**Efficacy and feasibility of saliva for assessment of vitamin A status in extremely preterm infants.**

**Background:**

Vitamin A is essential for vision, growth, healing, reproduction, cell differentiation, and immunocompetency. It plays an important role in the development and growth of lung by mediating alveolar formation and septation through the binding of its active metabolite, retinoic acid, to nuclear receptors. (Galambos 2008) The diseases arising from abnormal pulmonary development such as bronchopulmonary dysplasia (BPD) may be linked to vitamin A deficiency. (Shenai 1987, Frank 1988)

Extremely preterm infants (EPI) are at risk of vitamin A deficiency. Timely evaluation of vitamin A status of EPI may help to assess the risk of BPD and tailor vitamin A therapy based on the infant’s vitamin A status without exposing infants to the potential adverse effects of vitamin A.

Assessment of body vitamin A stores is complicated as during the initial phase of vitamin A deficiency, plasma retinol level remains within normal range at the cost of liver vitamin A stores. Therefore, plasma retinol value does not correlate well with liver stores until it becomes very low (< 0.35 mcmol/dL). Relative dose response (RDR) is a better tool to assess body vitamin A stores. (Underwood 1990) However, the test requires repeated blood sampling, which is an invasive procedure and also increases risk of anemia. Therefore, a minimally invasive method with least harm is urgently required.

Recent years have seen increasing use of saliva for diagnostic purposes. Use of saliva for the measurement of hormones and vitamins is gaining attention because of its ease of collection, painless nature and little potential for patient harm. Salivary cortisol level correlates well with the plasma cortisol. Hence, late night salivary cortisol level has become the method of choice for screening of patients with suspected Cushing’s syndrome. (Sakihara 2010) Saral et al have shown a good correlation between salivary and plasma retinol in adults. (Saral 2005) If salivary retinol correlated with plasma retinol in EPI, it will be useful to assess vitamin A status of EPI. In this study we aim to assess correlation between plasma and salivary retinol levels, and usefulness of salivary retinol levels to measure RDR in EPI.

**Hypothesis:**

We hypothesize that salivary retinol level will correlate with plasma retinol and will be useful to calculate RDR in EPI.

**Objective:** To compare 1) salivary retinol with the “gold standard” plasma retinol, and 2) RDR using saliva with the “gold standard” RDR using blood in EPI

**Study design and setting**: This is a prospective diagnostic accuracy study. The study will be conducted in a tertiary neonatal intensive care unit (King Edward memorial Hospital for Women).

**Inclusion criteria:**

1. EPI (Preterm infants less than 28 weeks of gestational age at birth): EPI are at highest risk of vitamin A deficiency as well as BPD. Therefore, assessment of vitamin A status will be important in this population.
2. Tolerating enteral feeds: For the measurement of RDR, we intend to administer vitamin A enterally. Preterm infants are kept nil per orally when they are systemically unwell, are not tolerating feeds or have / suspected to have gut pathology like necrotizing enterocolitis. All these conditions may affect enteral absorption of vitamin A and therefore RDR may not reflect true vitamin A status of the infant. Hence, we chose to include only those infants who are tolerating enteral feeds.
3. Post natal age between 21 and 35 days: First few weeks of postnatal life of EPI are the most vulnerable period for vitamin A deficiency because of the difficulties in the administration of adequate vitamin A through parenteral nutrition and difficulties in the establishment of enteral feeds. Therefore we chose to assess these infants for the assessment. The results of this assessment may be helpful to evaluate efficacy of vitamin A supplementation during first few weeks of life and guide subsequent therapy.

**Exclusion criteria:**

1. Infants with necrotizing eneterocolitis or major congenital gastrointestinal malformation: These infants will be excluded as the condition may contraindicate enteral medication and may also affect vitamin A absorption.
2. Critically unwell patient: These infants will be excluded as often associated paralytic ileus may affect enteral vitamin A absorption.
3. Systemic administration of corticosteroids within 7 days prior to the test: As systemic corticosteroids affect serum vitamin A levels, RDR may not reflect vitamin A status.
4. No parental consent

**Sample size**: A sample of convenience of 20 eligible infants will be recruited. An average 110 EPI are born every year at KEMH. The parents of eligible infants will be approached at 3 weeks of life for the consent. We expect to finish recruitment in 3 months period.

