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SHORT COMMUNICATION

Determination of salivary cortisol reference interval in a selected Malaysian population

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Abstract

Introduction: Salivary cortisol is used as an indicator of stress level and a recommended screening test for Cushing syndrome. The normal reference interval for salivary cortisol is dependent on the analytical methodology and the population studied and hence, establishment of a local population-based reference interval is recommended. *Materials and methods:* A total of 129 healthy blood donors and staffs of Penang General Hospital were recruited from June 2018-May 2019. Paired (morning and late-night) saliva samples were collected from individuals aged between 18 and 60 years old with no history of chronic medical illness. Salivary cortisol was assayed using electrochemiluminescence immunoassay technique. Non-parametric statistics were used for calculation of reference interval and 90% confidence intervals (90% CIs). *Results:* The reference interval for morning and late-night salivary cortisol was 2.09 – 22.63 nmol/L and <12.00 nmol/L, respectively. *Conclusion:* The locally-derived adult reference intervals for morning and late-night salivary cortisol concentration was determined and varied with previous studies emphasising the need in establishing individual laboratory reference interval.

Keywords: Salivary cortisol, reference interval, immunoassay, adults

INTRODUCTION

Cortisol regulates carbohydrate, fat and protein metabolism and is the main glucocorticoid hormone produced by the zona fasciculata of adrenal cortex. Physiologically, it exhibits diurnal secretion, demonstrating highest concentration in the morning and reaching a nadir in late evening. It is an indicator of plasma-free cortisol, and hence, preferred for an assessment of hypothalamic–pituitary–adrenal axis in physiological and pathological conditions.[1,2] Salivary cortisol measurements is widely used in psychological stress research as a biomarker of stress and the late-night salivary cortisol is a recommended screening tests for Cushing syndrome.[2,3] The non-invasiveness of the sample collection and being able to be collected in an outpatient setting is among its major advantage.[1] There are several techniques available for salivary cortisol measurements, including radioimmunoassay and more recently the liquid chromatography–tandem mass spectrometry assay.[1] Nevertheless, immunoassay method such as the electrochemiluminescence immunoassay (ECLIA) on automated analysers allows for ease of use in a clinical laboratory setting.[4] The Roche Cobas Elecsys Cortisol II assay and the IDS-isys cortisol assay specifically mentioned saliva as samples suitable for analysis.[5]

Prior to introducing the salivary cortisol test into routine use, the reference interval for this assay must first be determined from individuals demographically representative of the population most frequently tested by the laboratory.[1] Assay variations and the population characteristics are among factors influencing the reference range values. The aim of this study was thus to determine the reference ranges for the morning (0600h – 0800h) and the late-night (2300h – 2400h) salivary cortisol in a selected healthy Malaysian population.

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MATERIALS AND METHODS

A cross-sectional study was conducted at Penang General Hospital from June 2018 to May 2019. A minimum of 120 hospital staffs and blood donors aged between 18 – 60 years old without chronic medical illness were included by random sampling. The minimum number of subjects was based on the Clinical and Laboratory Standards (CLSI) guideline EP28-A3C in setting up a reference range of an analyte of interest.[6] The subjects were included if they did not demonstrate or have history of i) physical signs and symptoms suggestive of hypercortisolism or hypocortisolism ii) using steroid tablets, corticosteroid creams, oral contraceptives or drugs such as mitotane, phenytoin, phenobarbitone, rifampicin, itraconazole or fluoxetine, iii) chronic alcoholism or drug abuse, iv) psychiatric illness such as major depression, obsessive-compulsive disorders and acute depression, v) abnormal working hours and shift working system. Pregnant women were excluded from the study.

Subjects were interviewed with regards to their current and past medical history as well as details regarding medication, sleeping pattern and smoking history. The subjects' anthropometric measurements (height and weight) were taken and body mass index (BMI) was calculated.

Saliva sample collection

Each subject was briefed on saliva sample collection procedure. The samples was obtained by chewing a polyester swab of the Salivette® for approximately 2 to 3 minutes, performed before brushing of teeth but after rinsing of mouth. The swab was then placed into the Salivette® system and capped. Two saliva samples were required, with the morning sample collected upon waking between 0600h – 0800h, while the late-night sample collected prior to sleep between 2300h – 2400h. Subjects were asked to keep the sample refrigerated at 2 – 8°C and send it to the laboratory the following morning.

