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Salivary cortisol in preterm infants: Validation of a simple method for collecting saliva for cortisol determination

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Abstract

Background and aims: The increased use of salivary cortisol as a biomarker of stress and/or diurnal rhythms has facilitated research of Hypothalamic–pituitary–adrenocortical (HPA) function. Saliva collection remains problematic with preterm infants. The twofold purpose of this study is to 1) establish validity of the filter paper method for saliva collection and 2) apply the filter paper method for saliva collection to preterm infants.

Design and measures: Whole saliva was collected from six normal adult subjects to create a pool. Validation measures included comparison of levels obtained from whole saliva and filter paper, an evaluation of storage effects, assessing spiking recovery, and measurement of linearity of dilution. In the application study, saliva was collected every three hours, before feedings for three consecutive days from 26 hospitalized preterm infants. Diurnal variation in cortisol was examined using hierarchical linear modeling and individual calculation of diurnal pattern using an accepted technique.

Results: Validation studies revealed acceptable recovery of whole saliva from filters, no effect of room temperature storage of filters for up to six months, and acceptable linearity of dilution up to 4×. Saliva from preterm infants was easily collected. Only 2% of the samples were lost due to inadequate wetting of the filters. An inverse association was found between postconceptional age and one-minute APGAR scores and infant cortisol levels. Variable daily cortisol patterns and no discernable rhythm were found for this sample; however, four infants appeared to show a typical diurnal pattern.

Conclusions: The filter paper method is a valid method of saliva collection that is feasible to use with preterm infants.

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1. Introduction

Over the past decade there has been a dramatic increase in studies using salivary cortisol as a biomarker of stress and/or diurnal rhythms [1]. Saliva collection minimizes some of the potential confounds associated with blood sampling such as stress responses associated with the blood draw [2] or anticipation of a blood draw [3]. Collecting saliva instead of blood adds the convenience of self-sampling in the home and/or other naturalistic environments [4]. Saliva collection has particularly facilitated infancy studies, resulting in more practical examination of development of cortisol diurnal rhythm, factors that influence development of this rhythm, and acute stress responses.

Hypothalamic–pituitary–adrenocortical (HPA) axis function, correlation with clinical variables, and response to stress are important areas of study in preterm infants. Exploration of this field has been limited by small blood volumes that limit sample availability and preclude repetitive study. Salivary cortisol provides a minimally invasive alternative, with the additional feature that it reflects free cortisol rather than total cortisol that can be affected by plasma binding protein concentrations [5,6]. Salivary cortisol measures have been validated against serum cortisol measures in preterm infants [7].

Two methods of saliva collection typically have been used with preterm infants. A commercial device, the Salivette (Sarstedt, Inc) permits easy extraction of saliva from a small cotton plug [8] that subjects moisten in their mouths. The cotton then is returned to a holder and saliva is expressed by centrifugation in the holder. A second method involves aspirating saliva with a small plastic feeding tube or suction catheter attached to a syringe [9,10]. In both methods the saliva typically is frozen after collection until assay. A number of problems are associated with these collection methods. Collection using these approaches can take as much as 5–10 min [8–10]. Herrington et al. [11] reported a loss of 54% of the samples collected due to lack of adequate amounts of saliva collected with cotton plugs. Aspiration methods are rather intrusive and may damage the delicate mucous membranes of the preterm infant. This results in bleeding and contamination of the saliva samples with whole blood. Finally, many times freezing or refrigeration of samples may not be feasible.

An ideal method for infant saliva collection is one that is minimally intrusive, easy for a caregiver or family member to accomplish, and, most importantly, capable of yielding valid and reliable detection of cortisol in the saliva. Other important features include ease of transport and storage at room temperature.

