3-2015

Comparison of cortisol samples in the first two weeks of life in preterm infants

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Abstract

Background: Growing literature on negative childhood stress emphasizes the need to understand cortisol values from varying biomarker samples.

Objective: This work aimed to examine cortisol samples for usability, associations, and individual stability in neonates.

Subjects: The sample consisted of preterm infants (n = 31).

Materials and methods: Analyses on cortisol collected from cord blood and from saliva and urine samples on days 1, 7, and 14 included Spearman correlations and paired t-tests.

Results: Usability rates were 80.6% (cord blood), 85.9% (saliva), and 93.5% (urine). Salivary and urinary cortisol levels had significant correlation on day 1 only ($p = 0.004$). Significant differences in individual stability of cortisol concentrations existed except in urine on days 1 and 7 and in saliva on days 7 and 14.

Conclusions: Usability was highest for urine samples. We found little correlation between cortisol sample levels at each time; individual stability of cortisol concentrations was minimal. Interpretation of cortisol findings in all studies should be performed cautiously.

Keywords: cortisol; infant; preterm; stress.

Introduction

Toxic stress, fetal programming, allostatic load, and other models of stress that link early childhood experiences with adult diseases have become critical concepts in biomedicine. The essential theme of these concepts is that negative prenatal and neonatal experiences may be associated with structural and functional alterations in the brain, which in turn, may negatively impact long-term health (1–3). Preterm infants endure numerous and accumulating types of physical, psychological, and physiological stress during critical periods of brain and nervous system development (3, 4). Hence, the pivotal role that stress plays in driving negative outcomes in preterm infants must be further understood. Identifying and interpreting measurements of stress is fundamental to advancing scientific discovery in this area.

Research on stress often involves examining the activity of the hypothalamus-pituitary-adrenal (HPA) axis. The most common biomarker used to measure HPA axis activity in all populations is cortisol because of its major role in stress and neuroendocrine mechanisms. This steroid hormone can be measured in blood (i.e., plasma), urine, saliva, and hair using enzyme-linked immunosorbent assay (ELISA) and radioimmunoassay (RIA) techniques. Blood levels reflect total cortisol values, which include both the bound and unbound structures. In a homeostatic environment, 90% of cortisol in the body is bound to a carrier protein in the blood, leaving < 10% of unbound cortisol, referred to as “free” cortisol

(5). This unbound cortisol is considered the biologically active form of the hormone and can be excreted into the urine and saliva. Examining unbound cortisol levels following stressful stimuli reflects an individual's HPA activity and neuroendocrine response.

Non-invasive methods used to measure cortisol (i.e., saliva and urine samples) are preferable to invasive samples via venipuncture in the preterm infant population, yet usability rates for these samples range from 65% to 85% (6–11). Saliva is the most common biological substrate used in preterm infants because it can be collected noninvasively at multiple time points, and studies have demonstrated an association between salivary cortisol and plasma levels of unbound cortisol (12, 13). However, a recent systematic review using the Bland-Altman method of analysis found insufficient agreement between salivary and plasma cortisol levels in this population (14). The authors specifically examined the use of salivary cortisol as a measurement for adrenal insufficiency and concluded that salivary samples should not replace plasma samples in preterm infants.

The literature is inconsistent as to whether variations in cortisol levels are reflective of neonatal illness. Negative neonatal outcomes have been associated with both lower and higher levels of cortisol collected during the first week of life (15, 16). These unpredictable findings may be associated with the lack of standardized collection methods for cortisol measurements. Salivary collection methods used in previous studies range from 5 to 20 min of sample collection using various types of cotton swabs and aspiration techniques; some of these studies used saliva stimulation crystals (14, 17). Urinary samples have been collected via special diapers, cotton swabs, and urine bags and as a "spot" test or over a 24-h period (18–21). Given that different methods for collecting samples yields varying results, rigorous testing of cortisol collection methods and interpretation of findings in preterm infants is needed (6–8, 14).

