Non-invasive surrogate markers for plasma cortisol in newborn infants: Utility of urine and saliva samples and caution for venipuncture blood samples

Hisayoshi Okamura[1], Masahiro Kinoshita[2], Hiroki Saitsu[2,3], Hiroshi Kanda[2], Sachiko Iwata[1,2], Yasuki Maeno[2], Toyojiro Matsuishi[1,2], and Osuke Iwata[1,2,3]*

Monitoring plasma cortisol in neonates

1. Centre for Developmental and Cognitive Neuroscience, Kurume University School of Medicine, Kurume, Fukuoka, Japan

2. Department of Paediatrics and Child Health, Kurume University School of Medicine, Kurume, Fukuoka, Japan

3. Division of Pediatrics, St Mary's Hospital, Kurume, Fukuoka, Japan

*Corresponding author: Dr Osuke Iwata

Centre for Developmental and Cognitive Neuroscience

Department of Paediatrics and Child Health, Kurume University School of Medicine

67 Asahimachi, Kurume, Fukuoka 830-0011, Japan

E-mail: o.iwata@ucl.ac.uk

Tel: +81-942-31-7565 Fax: +81-942-38-1792

Conflicts of interest

The authors declare no support from any organisation for the submitted work; no financial relationships with any organisations that might have an interest in the submitted work in the previous 3 years; and no other relationships or activities that could appear to have influenced the submitted work.

This work was supported by the Japan Society for the Promotion of Science, The Ministry of Education, Culture, Sports, Science, and Technology (Grant-in-Aid for Scientific Research C24591533, Grant-in-Aid for Young Scientists B: 24730613 and B01-24119004, Constructive Developmental Science, Innovative Areas).

Key words

plasma cortisol; arterial blood; venipuncture blood; saliva; urine; non-invasive marker; newborn infant

Word count: 1,788

Figures (colour figure): 1 (0)

Tables: 1

Abstract

Context: Hypothalamus-pituitary-adrenal (HPA) function is associated with important physiological/pathological events in neonates. Plasma/serum cortisol levels have been used to assess HPA function. Several non-invasive surrogate markers have been used without sufficient validation.

Objective: The objectives of the study were to investigate whether plasma cortisol levels are correlated with those in saliva and urine and whether these correlations are affected by procedural pain at blood sampling.

Design, Setting and Patients: Fifty neonates were recruited from a tertiary neonatal intensive care unit. Saliva and urine samples were collected shortly before routine clinical blood sampling.

Main Outcome Measures: Cortisol levels were compared between plasma and non-invasive samples using linear regression analysis for the entire study population and groups, whose blood was obtained via indwelling arterial catheters (group A) or by venipuncture (group V). Predictive values of salivary/urinary cortisol for low plasma cortisol levels <2.0μg/dL were evaluated by receiver-operating characteristic analysis.

Results: Plasma cortisol showed linear correlations with salivary and urinary cortisol for the entire study population and patients in group A (all P <0.0002), but not in group V. Areas under

curves of salivary and urinary cortisol to predict low plasma cortisol levels were 0.87 (95% CI 0.78-0.97) and 0.84 (95% CI 0.74-0.95), respectively.

Conclusions: Cortisol levels from saliva or urine samples were shown to be useful surrogate markers for plasma cortisol levels in neonates. Caution is required in interpreting findings of plasma cortisol levels in young patients, when blood samples are obtained by venipuncture, because procedural pain may induce alteration of cortisol levels.

Introduction

A range of physiological and pathological conditions in neonates depend on the hypothalamus-pituitary-adrenal (HPA) function. Increased foetal plasma cortisol induces lung maturation and causes adaptation to extra-uterine stress (1). Cortisol administration ameliorates chronic lung disease and pressor-resistant hypotension in immature infants (2, 3). Further understanding of the HPA axis regulation may considerably improve the treatment of high-risk neonates. HPA function has primarily been assessed using plasma cortisol (4); for young patients, blood sampling under low-stress conditions is recommended to minimise pain-induced cortisol elevation (5).