The present study approached saliva collection from preterm infants in a manner suggested by several published papers [12,13] but without extensive validation provided in the methods. Specially-cut filter papers for absorbing the saliva from the infant's mouth were used in the present study. Herein we describe variations in salivary cortisol pattern throughout the day in preterm infants based on saliva collection using these calibrated and tested filters. Purposes of this study were 1) to establish validity of this method for saliva collection and 2) to apply the filter paper method for saliva collection to preterm infants. Specific questions included feasibility of saliva collection using filter papers

for preterm infants (e.g., collection of adequate amounts of saliva, minimal intrusiveness, ease of collection, and simplicity of portability and storage) and comparison of baseline cortisol levels, assessing development of diurnal pattern, and the relationship of associated demographic factors to extant studies of salivary cortisol in preterm infants.

2. Methods

2.1. Filter extraction and cortisol assay

2.1.1. Saliva pool

Saliva pools were created as needed from 6 healthy subjects (typically equal males and females) who provided 10–20 ml of saliva each. Saliva was collected prior to lunch using Trident Original Flavor gum to stimulate saliva flow if necessary. Samples were collected into 50 ml conical tubes and allowed to freeze. The samples were spun to remove mucins from the saliva, and individual fluid saliva samples were assayed for CORT. These samples then were pooled and placed in separate aliquots. This pool was run in duplicate as seven separate whole saliva aliquots. These pools were used in the validation assays described below. Whole saliva from the pools was always run as 6–10 separate aliquots in the cortisol assay described below to establish the cortisol level for the pool.

2.1.2. Filter extraction

After Whatman Grade 42; 2.4×9 cm filter paper was wetted with saliva and dried, it was carefully cut crosswise with a precision paper cutter to a fixed length. Then the cut end of the filter was placed in a 1.5 ml micro centrifuge tube to which the Salimetrics assay buffer (250–500 μ l) was added in proportion to the exact area of the filter added for extraction. The same filter area to assay buffer ratio was always maintained which varied in accordance with specific lots of filter. The tube containing the filter and buffer was shaken on a platform shaker for 24 h. after which buffer containing the extracted cortisol was added in duplicate to wells of the assay plate as described in the next section.

2.1.3. Salivary cortisol EIA

Salivary cortisol (CORT) concentration in the extraction medium or whole saliva was determined using a commercial high sensitivity EIA kit (Salimetrics, LLC) according to the manufacturer's directions and read at 450 nm on the microplate reader (Dynex MRX). The Salimetrics kit detects CORT levels over the range of 0.19–49.66 nm/l. Standard curves were fitted by a weighted regression analysis using commercial software (Revelation 3.2) for the plate reader. From these curves, unknown values were computed. This kit shows minimal cross reactivity (less than 4% or better) with other steroids present in the saliva. To the wells of the microtiter plate, 50 μ l of either whole saliva or extraction fluid was added in duplicate. Commercial controls and high and low laboratory controls were included on every plate for determination of inter- and intra-assay coefficients of variability, which are less than 6%, respectively. Based on dilutions described above, our detection limit was approximately 1.38 nm/L for the extraction media.

2.2. Validation procedures

Previous papers [12,13] that used a filter collection technique provided little information regarding the procedural steps, and no information with regard to assay validation. Therefore several basic concepts such as storage and shelf life after collecting samples on the filter paper, extraction recovery, and linearity of dilution of extracted samples must be addressed to ensure its validity.

2.2.1. Filter calibration

Whatman Grade 42; 2.4×9 cm filter paper was used for saliva collection and for the validation and application studies. Filter lot absorbency was individually calibrated using one of the saliva pools to which radiolabeled cortisol (100,000 cpm/100 µl final concentration) was added. The area equivalent to absorbing 100 µl of saliva based on tracer activity was determined using the radiolabeled cortisol (H^3 -cortisol) saliva pool. In brief, filters were soaked with the H^3 -cortisol saliva pool and allowed to dry for 24 h by hanging. After drying, different areas of filters were cut and extracted in assay buffer as described in the previous section (Filter extraction). Next, 100 µl replicates of the extraction buffer were added to scintillation vials containing counting fluid. Finally, 100 µl of the H^3 -cortisol saliva pool was added to similar vials of counting fluid. By calculating the ratio of counts/minute (CPM) of 100 µl of the radiolabeled saliva pool to the CPM of 100 µl of the extraction buffer, the absorbency characteristics of each filter lot was determined for a fixed area of the paper.

