Effects of Soybean Lipid Infusion on Unbound Free Fatty Acids and Unbound Bilirubin in Preterm Infants

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Objective To assess the effects of a soybean lipid emulsion infusions on levels of unbound (free) bilirubin (Bf) and unbound free fatty acids (FFAu) as well as changes in Bf and total serum bilirubin (TSB) during phototherapy in infants born preterm.

Study design Ninety-seven infants born preterm (birth weight: 500-2000 g; gestational age: 23-34 weeks) were enrolled to investigate the effect of 0, 1, 2, and 3 g/kg/d of intralipid infusion on Bf and FFAu. Pre- and postphototherapy TSB, FFAu, and Bf also were analyzed in 91 infants to assess the effects of phototherapy. FFAu levels were measured with the fluorescent probe ADIFAB2 and Bf by the fluorescent Bf sensor BL22P1B11-Rh during intralipid infusion and at start and end of phototherapy. TSB and plasma albumin were measured by the diazo and bromcresol green techniques, respectively. Bilirubin-albumin dissociation constants were calculated based on Bf and plasma albumin.

Results Bf and FFAu increased with increasing intralipid dosage across all gestational ages. TSB and Bf were correlated significantly when infants received 0 or 1 g/kg/d of intralipid but not at greater doses of intralipid (2 and 3 g/kg/d). Although phototherapy effectively reduced both TSB and Bf in the total phototherapy group (by 32% and 12%, respectively), it reduced TSB, but not Bf, in infants less than 28 weeks of gestation.

Conclusions Increasing intralipid doses result in increasing FFAu levels, which are associated with increased Bf independent of TSB. In infants born extremely preterm (<28 weeks of gestation), phototherapy effectively reduces TSB but not Bf. *(J Pediatr 2017;184:45-50).*

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More than 80% of infants develop some degree of hyperbilirubinemia during the first week of life.[1] Although the effects of jaundice typically are benign in infants born full term, infants born preterm are at increased risk for bilirubin encephalopathy.[2,3] Currently total serum bilirubin (TSB) levels are used as indicators for treatment with phototherapy to prevent progression to toxic levels. Levels of free, unbound bilirubin, however, predict the risk of bilirubin neurotoxicity more accurately than TSB.[4-6] Levels of free unbound bilirubin can be elevated relative to TSB by displacement of bilirubin from albumin binding sites by a variety of compounds including unbound free fatty acids (FFAu). Free unbound bilirubin crosses the blood-brain barrier and exerts its toxic effect. Direct determination of free bilirubin (Bf) may be important in assessing the toxicity risk of bilirubin and targeting therapy. Unfortunately, it is not measured routinely in the US because of lack of commercially available assay methods but is available in other countries, such as Japan.[7,8]

Most infants born with very low birth weight are treated with parenteral nutrition, including lipid infusions. Intralipid is an emulsion of soybean oil that is a major source of calories and essential fatty acids. Similar to bilirubin, serum free fatty acids are bound primarily to albumin in the circulation but are in equilibrium with a small unbound fraction that exerts biologic effects. A concern about the use of intralipid is that elevated levels of FFAu can displace bilirubin from albumin binding sites, resulting in increased Bf levels relative to TSB.[9,10] This effect may be more apparent in infants born very preterm because of lower albumin binding capacity for bilirubin.[11]

We have shown previously that both FFAu and Bf were highly elevated in infants with extremely low birth weight treated with intralipid at 3 g/kg/d.[12] In the present study, we hypothesized that (1) increasing doses of intralipid (from 1 to 3 g/kg/d) would be associated with increasing levels of FFAu and Bf, independent of TSB and (2) that phototherapy would be effective in lowering TSB but not Bf, particularly in infants born extremely preterm (<28 weeks of gestational age) during intralipid infusion.

Bf	Free bilirubin	K_d	Bilirubin-albumin dissociation constant
FFAu	Unbound free fatty acid	TSB	Total serum bilirubin

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Methods

Infants with birth weights between 500 and 2000 g and gestational ages of 23-34 weeks treated with intralipid were eligible for this observational study. Recruitment was carried out in the neonatal intensive care unit at Rutgers Robert Wood Johnson Medical School. The study was approved by the institutional review boards of Rutgers and The Torrey Pines Institute for Molecular Studies. Parental informed consent was obtained. Because the laboratory policy is to store all blood samples for 7 days, it was possible to obtain research samples earlier than the age of the infant at consent.