Salivary cortisol has been used as a surrogate marker of plasma cortisol (6), which has additionally been validated in preterm-born infants during the corticotropin-releasing hormone (CRH) stimulation test (7). This needs to be confirmed under physiological conditions, where the dynamic range of cortisol is approximately one third of that with CRH stimuli (8). Urinary cortisol has also been used to estimate plasma cortisol levels (9), the relevance of which also needs to be confirmed in neonates.

This study aimed to investigate (i) whether salivary and urinary cortisol reflect plasma

cortisol levels under physiological conditions, and (ii) whether the relationship in cortisol levels between non-invasive samples and blood samples differs according to the blood sampling procedure.

Materials and Methods

This study was conducted with the approval of the Ethics Committee of Kurume University School of Medicine. Written informed consent was obtained from a parent of each neonate.

Study population

Fifty neonates were recruited from a tertiary neonatal intensive care centre (Kurume University Hospital, Kurume, Fukuoka, Japan) between December 2011 and August 2013. Neonates who were in a life-threatening condition, too immature (body weight <1000g and/or corrected age <32 weeks), anaemic (blood haemoglobin <12g/dL and/or on erythropoietin therapy), and/or on cortisol supplementation, were not included. Clinical variables of participants, such as gestational age, birth weight, antenatal/postnatal corticosteroid administration, and delivery mode, were collected from the patient's record.

Sample collection

Sample collection was performed on the day when regular blood sampling was scheduled. In our unit, regular blood sampling is performed at 10:00h in the morning to allow sufficient time after the latest 3-hourly feeding at 07:00h. A rectangular cotton pad (5cm×6cm×0.3cm) was inserted within the diaper at 09:00h with maximum caution not to stimulate neonates. The

diaper was checked every 30min until urination was confirmed. The cotton pad was then secured within a sealable plastic bag. Saliva samples were collected using an absorbent swab stick (Sorbette; Salimetrics, State College, PA, USA) as previously reported (8). A sufficient amount of saliva sample was assumed when saliva oozed out of the swab end when pressed to the inner wall of the conical tube. A blood sample was then obtained either by venipuncture or via an indwelling arterial catheter when applicable. In our unit, arterial catheters are inserted for neonates with a gestational age of <26 weeks, unstable cardio-pulmonary conditions, or scheduled surgical operations. Blood sampling for a cortisol assay (0.6mL) was performed immediately after routine blood sampling (0.2–1.2mL). We aimed to collect saliva and blood samples within 10–15min after the urine was collected. All samples were then centrifuged at 4000rpm for 10min, and were kept at −80ºC until assayed. Repeated sampling from the same newborn was limited only to those who were healthy with a haemoglobin level of >15mg/dL, with an interval between samplings >2 weeks.

Cortisol assay

Minimum sample volumes required for the assay were 50μL (urine), 25μL (saliva) and 100μL (plasma). Salivary cortisol levels were determined by enzyme immunoassay (High Sensitivity Salivary Cortisol ELISA Kit; Salimetrics, Carlsbad, CA, USA). The limit of detection of this

assay in our laboratory was 0.19nmol/L, and the intra- and inter-assay coefficients of variation were 5.43% and 6.41%, respectively. Plasma and urinary cortisol were measured using a cortisol enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI, USA). The limit of detection of this assay in our laboratory was 1.24nmol/L, and the intra- and inter-assay coefficients of variation were 7.61% and 8.22%, respectively.