2.2.2. Storage effects

We stored filters, wetted using the saliva pool, at room temperature, for up to six months prior to assay. Thus filters were assayed immediately after soaking, one and two weeks later, and one month, three months, and six months after wetting and storage at room temperature. The frozen saliva pool was thawed and run in each assay for comparison purposes. Filters were compared to the same saliva pool used in wetting the filters that was stored at $-20^{\circ}C$ until the time of the assay. In addition we maintained a fluid saliva pool at room temperature for comparison to the pool maintained at $-20^{\circ}C$ and the filters maintained at room temperature.

2.2.3. Spiking recovery

We assessed recovery of several levels of CORT added to the saliva pool with a known concentration of CORT. To the saliva pool, CORT (Sigma) was added in three concentrations. Ten separate filter strips were individually dipped in each of four saliva pools (undiluted pool, undiluted pool +2.76 nm/l, undiluted pool +6.90 nm/l, and undiluted pool +13.80 nm/l), excess moisture on the strip was carefully wiped off, and the filters were allowed to dry for 24 h by hanging. Wiping was accomplished by placing the filter between plastic cut from a Ziploc bag and pulling the filter gently through the plastic applying constant light pressure, mimicking the manner in which we instruct adults to collect saliva and to wipe excess saliva from the filter with their lips. The filters were cut and extracted in 0.5 ml assay buffer for 24 h on a shaker platform. The extraction buffer (25 µl) was transferred to the assay plate as described above.

2.2.4. Linearity of dilution

To determine linearity of dilution of extracted filters, extractions from the filters previously wetted with the saliva pool were diluted 2×, 3×, 4× and 6× in assay buffer (six replicates per dilution).

2.3. Application of the filter paper method to preterm infants

2.3.1. Subjects

The study was conducted in a teaching hospital of a midsized metropolitan area, within a 40-bed Neonatal Intensive Care Nursery (NICU). The study was approved by the Colorado Multi-Institutional Review Board, and parental consent was obtained. Infants fit the following criteria: a) 32 through 35 weeks postconceptional age, b) singleton birth, c) no chromosomal anomalies, d) no major surgery, e) no inter-ventricular hemorrhage, e) no current illness, f) no mechanical ventilation at time of data collection, and g) no maternal drug or alcohol abuse. The target number of infants for enrollment was 26, including at least six infants from each of the postconceptional age weeks, 32–35.

2.3.2. Saliva collection

Either the first author (MN) or trained nurses collected saliva every three to four hours, immediately before feedings for three consecutive days. A log was kept for each infant showing activity level of the infant at collection time.

Saliva samples were collected from infants by folding the paper in half lengthwise and placing it on the infant's tongue for 30 s to three minutes, until the lower 1/5 to 1/4 was completely wetted. The furthest extent of the absorption of the saliva was outlined with pencil. The filter paper was dried by hanging the wet end down for three to four hours and the dried papers placed in a sealed plastic bag until all samples were collected for a particular infant. Filters were stored at room temperature until assayed. In some cases, the area on the filter from some of the infants was not sufficiently saturated to represent 100 µl of fluid saliva and extraction buffer was adjusted proportionately.

2.3.3. Salivary cortisol EIA

All samples from an infant were included in the same assay batch to minimize within subject inter-assay variance. Papers were cut based on the extent of wetting of the paper. Extraction buffer volume was adjusted appropriately to maintain a consistent paper area to buffer volume relationship.

