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Reference ranges for cortisol and α-amylase in mother and newborn saliva samples at different perinatal and postnatal periods

Ana García-Blanco[a], Máximo Vento[b], Vicente Diago[c], Consuelo Cháfer-Pericás[a,*]

[a] Neonatal Research Group, Health Research Institute La Fe, Valencia, Spain
[b] Division of Neonatology, University & Polytechnic Hospital La Fe, Valencia, Spain
[c] Woman Health Unit, University & Polytechnic Hospital La Fe, Valencia, Spain

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ABSTRACT

This paper describes a reliable analytical method based on Ultra High-Performance Liquid Chromatography coupled to tandem mass spectrometry to determine cortisol in saliva samples from healthy mothers (n = 87) and newborns (n = 65) at different time points: (i) 38 weeks of gestation, (ii) in the immediate postnatal period (48 h) after a term delivery and, (iii) 3 months after delivery. The procedure is characterized by a simple sample treatment employing a sample volume of 25 μL. In addition to this, salivary α-amylase was determined using a commercial kit.

We have proposed potential reference ranges in saliva for cortisol (0.7–35 nmol L^{-1}) and α-amylase (2–500 U mL^{-1}) in mothers, and for cortisol (0.1–56 nmol L^{-1}) and α-amylase (0.1–500 U mL^{-1}) in newborn infants. In addition, statistical differences between the two sensitive population groups (mothers and newborns) at the perinatal and postnatal periods were studied. A lower concentration for maternal cortisol was found at 38 weeks of gestation than at 48 h ($p = 0.048$) or 3 months after delivery ($p = 0.021$). Similar results were found for the α-amylase determinations. Hence, higher concentrations than could be expected from a chronic stress marker were found at 3 months after delivery than at 38 weeks of gestation ($p < 0.001$) or 48 h after delivery ($p < 0.001$).

We conclude that this analytical method could be applied to further clinical research on perinatal and postnatal stress, such as threatened preterm labor and/or parenting stress, respectively.

1. Introduction

Stressful events such as motherhood alter numerous human biological functions. Unpredictable and uncontrollable events can occur during the prenatal (e.g., pregnancy complications or delivery conditions) and postnatal (e.g., social rhythm alterations) periods causing stress in both mother and newborn. Of note, stressful events occurring to women during pregnancy or thereafter may have subsequent consequences upon their offspring's neurocognitive, behavioral, emotional and physical development [1]. It is important to highlight that some perinatal events can cause either acute or chronic stress [2]. However, further research is required to know the biological mechanisms that trigger each one of these types of maternal stress (MS) [3,4].

Alterations in the hypothalamic-pituitary-adrenal (HPA) axis and placenta are hypothesized to be one mechanism related to MS. However, several other biological mechanisms are likely to be involved [5]. For instance, while the HPA axis shows higher habituation to stress, the sympathetic adrenal medullary (SAM) axis acts under chronic stress conditions [2]. With regard to the HPA axis, cortisol is the glucocorticoid considered its primary end product. During pregnancy, women have naturally elevated levels of cortisol, since it is essential for fetal growth. However, an increased level of cortisol associated with stress could be harmful for both mother and newborn [6]. Thus, high cortisol levels could cross the placenta affecting fetal outcome in multiple ways and for a prolonged period of time [7,8]. With regard to the SAM axis, α-amylase is an enzyme highly sensitive to chronic stress that can affect to other biological systems that are vulnerable to stress such as the cardiovascular system [9]. Specifically, α-amylase is considered the main mediator of this axis by its precursor activity on adrenaline and noradrenaline neurotransmitters [2], which has been associated with prenatal psychopathology [10].

* Corresponding author at: Neonatal Research Group Laboratory, Health Research Institute La Fe, Avenida Fernando Abril Martorell 106, Valencia E46026, Spain.
E-mail address: m.consuelo.chafer@uv.es (C. Cháfer-Pericás).

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These stress markers can be determined in different kind of biofluids such as, amniotic fluid [11,12], umbilical cord plasma [13], maternal plasma [14], urine [15], and saliva [16–19]. Saliva is the most frequently employed biofluid to evaluate stress because it is simple to collect and this convenience allows for rapid analysis and subsequent early analysis of results for evidence of pathological conditions in the same donor. Recent literature has focused on improvement of the techniques used to determine cortisol and α-amylase in saliva samples. The saliva processing is complex and can involve a series of steps including deproteinization, liquid–liquid extraction, solid-phase extraction, dilution, evaporation, derivatization and/or digestion. The aim of a recent assay has been oriented towards obtaining a simple, sensitive and selective method [20].

Of the analytical techniques used to determine cortisol, immunoassay has been favoured for its commercial availability, simple sample treatment and cheap equipment. However, the technique is also characterized by high irreproducibility, high cross reactivity and low sensitivity [21,22]. In addition to this, saliva collection procedures can introduce a risk of interferences to immunoassays [23]. In order to improve the cortisol assay, the high-performance liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS) has been used [24–27], as well as other mass spectrometry-based techniques [28]. All of them are characterized by satisfactory selectivity and reproducibility, high throughput and the ability to simultaneously identify several analytes. A previous report has attributed nonlinear relations between immunoassays and LC–MS/MS due to immunoassay cross-reactivity with saliva matrix components [29].

