Chapter 3

Materials and Methods

In the first part of the study, serum vitamin A levels of 80 healthy full-term infants delivered at Srinagarind Hospital, Khon Kaen University were measured on day 2 of age to define values in Thai neonates compared to 40 VLBW premature infants and their mothers.

Sample size of the VLBW premature infants in the second part of the study to evaluate the effectiveness of vitamin A supplementation for prevention of BPD was calculated by using the statistical formula below.

$$n = \frac{2(Z_{alpha} + Z_{beta})^2 P(1-P)}{\Delta (P)^2}$$

$$P = (P_{treatment} + P_{control})/2$$

BPD in control group =0.6, BPD in treatment group is 0.32

alpha = 0.05

beta = 0.84

$Z_{alpha} = 1.65$, $Z_{beta} = 0.84$

$$n = \frac{2(0.84 + 1.65)^2 \, 0.46(1-0.46)}{(0.28)^2} = 39 \text{ /group}$$

Eighty premature infants weighing less than 1,500 g who received mechanical ventilation or oxygen supplementation at 24 hours of age admitted to Neonatology services of Srinagarind Hospital, Khon Kaen University between July 2004 and July 2007 were enrolled. To reduce the likelihood of early death that was unrelated to vitamin A status and to facilitate enrollment, we enrolled infants at 24 to 96 hours after birth. Exclusion criteria were infants with major congenital anomalies, congenital non- bacterial infection, terminal illness (as indicated by a pH below 6.80 or by the presence of hypoxia with bradycardia for more than two hours). The study was approved by the Khon Kaen University Ethics Committee for human research, and written informed consent was obtained from parent of each infant.

The infants were assigned to the vitamin A or control group by a research nurse using a randomization list (using sealed envelopes containing the treatment assignments). Vitamin A was given intramuscularly because of its poor absorption from gastrointestinal tract and unreliable delivery in crystalloid solutions as given in parenteral nutrition: a dose of 5,000 IU (0.1 ml) was given intramuscularly on Mondays, Wednesdays, and Fridays for four weeks in a 1 ml insulin syringe with a 26-gauge needle. The same dose was used regardless of birth weight, because the smallest infants have the highest incidence of bronchopulmonary dysplasia, the lowest vitamin A stores at birth, and the lowest enteral

intake. The vitamin A preparation (Chochola A, Esai co., Ltd, Japan) was refrigerated and shielded at all times from direct light. To avoid pain and potential side effects and to facilitate enrollment, control infants received a sham procedure rather than placebo injections. For each treatment, a screen was placed around the bed, a pacifier was used to minimize crying after injections, and the injection site was covered with an adhesive bandage (or gauze in areas where the skin was fragile). The same covering was placed on control infants. The research nurse removed the covering at the next treatment. With the small needle, the injection site was either not visible or visible only on close inspection.

To assess vitamin A supplementation under usual clinical circumstances, the attending physician of the neonatal intensive care unit retained responsibility for decisions regarding the use of therapies other than vitamin A, including parenteral nutrition, mechanical ventilation, and glucocorticoids.

Evaluation

All vitamins and enteral feedings provided to the subjects were recorded. Experienced personnel who were not involved in the infants' care assessed the infants for signs of potential vitamin A toxicity each week for four weeks. The examiners assessed the fontanelle, fronto-occipital circumference, and liver size and checked each infant for edema, cutaneous abnormalities, bony tenderness, lethargy, and irritability. They also

assessed whether any abnormal findings could be explained by factors other than vitamin A toxicity. If vitamin A toxicity was suspected, the attending neonatologist decided whether to continue treatment on the basis of clinical findings without knowledge of the infant's treatment assignment.