2.3.4. Data analysis

For samples collected from the infants, morning means and ranges of the salivary cortisol levels found in this study were compared to those reported in other studies of infants with similar postconceptional age in which other methods of salivary cortisol collection were used. Associations with demographic factors also were examined using hierarchical linear modeling [14]. This approach examined the possible associations between cortisol level and the collected demographic characteristics of the study sample. The overall pattern for all subjects was examined as well, i.e., the association between cortisol level and time. Hierarchical linear modeling permits taking into account the corre-

lation among the measurements at different times and different days for the same subject. At the first step, each of the following demographic characteristics was entered into the model separately to examine its unique association with the cortisol level: postconceptional age, postnatal age, hyperbilirubinemia, APGAR scores at 1 min, whether or not the mother received antenatal corticosteroids, and days since the mother received antenatal corticosteroids. The variables time and day were included in the model to assess if cortisol levels changed from time to time and from day to day. Although infants were fed every three hours, their schedules varied somewhat. Therefore, times were grouped into seven two-hour periods: 1) 0500–0700, 2) 0800–1000, 3) 1100–1300, 4) 1400–1600, 5) 1700–1900, 6) 2000–2200, and 7) 2300–2400 h.

For the second step, all the variables that were associated with cortisol at a significance level of 0.1 were entered into a final model to assess their association while adjusting the effects of other variables in the model. Stepwise model selection included choosing the model containing parsimonious predictors as well as the best correlation structure for the data. The selection was based on Akaike [15] information criteria (AIC). Due to the skewness of the cortisol distribution, the logarithm to the base 10 of cortisol was used to normalize the data.

Another method of analyzing cortisol diurnal rhythm was described by Krieger et al. [16], who defined the presence of a diurnal pattern as when all levels after the 0800 h level are less than 75% of the 0800 h level. Cortisol levels for each time period for each infant were aggregated and the Krieger criteria used to determine if any infants in this sample showed a diurnal pattern. Levels between 0600 and 0900 h were used for the morning peak level.

3. Results

3.1. Validation

Validity and reliability for the filter papers were assessed in four ways: 1) comparing cortisol levels obtained from whole saliva aliquots to levels obtained from filter papers wetted with the same saliva pool, 2) assessing cortisol levels obtained from filters stored at room temperature for up to six months compared to the saliva pool maintained at -20°C , 3) verifying spiking-recovery between whole saliva and extracted filter papers, and 4) verifying linearity of dilution.

3.2. Saliva pool

The average cortisol level of seven replicates of the saliva pool was 12.14 ± 0.28 nmol/L. The average cortisol level found from the saliva extracted from 32 filters wetted with the saliva pool was 11.31 ± 1.38 nmol/L or 92% recovery. Extractions of six filters wetted in the same saliva pool showed a standard deviation of 9.2%.

3.2.1. Storage effects

As indicated in Fig. 1, filters dried and maintained at room temperature compared favorably to the whole saliva pool maintained at -20°C , which was used to wet the filters. In contrast, cortisol levels of fluid saliva stored at room

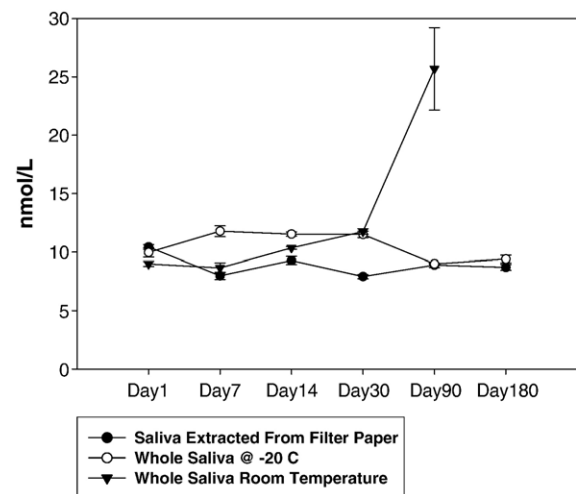


Figure 1 Comparison of salivary cortisol levels using filter paper versus whole saliva from 1 to 180 days.

temperature showed an increase beginning at two weeks. Thus filters can be stored at room temperature for up to six months and possibly longer without a loss of signal.