The growing literature on negative stress in early childhood affecting long-term health emphasizes the need to increase understanding of stress and cortisol levels from a variety of biomarker sources in preterm infants. We recently studied cortisol levels in preterm infants using cord blood and urine and saliva samples using the ELISA technique (22). The purpose of the current analysis was to examine cortisol values of blood, saliva, and urine samples in preterm infants (< 32 weeks of gestation) during the first weeks of life. The specific aims were to 1) evaluate cord blood and salivary and urinary samples for usability rates; 2) examine associations among cortisol levels in the cord blood, saliva, and urine on day 1 of life; 3) examine relationships between cortisol levels in the saliva and urine on day 7 and day 14; and 4) determine individual consistency in salivary and urinary cortisol levels among samples taken from the infants.

Materials and methods

This was a prospective, correlational, longitudinal design study on a convenience sample of preterm infants in a level III NICU located in the Midwestern US. Approval was received from the institutional review board. Infants with gestational age (GA) of < 32 weeks at birth were included in the study; exclusion criteria were major congenital anomalies. Infants were enrolled after informed consent was obtained by research personnel from a parent of an eligible infant. The day of consent was regarded as day 1 of the study for that infant and occurred within 24 h of birth and after admission to the NICU. Samples were obtained from the cord blood at birth and from the saliva and urine on days 1, 7, and 14 after birth. Further details of the methods used can be found in Moore et al. (22).

Cord blood

Cord blood was obtained at birth in this institution per hospital protocol. Extra blood that was not used for clinical purposes was used for this study. Plasma samples were processed and stored in a –80°C freezer in the hospital's clinical research center.

Saliva

Saliva samples were obtained during daytime hours (09:00–20:00 h) on days 1, 7, and 14 using a Salimetrics Infant Swab (Salimetrics LLC, State College, PA). We made efforts to collect saliva before, or at least 60 min after, a major stimulation or painful procedure (e.g., kangaroo care, endotracheal suctioning, etc.). The cotton swab was placed in the infant's buccal mucosa for 5 min. The swab was then visually inspected for blood. Oral care, by gently swabbing the mouth with a moistened towelette, was performed prior to an additional collection attempt with a limit of two attempts if visible blood was found on the swab. Collection was discontinued if infants displayed any stress cues (e.g., gagging, desaturation, jittering) during the procedure. We chose the above restrictions to minimize infant stimulation and staff burden. The swab was then placed in the Salimetrics storage tube, labeled with the study ID and sample number (day 1, 7, or 14), and stored in a –80°C freezer in the hospital's clinical research center.

Urine

Spot urine samples were collected on days 1, 7, and 14. Urine collection was performed by placing a sterile cotton ball on the perineal area inside the infant's diaper. At the next diaper change, the cotton ball was removed, placed into a 10 mL syringe, and squeezed into a 1.5 mL microcentrifuge tube, labeled with the study ID and sample number, and stored at –80°C. We made efforts to coordinate the urine collection within the time frame as the salivary collection. For example, the cotton ball was placed in the diaper when the morning vital signs were collected at 08:00 h. Research personnel collected the saliva sample and removed the urine-saturated cotton ball prior to the next set of vital signs at 12:00 h. If the cotton ball was found to be misplaced, a new cotton ball was placed in the diaper at 12:00 h and then removed during the afternoon set of vital signs at 16:00 h.

Cortisol

Plasma cortisol levels from the cord blood samples were determined by the hospital's laboratory services using RIA and hospital protocol. Cortisol levels from the salivary and urinary samples were completed at the Endocrine Bioservices Laboratory (Omaha, NE) using an enzyme immunoassay according to methodological details outlined in Moore (22). Briefly, salivary samples were diluted 1:4 in distilled, deionized water, and urine samples were diluted 1:80 with the same medium. Standards and quality control samples were included on each microtiter plate. Intra-assay variations in the cortisol assay for high and low concentration pools were as follows: salivary- 7.6% and 10.8% and urinary- 3.9% and 11.2%. Interassay variations in the cortisol assay for high and low concentration pools were as follows: salivary- 11.9% and 9.4%, and urinary- 14.3% and 4.4%. A creatinine level for each urine sample was identified using a modified Jaffe assay in a 1:20 dilution based on a standard dilution test (23); a ratio of µg cortisol per mg creatinine was reported.

