OPEN ACCESS Research Journal of Obstetrics and Gynecology

ISSN 1994-7925
DOI: 10.3923/rjog.2017.11.16

Technical Report
Evaluation of a Salivary Cortisol Elisa Kit in a Critical Obstetrical Unit

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Abstract

Background and Objective: In recent years the use of saliva samples for the diagnosis of several clinical entities has been constantly increasing. The aim of this study was to describe the correlation between salivary and serum cortisol levels with the bleeding volume in cases of obstetrical hemorrhage grades III-IV. **Methodology:** This was a pilot prospective cross-sectional study. Two groups were conformed, 1) Puerperal women with grade III-IV obstetric hemorrhage and (2) Healthy puerperal women. During the first 8 h of admission to the Obstetrical-ICU or Hospitalization Service, a blood and saliva samples for cortisol measures were taken simultaneously. Relationships between the measured variables were assessed using Spearman correlations. All tests were performed with the SPSS ver. 23 statistical software program (IBM SPSS Statistics Armonk, NY: IBM Corp.). **Results:** Eight patients were included, 4 with hemorrhage (mean age 31 years, SE: 2) and 4 with healthy pregnancies (mean age 26 years, SE: 2.7). Within the subgroup of patients with hemorrhage, the Spearman correlation for saliva and serum cortisol was of 0.800 ($p = 0.2$). The negative correlation between salival cortisol and hemorrhage was high but without reaching statistical significance ($r^2 = -0.800$, $p<0.200$). **Conclusion:** The saliva cortisol determination through an ELISA kit has a high correlation with serum cortisol in case of obstetric hemorrhage Grades III-IV which give a glimpse to think about a possible failure in the ACTH-cortisol axis.

Key words: ELISA technique, obstetric hemorrhage, saliva cortisol, serum and saliva correlation, Sheehan's syndrome

Received: September 21, 2016 Accepted: November 24, 2016 Published: December 15, 2016

Citation: Luis Emilio Reyes Mendoza, Cristian Fabian Layton Tovar, Emmanuel Alejandro Andrés Osorio and Hugo Mendieta Zerón, 2017. Evaluation of a salivary cortisol elisa kit in a critical obstetrical unit. Res. J. Obstet. Gynecol., 10: 11-16.

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Saliva is becoming an increasingly common human fluid for the study of several diseases[1,2]. However, some techniques used to quantify salivary hormones are very expensive[3]. Talking about cortisol in saliva, its levels are unaffected by salivary flow rate and are relatively resistant to degradation from enzymes or freeze-thaw cycles. Even more, studies consistently report high correlations between serum and salivary cortisol, indicating that salivary cortisol levels reliably estimate serum cortisol levels[4].

About alternatives to measure cortisol and saliva, the Salimetrics Salivary Cortisol ELISA Kit has been designed to standardize the quantitative determination of free cortisol concentrations in saliva samples. Another advantage of this kit is that it has also been formatted to minimize cross reactivity for related steroids[5].

Of particular concern, obstetric hemorrhage is one of the leading causes of maternal mortality worldwide[6]. Main causes of bleeding during labor and postpartum period may be caused by: (a) Uterine atony, (b) Accretism and (c) Retention of placental remains[7].

Severe obstetric hemorrhage is considered when vaginal bleeding exceeds 1,000 mL[8]. Due to the lack of a universal classification of obstetrical hemorrhage, the most common guideline is that of the American College of Surgeons (Table 1)[9]. Blood loss leads sequentially to cardiovascular instability, coagulopathy, decreased oxygen transport, decreased perfusion and cellular hypoxia. These alterations lead to the development of systemic inflammatory response and ultimately to multiple organ failure, which contributes to increased risk of death.

In developing countries, despite the high incidence of obstetric hemorrhage there is a low frequency of diagnosis of its hormonal failures, being Sheehan's syndrome the extreme, characterized by hypopituitarism that occurs as a result of ischemic pituitary necrosis due to severe postpartum hemorrhage[10]. In some cases, the diagnosis is not made until years later, when features of hypopituitarism, such as secondary hypothyroidism or secondary adrenal insufficiency, become evident[11].