3.2.2. Spiking recovery

Generally extraction recoveries exceeding 50% are considered adequate [13]. Recovery for CORT from extracted filters across the undiluted saliva pool and three spiked saliva samples concentrations averaged 91% for the spiked whole saliva samples and 86% for the spiked filters. The correlation between whole saliva and the extracted filters was 0.98 across the four cortisol levels. We have cross validated results based on the Salimetrics assay to results obtained for the same samples with mass spectroscopy and found that the two procedures were highly correlated ($r=0.98$).

3.2.3. Linearity of dilution

The average percent recovery compared to undiluted extraction buffer was 100.5% (± 21.1), 113.3% (± 25.3), 103.3% (± 33.4), and 195.5% (± 92.4), respectively for dilutions of 2 \times , 3 \times , 4 \times , and 6 \times . The higher dilution (6 \times) fell outside linear range of the standard curve and not surprisingly, the 6 \times dilution was considerably in error.

3.3. Application of the filter paper method

Seventy-two percent of parents who were approached consented their infants to be in the study. A total of 26 infants served as subjects. Demographic characteristics of participating infants are given in Table 1. No demographic differences were apparent between infants of consenting and nonconsenting parents. Thirteen infants were 32–33.9 weeks postconceptional age and thirteen infants were 34–35.9 weeks postconceptional age. Fifteen (58%) of the infants were Caucasian, seven (27%) were Hispanic, three (11%) were African American, and one (4%) was American Indian. Eleven infants (42%) were boys.

In addition to respiratory problems, eight infants (31%) had other complications (sepsis, twin to twin transfusion, thrombocytopenia, hypoglycemia or hyperglycemia) that

Table 1 Demographic characteristics of the study sample

	M	SD
Postconceptional age at collection time (weeks)	33.7	1.1
Postnatal age at collection time (days)	9.2	6.1
Birth weight (g)	1815.7	372.5
1 min APGAR	7	1.8
5 min APGAR	8.4	0.8
Days since administration of maternal antenatal steroids	19.8	17.6
Maternal age (years)	26.8	6.4
Percent of infants with hyperbilirubinemia	61.5	

were resolved. Sixteen infants (61.5%) had been treated for hyperbilirubinemia. Fifteen mothers (35%) were primigravida and 20 mothers (80%) were in a stable relationship with the infant's father. Seven mothers (27%) experienced delivery complications (severe pre-eclampsia or chorioamnionitis). Mothers of 21 infants (81%) received one to three doses of prenatal betamethasone 4 to 66 days before cortisol sampling.

The nursery was divided into four sections: two for infants requiring acute care (mechanical ventilation, post surgical care and emergency admission) and two for infants requiring non-acute care (feeding, oral medications, incubator care). Twenty-one infants were in the two non-acute areas of the nursery, four were in one of the acute rooms, but in a quiet corner and one was in an acute room in the midst of activity.

3.3.1. Saliva collection

Samples were collected every three hours from 5:00 AM through midnight, allowing the potential to collect a total of 546 samples. Of these potential samples, 138 (25.3%) were not obtained due to either staffing shortages (110 samples) or discharge of the infant before study completion (28 samples). Eight samples (<2%) had inadequate infant saliva for analysis. A total of 400 samples were finally assayed (73.3% of the total possible samples). Of these samples, four were below the limit of detection, and seven samples from one infant were omitted because of infant fussiness (5) or values above the upper limit of detection (2).

3.3.2. Comparison of findings with those of other studies

Salivary cortisol levels noted in this study were compared to cortisol levels reported in other papers that included infants of similar postconceptional age (see Table 2). In addition, we found a range of cortisol levels from 1.8 to 47.9 nmol/L, comparable to the range reported by Bettendorf et al. [10] which was 0.6 to 52.1 nmol/L.

