in a sterile, room temperature environment. After drying, 100 mg of milled teeth were weighed and placed into a 2 ml microcentrifuge tubes. Methanol was used to elute impurities from the sample and retain the cortisol in the stationary sorbent. One ml of methanol was added to each microcentrifuge tube, and the tubes were incubated at room temperature for 24 h with slow rotation to extract cortisol. After the 24 h period, samples were spun for 1 min in a microcentrifuge (1000 rpm) and a 0.6 ml aliquot of methanol extract was added to a new tube and dried at 38°C under a stream of nitrogen gas. This volume of methanol was chosen to avoid contamination of the supernatant with tooth particles. The dried extracts were reconstituted with 0.4 ml of phosphate buffer, provided in the assay kit, to analyze the cortisol levels.

All tubes were labeled throughout the experiment. Cortisol was recovered with a final elution of 90% methanol. The prepared samples were then subjected to analysis by using a cortisol assay kit (Salimetrics, high sensitivity salivary cortisol, enzyme immunoassay kit, no. 1-3002, State College, USA, 16803) according to the instructions provided by the manufacturer. Optical density was measured at 450 nm using a SpectraMax ELISA reader M2E (Molecular Devices, CA, USA). Saliva cortisol, for its high sensitivity, was used as the standard to validate the data of teeth cortisol. Immunoassay is the most common approach for analyzing cortisol in human saliva, hair or serum [2,14-16]. However, there is currently no methodological report. Even ELISA has not been approved for quantifying cortisol in teeth yet. Teeth cortisol levels were analyzed using the salivary enzyme-linked immunosorbent assay (ELISA) test method with a sensitivity of 0.003 μg/dL [5].

In this study, twenty-one wisdom teeth from 11 male and 10 female subjects (27-29 yrs) were used. Subjects were recruited to be the study group for the examination of potential gender differences in dental cortisol contents. Subjects were also asked to complete a short self-developed questionnaire related to their general health including; age, weight, diet, smoking habits, marital status, work status, pain intensity, general health and medication intake. The subjects were then screened to ensure no history of dental treatment 3 months prior to the study. Participants also confirmed that they were not afflicted with any systemic diseases, not taking any medications or supplements affecting cortisol activity, and attested to being nonsmokers. All procedures were performed as explained before.

Statistics

Statistical analysis was carried out using the GLM procedure of SAS software (version 9.1; SAS institute Inc., Cary, NC). The univariate procedure of SAS was used to check the normality of data. The result of this analysis showed that the data for all the measured characteristics were normally distributed.

Samples of low, medium and high concentration in two replicates were used to calculated intra-assay and inter-assay coefficients of variation (CV) according to [14]. The acceptable range was determined for intra-assay CV to 5% and inter-assay CV to 15%.

Results

Cortisol accumulation in teeth and validation of cortisol measuring assay

The results of the precursor study revealed irrefutable proof of the existence of cortisol within the dentin of the teeth (Table 1). Cortisol was found in all three fractions (wash, dry and residual extract) of the teeth. For this reason the current study was carried out.

We found that the commercially available ELISA kit, developed originally for evaluating cortisol in human saliva was efficient in measuring cortisol in dental samples followed by methanol preparation.

Items	Cortisol level (ng/mg of teeth)
Wash[1]	2.54 ± 1.13
Dry[2]	7.92 ± 0.91
Residual extract[3]	5.68 ± 2.73
Total[4]	10.46

[1]Wash tooth cortisol represents the level of cortisol in the isotonic sodium chloride plus wash buffer (iso-propanol);

[2]Dry tooth cortisol represents the level of cortisol in fine tooth grounds after washing with methanol;

[3]Residual extract indicates the level of cortisol in methanol extract with ground tooth particles remained in tube after removing 0.6 ml supernatant for dry tooth cortisol analysis;

[4]Total cortisol represents the sum of levels of cortisol in wash and dry part. Residual extract does not count for total cortisol as it is incorporated in the 100 mg of the dry part

Table 1: Teeth cortisol level (n=6, ± SD).

Dental cortisol contents in different gender

In this study, basal levels of cortisol and their relation to gender was investigated. It should be noted that they were similar in relation to weight, height, diet, health condition, and smoking habits. The comparative statistics of the cortisol levels among males and females can be seen in Table 2. The concentrations of cortisol did not differ significantly between male and female tooth samples ($p > 0.05$).

To validate the ELISA data, an in depth analysis of the intra-assay and inter-assay CV was performed. The intra-assay and inter-assay coefficients of variations were 2.05 and 12.35, respectively. This finding is consistent with earlier reports for hair cortisol [3,10]. The results of our first experiment might be owing to the similar mechanism in which cortisol is transported and stored in both tooth and hair follicle cells. The absence of significant variations in dental cortisol levels suggests the potential for teeth to be considered as an unbiased biomarker for i.e. psychobiological studies.