In the case of α-amylase kinetic assays, different substrates have been evaluated (β-2-chloro-4-nitrophenylmaltopentaoside, 4-nitrophenyl-maltopentaoside) to determine the enzyme activity and to improve the method sensitivity [30].

To our knowledge, this paper describes the first analytical method developed to determine cortisol and α-amylase in saliva samples from healthy pregnant women and offsprings at prenatal (38 weeks of gestation, 48 h after delivery) and postnatal (3 months after delivery) periods. The aim of this work is to establish the foundations for reference ranges for cortisol and alpha-amylase using non-invasive techniques.

2. Materials and methods

2.1. Sample collection and storage

Saliva samples were obtained from pregnant women (n = 87) at the University and Polytechnic Hospital La Fe, Valencia (Spain). All participants were between 18 and 40 years old, had medium-high socioeconomic status and a stable partner. They did not exhibit fertility problems, any medical conditions (e.g.: diabetes mellitus, body mass index >25) or obstetric complications (e.g. placenta abruption, preeclampsia, intrauterine growth restriction, clinical signs of infection, obstructed labor) during pregnancy, and had not been exposed to teratogenic substances nor had any history of mental disorder or previous traumas (corroborated by the Traumatic Experience Questionnaire [31]), which may have previously modify their stress response system.

The Ethics Committee at the Health Research Institute La Fe approved this study, and informed consent was obtained from participants. The demographic characteristics of participants in this study are summarized in Table 1.

Saliva samples were collected in sterile bottles at 38 weeks of gestation (evaluation #1), at the day of discharge, this is approximately 48 h after delivery (evaluation #2) and at 3 months after delivery (evaluation #3). All samples were collected between 10 and 12 a.m. (minimum 1 h after breakfast). Then, the samples were aliquoted into 1.5-mL tubes. Finally, samples were stored at −80 °C until analysis.

Table 1
Characteristics of studied population.

	Control group (n = 87)
Maternal age (years), mean ± SD	32 ± 5
Gravida (n), mean ± SD	2 ± 1
Parity (n), mean ± SD	0.6 ± 0.7
Gestational age at delivery (weeks), mean ± SD	40 ± 1
Gender: boys/girls, n (% boys)	42/45 (48)
Birth weight (g), mean ± SD	3300 ± 400

Data are expressed as mean ± SD (standard deviation) for parametric continuous variables and number of cases (percentages) for categorical variables.

In addition to this, saliva samples were obtained from the corresponding healthy term newborn infants (n = 65) at the day of discharge, this is approximately 48 h after delivery (evaluation #2) and at 3 months after delivery (evaluation #3) employing cellulose spears (Eyetec, North Yorkshire, United Kingdom). All the samples were collected from 10 to 12 a.m. They were inserted in 1.5-mL tubes and centrifuged at 5000*g* for 5 min at room temperature. Finally, the samples were stored at −80 °C until analysis.

Fig. 1 shows the process for samples collection, and the number of samples analyzed in each evaluation time.

2.2. Reagents and materials

Standards of cortisol and sulfadimethoxine were purchased from Sigma-Aldrich Química SA (Madrid, Spain). Salivary α-amylase kits were from Salimetrics (Suffolk, United Kingdom). Formic acid (98%) and ethyl acetate (analytical grade) were from Panreac (Barcelona, Spain). Methanol (MeOH, HPLC grade) was from J.T. Baker (Avantor Performance Materials B.V., The Netherlands). Centrifuge (Biocen22R) was from OrtoAlresa (Madrid, Spain). Ultrasonic bath (Sonorex digitec) was from Bandelin (Berlin, Germany). Vortex mixer was from Velp Scientifica (Usmate, Italy). Thermomixer HLC from Ditabis (Pforzheim, Germany). Speed vacuum concentrator (mi Vac) was from Genevac LTD (Ipswich, United Kingdom). 96-well sample plates (Acquity UPLC 700 μL) were from Waters (Barcelona, Spain). Microplate reader for 96 well plates (Halo LED 96) was from Dynamica Scientific Ltd. (London, United Kingdom).

2.3. Solutions

A stock solution of cortisol standard (7500 μmol L^{-1}) was prepared in methanol, and a stock solution of sulfadimethoxine standard (6 mmol L^{-1}) was prepared in sodium hydroxide (0.5 mol L^{-1}), and kept at −20 °C. A 22.6 μmol L^{-1} working solution of cortisol was prepared in H_2O(0.1% (v/v) HCOOH, pH 3):CH_3OH (85:15 v/v) and kept at −20 °C. The calibration curve was prepared daily by serial dilution in H_2O(0.1% (v/v) HCOOH, pH 3):CH_3OH (85:15 v/v) at concentrations ranging from 0.6 to 1200 nmol L^{-1} of cortisol. A 600 nmol L^{-1} working solution of sulfadimethoxine was prepared in H_2O(0.1% (v/v) HCOOH, pH 3):CH_3OH (85:15 v/v) and kept at −20 °C.

2.4. Sample treatment

Saliva samples were thawed on ice and homogenized. The sample treatment to determine cortisol was based on previous work by Jensen et al. [25]. However, some minor modifications were required due to the low sample volumes available from newborns. First, 25 μL of each sample were added to 75 μL of H_2O(0.1% (v/v) HCOOH, pH 3):CH_3OH (85:15 v/v). Then, a liquid–liquid extraction (LLE) was carried out by adding 200 μL of ethyl acetate. The mixture