Data analysis

To minimise the bias caused by the sample collection, analysis was performed only for patients with a complete data set of plasma, salivary and urinary cortisol. The correlations among plasma, salivary and urinary cortisol of the overall samples were assessed using the Pearson's correlation coefficient. To examine the effect of procedural pain on the relationships between salivary/urinary cortisol and plasma cortisol, correlation analyses were repeated for groups of newborns, whose blood was collected by venipuncture (group V) or via an indwelling arterial catheter (group A) (Bonferroni-corrected for two groups). Baseline characteristics and cortisol levels of neonates were compared between groups using the Student's t-test and $\chi 2$ test where applicable (Bonferroni-corrected for 10 clinical variables and three sample types). Finally, threshold values of salivary/urinary cortisol to predict low plasma cortisol levels of <55nmol/L (2.0μg/dL: determined based on the reference range in term-born neonates, below which

dynamic tests are recommended (5)) were assessed using the receiver-operating characteristic (ROC) analysis.

Results

Data profile

– Overall study population:

Two neonates were excluded because of insufficient saliva volume. Two other newborns were studied twice each with intervals of 21 and 16 days. Eventually, 50 complete sample sets from 48 neonates were analysed. Indications for hospitalisation for these neonates were low birth weight (n=33), congenital blood disease (n=4), maternal gestational diabetes mellitus (n=4), congenital heart disease (n=3), neonatal encephalopathy (n=2), infection (n=2), maternal hyperthyroidism (n=1) and suspicion of metabolic disease (n=1) (see Table 1 for other profiles). Urinary cortisol levels were the highest, which were followed by those in plasma and salivary samples (all difference P <0.0003) (Table 1).

– Group characteristics:

Neonates in group A had a smaller birth weight, gestational age and Apgar scores at 1 and 5min compared with those in group V (P <0.05, 0.001, 0.001 and 0.05, respectively) (Table 1). More newborns in group A were administered postnatal corticosteroids and had more days of respiratory support compared with those in group V (P <0.01 and 0.005, respectively); other variables were similar between the two groups.

Relationships between plasma, salivary and urinary cortisol

– Overall study population:

Plasma cortisol levels were positively correlated with salivary (r=0.71, P <0.0002) and urinary (r=0.61, P <0.0002) cortisol levels. Salivary and urinary cortisol levels also showed a linear correlation between each other (r=0.68, P <0.0002) (Figure 1A-C).

– Group analysis:

Plasma cortisol levels were positively correlated with salivary and urinary cortisol levels in group A (r=0.82 and 0.67 respectively; both P <0.0002), but not in group V (r=0.43 and 0.42, respectively) (Figure 1A-C).

– Receiver-operating characteristic analysis:

For the entire study population, mean areas under ROC curves (AUCs) to predict low plasma cortisol levels were 0.87 (95% CI 0.78-0.97) and 0.84 (95% CI 0.74-0.95) for salivary and urinary cortisol, respectively (Figure 1D). The optimal sensitivity and specificity were obtained with cut-off values of 12.7 nmol/L (sensitivity 0.76, specificity 0.83) for salivary cortisol and 109.0 nmol/L (sensitivity 0.76, specificity 0.83) for urinary cortisol. In group A, AUCs improved

to 0.94 (95% CI 0.85-1.00) for salivary cortisol and 0.87 (95% CI 0.73-1.00) for urinary cortisol, whereas, in group V, the AUC decreased to 0.77 (95% CI 0.58-0.95) for salivary cortisol and 0.80 (95% CI 0.62-0.98) for urinary cortisol (Figure 1, E and F).

Discussion

Salivary and urinary cortisol is well-established surrogate markers for plasma cortisol in adults and adolescents (6, 9). Consistent to a previous observation in immature neonates under CRH stimulation (7), we observed a robust linear correlation between salivary and plasma cortisol levels under physiological conditions, thus expanding the utility of salivary cortisol for virtually all neonates. In a previous study, sufficient saliva samples for the assay were collected only in 46% of infants (10), which contrasts with a high success rate of 96% in our study. This might be associated with our protocol to check the approximate sample volume during collection using a simple manoeuvre (see “Sample collection” for detail). However, saliva collection may still be burdensome for longitudinal studies over days. In our study, we additionally assessed urinary cortisol levels, which showed a marked correlation with plasma cortisol values. Urine collection using cotton pads or disposable diapers is simple and non-invasive (11). The use of urinary samples provides additional information, such as total cortisol secretion per day when serially collected (12). However, this technique might not be optimal when the exact timing of changes in cortisol levels is important, because urinary cortisol is likely to reflect plasma cortisol levels when urine is extracted from blood, rather than when urine is collected (13). Urinary cortisol levels may also be affected by the amount of urination; correction of urinary cortisol for creatinine may improve the reliability of

