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Bilirubin Binding Capacity in the Preterm Neonate

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SYNOPSIS

Total serum/plasma bilirubin (TB), the biochemical measure currently used to evaluate and manage hyperbilirubinemia, is not a useful predictor of bilirubin-induced neurotoxicity in premature infants. Altered bilirubin-albumin binding in premature infants limits the usefulness of TB in premature infants. In this article, bilirubin-albumin binding, a modifying factor for bilirubin-induced neurotoxicity, in premature infants is reviewed.

Keywords

total serum bilirubin; unbound bilirubin; bilirubin:albumin molar ratio; bilirubin-albumin binding affinity; bilirubin-induced neurotoxicity

INTRODUCTION

The primary goal of the evaluation and management of unconjugated hyperbilirubinemia in premature infants is to prevent acute and chronic bilirubin-induced neurotoxicity, a spectrum of neurodevelopmental disorders including, but not limited to, central apnea, sensorineural deafness, auditory neuropathy spectrum disorder (ANS), language disorders, autism, upward gaze palsy, and athetoid cerebral palsy (CP).[1–10] It is generally believed that premature infants are at increased risk for bilirubin-induced neurotoxicity compared to term infants.[4,11] However to date, the evidence-based management of hyperbilirubinemia to prevent bilirubin-induced neurotoxicity remains elusive in premature infants <35 weeks gestational age (GA).[12] Consensus-based management guidelines for hyperbilirubinemia using total serum/plasma bilirubin (TB) levels, GA, and clinical risk factors for premature infants <35 weeks GA were recently published.[11] The TB level, the traditional parameter to evaluate and manage hyperbilirubinemia in premature infants, has not been a useful predictor of acute and chronic bilirubin-induced neurotoxicity.[4,13–19] This is not surprising because several biochemical and physiological factors are involved in the

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pathogenesis of bilirubin-induced neurotoxicity.[20] More specifically, bilirubin-albumin binding limits the use of TB as an indicator of the amount of bilirubin in vascular and extravascular compartments, the magnitude of ongoing bilirubin production/excretion mismatch, and the overall risk of bilirubin toxicity in premature infants. There is growing evidence that non-albumin bound (unbound) or free bilirubin (UB), resulting from altered bilirubin-albumin binding, is a more sensitive and specific biochemical measure of acute and chronic bilirubin-induced neurotoxicity than TB. In this article, bilirubin-albumin binding, a modifying factor for bilirubin-induced neurotoxicity, in premature infants is discussed. The endogenous and exogenous factors which may influence the bilirubin-albumin binding in premature infants is also examined. Furthermore, existing evidence for the role of bilirubin:albumin (B:A) molar ratio (BAMR) and UB as predictors of bilirubin-induced neurotoxicity in premature infants is provided.

Bilirubin-Albumin Biochemical Structure and Binding

The predominant native form of unconjugated bilirubin in its usual form (*4Z,15Z-IX α* isomer) has its two rigid dipyrrole units internally hydrogen bonded to each other such that no polar (or ionizable) groups are exposed making it quite insoluble in water at a neutral pH. [21,22] Because of its low solubility in water, free unconjugated bilirubin can form detrimental dimers and higher aggregates.[22] Solubility increases with increasing pH due to the ionization of the acidic groups on the molecule.[21,22] The hydrophobic native form of unconjugated bilirubin can readily cross the phospholipid layers of biomembranes, including the blood-brain barrier and neuronal cell membranes.[23] This native bilirubin or free unconjugated bilirubin (*4Z,15Z-IX α* isomer) is neurotoxic.[24,25]

Fortunately, native bilirubin, primarily a product of hemoglobin (Hgb) degradation, exists in the blood mostly bound to albumin, preventing it from crossing intact blood-brain barrier. Albumin serves as a vehicle for the transport of bilirubin to the liver, where the bilirubin dissociates from albumin and enters the hepatic cell for conjugation. More importantly, albumin is a highly abundant serum protein (0.6 mM), which comprises 50%–60% of the total plasma protein in humans. Moreover, albumin is a 66-kDa monomer containing three homologous helical domains (I–III), each divided into A and B subdomains.[26] It is therefore not surprising that, in addition to bilirubin, albumin binds a wide variety of endogenous ligands, including non-esterified fatty acids and heme, all of them acidic and lipophilic compounds, with high-affinity at multiple sites.[27–29] Among exogenous substances, many commonly used drugs with acidic or electronegative features (*e.g.* ibuprofen) also bind to human serum albumin (HSA), usually at one of two primary sites located in subdomains IIA and IIIA.[27]

