Bilirubin Albumin Binding and Unbound Unconjugated Hyperbilirubinemia in Premature Infants

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Objective To evaluate the associations between unbound bilirubin (UB) and total serum bilirubin (TSB), bilirubin:albumin molar ratio (BAMR), and bilirubin albumin binding affinity (Ka) as a function of gestational age (GA) in infants born at 24-33 weeks GA.

Study design In a prospective observational study, TSB and UB were measured twice daily at least 8 hours apart during the first postnatal week. Serum albumin was measured to calculate BAMR on each day. The highest UB on each day, corresponding TSB, and serum albumin were used to calculate the Ka on each day.

Results For the 166 infants studied, peak UB significantly correlated with concomitant Ka (r = –0.44, $P = .001$) but not with concomitant TSB or BAMR after adjusting for GA. On multiple regression analyses, there was a significant association of concomitant Ka (–0.06, 95% CI –0.08 to –0.04, $P = .0001$), but not concomitant TSB or BAMR with peak UB after controlling for GA, birth weight, race, and sex. GA group was a significant effect modifier for the association between Ka and peak UB (0.03, 95% CI 0.02-0.04, $P < .001$). Interaction analyses showed the association between concomitant Ka and peak UB was significant for the 24-30 weeks GA group infants, but not for the $30^{1/7}$-33 weeks GA group infants.

Conclusions Peak UB was primarily associated with a decrease in binding affinity in infants ≤30 weeks GA. Interventions aimed at improving binding affinity may be important in decreasing the risk of bilirubin-induced neurotoxicity. *(J Pediatr 2017;■■:■■-■■).*

During the first postnatal week, premature infants are at increased risk of bilirubin-induced neurotoxicity at lower concentrations of total serum bilirubin (TSB) than term infants.[1,2] The increased risk for bilirubin-induced neurotoxicity is due to increased susceptibility of premature neuronal cells to bilirubin injury when unbound bilirubin (UB, bilirubin not bound to protein),[3-5] but not bilirubin bound to albumin, crosses the intact blood brain barrier and the neuronal cell membrane.[6] The critical role of UB in the pathogenesis of bilirubin-induced neurotoxicity has been corroborated by several studies demonstrating that peak UB is a better predictor of abnormal neurologic outcomes than TSB in premature and term infants.[5,7-13] Therefore, interventions targeted to prevent or resolve unbound unconjugated hyperbilirubinemia will be important in reducing bilirubin-induced neurotoxicity.[14]

Serum or plasma UB concentrations depend on TSB concentrations, the bilirubin:albumin molar ratio (BAMR, a measure of bilirubin binding capacity of albumin), and the bilirubin-albumin binding affinity (Ka, strength of bilirubin binding to albumin).[3] Although serum albumin levels and bilirubin binding capacities are lower in preterm compared with term infants, there is little information on bilirubin-albumin binding during the first postnatal week as a function of gestational age (GA).[13,15-17] More importantly, little is known about the primary underlying mechanism for unbound unconjugated hyperbilirubinemia as a function of GA in premature infants. Such information is necessary to provide interventions to prevent or resolve unbound hyperbilirubinemia and to decrease the risk of bilirubin-induced neurotoxicity in premature infants. The objective of this study was to evaluate the underlying mechanism of unbound hyperbilirubinemia in infants ≤33 weeks GA and to determine if unbound hyperbilirubinemia during the first postnatal week is associated with an increase in TSB, increase in BAMR, or a decrease in Ka and if these associations are modified by GA.

Methods

This was a prospective observational study involving infants born at 24-33 weeks GA. The study was approved by the local institutional research review board. Informed consent was obtained from the parents of each subject.

AUC	Area under the curve
BAMR	Bilirubin:albumin molar ratio
GA	Gestational age
Ka	Bilirubin albumin binding affinity
TSB	Total serum bilirubin
UB	Unbound bilirubin

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Premature infants born at 24-33 weeks GA and admitted to the neonatal intensive care unit within 12 hours after birth were eligible. GA was assessed by obstetrical dating criteria, or when obstetrical data was inadequate, by Ballard examination. Infants with craniofacial malformations, chromosomal disorders, TORCH infections (toxoplasmosis, rubella, cytomegalovirus, syphilis, herpes, or HIV infections), family history of congenital hearing loss, or conjugated hyperbilirubinemia (direct bilirubin ≥2 mg/dL) during the first postnatal week were excluded. In addition, infants who died within 3 days after birth were excluded.