Salivary cortisol measurement

In the laboratory, samples were centrifuged at 1000g for 2 minutes and stored at -20°C until analysis. No pre-treatment was required for the centrifuged saliva sample. Samples were analysed in batches using the Roche Elecsys Cortisol II assay on Cobas e801 analyser, based on the ECLIA method. The measuring range for the salivary cortisol was 1.5 – 1750 nmol/L, defined by the limit of detection (LOD) and the maximum of the master curve. The LOD for the assay is 1.5 nmol/L and the limit of quantitation is 3.0 nmol/L.[5]

Statistical analysis

According to CLSI guidelines EP28-A3c: Defining, Establishing and Verifying Reference Interval in the Clinical Laboratory, the reference interval is defined as interval between and including an upper and lower reference limit, which are enclosed a specified percentage (usually 95%) of the values of the population from which the reference subjects are recruited.[6]

The lower and upper reference limits are estimated as the 2.5th and 97.5th percentiles of test results distribution of the reference population. With the inclusion of reference subjects of a minimum of 120, the reference interval established has a confidence interval of 90%. All laboratory data were stored and analysed using the Statistical Package for Social Science (SPSS) software version 22.0.

Ethics

Ethical approval to conduct the study was obtained from the Malaysian Research Ethical Committee (MREC) Ministry of Health.

RESULTS

Characteristics of study population

A total of 129 healthy adults (36 males and 93 females) aged between 19 and 60 years were included in the analysis with the demographic characteristics presented in Table 1. The mean age was 32 ± 9 years old with the majority (69%) being Malays. The mean BMI was 25.7 ± 5.5 kgm^{-2} with 18.6% were classified as obese. The majority (93%) were non-smokers.

Early morning and late-night salivary cortisol concentration

Table 2 shows the percentages of results for the morning and the late-night salivary cortisol concentration. The morning salivary cortisol concentrations showed a normal distribution, while the midnight salivary cortisol concentrations were not normally distributed, even after log transformation as a result from the greater number of samples (67.4%) with concentration < 1.5 nmol/L. The mean ± SD for morning salivary cortisol was 10.87 ± 5.39 nmol/L while the median (IQR) for midnight salivary cortisol was 1.52 (0.40) nmol/L.

TABLE 1: Demographic characteristics of study participants (n=129)

Demographic Characteristics	n (%)
Age	
18 – 30 years	57 (44.2)
31 – 40 years	52 (40.3)
41 – 50 years	12 (9.3)
51 – 60 years	8 (6.2)
Gender	
Male	36 (27.9)
Female	93 (72.1)
Ethnicity	
Malay	89 (69.0)
Chinese	22 (17.0)
Indian	9 (7.0)
Others	9 (7.0)
BMI (kgm^{-2})*	
< 18.5 (Underweight)	9 (7.0)
18.5 – 22.9 (Normal)	41 (31.8)
23.0 – 27.4 (Pre-Obese)	34 (26.4)
27.5 – 34.9 (Obese I)	37 (28.7)
35.0 – 39.9 (Obese II)	6 (4.6)
≥ 40 (Obese III)	2 (1.5)
Smoking	
Yes	9 (7.0)
No	120 (93.0)

*Classification of weight by BMI based on the Ministry of Health Clinical Practice Guidelines on Management of Obesity[7]

TABLE 2: Distribution and mean of salivary cortisol concentration

Salivary cortisol concentration (nmol/L)	Morning n (%)	Late-night n (%)
Mean ± SD or median (IQR)	10.87 ± 5.39 [a]	1.52 (0.40) [b]
<1.5	1 (0.8)	87 (67.4)
1.5 – 3.0	6 (4.7)	21 (16.2)
3.1 – 4.5	5 (3.9)	6 (4.7)
4.6 – 6.0	8 (6.2)	8 (6.2)
6.1 – 7.5	15 (11.6)	1 (0.8)
7.6 – 9.0	19 (14.7)	0 (0.0)
9.1 – 10.5	15 (11.6)	1 (0.8)
10.6 – 12.0	16 (12.4)	2 (1.6)
12.1 – 13.5	10 (7.7)	1 (0.8)
13.6 – 15.0	7 (5.4)	2 (1.6)
15.1 – 16.5	5 (3.9)	0 (0.0)
16.6 – 18.0	6 (4.6)	0 (0.0)
18.1 – 19.5	6 (4.6)	0 (0.0)
19.6 – 21.0	5(3.9)	0 (0.0)
21.1 – 22.5	2 (1.6)	0 (0.0)
22.6 – 24.0	1(0.8)	0 (0.0)
24.1 – 25.5	0 (0.0)	0 (0.0)
25.6 – 27.0	1 (0.8)	0 (0.0)
27.1 – 28.5	0 (0.0)	0 (0.0)
28.6 – 30.0	1 (0.8)	0 (0.0)