There are at least two types of binding sites for bilirubin and its photoisomers on albumin. [30,31] The strongest and key binding site for native bilirubin has a very high binding constant (affinity), $\sim 1.4 \times 10^7$ L/mol at 37°C, and can be considered a specific site.[32,33] Other secondary sites have at least a ten-fold lower binding affinity.[34] Recent studies indicate that there are site-to-site interactions or allosteric interaction within albumin.[35–37] It is known that fatty acid and drug binding to albumin induces conformational changes in albumin which may then influence bilirubin-albumin binding.[29,35–37]

Bilirubin-Albumin Binding Capacity (BBC) and Affinity

Bilirubin-albumin binding is a function of the concentrations of bilirubin and albumin, and the binding affinity for bilirubin (strength of bilirubin binding to albumin). The fraction of UB increases significantly as the TB approaches the binding capacity of albumin (1 g HSA binds ~8 mg of bilirubin in term infants and much less in premature infants) or when the apparent binding affinity decreases.

Because of the large quantity of albumin and its' very high affinity for bilirubin, the serum UB level is essentially controlled by the BBC in healthy late preterm and term neonates. [33,38] Binding capacity is generally defined as the concentration of the bound ligand at infinite ligand concentrations.[39] For BBC at a high-affinity binding site, consideration has to be given to the following equilibrium:

Where 'R' represents the concentration of albumin with an empty high-affinity site or the reserve albumin binding capacity and 'B' represents the concentration of albumin with bilirubin bound to a high-affinity site. The total BBC is the total amount of albumin capable of strongly binding bilirubin (R + B) at the high-affinity site. The relationship assumes that the binding protein, albumin, has specific, non-interactive, and independent binding sites. [34,40] The relationship does not account for bilirubin binding to low affinity sites and other proteins or possible bilirubin displacement by competitors. Furthermore, allosteric interaction, aforementioned, may alter the total BBC in premature infants. In addition, problems with bilirubin aggregation and solubility as UB increases make it impossible to obtain complete binding isotherms (*i.e.*, binding isotherms in which the albumin-bound bilirubin reaches a level that changes little with additional increases in TB).[40,41] Several methods have been used to measure total and reserve BBC and provide indirect estimation of UB; however, each method is associated with some limitations.[42] It may be more helpful to consider the albumin concentration (or BAMR) as a practical "bilirubin binding capacity" while newer and better technology to measure BBC in premature infants is being developed and validated for research and clinical use.[42]

It is also important to recognize that bilirubin-albumin binding is reversible and that there is a dynamic equilibrium with bilirubin binding onto albumin and dissociating off continuously at normal serum pH and temperature.[43] As bilirubin leaves the blood compartment and enters the tissues, more bilirubin is dissociating from the albumin-bound pool. The bilirubin exits into the tissues at a rate that is proportional to the UB concentration. Thus, serum UB, TB, and albumin concentrations are related by the law of mass action and characterized by an affinity or binding constant, K as shown in the equation below:

$$\frac{TB-UB}{K ([\text{Albumin}] - TB + UB)} = UB$$

The law of mass equation indicates that the UB is inversely proportional to albumin and its intrinsic ability to bind bilirubin (K). The relationship clearly explains the influence of

bilirubin- albumin binding (BBC and affinity) on UB and the risk for bilirubin-induced neurotoxicity. The relationship also underscores the importance of evaluating bilirubin-albumin binding in addition to TB to indirectly determine the risk for bilirubin-induced neurotoxicity.

If UB level is available, the bilirubin-binding constant (K), a measure of the strength of the binding or binding affinity, can be calculated using the mass equation as:

$$\frac{TB-UB}{UB ([Albumin]-TB+UB)}=K$$

Bilirubin-Albumin Binding as a Function of GA and Postnatal Age

Limited data is available on the ontogeny of BBC and affinity in premature infants. There is general agreement that serum albumin levels and BBC is lower in premature compared to term infants.[11,12,44,45] Bender, *et al.*[46] using the 5th-day blood sample demonstrated that BBC at a high-affinity site improves with increasing GA in infants <31 wks GA. However, the findings were based on a single peroxidase concentration and several tenuous assumptions of bilirubin-albumin binding.[40] Similarly, Lamola, *et al.*[47] showed positive correlation (r=0.53) between GA and BBC measured by two different versions of the hematofluorometric instrument in three different cohorts of premature and term infants. They reported an increase in BBC by 1 mg/dL/week with increasing GA.[47] Interestingly, there was no association of BBC with sepsis in their cohort.[47] The hematofluorometric instrument assumes a single non-interactive, high-affinity binding site on albumin.[42] Cashore, *et al.*[48] using the Sephadex method reported that BBC remains unchanged during the first 10 days of life in healthy preterm and term infants; however, BBC is highly variable in sick infants.

