Chapter 6

Beyond Plasma Bilirubin: the Effects of Phototherapy and Albumin on Brain Bilirubin Concentrations in Gunn Rats

F.J.C. Cuperus [1*]
A.B. Schreuder [1*]
D.E. van Imhoff [2]
L.Vitek [3,4]
J. Vanicova [3]
R. Konickova [3]
C.E. Ahlfors [5]
C.V. Hulzebos [2]
H.J. Verkade [1]

**Both authors contributed equally*

[1]Pediatric Gastroenterology and Hepatology, Department of Pediatrics, Center for Liver, Digestive, and Metabolic Diseases, Beatrix Children's Hospital- University Medical Center Groningen, University of Groningen, The Netherlands.
[2]Neonatology, Department of Pediatrics, Beatrix Children's Hospital - University Medical Center Groningen, The Netherlands.
[3]Institute of Clinical Biochemistry and Laboratory Diagnostics, 1st Faculty of Medicine, Charles University, Prague, Czech Republic.
[4]4th Department of Internal Medicine, 1st Faculty of Medicine, Charles University, Prague, Czech Republic.
[5]Stanford University, School of Medicine, Stanford, California, USA

Submitted

6.1 Abstract

Severe unconjugated hyperbilirubinemia, as occurs in Crigler-Najjar disease and neonatal jaundice, carries the risk of neurotoxicity. Neurotoxicity is caused by the ability of free bilirubin (UCB_{free}), the non-protein bound fraction of unconjugated bilirubin (UCB), to translocate into the brain. We hypothesized that higher plasma protein levels would decrease this free bilirubin fraction and thereby prevent its deposition in the brain. Accordingly, we assessed the effect of albumin treatment on plasma UCB_{free} and brain bilirubin levels during phototherapy in Gunn rat models of chronic and acute jaundice. As a chronic model, resembling patients with Crigler-Najjar disease, we treated adult hyperbilirubinemic Gunn rats for 16 days with phototherapy or with phototherapy + human serum albumin (HSA; 2.5 g/kg *i.p.* at t=14 days). As an acute model, resembling neonatal jaundice, we induced hemolysis in adult hyperbilirubinemic Gunn rats by acetylphenylhydrazine, and then treated these animals with phototherapy or with phototherapy + HSA (2.5 g/kg *i.p.* at t=0h) for 48h. In the chronic model, phototherapy decreased plasma UCB_{free} by 55% and decreased brain bilirubin levels by 45% (each $p<0.001$). Adjunct HSA treatment enhanced the efficacy of phototherapy; it decreased plasma UCB_{free} and brain bilirubin levels by 88% and 67%, respectively (each $p<0.001$). In the acute model, phototherapy decreased plasma UCB_{free} by 31% ($p<0.001$), but failed to decrease brain bilirubin. Adjunct HSA decreased plasma UCB_{free} by 76% ($p<0.001$) and completely prevented the deposition of bilirubin in the brain during hemolytic jaundice. This chapter demonstrates that adjunct treatment with albumin decreases brain bilirubin levels in phototherapy-treated Gunn rats, during both chronic and acute jaundice. We hypothesize that albumin decreases these levels by lowering UCB_{free} in the plasma. Present results underline the need to evaluate the use of albumin as an adjunct treatment to phototherapy in Crigler-Najjar patients and in hyperbilirubinemic neonates.

6.2 Introduction

Crigler-Najjar patients and hemolytic neonates suffer from unconjugated hyperbilirubinemia.[1] Severe unconjugated hyperbilirubinemia can lead to brain damage. This damage is mediated by the ability of "free" bilirubin (UCB_{free}), the small (<1%) fraction of unconjugated bilirubin (UCB) not bound to plasma proteins, to cross the blood-brain barrier (BBB).[2-6] Within the brain, bilirubin disrupts several vital cell functions and induces apoptosis and necrosis. Bilirubin-induced neurotoxicity may eventually lead to kernicterus and even death.[3,7,8]

Severe unconjugated hyperbilirubinemia is conventionally treated by phototherapy, which converts UCB into photo-isomers that can readily be excreted in the bile.[9] Phototherapy, however, has some disadvantages. Crigler-Najjar patients, who suffer from permanent inherited unconjugated hyperbilirubinemia, may need up to 16h of treatment per day. In spite of this intensive regimen, up to 25% of these patients will eventually develop brain damage.[10,11] Phototherapy is more effective during neonatal hemolytic jaundice, but may still require additional, potentially dangerous, exchange transfusions.[12] The efficacy of phototherapy is often estimated by its hypobilirubinemic effect. Plasma bilirubin levels, however, correlate poorly with the occurrence of brain damage in individual patients.[6] The reason for this poor correlation lies in the inability of protein-bound bilirubin (>99% of total plasma bilirubin) to leave the circulation.[2,3,5,6] Only UCB_{free} is able to translocate across the BBB, and thus plays a key role in the pathogenesis of bilirubin-induced brain damage. UCB_{free} concentrations, however, are not routinely evaluated in phototherapy-treated patients. The main reason for this lies in the inaccuracy of the commercial test, most notably caused by a 42-fold sample dilution that alters bilirubin-albumin binding.