Statistical analyses

All analyses were conducted using natural log transformed data because raw data were not normally distributed. Spearman correlation was used to assess the relationships among cortisol measures on days 1, 7, and 14. Paired t-tests were used to determine whether salivary and urinary cortisol concentrations differed between days 1 and 7 and between days 1 and 14. The percent of missing data was calculated for all anticipated samples and for each type.

Results

A total of 31 infants with a mean gestational age of 29.0 weeks were enrolled in the study. Twenty-seven out of 217 (12.4%) anticipated samples (cord blood, saliva, and urine) were not collected for the entire study. Unavailable cord blood accounted for six (19.4%) of the missing samples. One salivary and one urinary sample were missing on day 14 due to an infant death on day 8. For the remaining 184 collected salivary and urinary samples, 19 (10.3%) were insufficient for cortisol assay. Thirteen (68.4%) of these were salivary and six (31.6%) were urinary samples. Inadequate salivary samples were from bloody samples or inadequate volumes on days 1 (n = 3), 7 (n = 6), and 14 (n = 4). Three infants displayed stress cues resulting in collection times < 5 min. The missing urinary samples on days 1 (n = 3), 7 (n = 2), and 14 (n = 1) were from procedural errors or inadequate volume. Final sample usability rates were 80.6% for cord blood (n = 25/31), 85.9% for saliva (n = 79/92), and 93.5% for urine (n = 86/92).

Table 1 summarizes the associations of cortisol levels among samples in the cord blood, saliva and urine on day 1 of life (aim 2) and in the saliva and urine on day 7 and day 14 (aim 3) using Spearman correlations and p-values. Infants with high levels of cortisol in the saliva also had high levels of cortisol in the urine on day 1 ($p = 0.004$). No significant associations were found between cortisol levels measured in cord blood and cortisol measured in the saliva or urine on day 1 or between cortisol levels in the urine and saliva on days 7 and 14.

Table 2 summarizes the individual stability of cortisol concentrations in the saliva and urine. In saliva, significant differences were found between cortisol levels on days 1 and 7 ($p = 0.002$) and days 1 and 14 ($p = 0.002$), but no difference was found between cortisol levels on days 7 and 14. In urine, significant differences were found between cortisol levels on days 1 and 14 ($p = 0.039$) and on days 7 and 14 ($p = 0.025$), but not on days 1 and 7.

Discussion

This is the first known study to compare sample collection methods and cortisol values among biomarker samples obtained from preterm infants with GA < 32 weeks during the first 2 weeks of life in an NICU. Samples were compared for usability rates and associations among cortisol levels in different biological samples. Individual stability of cortisol concentrations was also analyzed. Usability rates were highest for urinary collection methods compared with salivary collection methods. A significant positive correlation was found between salivary and urinary cortisol on day 1, but not on days 7 and 14 with little individual stability.

Unavailable samples were the primary reason for missing cord blood values. Due to the fact that our research team was not directly involved in collecting the cord blood, our discussion for usability rates focused on the non-invasive collection methods. Sample usability rates were highest for urine (93.5%) compared with saliva (85.9%). Our usability rates for spot-urine samples were higher than reported in previous studies (9–11). The major reasons for missing urine data included stool contamination and

misplacement of the cotton ball leading to insufficient amount of urine. The infants did not display any signs of discomfort from the cotton ball. This result is consistent with previous studies.

The usability rate for salivary samples was consistent with previous studies at 86% (6, 8). Mitchell et al. (17) reviewed various techniques to determine the best salivary collection method using a sorbette. The suggestions for success included keeping the sorbette in the buccal mucosa for 20–25 min and avoiding potential contamination when collecting during feedings. Collecting saliva for 20 min may not be ideal for the unstable infant, as observed in the three infants who tolerated the procedure for < 5 min only in our study. The standard feeding practice for infants < 32 weeks of gestation in many NICU units is to provide continuous enteral feedings. Therefore, GA, feeding protocols, and stability of the patient must all be considered before using the salivary collection methods discussed in Mitchell et al. (17).

Overall, we preferred the urinary collection method over the salivary collection method because of the higher usability rates and decreased perceived infant stimulation. Experience with both methods will likely improve usability rates, but missing data associated with salivary samples due to infant distress and blood contamination will still be difficult to control. Thus, we recommend obtaining urine samples in this population and testing reliability of collection methods.