3.3.3. Diurnal pattern and factors associated with cortisol levels

Results of hierarchical linear modeling indicated that the variable day was significant, suggesting that the measured cortisol values changed depending on the day the samples were taken. For example, on day 1, the average log10

Table 2 Study comparison of salivary cortisol levels in preterm infants

Study	Postnatal age (days)	Time of collection	Relationship to feeding	Collection method	Assay method	Mean AM levels (nmol/L)
Current study (n=26)	3–27	5–9 AM	Immediately before (3 h past last feeding)	Filter paper	ELISA	8.0 (5/6 AM) 8.5 (8/9 AM) median = 7.3
Antonini et al. [9] (n=9)	14	8–9 AM	?	Feeding tube	RIA	8.0 (approximate) ^a
Davis et al. [8] (n=18)	3–6	6–9 AM	2 h after feeding	Cotton applicator	Fluorescence immunoassay	16.7 (approximate) ^a
Herrington et al. [11] (n=19)	5–24	1–6 AM	Immediately before (3 h past last feeding)	Cotton applicator	ELISA (Salimetrics lab)	17.3 (approximate) 2.2 (approximate) ^a
Bettendorf et al. [10] (n=10)	15–39	6 AM	Immediately before (3 h past last feeding)	Feeding tube	ELISA (Study lab) RIA	Median 5.5

^a Approximate values were obtained from graphs.

cortisol concentration was 0.16 log unit higher than that on day 3, while that value on day 2 was not much different from day 3. Time was not significant. We then examined the demographic variables associated with cortisol while adjusting the variable “Day” in the model. The best model included Day, one-minute APGAR scores, and postconceptional age as predictor variables, with the correlation structure assuming the same correlation among different measurements for the same subject. The calculated correlation equaled 0.3. The fixed effects parameter estimates are summarized in Table 3.

The estimated coefficients for APGAR and postconceptional age were both inversely associated with the outcome variable, the logarithm of cortisol. Lower cortisol values were associated with higher one-minute APGAR scores after adjusting the postconceptional age and the days the measurements were taken. The same was true for postconceptional age; lower cortisol values were associated with higher postconceptional age. The variable day was significant, suggesting that the cortisol values fluctuated from day to day. The cortisol values were not significantly associated with the variable “Time” or any functional format of time, indicating that there was no rhythm pattern for the sample as a whole.

After applying the Krieger [16] method to the aggregated daily levels, only four infants, or 15% of the sample, showed a typical diurnal pattern. These infants ranged from 4 to 18 days of age. No unifying factor that might be associated with the appearance of the typical diurnal pattern was apparent among these infants. Although they did not display the typical cortisol diurnal pattern with the highest levels in the early morning, 12 infants (46%) showed a time-skewed diurnal pattern. Ten of these infants had a single peak time (level higher than 75% of other daily levels) and two showed a bi-phasic pattern (two peaks). Fig. 2 shows cortisol levels for infants whose patterns exemplify a typical diurnal pattern, while Fig. 3 presents examples of time-skewed diurnal patterns and a pattern lacking diurnal rhythm.

4. Discussion

Aims of this study were to first establish the validity of using filter paper for saliva collection, and second to apply the filter paper for collecting saliva for cortisol assessment in preterm infants. Results indicated that the filters provide a valid means for saliva collection for cortisol assessment that could easily be applied to preterm infants in a neonatal intensive care nursery.