The administration of intralipid was determined by the clinical team, usually commencing at a dose of 1 g/kg/d on the second day of life and advancing it daily to a maximum of 3 g/kg/d. The decision to withhold, reduce, or terminate the lipid infusion was determined by the clinical staff and resulted in some infants receiving fewer than the projected doses. The decision to initiate and terminate phototherapy was determined by the clinical staff and based on published recommendations.[13] Phototherapy devices used were neoBLUE LED (Natus Medical Inc, Pleasanton, California) positioned above the infants for those in incubators and fluorescent bulbs housed in the phototherapy attachment for those infants in warmer beds. Irradiance levels ranged between 15 and 30 $\mu w/cm^2/nm$.

Blood Sampling

Plasma samples were obtained before intralipid infusion (intralipid 0), during infusion of 1, 2, and 3 g/kg/d (intralipid 1, intralipid 2, intralipid 3), and before and after phototherapy. Samples were obtained from residual blood drawn for clinical indications during those intralipid dosing windows. These samples were collected immediately after completion of the requested clinical test, processed, and frozen. The lack of ordered clinical samples or inadequate residuals resulted in an incomplete set of research samples for some infants. Deidentified samples were stored at −70°C and shipped to Fluoresprobe Sciences for determination of Bf and FFAu.

Bf, FFAu and TSB Measurements

FFAu and Bf were measured in the same plasma sample with the use of fluorescently labeled mutants of fatty acid binding proteins.[14-16] The Bf probe (BL22P1B11-Rh) binds unconjugated bilirubin with high affinity (bilirubin-albumin equilibrium dissociation constant [K_d] = 16 nM) but is poorly sensitive to FFA (K_d > 3000 nM), conjugated bilirubin (K_d > 300 nM), bilirubin photoisomers, bilirubin oxidation products, ibuprofen, and indomethacin.[14] FFAu concentrations were determined with the ADIFAB2 probe (FFA Sciences LLC, San Diego, California).[12,15] Fluorescence was assessed at 2 emission wavelengths: 457 and 550 nm for the FFAu probe and 525 and 580 nm for the Bf probe (excitation = 375 nm for both). The ratio of fluorescence at the 2 wavelengths, together with probe characteristics, yielded the FFAu and Bf concentrations. Fluorescence was measured at 22°C by the use of handheld ratio fluorometers in which plasma sample volumes were 4 μL for FFAu and 25 μL for Bf measurements. After dilution, the total volume was 200 μL so that FFAu was measured at 50-fold and Bf at 8-fold dilution of the plasma sample.[14,15]

The accuracy of the Bf probe measurements was confirmed previously by comparison with the peroxidase assay in adult plasma supplemented with bilirubin as well as in bilirubin-albumin in vitro measurements.[14] The Bf probe has an average coefficient of variation of 3% for repeated measurements of aqueous bilirubin, bilirubin-albumin complexes, and bilirubin-spiked adult plasma over a Bf range from 1 to >350 nmol/L.[14] Measurements with the Bf probe, in contrast to the peroxidase method, determine the Bf concentration directly in a single measurement and are insensitive to substances that can interfere with the peroxidase measurement.[14]

TSB was measured via the diazo method at the Robert Wood Johnson Medical School clinical laboratory. Plasma albumin was measured in infants before intralipid infusion by use of the bromocresol green albumin assay (Sigma-Aldrich, St Louis, Missouri) method, which was standardized with fatty acid free human serum albumin from 72 to 722 μM (coefficient of variation< 4%). Infant plasma (5 μL) was diluted into 200 μL of the bromocresol green reagent, and absorbance was measured at 620 nm. We calculated the K_d, which is the inverse of the binding constant K_a using equation,[1]

$$K_d = \frac{B_f A_t}{(TSB - B_f)} - B_f$$

where A_t is the measured albumin concentration and K_d is in mol/L or nM.

Statistical Analyses

Changes in TSB, Bf, and FFAu as a function of increasing dose of intralipid or of phototherapy were analyzed by repeated measures analysis and verified by the generalized estimating equation semiparametric regression method. Multiple regression analysis was performed to evaluate the correlation between FFAu and TSB with Bf with increasing dose of intralipid, taking into account confounding variables including birth weight, gestational age, sex, race, and 5-minute Apgar. The analysis was verified by nonparametric Spearman correlation. The analyses were performed with XLSTAT (AddinSoft, New York, New York) and verified with SAS Software (SAS Institute, Cary, North Carolina). *P* values < .05 were considered statistically significant.

Results

Effects of Increasing Dose of Intralipid on TSB, Bf, and FFAu

The study population included 97 infants with a mean birth weight of 1315 ± 435 g and gestational age of 29.2 ± 3.1 weeks. Fifty-two percent of infants were male. Fifty-two percent of the infants were white, 30% were black, 17% were Asian, and 1% were Hispanic. For 48 infants, measurements of Bf, FFAu, and TSB were obtained before the intralipid infusion (intralipid 0) and at all 3 intralipid dosages, intralipid 1, intralipid 2, and intralipid 3. Of the remaining infants, 32 had measurements