this technique (14). These limitations need to be considered for the use of urine cortisol in routine clinical practice, where considerable intra- and inter-patient variability is observed in the amount and interval of urination.

Our secondary analysis showed that robust linear correlations between plasma and salivary/urinary cortisol levels were observed only for neonates, whose blood was sampled via an indwelling arterial catheter. Painful procedures induce sympathetic stimulation and subsequent increase in circulating adrenocorticotropic hormone within less than a few minutes (15). Our findings indicate that assessment of plasma cortisol using venipuncture blood might not be reliable, at least for neonates. Studies in the 1980–90s observed an overt diurnal rhythm in plasma cortisol only after a few months of birth (16, 17). Other studies failed to reduce a cortisol response to painful procedures by analgesia (18). Although these findings were attributed to immature HPA functioning, recent studies, which used salivary cortisol markers, showed that diurnal cortisol rhythms were present even shortly after birth (8); other studies have shown that non-nutritive sucking, maternal skin-to-skin contact and gentle sensory stimuli reduced excessive adrenal responses to procedural pain in infants (19, 20). Taken together, blood sampling via venipuncture may induce a prompt elevation in cortisol according to the response of each neonate, rendering these invasive samples unsuitable for assessment of adrenal

function at a young age. Current understanding of the developing HPA axis may carefully be re-appraised when knowledge is obtained from studies that use invasive blood samples.

Limitations of the study

Because of ethical reasons, arterial and venous blood samples were not obtained from the same patient. Therefore, caution for cortisol levels of venipuncture blood is only suggested from indirect observations. Although we expected pain-induced elevation of plasma cortisol in some group V patients, cortisol levels were relatively higher in group A. This finding could be explained by the greater severity of condition and postnatal corticosteroid administration for newborns, who required an arterial catheter. Finally, our study population was recruited from a neonatal intensive care centre, whose clinical background and environmental factors were different from their healthy peers.

Conclusions

Salivary and urinary cortisol levels can be used for both snap-shot and serial monitoring of plasma cortisol levels in neonates. Blood samples obtained by painful procedures may not be suitable for the assessment of resting plasma cortisol levels. Specific conditions, where cortisol levels of venipuncture blood become unreliable, need to be identified.

Acknowledgements

The authors thank the patients who participated in the study and their parents for their cooperation, the nurses of the Neonatal Intensive Care Unit, Kurume University Hospital for their support, Dr Yuko Araki for her guidance in statistical analysis, Drs Mitsuaki Unno, Naoko Hara, Junichiro Okada and Akiko Hirose for their contribution to data collection and Ms Chihoko Urata, Chiho Yoshii and Chiaki Ueno for their consistent support and encouragement.