[a]Mean ± SD, [b]median (IQR)

Reference range for early morning and late-night salivary cortisol

The reference interval of the morning salivary cortisol concentration is 2.1 – 22.6 nmol/L, based on the 2.5th and 97.5th percentile. For the late-night salivary cortisol, the majority (67.4%) had concentrations < 1.5 nmol/L. As only elevated midnight salivary cortisol is of medical importance of suspecting Cushing syndrome the reference interval obtained can be redefined as < 12.0 nmol/L, based on reference value at 97.5th percentile.

DISCUSSION

The reference interval for the morning salivary cortisol concentration was 2.1 – 22.6 nmol/L. This was similar to another study which had reported a reference interval (2.5th to 97.5th percentiles) of 1.50 – 22.02 nmol/L on waking and 1.50 – 20.87 nmol/L one-hour post waking, which was measured using a second generation Roche Cobas Cortisol assay on Modular Analytics E170.[8] The Canadian study involved 134 healthy subjects and had a similar mean age (32 years old) to our study. The reference interval obtained in the current study was also similar to the values provided by Roche for their Elecsys Cortisol II assay with reported morning salivary cortisol (6 to 10 am) of <20.3 nmol/L and <24.1 nmol/L for 95th and 97.5th percentile values, respectively.[5] In contrast, using a first-generation Roche Cobas Cortisol assay the reference interval was 6.14 - 33.19 nmol/L and 5.42 - 28.06 nmol/L on waking and one-hour post waking, respectively.[8] The median salivary cortisol concentrations obtained using first-generation assay were also consistently doubled the value of the second-generation assay, emphasising the need to determine assay specific reference range.[8]

For the late-night salivary cortisol, the determined reference interval (97.5th percentile) was <12.0 nmol/L with the majority of subjects had values < 1.5 nmol/L consistent with previous report.[5] Similarly, a reference interval study which had used the same assay reported a midnight salivary cortisol of <7.56 nmol/L and < 11.3 nmol/L for 95th and 97.5th percentile values, respectively.[5] In contrast, a lower reference interval of 1.5 – 6.28 nmol/L was obtained in the study in Canada whilst others have reported a value of <8.9 nmol/L or <8.3 nmol/L as values typically found in healthy volunteers.[4,8,9] It is recommended that two measurements of the late-night salivary cortisol be performed as initial testing for Cushing syndrome, with levels between 2 and 20 times the upper limit of the reference range, establishes the diagnosis with 90–95 % certainty.[10] This reiterates the importance of establishing assay specific and local population specific reference interval for this purpose. Nevertheless, the sensitive and specificity of late-night salivary cortisol cut-off values for diagnosis of Cushing syndrome needs to be further determined in subjects with Cushing syndrome.

This study has established the reference interval for the morning and the late-night salivary cortisol for healthy Malaysian population, based on minimum requirements as recommended by CLSI guidelines. However, an important limitation about home-based saliva sampling is the potential error in sampling time and sampling procedures despite subjects being briefed on proper sample collection.[11] Furthermore, studies have shown that emotional factors, particularly stress have a positive association with salivary cortisol level.[12] In some subjects, the need to obtained saliva samples at 2300h, may disrupt their routine sleeping time, and contribute to stress resulting in higher salivary cortisol concentration in this study.

In this study, a reference interval of 2.1 – 22.6 nmol/L and < 12.0 nmol/L for the morning and the late-night salivary cortisol respectively was established for the Malaysian population in Penang, Malaysia. The findings of this study reaffirmed the need to establish locally-derived reference intervals for morning and late-night salivary cortisol concentration.

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