On the other hand, critical illnesses are often accompanied by hypercortisolemia and low levels of corticotrophin[12]. In recent years, it has been spread using saliva samples for the diagnosis of various clinical entities being a body fluid that can be used to detect the presence and determine concentrations of a wide variety of antibodies, drugs, hormones and tumor markers[13,14]. The salivary concentrations of free soluble steroids such as cortisol, reflect about 10% of plasma concentrations[15].

Table 1: Classification of postpartum hemorrhage

Hemorrhage class	Blood loss (mL)	Percentage lost (%)
1	<750	<15
2	750-1500	15-30
3	1500-2000	30-40
4	>2000	>40

American College of Surgeons[9]

Corticotropin deficiency can cause weakness, fatigue, hypoglycemia, or dizziness. Diagnosis of panhypopituitarism is straightforward, but partial deficiencies are often difficult to elicit[16]. The aim of this study was to analyze the reliability of a saliva cortisol ELISA kit and its correlation with the serum values in cases of obstetrical hemorrhage grades III-IV.

MATERIALS AND METHODS

Subjects: Two groups were conformed, (1) Puerperal women with grade III-IV obstetric hemorrhage and (2) Healthy puerperal women. Women prescribed with steroids, or with autoimmune diseases were excluded from the study. Files with incomplete information were discarded from the final analysis.

Study design: This was a pilot prospective cross-sectional study conducted at the "Mónica Pretelini Saenz" Maternal-Perinatal Hospital (HMPMPS), Health Institute of the State of Mexico (ISEM), Toluca, Mexico, from February, 2016-June, 2016.

Anthropometric measurements: Weight (kg) and height (m) were calculated in a mechanical column scale (SECA). Birth weight was measured with a calibrated digital scale (TANITA 1582, Tokyo, Japan) to the nearest±0.1 kg. Length was measured with an infantometer (SECA 207, Germany) to the nearest±0.1 cm. In the obstetrical intensive care unit (O-ICU) height was measured with a measuring tape, weight with an electric bed (Hill-Rom, Total Care) and the blood pressure with an electronic monitor (Infinity Delta XL, Drager, USA.). Patients that required aminergic support were monitored with a Niccomo™ equipment (Medis, GmbH Ilmenou, Germany).

Laboratorial studies: In the morning, blood samples were collected into Vacutainer tubes and centrifuged to separate serum from plasma. Glucose (mg dL^{-1}) was measured (Dimension Rx L Max, Dade Behring) at the Clinical Laboratory of the HMPMP according to standardized procedures recommended by the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC).

Cortisol: During the first 8 h of admission to the O-ICU or Hospitalization Service, a blood and saliva samples for cortisol measures were taken simultaneously. Saliva samples from control patients were obtained in sterile flasks asking patients to spit after 2 min. In the case of patients admitted to the O-ICU, saliva samples were taken using an insulin syringe, sucking on the cheek. Salivary cortisol was quantified at the Research Laboratory of Ciprés Grupo Médico S.C. (CGM) with an ELISA kit (Salimetrics, USA), following the next manufacturer's instructions:

- After preparing the plate layout pipette 24 mL of assay diluent into the disposable tube (scale down proportionally if using less than the entire plate)
- Pipette 25 µL of standards, controls and saliva samples into appropriate wells. Pipette 25 µL of assay diluent into 2 wells to serve as the zero and pipette 25 µL of assay diluent into each Non-Specific Binding (NSB) well
- Dilute the enzyme conjugate 1:1600 by adding 15 µL of the conjugate to the 24 mL tube of assay diluent (scale down proportionally if not using the entire plate). Conjugate tube may be centrifuged for a few minutes to bring the liquid down to the tube bottom. Immediately mix the diluted conjugate solution and add 200 µL to each well using a multichannel pipette
- Mix plate on a plate rotator for 5 min at 500 rpm and incubate at room temperature for a total of 1 h
- Wash the plate 4 times with 1X wash buffer. A plate washer is recommended. However, washing may be done by gently squirting wash buffer into each well with a squirt bottle, or by pipetting 300 µL of wash buffer into each well and then discarding the liquid over a sink. After each wash, the plate should be thoroughly blotted on paper towels before turning upright. If using a plate washer, blotting is still recommended after the last wash
- Add 200 µL of TMB Substrate Solution to each well with a multichannel pipette
- Mix on a plate rotator for 5 min at 500 rpm and incubate the plate in the dark (covered) at room temperature for an additional 25 min
- Add 50 µL of 3 M stop solution with a multichannel pipette
- Mix on a plate rotator for 3 min at 500 rpm. If green color remains, continue mixing until green color turns to yellow. Be sure all wells have turned yellow. Wipe off bottom of plate with a water-moistened, lint-free cloth and wipe dry and finally read in a plate reader at 450 nm within 10 min of adding 3 M stop solution