**Study protocol**:

1. Approval from the hospital ethics committee will be taken.
2. Informed consent will be taken by AR from the parents of the eligible infants (Supplement 1 – Parental consent form).
3. For serum retinol estimation, blood sample (1 mL from first five recruited patients and 0.5 mL from subsequent 15 patients) will be taken in a lithium heparin tube for baseline plasma retinol (B0) estimation. Capillary, venous or arterial samples are acceptable, as method of collection does not influence plasma retinol values significantly (Kennedy 1997). Whenever possible, the blood sampling will be performed along with the routine blood investigations to avoid additional prick to the infant. The tube will be labelled and wrapped with aluminium foil to protect the sample from light. Plasma will be obtained immediately by centrifugation at 3000 rpm for 5 min and will be stored at -80 °C till further analysis by AR at KEMH.
4. 1 mL of saliva will be collected (S0) using SalivaBio Infant’s Swab (SIS)® (salimetrics® USA). SIS is recommended for the collection of saliva from infants less than 6 months of age. One end of SIS will be held in infant’s mouth under the tongue securely for up to 3 minutes, till half of the swab gets saturated with saliva. This will be followed by holding the other end in infants mouth to saturate the swab fully. The swab is then folded and placed in a swab storage tube and centrifuged for 15 minutes at 3000 rpm to separate saliva from swab (AR). The saliva will be aspirated and stored in a glass tube wrapped with aluminium foil at -800C till further analysis (AR).
5. After collection of B0 and S0 samples 2000 IU retinyl palmitate (Vitamin A Nepalm ® at dose of 0.04 mL/kg diluted with water for injection to make total volume of 0.5 mL) will be administered through gastric tube (GT) to the infant. GT placement is a part of routine management of premature infants. The feed will be given after instillation of the medication. The GT will not be aspirated or changed for 3-6 hours after the medication. If GT change is needed, it will be done prior to administration of the study medication.
6. Five hours after administration of vitamin A, second blood (B5) and saliva (S5) samples will be collected and stored using the same technique employed for collection and storage of samples B0 and S0 (AR).
7. An additional saliva sample (S7) will be collected at 7 hours after administration of the vitamin A dose.
8. Serum retinol measurement: Serum retinol will be measured using high performance liquid chromatography (HPLC). It has been widely used for measurement of retinol in plasma. It has also been used for retinol analysis in breast milk, parenteral nutrition, tissue samples as well as saliva. We intend to use mass spectrometer (MS) in addition to the UV detector for better identification and quantification of retinol. The analysis will be performed at School of Chemistry and Biochemistry at University of Western Australia by AR under guidance of Assistant Professor Michael Clarke. A/Prof Michael Clarke has extensive experience in use of HPLC and MS. AR will undergo required training and credentialing before commencing vitamin A analysis. To ensure accuracy of measurement, first five patient’s samples will be analyzed in duplicates.
9. Calculation of Relative Dose Response (RDR)