In a large prospective study using the modified peroxidase method, Amin, *et al.*[49] reported that in more mature preterm infants (>30 wks GA), the binding affinity improves over the first week, while in infants <30 wks GA, bilirubin-binding affinity varies within and between infants, but overall, decreases during the first postnatal week. This variability in bilirubin binding affinity in premature infants has been previously reported using the Sephadex method.[45] Several exogenous and endogenous factors described below may contribute to this variability in bilirubin binding affinity in premature infants.

Clinical Risk Factors and Bilirubin-Albumin Binding

Various clinical factors such as hypothermia, hypoxia, acidosis, asphyxia, and sepsis have been postulated to explain the occurrence of bilirubin-induced neurotoxicity at much lower TB levels in premature infants.[4,12] These factors are thought to increase the risk of bilirubin-induced neurotoxicity by not only affecting the bilirubin-albumin binding; but also, by facilitating bilirubin entry into the brain, and/or neuronal uptake of bilirubin.[4] In an earlier study involving 76 infants, reserve albumin concentration determined by dialysis with ¹⁴C-monoacetyl diamino diphenyl sulfone was decreased and the bilirubin toxicity index was increased in the presence of clinical risk factors (hypoxia, acidosis, or sepsis), suggesting an increased risk of bilirubin-induced neurotoxicity in the presence of these risk

factors.[50] Maisels, *et al.*[51] evaluated the influence of acid–base status on bilirubin–albumin binding in 254 low birthweight (LBW) infants at the ages of 3, 4, 5, and 8 days and reported a weak, but significant, correlation between metabolic acidosis and the bilirubin:reserve albumin ratio as well as the bilirubin toxicity index. A decrease in bilirubin–albumin binding and a doubling of the toxic potential of bilirubin occurred with shifts to a metabolically acidic state with a base deficit of 10.[51] In another study, involving 11 late preterm infants, the correction of a metabolic acidosis (pH 7.12 to 7.34) during the first postnatal day resulted in an improvement of apparent bilirubin–albumin binding affinity and a decrease in UB levels.[52] Although the exact mechanism is unclear, neonatal sepsis by decreasing albumin production and increasing free fatty acid (FFA) concentration, a known bilirubin displacer, may adversely influence bilirubin–albumin binding.[53,54] Ritter, *et al.*[55] reported in a small prospective study involving premature infants that prolonged hypoxia ($\text{PaO}_2 < 50$ mm Hg), prolonged acidosis (pH < 7.1) and prolonged hypothermia ($< 35.5^\circ\text{C}$) occurring prior to peak UB levels were significant clinical risk factors for kernicterus. Both hypoxia and hypothermia may also increase concentrations of FFA, known bilirubin displacers. Although Kim, *et al.*[56] failed to identify a single or combination of risk factors to be useful in predicting the development of kernicterus in premature infants, the incidence of severe acidosis and hypoxic encephalopathy was higher in infants with kernicterus ($n=27$) compared to normal infants ($n=103$). Compared to these studies, Turkel, *et al.*[57] in a small retrospective matched control study of 64 infants, failed to identify any clinical risk factor associated with kernicterus in premature infants. Clearly, the role of these clinical risk factors in the pathogenesis of bilirubin-induced neurotoxicity needs to be further evaluated in premature infants. In the meantime, the weak evidence derived from observational studies warrants a more aggressive management of hyperbilirubinemia in the presence of one or more clinical risk factors as also recently recommended by the expert committee.[12]

Phototherapy and Bilirubin-Albumin Binding

Phototherapy is the mainstay of therapy in premature infants. In jaundiced newborns, bilirubin photoisomers, mainly *4Z,15E*-bilirubin and *Z*-lumirubin, comprise ~10% of TB before phototherapy, and increase to as much as ~30% during phototherapy.[58–60] Earlier *in vitro* studies reported that the photoisomers bind much less avidly to plasma proteins than to native *4Z,15Z*-bilirubin, and UB levels should therefore increase during phototherapy.[61,62] However, the issue regarding neurotoxic properties of photoisomers is still an unresolved issue.[63] If *4Z,15E*-bilirubin is not neurotoxic, UB measured by the peroxidase method may overestimate the toxic UB fraction by up to 30%.[58,59,63] However, if it is neurotoxic, UB remains an “accurate” measure of toxic bilirubin isomers even during phototherapy. The available, but limited, literature also suggests that the peroxidase test commonly used to measure UB appears to be more specific for native *4Z,15Z*-bilirubin than the photoisomers produced by phototherapy.[62,64] Although a recent *in vitro* study reported a disproportionate decrease in UB relative to the decrease in the concentration of *4Z,15Z*-bilirubin, the clinical studies performed thus far suggest little change with phototherapy in bilirubin–albumin binding as assessed by the peroxidase test.[58,65–67] This interaction with photoisomers will need to be investigated with any newer assays of bilirubin–albumin binding that may be developed and used in the future.[42]