Validity of the filter paper method for collection of salivary cortisol was demonstrated by 92% recovery from the filters, no effect of storage for up to six months, acceptable

Table 3 Parameter estimates from hierarchical linear modeling

Fixed effect	Estimate	Std error	P-value
Day (day 3 as reference)			
Day 1	0.16	0.05	0.004
Day 2	0.001	0.05	
One minute APGAR	-0.11	0.03	0.002
Postconceptional age	-0.18	0.06	0.003

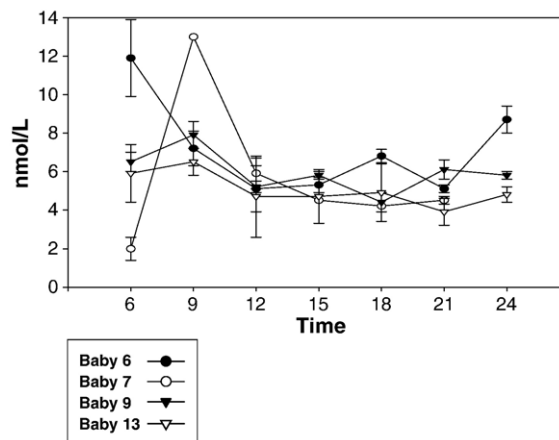


Figure 2 24-hour cortisol levels of infants displaying diurnal patterns.

spiking recovery in the extracted saliva, and linearity of dilution up to 4×. Even though the higher dilution (6×) fell outside the linear range of the standard curve, we are convinced that the assay buffer allows acceptable linearity of dilution for the filter strips.

Most importantly, the method was easily and successfully used to collect saliva from hospitalized preterm infants between 32–35 weeks postconceptional age, with <2% loss of samples due to inadequate saliva. Salivary cortisol levels obtained were within the range of those reported in other studies. An inverse association was found between postconceptional age and infant cortisol levels, and one minute APGAR scores and infant cortisol levels. Infants displayed variable daily cortisol patterns and no discernable rhythm in the hierarchical modeling analysis, but four infants displayed a typical diurnal pattern using the Krieger criteria [16].

Validation studies indicated that cortisol levels from dried saliva extracted from filter papers are similar to levels found in whole saliva. Extracted samples showed the expected linearity of dilution as well as adequate recovery. It is important to note that the level of frozen whole saliva

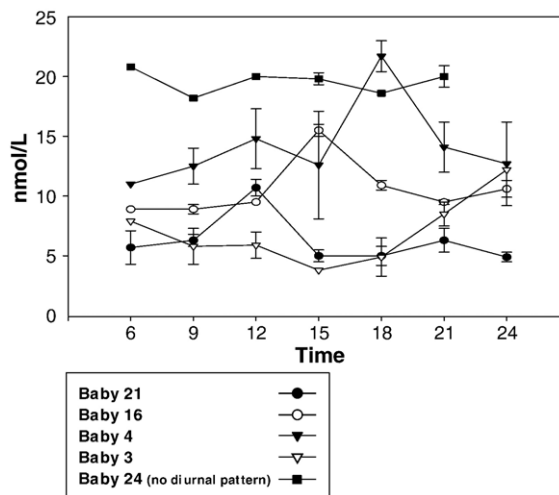


Figure 3 24-hour cortisol levels: examples of infants displaying time-skewed diurnal patterns and no diurnal pattern.

and saliva that was dried on filter paper kept at room temperature before extraction were similar. This affords a unique advantage to this collection approach, e.g., the lack of a need for refrigeration. Thus, salivary cortisol levels obtained using filter papers represent a valid alternative to other methods of collection.

Infants sucked on the filter paper without difficulty. Although infant distress has not been reported with other saliva collection methods, time to collect the saliva has been reported to take up from five to ten minutes [8–11]. Time to collect saliva in this current study was 30 s to three minutes. Even if not distressed by saliva collection, infants become aroused. A goal of care for hospitalized infants is to allow adequate rest periods between feedings. Thus, minimizing saliva collection time is desirable, especially when obtaining multiple samples. Brief or no arousal during multiple sample collection during a short time period also will be less likely to affect levels of subsequent samples.