* Blood RDR = (B5 – B0) X 100/B5
* Saliva RDR = (S5 – S0) X 100/S5
* During the initial phase of VA deficiency, plasma retinol level remains within normal range at the cost of liver vitamin A stores. Plasma retinol value does not correlate well with liver stores until it becomes very low (< 0.35 mcmol/dL) RDR reflects vitamin A status of an individual better than plasma retinol. RDR test is based on the principle that during vitamin A deficiency status apo-retinal binding protein (RBP) accumulates in the liver. By giving a challenge dose of vitamin A, the retinol will bind to the excess of RBP in the liver and shipped out in the plasma as the holo-RBP-retinol complex resulting in increase in plasma retinol concentration. The proportion of rise in the plasma retinol concentration directly correlates with the severity of liver vitamin A depletion. RDR value of > 20 % indicates deficient liver vitamin A stores.
* RDR can be calculated either using oil soluble or water soluble preparation of vitamin A. Many studies have used Aquasol A® (Hospira® Worldwide, Inc, USA) preparation of vitamin A via intramuscular or enteral route for calculation of RDR. Aquasol A® is a water soluble vitamin A preparation containing retinyl palmitate as an active ingredient and polysorbate 80 as a solubilizer / emulsifying agent, chlorobutanol as a preservative, and citric acid and sodium hydroxide to adjust pH. However, this formulation is not readily available in Australia. The dose used for RDR measurement in preterm infants ranges from 2000 IU/kg (Mactier 2012, Landman 1992, Ambalavanan 2003, Tyson 1999, Shenai 1987) to 5000 IU (Weinman 2007, Woodruff 1987) and 5000 IU/kg (Zachman 1996).
* Vitamin A Nepalm® (Lexphar® Laboratoire) is a water soluble vitamin A product accessible under Special Access Scheme (SAS) in Australia. It contains retinyl palmitate 100000 IU/2 mL as an active ingredient. It contains following excipients:

1. Polyoxyethylene hydrogenated castor oil 40 (Cremophor RH 40): Cremophor 40 is a nonionic solubilizer and emulsifying agent obtained by reacting 1 mole of hydrogenated castor oil with 40 moles of ethylene oxide. It has not been associated with any developmental toxic or teratogenic or mutagenic adverse effects in vivo and animal studies. It is used as a solubilizer for various fat-soluble vitamins, and essential oils preparations. (Product information)
2. Sodium benzoate: Sodium benzoate has the chemical formula NaC7H5O2. It is widely used as a preservative in food and medicines. It is designated as generally recognized as safe (GRAS) by the Food and Drug Administration (FDA).
3. Hydrochloric acid to adjust pH to 5, alpha tocopherol and water for injection.

Vitamin A Nepalm® is used intramuscularly in patients with vitamin A deficiency when enteral route is not possible, patients with conditions causing malabsorption of vitamin A and vitamin A supply during elemental enteral nutrition. Its dose in adult is 100,000 IU and in children 50,000 IU. (Vitamin A Nepalm® Product information)

**Adverse effect monitoring**: All the infants will be monitored for possible adverse effect of vitamin A administration.

Palpation of anterior fontanel (AF): AF is palpated with infant in a quite state and held in the sitting position. Normal AF is flat, flushed with the skin and soft. (Cloherty, Manual of Neonatal care 7th Ed) A tense and bulging AF may indicate vitamin A adverse effect. The infants with tense and bulging AF with no known cause will have cranial ultrasound examination.

Liver size: Liver will be palpated and recorded in cm below costal margin in the mid-clavicular line.

Skin changes: Desquamation of skin particularly palm and sole, mouth or lip fissures.

**Statistical analysis**: The data will be statistically analyzed using SPSS statistical package (SPS, version 9.0, Chicago, IL, USA). Correlation between serum and salivary vitamin A levels, and blood and saliva RDR values will be tested using Pearson r correlation analysis. Statistical significance will be defined as p < 0.05.

**Results**:

Results will be displayed as scatter plot of:

1. Serum vitamin A (microgram/mL) on X axis versus salivary vitamin a levels (nanogram/mL) on Y axis; and
2. Blood RDR (%) on X axis versus saliva RDR (%) on Y axis.

Pearson correlation value of >0.8 will signify strong correlation between the two variables.

**Justification of project**:

Bronchopulmonary dysplasia is an important morbidity associated with preterm birth with long term health consequences. There is evidence to suggest Vitamin A deficiency in preterm infant is associated with increased risk of BPD. (Shenai 1999) Therefore, it is essential for clinicians to assess vitamin A status of preterm population so that vitamin A deficiency is timely diagnosed and corrected. Current measure of assessment of vitamin A status of infants (RDR) involves multiple blood samples from the preterm infants who are also at risk of anemia. If saliva could be used to assess vitamin A status, the procedure would not cause discomfort and iatrogenic blood loss worsening anemia. This will make the test more acceptable and widely used by clinicians across the world.