Exogenous Drugs and Bilirubin-Albumin Binding

Several therapeutic drugs and preservatives used in preparing these drugs have been studied and shown in *in vitro* studies to displace bilirubin from albumin binding sites with different levels of activity (Table 1).[68–72] Of the drugs studied, the most potent bilirubin displacers are sulfamethoxazole, sulfisoxazole, sulfadiazine, and ceftriaxone. The potential for abnormal binding may persist for the first 10 to 12 wks in premature infants.[39] Therefore, drugs with a strong bilirubin displacing effect should be avoided as long as there is a risk for bilirubin-induced neurotoxicity in premature infants. Also, any new drugs with acidic or electronegative features and high protein binding should be evaluated for a bilirubin displacing effect before routine clinical use in neonates, as was considered for ibuprofen use in premature infants.

Several *in vitro* studies performed using bilirubin-albumin solution or pooled newborn sera have shown that the risk of a ibuprofen-induced bilirubin displacing effect depends on the plasma concentration of ibuprofen, BAMR, and the intrinsic ability of albumin to bind ibuprofen and bilirubin.[73–75] Ahlfors, *et al.*[74] demonstrated, using the peroxidase method, that a significant increase in UB concentration was only observed at ibuprofen concentrations >50 µg/mL in two pooled newborn sera with BAMRs of 0.3 and 0.36. Similarly, Cooper-Peel, *et al.*[75] demonstrated that with a reverse displacement method using cord sera with a BAMR of 0.5 that there was none to minimal bilirubin displacing effect with ibuprofen concentrations <50 µg/mL. However, there was an increase in UB by a factor of 4 with an ibuprofen concentration of 154 µg/mL (750 µmol/L). Compared to *in vitro* studies, Amin, *et al.*,[76] using the modified peroxidase method, reported that intravenous (IV) ibuprofen, when used at the recommended dosage for the management of patent ductus arteriosus (PDA) after the first 2 postnatal days, is not associated with a bilirubin displacing effect in relatively stable premature infants (<30 wks GA) with a BAMR <0.5. This finding was subsequently confirmed by Desfrere, *et al.*[77] in stable premature infants (<32 wks GA) with mild to moderate hyperbilirubinemia and a BAMR <0.3. In summary, the accumulated evidence from clinical studies in premature infants suggests that clinically significant displacement is unlikely if IV ibuprofen is used with the recommended dosage after the first 2 postnatal days and with a BAMR <0.5. However, until further data is available, caution is warranted in using IV ibuprofen in infants with a BAMR >0.5 and during the first 2 postnatal days when the plasma levels of ibuprofen may be higher.[78]

Intralipid and Bilirubin-Albumin Binding

The clearance of FFA is slower in premature than in term infants, and intralipid (IL) intake in premature infants may result in elevated FFA levels.[79–81] FFA bind to albumin at three sites. The first two moles of FFA preferentially bind to its first two binding sites. The third binding site of FFA to albumin is also the high affinity site of bilirubin to albumin. When the FFA:albumin molar ratio is 4:1, FFA compete with bilirubin at the primary site of bilirubin (also the third binding site of FFA) resulting in displacement of bilirubin.[82] Earlier studies that demonstrated the adverse effects of IL on bilirubin-albumin binding were based on indirect estimation of UB or using a single peroxide concentration in more mature infants. [34,79,82–91] The effect of a gradual increase in IL (20%) intake from 1.5 to 3.0 g/kg/day during the first postnatal week on bilirubin-albumin binding, as measured by the modified

peroxidase test using an UB analyzer, was evaluated in 62, 24 to 33 wks GA infants.[92] At each IL dose >1.5 g/kg/day, there was statistically significant lower binding affinity in infants ≤ 28 wks GA ($n=32$) compared to infants >28 wks GA ($n=30$). There were also significantly higher UB concentrations, despite significantly lower TB concentrations, at each IL dose 1.5 g/kg/day in ≤ 28 wks GA infants compared to infants >28 wks GA. The cumulative frequency of elevated UB concentrations (>1 $\mu\text{g/dL}$ or >17.1 nmol/L, a level that may be associated with neurotoxicity in premature infants) as a function of IL intake was inversely related to GA and was significantly different between 2-wk GA groups: 24–26, 26^{1/7}–28, 28^{1/7}–30, and 30^{1/7}–32 wks GA infants (Table 2). The amount of IL intake that is tolerated without decreasing bilirubin binding affinity and secondary increase in UB increases with GA. The IL intake of 1.5 and 2.0 g/kg/day was associated with UB concentrations >1 $\mu\text{g/dL}$ in 24–26 and 26^{1/7}–28 wks GA infants, respectively.