References

1. **Bolt RJ, van Weissenbruch MM, Lafeber HN, Delemarre-van de Waal HA** 2002 Development of the hypothalamic-pituitary-adrenal axis in the fetus and preterm infant. J Pediatr Endocrinol Metab 15:759-769
2. **Doyle LW, Davis PG, Morley CJ, McPhee A, Carlin JB** 2006 Low-dose dexamethasone facilitates extubation among chronically ventilator-dependent infants: a multicenter, international, randomized, controlled trial. Pediatrics 117:75-83
3. **Okada J, Iwata S, Hirose A, Kanda H, Yoshino M, Maeno Y, Matsuishi T, Iwata O** 2011 Levothyroxine replacement therapy and refractory hypotension out of transitional period in preterm infants. Clin Endocrinol (Oxf) 74:354-364
4. **Kudielka BM, Buske-Kirschbaum A, Hellhammer DH, Kirschbaum C** 2004 HPA axis responses to laboratory psychosocial stress in healthy elderly adults, younger adults, and children: impact of age and gender. Psychoneuroendocrinology 29:83-98
5. **Donohoue P, Wolfsdorf J, Pierrat A** 2014 Diagnosis of adrenal insufficiency in children. In: UpToDate, Post TW (Ed), UpToDate, Waltham, MA. Accessed on June 12, 2014.
6. **Voegtline KM, Granger DA** 2014 Dispatches from the Interface of Salivary Bioscience and Neonatal Research. Frontiers in endocrinology 5:25
7. **Matsukura T, Kawai M, Marumo C, Iwanaga K, Yoshida K, Shibata M, Niwa F, Hasegawa T, Heike T** 2012 Diagnostic value of salivary cortisol in the CRH stimulation test in premature infants. J Clin Endocrinol Metab 97:890-896
8. **Iwata O, Okamura H, Saitsu H, Saikusa M, Kanda H, Eshima N, Iwata S, Maeno Y, Matsuishi T** 2013 Diurnal cortisol changes in newborn infants suggesting entrainment of peripheral circadian clock in utero and at birth. J Clin Endocrinol Metab 98:E25-32
9. **Morineau G, Boudi A, Barka A, Gourmelen M, Degeilh F, Hardy N, al-Halnak A, Soliman H, Gosling JP, Julien R, Brerault JL, Boudou P, Aubert P, Villette JM, Pruna A, Galons H, Fiet J** 1997 Radioimmunoassay of cortisone in serum, urine, and saliva to assess the status of the cortisol-cortisone shuttle. Clinical chemistry 43:1397-1407
10. **Herrington CJ, Olomu IN, Geller SM** 2004 Salivary cortisol as indicators of pain in preterm infants: a pilot study. Clinical nursing research 13:53-68
11. **Alam MT, Coulter JB, Pacheco J, Correia JB, Ribeiro MG, Coelho MF, Bunn JE** 2005 Comparison of urine contamination rates using three different methods of collection:

clean-catch, cotton wool pad and urine bag. Annals of tropical paediatrics 25:29-34

12. **Siamopoulou-Mavridou A, Mavridis AK, Vizandiadis A, Harsoulis P** 1986 Free urinary cortisol immunoreactive levels in premature and full term infants. Acta Paediatr Scand 75:919-922
13. **Lasley BL, Mobed K, Gold EB** 1994 The use of urinary hormonal assessments in human studies. Ann N Y Acad Sci 709:299-311
14. **Heckmann M, Hartmann MF, Kampschulte B, Gack H, Bodeker RH, Gortner L, Wudy SA** 2005 Assessing cortisol production in preterm infants: do not dispose of the nappies. Pediatr Res 57:412-418
15. **Meeran K, Hattersley A, Mould G, Bloom SR** 1993 Venepuncture causes rapid rise in plasma ACTH. The British journal of clinical practice 47:246-247
16. **Mantagos S, Moustogiannis A, Vagenakis AG** 1998 Diurnal variation of plasma cortisol levels in infancy. J Pediatr Endocrinol Metab 11:549-553
17. **Vermes I, Dohanics J, Toth G, Pongracz J** 1980 Maturation of the circadian rhythm of the adrenocortical functions in human neonates and infants. Hormone research 12:237-244
18. **Stang HJ, Gunnar MR, Snellman L, Condon LM, Kestenbaum R** 1988 Local anesthesia for neonatal circumcision. Effects on distress and cortisol response. JAMA 259:1507-1511
19. **Cong X, Ludington-Hoe SM, Walsh S** 2011 Randomized crossover trial of kangaroo care to reduce biobehavioral pain responses in preterm infants: a pilot study. Biological research for nursing 13:204-216
20. **South MM, Strauss RA, South AP, Boggess JF, Thorp JM** 2005 The use of non-nutritive sucking to decrease the physiologic pain response during neonatal circumcision: a randomized controlled trial. Am J Obstet Gynecol 193:537-542; discussion 542-533