Study Outcomes

Bronchopulmonary dysplasia was defined as the need for oxygen at 36 weeks' postmenstrual age (gestational age confirmed by certain last menstrual period, early assessment by ultrasonography in the first trimester of pregnancy or calculated at birth according to the original method of Ballard et al.). Radiologic findings were not assessed, because the need for oxygen at 36 weeks' postmenstrual age is predictive of later pulmonary complications and because we wished to avoid unnecessary exposure to radiation, expense, variability in interpretations, and the logistic problems of interpretations. Sepsis was defined on the basis of a positive blood culture and treatment with antibiotics for at least five days (unless the infant died within five days). Other outcomes measurement were necrotizing enterocolitis (NEC) > stage 2 according to Bell's criteria, patent ductus arteriosus (PDA) diagnosed by cardiologists using echocardiography, retinopathy of prematurity (ROP) evaluated by ophthalmologists, severe intraventricular

hemorrhage (IVH) grade 3 or 4 diagnosed by radiologists using cranial ultrasonography, duration of intubation, days of oxygen therapy, and length of stay.

Collection of serum samples

0.5 mL of blood samples from VLBW premature infants were obtained from peripheral venous blood or drawn from arterial or venous catheter on day 1 before and on day 7, 14 and 28 in control group and after vitamin A supplementation in vitamin A supplemented group. Blood samples in full term infants were obtained from peripheral venous blood on day 2 of age at the same time of routine neonatal screening for hypothyroidism. All samples were wrapped with aluminum foil to prevent photodegradation of vitamin A by light.

Serum samples were obtained from the spontaneous coagulation of blood. The blood was then centrifuged at 2,500 rpm at 4°C for 10 min to obtain serum. Hemolyzed samples were excluded. The serum was stored at −20°C until analysis.

Determination of vitamin A

Vitamin A levels were measured by reverse-phase HPLC system with a dual wave length spectrophotometric detector according to a method of Thurnham et al.

Vitamin A was extracted from serum samples according to the following procedure. In a glass tube (10 x 0.75 cm), 100 mL of serum sample was mixed briefly

with 200 mL of 0.1 μmol/L SDS reagent for 1 min. Next 200 ml of ethanol contained 42 μmol/L of tocopherol acetate (as internal standard) was added to the sample. N-Heptane contained 0.5 g of BHT per liter was then added, mixed vigorously for 2.5 min and centrifuged at 5000 rpm for 10 min at 20°C to separate the organic phase from the aqueous phase. 700 μL of heptane layer was transferred to a 10 x 0.75 cm glass tube and evaporated under nitrogen at 40°C. The residue was reconstituted with 250 mL of freshly prepared mobile phase. Samples were protected from photodegradation by extracting under dimmed natural lighting, excluding direct sun and fluorescent light at all times.

The HPLC system for vitamin A analysis consisted of a pump (model Waters 501) with a 20 μL injector (model 7125, Rheodyne), a C18 ODS-2 Spherisorb column (diameter 5 micron, 100x4.6 mm, Waters), and a UV detector (model Lambda-Max 481). The area under the main peaks was calculated quantitatively by an integrator (model Waters 746 Data Module).

The mobile phase was consisted of 200 mL acetonitrile, 200 methanol and 50 mL dichloromethane. It was filtered under a vacuum pump using a 2 micron filter paper (Cat. No.3400, Sartorius, Germany). 0.01 g BHT was added to the mobile phase and it was degassed by sonication for 30 min. It was then delivered to the C18 ODS-2

Spherisorb column at a flow rate of 1 mL/min by an isocratic pump. The column was equilibrated with the mobile phase for 5 column volume. 20 μL of sample was injected into the column using a Rheodyne injector at a flow rate of 1 mL/min. The area under the peak was calculated by an integrator/Clarity program.

Statistical analysis

Data were analyzed with Strata software and the two groups were compared with the use of Student's t-tests, chi-square analysis, and Fisher's exact tests where appropriate.

Safety of Vitamin A Supplementation

Signs of potential vitamin A toxicity that could not be explained by other factors (e.g., post-hemorrhagic hydrocephalus causing a full fontanelle) were not identified in all 31 premature infants enrolled in the study.