The causal relationship between IL intake and bilirubin-albumin binding variables as measured by the modified peroxidase method was subsequently evaluated in 24–33 wks GA infants.[93] In ≤ 28 wks GA infants, there was a statistically significant association between IL intake and FFA or FFA:albumin molar ratio. FFA:albumin molar ratio also predicted bilirubin-albumin binding affinity. As the FFA:albumin molar ratio increased with increasing IL intake, bilirubin-albumin binding affinity decreased. Binding affinity predicted UB, and a decrease in binding affinity correlated significantly to an increase in UB. However, in infants >28 wks GA, IL intake was not significantly associated with FFA concentrations or FFA:albumin molar ratios. Therefore, FFA or FFA:albumin molar ratio is not a mediator of any increase in UB with IL intake in infants >28 wks GA. These findings provide an explanation for why higher IL intake may not be associated with an increase in UB concentrations in infants >28 wks GA. The available evidence suggests that IL intake should be appropriately limited in infants ≤ 28 wks GA during the first postnatal week when unconjugated hyperbilirubinemia is common.[90,92,93]

BAMR and Risk for Bilirubin-Induced Neurotoxicity

The consensus-based guidelines for the management of hyperbilirubinemia recommends measurement of serum albumin in premature infants.[11] Scheidt, *et al.*[18] reported the neurodevelopmental outcomes at 6 yrs in 224 infants <2000 g who participated in the National Institute of Child Health and Development (NICHD) Neonatal Research Network (NRN) randomized phototherapy trial. There was no significant association of TB or BAMR with neurodevelopmental outcomes, including cerebral palsy and intellectual disability after controlling for neonatal risk factors.[18] Similarly, Cashore, *et al.*[94] in a small retrospective study reported no significant difference in TB, albumin, or BAMR between 5 premature infants (mean GA=29.2 wks) with autopsy-proven kernicterus compared to 8 premature infants (mean GA=28.1 wks) without kernicterus.

However, several others have reported on the possible role of albumin or BAMR in premature infants. Kim, *et al.*[56] in a retrospective matched control study involving premature and term infants (24 to 42 wks GA) reported no significant differences in peak TB between 27 infants (mean GA=32 wks) with autopsy findings of kernicterus and 103 control infants (mean GA=31 wks) without kernicterus. In a subset of the study population, the

mean serum albumin concentration (2.0 ± 0.8 g/dL) in the 14 infants with kernicterus was significantly lower compared to the 33 control infants (2.6 ± 0.5 g/dL), indicating that peak BAMR was higher in infants with kernicterus compared to control infants.[56] Ritter, *et al.* [55] also reported no significant difference in peak TB and albumin levels between 7 premature infants (mean GA=28.3 wks) with autopsy-proven kernicterus and 23 premature infants (mean GA=28.5 wks) without kernicterus; however, calculated peak BAMRs were higher in infants with kernicterus compared to those without kernicterus (0.29 vs. 0.25). Amin, *et al.*, [95] using sequential auditory brainstem evoked response (ABR) during the first two postnatal weeks, compared TB and BAMRs as predictors of acute bilirubin encephalopathy (ABE), as defined by abnormal ABR maturational changes in 143, 28–32 wks GA infants. There was no significant difference ($p=0.98$) in mean peak TB between infants with normal ABR maturation and abnormal ABR maturation. Although BAMRs did not significantly predict abnormal ABR maturation, the trend ($p=0.19$) was better than that for TB. In a subset of 45 premature infants, in whom UB was measured, peak BAMR, but not peak TB, was significantly higher in infants with abnormal ABR maturation compared to infants with normal ABR maturation.[95] In a recent study involving late preterm and term infants with severe hyperbilirubinemia in Egypt, both TB and BAMR were significantly associated with bilirubin-induced neurotoxicity; however, BAMR failed to provide any advantage over TB for predicting bilirubin-induced neurotoxicity.[96] A recent multicenter randomized trial in the Netherlands studied the usefulness of BAMR combined with TB compared to TB alone in the management of hyperbilirubinemia and reported no additional benefit of using BAMR in the management of hyperbilirubinemia in infants 32 wks GA. However, in a subgroup analysis of infants with >1000 g, the use of BAMR, in addition to TB, was associated with reduction in mortality compared to TB alone.[97] Furthermore, a recent case series of 5 sick premature infants who developed MRI findings of kernicterus with peak TB concentrations ranging from 8.7 to 11.9 mg/dL and serum albumin concentrations ranging from 1.4 to 2.1 g/dL (BAMR >0.47) underscores the importance of measurement of serum albumin in premature infants.[98]