Unbound Bilirubin and Bilirubin-Albumin Binding

Blood samples to measure TSB (mg/dL) and UB (μg/dL) were drawn twice daily at least 8 hours apart starting at 24 hours after birth and continuing through the first postnatal week. The blood samples were collected in special amber colored serum separator tubes to protect them from light exposure. TSB was measured by the colorimetric method in the clinical chemistry laboratory. The same aliquot of blood was also used to measure UB. Each sample was centrifuged and analyzed immediately for UB or stored in a −80°C freezer for not more than a month before analysis of UB.[18] The validated modified peroxidase test was performed to measure UB using the Food and Drug Administration approved Arrows UB analyzer UA-1 (Arrows Company, Ltd, Osaka Japan) with 2 enzyme concentrations of precalibrated peroxidase (Arrows Reagent Kit, Arrows Company, Ltd).[19] The first order rate constant for the oxidation reaction or the peroxidase enzyme activity of 1 was confirmed using bilirubin standard (0.67 mg/mL) and albumin-free solution (ie, total bilirubin = UB) with each batch of peroxidase enzyme before UB measurement. The samples were analyzed for UB by the same investigator blinded to GA. Serum albumin concentrations (g/dL, multiply by 151 to convert to μmol/L) were measured using the same aliquot of blood using the Bromocresol Green method and used to calculate BAMR. The highest UB on each day, the corresponding TSB, and serum albumin were used to calculate the bilirubin binding affinity (Ka, L/μmol) on each day using the law of mass action equation described in the literature and shown below.[3]

$$Ka = \frac{TSB - UB}{UB\,(Albumin - TSB + UB)}$$

Phototherapy was used according to the institutional guidelines for the management of unconjugated hyperbilirubinemia in premature infants that was based on TSB concentrations, birth weight, and presence of clinical factors that increase the risk for bilirubin-induced neurotoxicity.

Subjects were subgrouped into GA groups based on GA at birth: (1) 24-26 weeks GA; (2) $26^{1/7}$-28 weeks GA; (3) $28^{1/7}$-30 weeks GA; (4) $30^{1/7}$-32 weeks GA, and (5) $32^{1/7}$-33 weeks GA.

Statistical Analyses

Statistical analyses were performed with SAS (v 9.4; SAS Institute Inc, Cary, North Carolina). Longitudinal analyses were performed to evaluate the association between each bilirubin-albumin binding measure (TSB, BAMR, and Ka) and UB across the time points using a linear mixed model. Because peak UB has been associated with bilirubin-induced neurotoxicity, peak UB was used as an outcome variable for further analyses. Partial Pearson correlation coefficients between peak UB and the other bilirubin-albumin binding measures (TSB, BAMR, and Ka) were calculated. Multiple linear regression analyses were carried out to evaluate the association between each bilirubin-albumin binding measure (TSB, BAMR, and Ka) and peak UB after controlling for GA, birth weight, sex, and race. Furthermore, the interaction effects with GA were tested and the associations were examined by each GA group. In addition, receiver operating characteristic curve analyses were used to compare the area under the curves (AUCs) of TSB, BAMR, and Ka for predicting the elevated UB level (upper 75th percentile of peak UB).

Results

A total of 345 infants born at 24-33 weeks GA were admitted to the neonatal intensive care unit over 3 years. Twelve infants (5 with major congenital malformations, 2 with TORCH infections, and 5 infants who died within the first 5 days) met exclusion criteria. A total of 167 infants ≤33 weeks GA were enrolled after obtaining consent. One infant subsequently withdrew from the study. A total of 166 infants ≤33 weeks GA were studied, with the following GA distribution: 24-26 weeks (n = 27); $26^{1/7}$-28 weeks (n = 37); $28^{1/7}$-30 weeks (n = 46); $30^{1/7}$-32 weeks (n = 28); $32^{1/7}$ -33 weeks (n = 28). The mean GA of study subjects was $28^{6/7}$ weeks (range, 24-33). The mean birth weight was 1211 g (range, 450-2595g). The majority of infants were male (n = 85, 51%), Caucasian (n = 105, 63%), and received phototherapy (n = 161, 97%) as per institutional guidelines for the management of unconjugated hyperbilirubinemia.

The daily UB and corresponding bilirubin albumin binding measures: TSB, BAMR, and Ka as a function of GA subgroups during the first postnatal week are shown in **Figure 1**. GA was negatively correlated to UB, but positively correlated to TSB, BAMR, and Ka. For infants 24-30 weeks GA, UB peaked on days 5 and 6 when TSB and BAMR were decreasing. For infants >30 weeks GA, UB peaked on days 3 to 4 and then gradually decreased with decrease in TSB and BAMR by the end of the first postnatal week. The Ka (binding affinity) was variable but decreased during the first postnatal week in infants ≤32 weeks GA, and for infants >32 weeks GA, Ka gradually improved over the first postnatal week. Using longitudinal regression analyses controlling for GA, birth weight, race, and sex, there was a significant association of TSB, BAMR, and Ka with UB across the time points during the first postnatal week ($P = .0001$).

The mean ± SD of peak TSB (mg/dL), peak BAMR, and peak UB (μg/dL) during the study period were 7.6 ± 2.6, 0.29 ± 0.09, and 1.9 ± 2.2, respectively. Peak UB correlated negatively with concomitant Ka (r = −0.44, $P = .001$), controlling for concomitant TSB, BAMR, and GA using a Pearson partial correlation analysis. There was no correlation between peak UB and concomitant TSB (r = 0.016, $P = .84$), controlling for concomi-

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