**Actual and potential risks to participants and mitigation of risk:**

1. Risk associated with blood collection:

* Pain and discomfort: Blood collection is a common procedure and is routinely done in NICU for clinical purpose. It is a painful procedure and therefore can cause discomfort to infant. Therefore, whenever possible blood collection for the study will be coordinated with the routine blood sampling for clinical purpose to avoid additional prick to the infant. It is a routine practice in the nursery to administer few drops of sucrose before blood collection to reduce discomfort.
* Anemia: Repeated blood sampling can increase risk of anemia in preterm infants. For this study we will require total 2 mL of blood from first five recruited infants for duplicating the measurements to confirm accuracy and repeatability of the results. However, for rest of the 15 infants we plan to collect only 1 mL of total blood.

1. Risk associated with saliva collection:

* Chocking or swallowing of saliva collection swab: Saliva collection will be done with a proprietary, especially designed collection swabs (SalivaBio® Infant Collection Swabs, SIS) for infants less than 6 months age. SIS is made from a durable polymer and it withstands chewing. It is 90 mm long, long enough to hold one end while other end is placed in infant’s mouth, thus eliminating any chocking hazard.

1. Risk associated with vitamin A administration:

* Aspiration: To avoid aspiration of vitamin A administered through gastric tube, prior aspiration of gastric tube will be done to confirm acidic pH.
* Osmolarity of preparation: High Osmolarity of gastric content may increase feed intolerance and risk of necrotizing enterocolitis. We plan to dilute the vitamin A Nepalm® formulation ten fold before administration. This will reduce the osmolarity of final solution significantly. In addition we plan to check osmolarity of the final solution and confirm it to be within acceptable range. In addition administration of feed along with the vitamin A dose will further decrease osmolarity of the medication.
* Dosage used: We plan to use a single dose of 2000 IU of vitamin A. The dose is within the range used by other studies. The dose of vitamin A used for RDR measurement in preterm infants ranges from 2000 IU/kg (Mactier 2012, Landman 1992, Ambalavanan 2003, Tyson 1999, Shenai 1987) to 5000 IU (Weinman 2007, Woodruff 1987) and 5000 IU/kg (Zachman 1996).
* Preparation used: Water soluble preparation of vitamin A (Aquasol A) has been used extensively in preterm infants either intramuscularly or orally. However it is not readily available in Australia. Vitamin A Nepalm® is a similar water soluble preparation which uses emulsifiers to solubilize retinyl palmitate. It is commercially available in Australia and indicated for clinical use in children and adults.

Budget:

|  |  |  |
| --- | --- | --- |
| Item | Unit | Cost ($) |
| SalivaBio SIS | 100 | 514 |
| Blood collection tubes  (Minicollect® 0.8 mL Light Green Lithium Heparin with Gel Separator - 450479) | 100 | 112 |
| Aluminum Foil | 1 | 10 |
| Centrifugation, storage of serum and saliva | 40 samples | --- |
| Vitamin A assay  ($ 28/sample) | 100 | 2800 |
| Stationary (parent information sheet, consent forms, etc) | 20 each | 40 |
| Total | | 3476 |

Fig 1: Flow diagram summarizing study protocol

Assessment of patients for eligibility at 3 to 5 weeks of post-natal age

Samples centrifuged, wrapped in aluminium foil and stored at -800C till further analysis

Samples centrifuged, wrapped in aluminium foil and stored at -800C till further analysis

Vitamin A administration

Blood and saliva collection

Informed parental consent

Patient Eligible

Blood and saliva collection after 5 hours

Blood and saliva collection after 7 hours

Samples centrifuged, wrapped in aluminium foil and stored at -800C till further analysis

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