Only salivary and urinary cortisol levels on day 1 were correlated. Cord blood cortisol levels were not related to either saliva or urine cortisol levels on day 1. This finding is not consistent with the literature (13). Chou et al. collected saliva within 15 min of delivery in infants (n = 51) with a mean GA of 34 weeks and found an association between saliva and cord blood levels. Our study collected saliva within the first 24 h of life in preterm infants with mean GA < 32 weeks. Given that the birth process itself is known to affect cortisol levels, lack of association in our study was likely due to variability of collection times after birth. However, as stated above, blood contamination from delivery and intubation attempts was an obstacle for saliva collection within the first 24 h of life.

Although cortisol levels in the saliva and urine were associated on day 1, the relationship was not present on days 7 and 14. This unexpected finding may be explained by findings of Iwata et al. (24). In their study, salivary cortisol was collected every 3 h over a period of 24 h from infants (n = 27) with a mean GA of 36 weeks at birth and mean 5 days postnatal age. Iwata et al. (24) showed a circadian pattern during the first 5 days of the infants' life, which correlated with time of birth and maternal circadian rhythms. The absence of circadian patterns after 5 days was discussed as due to the decreased influence of maternal circadian rhythms and re-entrainment of individual rhythms over time (24). This change in infant circadian rhythm may explain our findings that cortisol concentrations were more correlated between samples on day 1 vs. day 7 or day 14. Of note, the cord blood cortisol levels in our study were not associated with either the urine or saliva on day 1, which would dispute Iwata's findings that a maternal hormonal influence existed on day 1. This inconsistent finding may derive from the variability in collection times of saliva and urine on day 1 in our study. Cord blood was collected at birth, while the saliva and urine were collected within 24 h after birth based on timing of the parental consent and time of day (09:00–20:00 h). Therefore, the difference in collection timing for the cord blood vs. the urine and saliva on day 1 would affect correlations between cortisol levels in our study. A weakness of the study of Iwata et al. (24) was that the cortisol levels were measured only once for each infant and, therefore, no longitudinal data were available for each infant. The differences between the design of our study compared with previous designs complicate comparisons. Therefore, our results remain inconclusive.

The individual cortisol levels of each infant in our study did not demonstrate stability. Significant differences between individual stability of cortisol concentrations existed, except in the urine on days 1 and 7 or in the saliva on days 7 and 14. In other words, individual cortisol levels were not stable or predictable. An infant with lower levels of cortisol on day 1 may or may not have lower levels of cortisol on day 14. These results may explain why comparisons between outcome variables with cortisol levels in previous studies display mixed results and remain inconclusive. For example, studies in preterm infants have reported that chronic lung disease is associated with low serum cortisol levels between days 15 to 19 after birth (25), high cortisol levels on day 14 after birth (26), and no association with cortisol levels during the first week after birth (15). Together with our results, this finding suggests that cortisol levels are not stable or predictable; hence, comparing results between studies may be problematic.

Although the unpredictable and unstable cortisol levels in the first weeks of life may be attributed to an immature nervous system, prenatal determinants, such as stress and nutrition, are likely to play a role in this developing complex system in utero. For example, a lack of dysregulation and variability in individual infants during the first weeks of life may suggest a blunted HPA response as "programmed" from maternal hormonal influences. The concepts of fetal programming and brain plasticity may explain the dysregulation in cortisol levels and neonatal outcomes. Infants with negative outcomes may have higher levels of cortisol at baseline but display a blunted acute response to stress that could mimic hypocortisol levels when comparing cortisol levels within a population of infants. An example of this phenomenon has been identified in a recent study by O'Connor (1). Infants (n = 125) exposed to increased levels of prenatal cortisol were more likely to have higher cortisol levels at 17 months of age. When exposed to a separation-reunion stress event, these infants showed a blunted cortisol response to stress. Hence, a gap in the literature exists in terms of determining whether a similar phenomenon exists in preterm infants during the first weeks of life.