Table 1: Background clinical variables

	Overall subjects (n=50)	Group-A (n=25)	Group-V (n=25)
Female	23 (46%)	7 (28%)	16 (64%)
Birth weight (g)*	1897 (833)	1545 (800)	2248 (721)
Gestational age (week)***	34.1 (4.7)	31.0 (4.7)	36.8 (2.6)
Posnatal age (day)	7.1 (7.8)	7.2 (7.5)	7.1 (8.3)
Apgar score at 1 min.***	6.9 (2.3)	5.4 (2.4)	7.7 (1.4)
Apgar score at 5 min.*	8.2 (1.5)	7.6 (1.8)	8.8 (0.8)
Cesarean section	25 (50%)	15 (60%)	10 (40%)
Antenatal corticosteroid	16 (32%)	12 (48%)	4 (16%)
Postnatal corticosteroid *	7 (14%)	7 (28%)	0 (0%)
Days on respiratory support[a**]	18.4 (28.7)	32.1 (33.2)	2 (3.9)
Cortisol level (nmol/L)			
Plasma	64.7 (54.3)	76.1 (61.1)	53.3 (47.4)
Saliva**	15.2 (10.3)	20.7 (13.7)	9.7 (6.9)
Urine	113.5 (74.5)	134.7 (90.2)	92.3 (58.8)

Values are shown as the mean (standard deviation) or the number of cases (percent).

[a] Including nasal continuous positive airway pressure, mechanical ventilation and oxygen supplemental care

*$p < 0.05$, **$p < 0.005$ and ***$p < 0.001$ from between-group comparisons corrected for multiple comparisons over 10 clinical background variables and three sample types using the Bonferroni correction.

Figure legend

Figure 1: Relationships among plasma, salivary and urinary cortisol levels

(A-C) Scatter plot showing the association among plasma (arterial or venous), salivary and urinary cortisol levels. Within the overall population, plasma, salivary and urinary cortisol levels showed linear correlations between each other. Plasma cortisol levels showed strong linear correlations with salivary (A) and urinary (B) cortisol levels (r = 0.82 and 0.67 respectively; both $P < 0.0002$) in group A (obtained via an indwelling arterial catheter), but not in group V (obtained by venipuncture; r = 0.43 and 0.42; P = 0.062 and 0.068, respectively).

(D-E) Receiver-operating characteristic (ROC) curves of salivary and urinary cortisol to predict low plasma cortisol levels <55nmol/L. (D) Overall study population: Areas under curves (AUC) were 0.87 (95% CI 0.78-0.97) for salivary cortisol and 0.84 (95% CI 0.74-0.95) for urinary cortisol. Optimal cut-off values for salivary and urinary cortisol were 12.7nmol/L (sensitivity 0.76, specificity 0.83) and 109.0nmol/L (sensitivity 0.76, specificity 0.83), respectively. (E) Neonates in group A: AUC improved to 0.94 (95% CI 0.85-1.00) for salivary cortisol and 0.87 (95% CI 0.73-1.00) for urinary cortisol. Optimal cut-off values for salivary and urinary cortisol were 14.6nmol/L (sensitivity 1.00, specificity 0.83) and 136.9nmol/L (sensitivity 0.77, specificity 0.92), respectively. (F) Neonates in group V: AUC was 0.77 (95% CI 0.58-0.95) for salivary cortisol and 0.80 (95% CI 0.62-0.98) for urinary cortisol; optimal

cut-off values for salivary and urinary cortisol were 9.5nmol/L (sensitivity 0.88, specificity 0.71) and 109.0nmol/L (sensitivity 0.75, specificity 0.82), respectively.

Symbols: △, Group A; ×, group V. Regression lines: bold, whole population; grey, group A; broken, group V.

Figure 1