In summary, the existing evidence suggests that BAMR is better than TB as a predictor of bilirubin-induced neurotoxicity in premature infants, and serum albumin should be routinely measured and used for the management of hyperbilirubinemia in premature infants. Future prospective bilirubin toxicity studies in premature infants should continue to evaluate the usefulness of BAMR as a function of GA.

Bilirubin-Albumin Binding and Neurodevelopmental Outcomes in Premature Infants

The findings of earlier studies correlating bilirubin-albumin binding test results with poor neurological outcomes were very promising.[99–103] A highly significant association of low reserve BBC with abnormal communication skills and language functions at 7 yrs of age after controlling for known confounders was reported in 30–41 wks GA infants with moderate to severe hyperbilirubinemia.[10] These earlier studies of bilirubin-albumin binding status and neurodevelopmental outcomes involved binding tests such as the Sephadex gel filtration method for BBC, dye binding method for reserve BBC, and salicylate saturation index for bilirubin binding affinity.[42] Despite encouraging results, these bilirubin-albumin binding tests failed to find clinical application, most likely due to the

disappearance of kernicterus around the same time period. However, several factors including the development of the peroxidase method to measure UB, re-appearance of kernicterus in developed countries with early newborn discharge, acknowledgement of the high prevalence of kernicterus in the developing world, and identification of ABR as a valid objective assessment tool to evaluate bilirubin toxicity during the neonatal period in premature infants renewed interest in the field.[42,95,104]

Cashore, *et al.*[94] reported a significantly lower BBC, lower bilirubin binding affinity, and higher UB levels measured using the peroxidase method on 5 premature infants with autopsy-proven kernicterus compared to 8 premature infants without kernicterus. In the same year, Ritter, *et al.*[55] reported higher UB concentrations in 7 infants with kernicterus compared to 23 infants without kernicterus in a prospective study. Since then several studies have evaluated the usefulness of UB as a predictor of bilirubin-induced neurotoxicity in premature infants.[2,3,95,105,106] Nakamura, *et al.*[105] in a prospective study compared peak TB and peak UB in LBW infants as predictors of kernicterus, which was defined as one of the following: signs of ABE, autopsy findings of kernicterus, infants surviving with choreoathetosis, spastic paresis, and sensorineural hearing loss. Both peak TB and peak UB levels were higher in 5 VLBW (<1500 g) infants with kernicterus compared to 45 VLBW infants without kernicterus. Similarly, both peak TB and peak UB levels were significantly higher in 7 LBW (1500–2499 g) infants with kernicterus compared to 81 LBW infants without kernicterus. However, on comparing receiver operator characteristic (ROC) curves, the sensitivity and specificity of UB was better than TB in both the groups. In the VLBW group, the sensitivity and specificity for kernicterus was 100% and 96%, respectively, for a UB level of 0.8 µg/dL. In the LBW group, the sensitivity and specificity for kernicterus was 100% and 98%, respectively, for a UB level of 1.0 µg/dL.

More recent studies have used ABR to evaluate bilirubin-albumin binding variables in premature infants. Amin, *et al.*[95] in a prospective study involving 28–32 wks GA infants reported that peak UB, but not peak TB, was significantly higher in 25 infants with abnormal ABR maturation compared to 20 infants with normal ABR maturation. Furthermore, and more importantly, premature infants with abnormal ABR maturation had more concurrent apnea and bradycardia and required more prolonged respiratory support and methylxanthine therapy.[3] Apnea was more closely associated with serum UB levels than TB.[3] The later association has been recently reconfirmed in a prospective study involving 100, 27–33 wks GA premature infants.[2] Premature infants were divided into two groups based on the median UB concentration of 0.92 µg/dL (15.73 nmol/L) in this subject population. Although there was no difference in peak TB between the two groups, the high UB group (mean UB=2.3 µg/dL) had significantly higher frequency of central apnea during the first 2 postnatal weeks compared to the low UB group (mean UB=0.64 µg/dL) with the adjusted incidence rate ratio of 1.9 (95% confidence interval [CI]: 1.2–3.2). Ahlfors, *et al.*[106] in a retrospective study involving 97 infants <35 wks GA reported that UB (odds ratio [OR]: 2.25; 95% CI: 1.07 to 4.72) and not TB (OR: 0.98; 95% CI: 0.84 to 1.15) was strongly associated with abnormal automated ABR screening tests performed prior to discharge. Using ROC curves, both Amin, *et al.*[95] and Ahlfors, *et al.*[106] independently reported that UB is a more specific and sensitive predictor of bilirubin-induced neurotoxicity than TB in premature infants (Figures 1a and b). Ahlfors, *et al.*[106] also reported that the sensitivity