The concepts of fetal programming and brain plasticity also may explain the inconclusive results from studies examining the benefits of kangaroo care in preterm infants (27, 28). Infants in previous studies consistently showed positive behavioral responses to kangaroo care using pain scales and vital signs. The corresponding cortisol levels, however, were not reliably associated with kangaroo care. Some of the infants had decreased cortisol levels, while others had no changes or increased levels of cortisol after kangaroo care. Research examining the effects of prenatal cortisol exposure in utero and how it relates to the preterm infant's baseline HPA function and acute stress responses is essential in understanding stress and interpreting cortisol levels in this population.

Our results also support the need to study the relationship between additional physiological factors on the developing HPA axis. Cortisol binding globulin plays a major role in salivary cortisol levels and HPA activity. Salivary cortisol levels that measure the degree of an HPA response to stress would be directly affected by cortisol binding globulin levels, which also have various confounding factors (e.g., gonadal hormones) (5). Confounding factors, such as conjugation of cortisol with carrier proteins and the time-course of cortisol clearance between samples, also need to be considered (7).

Our study has several limitations. Our sample size was small and the correlational analyses may not have been adequately powered because this was not the primary aim of the study. Variability in collection times, especially on day 1, likely affected cortisol levels; hence, comparative analyses of cortisol levels across different biological matrices may not accurately portray actual relationships. Additional physiologic factors, such as cortisol-binding globulin, were not measured in this study.

Further understanding of cortisol levels based on sample source and the interpretation of cortisol levels for use in clinical research is essential in advancing the science of stress research in early childhood. Further research is also needed on the effects of maternal factors and physiologic measures (e.g. cortisol-binding globulin) as they relate to HPA axis development and function in the neonatal population. Our findings suggest that comparison analyses with demographic or medical variables may have differing outcomes based on the biomarker sample used. Comparing cortisol levels obtained from different sources should be interpreted with caution. Literature discussions often compare a study's findings with previous results regardless of the sample source. These comparisons may result in further confusion and inaccurate interpretations of outcomes. Researchers and clinicians must be aware of these potential confounding factors when interpreting data using cortisol as a measurement of stress. In the population of neonatal infants, a urinary collection method may be preferable to salivary methods due to the minimal stimulation to the infant and decreased sample contamination. However, more methodological studies are needed to improve reliability and provide a standardization of sample collection for non-invasive samples.

Conclusion

Cortisol samples were collected from the cord blood, urine, and saliva in preterm infants with mean GA < 32 weeks at birth and on days 1, 7, and 14. Usability rates were highest for urinary samples. Our study found that levels of cortisol in salivary and urinary samples collected on the same day were not highly correlated, and individual stability of cortisol levels in the urine and saliva was minimal. Standardization of collection methods is needed and comparisons between study findings using cortisol levels should be interpreted with caution.

Acknowledgments: The authors wish to express gratitude to Andrew Birnie, MS and Ann M. Berger, PhD, APRN, AOCNS, FAAN for their contributions to this manuscript.

Funding support: The authors wish to acknowledge the support provided by the University of Nebraska Medical Center Clinical Research Center, the Foundation for Neonatal Research and Education, and the Sigma Theta Tau-Gamma Pi Chapter.

Table 1 Correlations between cortisol levels and samples collected from cord blood samples at birth and in the saliva and urine on days 1, 7, and 14 in preterm infants with GA <32 weeks.

Cortisol sample	Spearman correlation	p-Value
Cord blood at birth and day 1 urine	0.272	0.208
Cord blood at birth and day 1 saliva	0.156	0.468
Day 1 urine and day 1 saliva	0.577	**0.004**
Day 7 urine and day 7 saliva	0.124	0.563
Day 14 urine and day 14 saliva	0.115	0.567

Table 2 Individual stability of cortisol levels measured in the saliva and urine samples collected on days 1, 7, and 14 from preterm infants with GA <32 weeks.

Cortisol sample	Mean difference	Standard error	p-Value
Collection day			
Saliva			
Day 1 and day 7	0.965	0.269	**0.002**
Day 1 and day 14	0.962	0.277	**0.002**
Day 7 and day 14	−0.121	0.182	0.512
Urine			
Day 1 and day 7	0.299	0.246	0.236
Day 1 and day 14	0.572	0.263	**0.039**
Day 7 and day 14	0.289	0.121	**0.025**

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