Statistical analysis: All data were assessed using non-parametric statistics. Results were presented as media±Standard Error (SE) for continuous variables. Relationships between the measured variables (i.e., pre-test values, relative changes) were assessed using Spearman correlations. Significance was set at $p<0.05$. All tests were performed with the SPSS ver. 23 statistical software program (IBM SPSS Statistics Armonk, NY: IBM Corp.)[17].

Ethics: The study was approved by the Research Committee of the HMPMPS (code 217B500402015075). The process complied with the ethical principles of the Declaration of Helsinki (Fortaleza, Brazil) and written informed consent was obtained from all participating subjects.

RESULTS

Eight patients were included in this pilot study, 4 (mean age 31 years, SE: 2) with hemorrhage, 3 with grade IV and one grade III and 4 (mean age 26 years, SE: 2.7) with healthy pregnancies. The general characteristics of the patients are shown in Table 2.

From the 4 patients with Grade III-IV hemorrhage, 3 required aminergic support. Interestingly, the patient with the highest value of serum cortisol and salival cortisol did not required aminergic drugs. The blood transfusion requirements in this group are depicted in Table 3.

Unexpectedly, the Spearman correlation between serum and saliva cortisol taking into account the 8 samples was low, of 0.443 ($p = 0.272$). Within the subgroup of patients with hemorrhage, the Spearman correlation for saliva and serum cortisol was of 0.800 ($p = 0.2$) with a significant negative correlation between serum cortisol and the volume of hemorrhage ($r^2 = -1.000$, $p<0.001$). The negative correlation between salival cortisol and hemorrhage was high but without reaching statistical significance ($r^2 = -0.800$, $p<0.200$).

Glucose had a negative but not significant correlation with serum ($r^2 = -0.400$, $p = 0.60$) and saliva cortisol ($r^2 = -0.200$, $p = 0.80$). Finally, the Cardiac Output (CO), Cardiac Index (CI), Systemic Vascular Resistance (SVR), Systemic Vascular Resistance Index (SVRI) and Stroke Index (SI), parameters evaluated with the Niccomo™ equipment (Medis, GmbH Ilmenou, Germany), did not show any significant correlation with any other variable.

Table 2: General characteristics of the patients

Characteristics	Puerperium with Grade III-IV hemorrhage	Puerperium without hemorrhage
Age	30.5 (SE: 2, range: 26-37)	14 (SE: 2.7, range: 18-35)
BMI	29.5 (SE: 1.4, range: 25.1-34)	28.6 (SE: 2.2, range: 25.2-34.7)
Serum cortisol	12.2 (SE: 1.7, range: 4.4-14.8)	13.65 (SE: 1.32, range: 7.1-14.8)
Salival cortisol	1.28 (SE: 1.1, range: 0.01-6.41)	0.09 (SE: 0.00055, range: 0.090.1)

BMI: Body mass index, SE: Standard error

Table 3: Blood transfusion requirements in the patients with obstetric hemorrhage

	Blood requirements			
Obstetric hemorrhage patients	Globular package	Fresh frozen plasma	Platelet apheresis	Cryoprecipitate
Media	10	10.5	2	14
Minimum	8	5	0	8
Maximum	18	27	4	42
Standard error (range)	±1.8	±3.9	±0.8	±6.2

DISCUSSION

The confirmation of a good correlation between saliva and serum cortisol has been probed in several clinical settings, sports[18], newborn infants[19], chronic diseases[20], etc., but the information related to pregnancy is really scarce[21].

Puerperal women that suffer obstetrical hemorrhage have the risk to require medical attention in an Intensive Care Unit (ICU). The importance of an organized, team approach in the scheme of management of women with obstetric hemorrhage cannot be overemphasized. Some drugs prescribed in the management of obstetric hemorrhage are ergometrine, oxytocin, rectal use of misoprostol[22] and some patients with no response to volume will receive aminergic support[8].