and specificity of the UB:TB ratio was better compared to UB, suggesting that the risk of bilirubin-induced neurotoxicity may not be a function of UB alone, but a combination of both UB and TB, which determines the miscible pool of bilirubin and the tendency to enter tissues. In a recent study (unpublished, Amin, *et al.*) involving late preterm and term infants with severe hyperbilirubinemia, peak UB, but not peak TB, was shown to be associated with bilirubin-induced auditory dys-synchrony, a more significant and persistent neurological outcome (Table 3 and Figure 1c).

Although a well-designed longitudinal study of the association of UB with long-term neurological outcomes is lacking, an increasing serum UB level measured once at ~5 days of age using the peroxidase method in ELBW infants was associated with a higher risk of death or adverse neurodevelopmental outcomes regardless of clinical status.[107] Overall, these early outcome studies in premature infants provide ample evidence that UB, as measured by the peroxidase method using the UB analyzer, is associated with bilirubin-induced neurotoxicity (Table 3). Secondly, these early outcome studies in premature infants suggest that a UB concentration >1 hg/dL measured by the modified peroxidase method using the UB analyzer may be associated with bilirubin-induced neurotoxicity in very premature infants 34 wks GA. However, longitudinal studies are urgently needed to establish the critical level of UB that may be associated with acute and/or chronic bilirubin-induced neurotoxicity in premature infants.

SUMMARY

In summary, both exogenous and endogenous factors, including certain clinical factors such as metabolic acidosis, hypoxia, sepsis, hypoalbuminemia, and hypothermia that adversely influence bilirubin-albumin binding explains variability in bilirubin-albumin binding in premature infants. The variability in bilirubin-albumin binding limits the usefulness of TB as a biochemical marker of bilirubin-induced neurotoxicity in premature infants. Due to lack of valid measures to evaluate other risk factors for bilirubin-induced neurotoxicity, such as genetic susceptibility, neuronal susceptibility, and blood-brain barrier integrity, direct measurement of UB by the modified peroxidase method provides the best risk assessment of bilirubin-induced neurotoxicity for premature infants. In most recent clinical studies that demonstrated significant association between UB and neurological outcomes in term and premature infants, UB analyzer, an FDA-approved semi-automated instrument, was used to measure UB by the modified peroxidase method. Therefore, any newer technologies of direct and indirect measurement of UB will be required to be standardized and validated against the gold standard, modified peroxidase method using the UB analyzer before use in neonatal outcome studies.[42] Until the availability of newer assays of bilirubin-albumin binding for clinical use, the BAMR in addition to TB with consideration to known clinical risk factors, may be used as a reasonable substitute for the management of hyperbilirubinemia in premature infants.

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Best Practices

What is the current practice?

- TB is primarily used to evaluate and manage 34 wks GA infants with unconjugated hyperbilirubinemia.

Recommendations

- Clinicians should routinely consider assessment of clinical risk factors such as hypoxia, acidosis (including perinatal asphyxia), sepsis, and hypothermia in the clinical algorithm for the management of hyperbilirubinemia.
- Albumin levels should be routinely measured in 34 wks GA infants with unconjugated hyperbilirubinemia.
- In the presence of hypoalbuminemia (low BBC) and/or presence of known clinical risk factors (such as hypoxia, acidosis, etc.) that may negatively influence binding affinity, treatment (phototherapy and exchange transfusion) should be considered at a much lower TB level than otherwise used in the absence of these factors.
- Hypoxia, acidosis, sepsis, and hypothermia should be prevented and/or treated aggressively in premature infants with unconjugated hyperbilirubinemia.
- As discussed in the article and shown in Table 2, IL intake should be appropriately limited during the first postnatal week in 28 wks GA infants to decrease the incidence of elevated UB and secondary risk of bilirubin-induced neurotoxicity.