Salivary cortisol levels obtained in this study were within the range of some studies [9,10] but not others [8]. Methodological differences most likely contributed to level variations. In one study yielding higher levels [8], saliva was collected two hours after a feeding versus three hours in the current study. Thus, the sleep cycle was interrupted at different times in the two studies. Laboratory differences also may contribute to variations in levels. In the other study, two labs were used. Although values obtained by the two laboratories differed considerably, patterns were the same. It is of note that salivary cortisol levels comparing our mass spectroscopy results and our Salimetrics results were quite comparable. In contrast, comparison of a second commercial EIA procedure (DSL, Inc.) to the Salimetrics and mass spectroscopy results noted 140% higher levels for the DSL assay. Thus assay variations could easily account for the differences noted across laboratories.

Settings for all studies were NICUs. The noise and activity level of the nurseries, however, were not discussed in previous papers and may have differed considerably across studies. The infant with the highest cortisol levels in the present study was in an area of the nursery with greater noise and activity. Study of the effects of noise and activity on cortisol levels of hospitalized preterm infants is needed.

A similar inverse association was found in our study between cortisol levels and postconceptional age as reported by others [17]. The lack of association between baseline cortisol levels and antenatal steroids in preterm infants more than two days of age also is similar to findings of others [18,19]. The inverse association between one-minute APGAR scores and cortisol levels in this study suggests that infants with higher levels were stressed either prenatally or by the birth process. Detailed prenatal information was not available, but infants were healthy and required no or minimal oxygen at collection time as inclusion criteria. No association was found between postnatal age and cortisol levels as previously reported [17]. Wittekind et al. [20] reported decreasing values with postnatal age, but the drop in levels did not occur until the 4th week of life. Only one infant in our study was four weeks of age and consequently we did not examine longitudinal decrease in cortisol levels past three weeks.

There was considerable variability in cortisol levels in the infants in the present study as noted by others [21]. No stable cortisol diurnal pattern was identified using hierarchical linear modeling. However, when individual rhythms were examined using the Krieger et al. method [16], four infants (15% of sample) two weeks of age and younger showed a typical diurnal pattern and 46% of infants showed a time-skewed diurnal pattern. Antonini et al. [9] found indications of a cortisol diurnal rhythm at 2 weeks of age in 25% of a sample of preterm infants. Both Antonini et al. [9] and de Weerth et al. [21] reported an association between the development of circadian sleep cycle and the development of a stable diurnal circadian cortisol pattern. Since infants in the current study were awakened every three to four hours throughout the day and night for feedings, the development of a circadian sleep cycle may have been disrupted, yet several infants showed a diurnal pattern. Although it was beyond the scope of this study to investigate stability in diurnal cortisol patterns, it is possible that the diurnal patterns observed in infants in this study were transitory. De Weerth et al. [21] found variability in diurnal patterns in infants up to 5 months of age. The effect of disrupted sleep cycle, health, extended hospitalization, and a host of other factors on the development of a diurnal cortisol pattern needs investigation.

5. Summary

The filter paper collection method represents a valid method for saliva collection for cortisol determination. Furthermore, it is adaptable for use with at-risk preterm infants. Collection time is relatively brief, resulting in minimal or no infant arousal during collection. Because the papers can be stored at room temperature for up to six months, refrigeration is unnecessary. Differences in collection, settings, and laboratories make comparison of actual levels difficult across studies and may have contributed to the lack of close correspondence between salivary cortisol levels reported in various papers. Levels observed using the filter paper method were within the range of other methods. Associations between demographic factors and cortisol levels were similar to those reported elsewhere. No diurnal pattern was found in the preterm infant sample as a whole, but four infants displayed individual typical diurnal patterns. Although cutting, extraction, and calibration of the filters is more involved, saliva collection, which is a real difficulty in this population and generally out of control of the experimenter, is considerably simpler using the filters. Using this method for collection, longitudinal studies of cortisol levels in preterm infants investigating prenatal, demographic, and environmental factors will improve understanding of how the typical cortisol pattern develops.

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