	Cortisol level (ng/mg of teeth)			
Item	**Female**	**Male**	**SEM**	**p - value**
Wash	2.57	2.83	0.35	0.65
Dry	7.28	9.79	1.43	0.25
Residual extract	7.55	6.61	1.53	0.41
Total	10.13	12.59	1.71	0.13

Table 2: Teeth cortisol level in different genders (n=19, 11 males/10 females).

Discussion

In past studies, cortisol profiles have been measured in saliva, urine or hair and have been used as a biomarker to evaluate stress and endocrine disorders [10,15,16]. Salivary cortisol profiles were initially considered to be a better index of adrenocortical active hormone than serum cortisol, though in recent years almost all cortisol sources and extraction methods have been challenged due to the high cost and low reliability of said methods [17]. As it stands, salivary and urine cortisol levels often can fluctuate due to gender (menstrual cycle, pregnancy, etc.), nutrition or different medical treatments. Despite that cortisol measurements in urine and saliva can be an efficient indicator of free cortisol effects on target tissue [7], interpretation of the data will be less reliable over a long period of time, considering that multiple samples are needed for salivary or urine analysis in order to study chronic stresses. It should be noted that the units of measurement used when assessing cortisol levels in saliva are different than those which are used to measure cortisol in hair and teeth. Positive correlations have also been observed between stress and accumulation of cortisol in the hair of humans [3], wool [10], urine [4] and feces [18].

Blood cortisol levels showed a linear correlation with saliva in acute stress for both humans and animals [3]. A dental cortisol profile may be used for tracing endogenous free cortisol levels owing to its closer incorporation with the blood streams particularly to study in patients under chemotherapy treatments or postmortem.

This current study is the first report on the existence and attempted measurement of cortisol in teeth. The goal is that these measurements would be considered as a new tool to assess long-term exposure to the stress and comparative endocrine studies. The minimal differences of tooth cortisol in the subjects' teeth could be partly due to the currently still unidentified mechanism by which some traces of cortisol are incorporated into the teeth. In addition considering the remarkably slower growth rate of teeth compared with nails and hair, dental cortisol profiling gives a much more reliable means of prolonged stress conditions. The difference in the rate of growth between hair and teeth would yield minimal differences. Dental cortisol is possibly delivered via the blood stream and saliva.

As it has been suggested for hair and nail cortisol [11], dental cortisol contents might remain steady even after extraction from the body. Since teeth are a much harder substrate, cortisol would remain trapped within the layers of the teeth and could be detected even after cell death. The authors should note that it is possible that under circumstances of chronic stress conditions, dental cortisol content may vary due to different gender effects or based on the severity of prolonged stress conditions in which gender response may be different. However, different treatment groups and that of possible gender-difference may be examined in further studies.

The results of this study will open a new window to the scientific community for those who are interested in endocrine science and human health and trying to correlate it with human stress levels. In addition, literature evidence shows that cortisol is present in biological samples and is preserved over centuries [2] and can subsequently be detected in fossils. Therefore, current data suggests that this method can be used for cortisol measurements in archeobiological research in order to study stress levels and psychological conditions during the lifespan of past humans and animals. However, the issue of whether or not teeth cortisol levels remain steady over a long period of time has yet to be explored.

Indeed, further investigations are required to determine the association between levels of cortisol in teeth and various clinical characteristics in a large population of patients with chronic stress. Further analyses must be performed to fully understand on dental cortisol accumulation in correspondence with the human stress response. Moreover, cross-sectional studies of clinical objectives and subjective parameters would shed light on locating potential predictors for long-term change in tooth cortisol levels within patients. Comparative cortisol profiling of teeth, hair, saliva and blood would make a platform for researchers to select the appropriate method and substrate for evaluating cortisol in relevant studies.

In conclusion, this finding suggests that extracted teeth from common dental clinics can be considered as a new substrate for endocrine and psychological investigations. The current method could also be used as a new approach to assess cortisol levels in child's milk teeth for patients under chemotherapy treatments, pediatric endocrinology, psychobiology, behavioral, archeological, and forensics studies.

Informed consent

Informed consent was obtained from all individual participants included in the study.

Ethical approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the Hallym University and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Authors' contributions

JGN was responsible for design of study, carried out the experiments and drafting the manuscript. CJ performed subjects' physical examination and the collection of the teeth. HS contributed in writing the manuscript and critical revisions. KIL have commented and revised the manuscript. JL participated in the design of the research, contributed to the manuscript edition, and obtained funds for the work. The authors have read and approved the final manuscript

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