KEY POINTS

- Bilirubin-albumin binding limits the use of total serum/plasma bilirubin (TB) as an indicator of bilirubin-induced neurotoxicity in premature infants.
- Bilirubin binding capacity (BBC) and affinity are low and variable in premature infants.
- Specific exogenous drugs, intravenous lipids, and clinical factors such as metabolic acidosis, hypothermia, hypoxia, and sepsis may adversely affect bilirubin-albumin binding.
- Bilirubin:albumin molar ratio (BAMR), in conjunction with TB, may be useful as a marker of bilirubin-induced neurotoxicity in more mature preterm and late preterm infants.
- Unbound bilirubin (UB) is a better vascular gauge of hyperbilirubinemia “severity”, *vis-à-vis* bilirubin-induced neurotoxicity, than the conventionally measured TB in premature infants.

Figure 1.

Receiver operator characteristics (ROC) curves of total serum/plasma bilirubin (TB) and unbound bilirubin (UB) in premature infants as predictors of abnormal auditory brainstem-evoked response (ABR) maturation during the first week (**a***); ROC curves of TB, UB, and UB:TB ratio (UBRATIO) as predictors of an abnormal automated ABR at discharge in premature and term infants (**b****); and ROC curves of TB, bilirubin:albumin molar ratio (BAMOLAR), and UB as predictors of auditory dys-synchrony in late preterm and term infants (**c**). The straight line in **b** and **c** is the expected curve (unity) if the variable has no predictive value (area under unity curve=0.5). The areas under the UB or UB:TB are significantly greater than that under the TB and BAMOLAR curves. (*Pediatrics 2001; 107:664–70; **J Perinatol 2009; 29:305–9; with permission)

Table 1**Drugs and Bilirubin Displacing Effect**

Drugs with Moderate to Strong Displacing Effect	Drugs with Minimal Displacing Effect	Drugs with No Displacing Effect
Sulfamethoxazole	Nafcillin	Aminoglycosides
Sulfisoxazole	Ampicillin	Insulin
Sulfadiazine	Oxacillin	Dopamine
Ceftriaxone	Methicillin	Dobutamine
Cefotetan	Furosemide	Morphine
Moxalactam	Phenobarbital	Fentanyl
Dicloxacillin	Papavarine	Indomethacin
Carbenicillin	Caffeine	Vitamins
Cefazolin	Aminophylline	Diazepam
Ibuprofen	Cefotaxime	Thyroxine

Table 2

Incidence of Elevated Unbound Bilirubin (EUB) Concentration (>1 µg/dL) as a Function of Intravenous Lipid (IL) Intake in 24–33 Wks Gestational Age (GA) Infants

	24–26 wks GA (n=11)	26 ^{1/7} –28 wks GA (n=21)	28 ^{1/7} –30 wks GA (n=11)	30 ^{1/7} –32 wks GA (n=11)	32 ^{1/7} –33 wks GA (n=8)	p
n (%) subjects with EUB @ IL 1.5 g/kg/day	4 (36)	1 (5)	0 (0)	0 (0)	0 (0)	0.015
n (%) subjects with EUB @ IL 2 g/kg/day	5 (45)	5 (24)	0 (0)	0 (0)	0 (0)	0.009
n (%) subjects with EUB @ IL 2.5 g/kg/day	6 (55)	7 (33)	0 (0)	0 (0)	0 (0)	0.001
n (%) subjects with EUB @ IL 3 g/kg/day	5(63)	10 (77)	0 (0)	0 (0)	0 (0)	<0.001

n, number of subjects

Unbound Bilirubin (UB) and Neurodevelopmental Outcomes in Premature Infants

Table 3

Study	GA or Birthweight (n)	Mean Unbound Bilirubin ($\mu\text{g/dL}$) [#]		
		No KI or Normal ABR	ABR Changes	Overt KI
Ritter (1982)	26–32 wks GA (30)	0.65 $\pm$ 0.05	Not Done	1.10 $\pm$ 0.26
Cashore (1982)	1500 g (13)	0.76 $\pm$ 0.58	Not Done	1.60 $\pm$ 0.52
Nakamura (1992)	1500–2499 g (88) <1500 g (50)	0.54 $\pm$ 0.2 0.46 $\pm$ 0.17	Not Done	1.30 $\pm$ 0.24 1.13 $\pm$ 0.34
Amin (2001)	28–32 wks GA (45)	0.40 $\pm$ 0.15	0.62 $\pm$ 0.2	None
Ahlfors (2009)	24–41 wks GA (191)	0.93 $\pm$ 0.70	1.76 $\pm$ 1.31	None
Amin (Unpublished)	34 wks GA with severe hyperbilirubinemia (44)	1.7 $\pm$ 0.8	4.2 $\pm$ 1.7	None

[#] Mean $\pm$ SD; n, number of subjects; KI, kernicterus; ABR, auditory brainstem-evoked response; GA, gestational age