The repercussion of the obstetrical hemorrhage is of a long lasting phenomenon. For example, in a study by Dokmetas *et al.*[23], to determine the clinical characteristics of Sheehan's syndrome in 20 patients (mean age 60.15±3.41 years) with typical obstetric history of massive hemorrhage at delivery, the mean duration between time of diagnosis and date of the last delivery was 26.82±2.52 years (range 2-40 years). Even more, according to the hormonal values, 11 (55.0%) had adrenal failure. In many medical units of developed countries that attend cases of obstetrical hemorrhage, the patients are stabilized, discarded but not studied of the possibility for a hormonal deficit due to the delay of signs and symptoms of such a thing[24].

For the confirmation of a partial hormonal deficit, stimulation tests (insulin-induced hypoglycemia or metyrapone stimulation test) are often necessary for diagnosis in the acute phase or in situations where a partial deficiency is suspected[25]. Unfortunately these tests are not performed as should be in all cases of obstetrical hemorrhage and women are discarded without the confirmation of a partial or complete pituitary hormonal deficit. Most patients usually present it months to years later, with a history of failure of postpartum lactation, failure to resume menses and other signs of panhypopituitarism. In mild forms of the disease, patients may remain undetected and do not receive treatment for many years. Early diagnosis and appropriate treatment are important to reduce the morbi-mortality of the patients[10]. Surprisingly, the information discussing the hypothalamic-adrenal axis changes in case of hemorrhage is scarce[26,27] which leads and empty niche for research.

The measurement of cortisol in saliva is advisable in patients with abnormal Cortisol Binding Globulin (CBG) levels such as pregnancy[28]. In this line, the data of this study show a better clinical application of the salival cortisol with the volume of hemorrhage than with serum cortisol, showing that with a higher bleeding the saliva cortisol was lower and that in case of high values of this hormone, the aminergic support was not necessary.

The low correlation between the eight serum and saliva cortisol samples reported in this first approach is consistent with data emerging from previous studies[29,30] that have found low to moderate correlations in the same comparison.

In comparison with the costs of the serum and urinary cortisol, the saliva cortisol is cheaper, 8.7$ versus 12.6$ and 15.8$ of the first two. Of particular concern is the speed to get a result, in this line of concern, the urinary cortisol is the worst option in a critical moment while the saliva and serum cortisol can be processed immediately.

A limitation of this study, besides the low number of patients, is that visual estimation is not optimal for measurement of obstetrical hemorrhage[31].For that reason the criteria to test the ELISA kit was hemorrhage Grade III-IV as corroborated as corroborated with the need of considerable amount of blood derivatives. Notwithstanding, as the initial focus of this research was to evaluate the usefulness of a saliva cortisol ELISA kit in an intensive care setting, the targeted has

been reached partially showing a high correlation between serum and saliva cortisol in case of obstetrical hemorrhage.

CONCLUSION

It is concluded that the determination of saliva cortisol with an ELISA kit might be an easier, cheaper and reliable tool to evaluate the hypophysis-adrenal axis in obstetrical intensive care units than the serum or urine cortisol. Secondly, a negative correlation between saliva or serum cortisol and the volume of hemorrhage might contribute to improve the perspicacious clinical analysis of a case of adrenal failure.

SIGNIFICANCE STATEMENT

This study analyzes and proposed the use of saliva cortisol as measured by the ELISA technique as a useful laboratorial test within a critical medical setting. The strongest point of this alternative is the cost, thus being an affordable technique for most of the countries. This study states the reliability of the ELISA saliva cortisol kit in relation to the volume of hemorrhage which means an easy and cheap study to evaluate a possible failure in the hypophysis-adrenal axis in puerperium.

ACKNOWLEDGMENTS

Authors thank to Residents of the Obstetrical Intensive Care Unit of the "Mónica Pretelini Sáenz" Maternal-Perinatal Hospital (HMPMPS) and Lizbeth Campos Reyes, Faculty of Sciences, Autonomous University of the State of Mexico (UAEMex) for their help to develop this study and Maggie Brunner M.A., former editor of Archives of Medical Research, Instituto Mexicano del Seguro Social (IMSS), for her excellent help with the English style correction. Source of support: Ciprés Grupo Médico S.C. (CGM), grant 2016-01.

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