We reasoned that decreasing UCB_{free} in the plasma could prevent bilirubin deposition in the brain. Human serum albumin (HSA) infusion could, theoretically, achieve this goal by providing additional binding sites for UCB_{free} in the plasma. Interestingly, HSA treatment has been used in severely jaundiced neonates.[13-17] Its efficacy, however, has been difficult to establish. This difficulty is partly due to the technical shortcomings of the available UCB_{free} measurement, but mainly results from the obvious inability to assess brain bilirubin levels in humans. Recently, Ahlfors *et al.* developed an automated UCB_{free} test with minimal sample dilution, while Zelenka *et al.* developed a highly sensitive method for tissue bilirubin determinations.[18,19] We now use these techniques to evaluate the effect of HSA treatment on plasma UCB_{free} and brain bilirubin levels in two well-established animal models. As a chronic model, resembling patients with Crigler-Najjar disease, we treated adult Gunn rats with

long-term phototherapy or with photoherapy + HSA.[20] As an acute model, resembling severe hemolytic jaundice, we induced hemolysis by 1-acetyl-2-phenyl-hydrazine (APHZ) in adult Gunn rats, and then treated these animals for 48h with phototherapy or phototherapy + HSA.[21] We now demonstrate that HSA treatment decreases plasma UCB_{free} and brain bilirubin levels in phototherapy-treated Gunn rats, both during chronic and acute jaundice. We speculate that HSA and phototherapy work *in tandem*: HSA binds UCB_{free} within the plasma, and phototherapy then promotes its excretion *via* the bile. Our results underline the need to evaluate the use of HSA as adjunct to phototherapy in randomized clinical trials.

6.3 Animals, materials, and methods

6.3.1 Animals

Homozygous male Gunn rats (RHA/jj; 225-340g, aged 10-12 weeks) from our breeding colony were kept in an environmentally controlled facility and were fed *ad libitum* with free access to water. Food intake, fluid intake, and body weight were determined regularly. The Animal Ethics Committee of the University of Groningen (Groningen, The Netherlands) approved all experimental protocols.

6.3.2 Materials

Hope Farms BV (Woerden, The Netherlands) produced the semi-synthetic control diet (code 4063.02), containing 13 energy% fat and 5.2 wt% long-chain fatty acids.[22] Gunn rats were fed this diet during a 5-week run-in period, and during the experiments.[22] HSA (Albuman®; 200 g/L) was purchased from Sanquin (Amsterdam, The Netherlands). APHZ, horseradish peroxidase type 1, D-glucose, glucose oxidase, hydrogen peroxide, and UCB were purchased from Sigma Chemical Co. (St. Louis, MO). Commercial UCB was further purified according to the method of Ostrow *et al.*[23] Phototherapy was administered continuously to Gunn rats (shaven on flank and back) *via* two blue phototherapy lamps (Philips, TL-20W/03T) suspended in a reflective canopy 20 cm above the cage. The phototherapy dose (17 $\mu W/cm^2/nm$; 380-480 nm) was measured by an Elvos-LM-1010 Lux meter at 20 cm distance.[24]

6.3.3 Methods

Permanent unconjugated hyperbilirubinemia

Adult Gunn rats were randomized to receive either no treatment (n=7) or phototherapy (17 μW/cm²/nm; n=14) for 16 days. At 14 days, we randomized the phototherapy-treated rats to receive either NaCl 0.9% (w/v; n=7) or HSA (2.5 g/kg, n=7) by means of a single *i.p.* injection. We determined plasma bilirubin levels from tail vein blood at T=0, 14, and 16 days, and plasma UCB_{free} at T=16 days under isoflurane anesthesia. After 16 days, all animals were exsanguinated *via* the descending aorta and flushed *via* the same port with 100-150 ml NaCl 0.9% under isoflurane anesthesia. The brain, liver, and aliquots of visceral fat were subsequently harvested for the determination of tissue bilirubin levels. These samples were rinsed 2 times in phosphate buffered saline, snap frozen in liquid nitrogen, and immediately stored (wrapped in aluminum foil) at -80 °C until analysis.[19]

Acute unconjugated hyperbilirubinemia

Adult Gunn rats received a single APHZ injection *i.p.* (15 mg/kg BW; T=-24h) to induce hemolysis. We then randomized these animals after 24h (T=0h) to receive either no treatment (n=6), phototherapy + NaCl 0.9% (17 μW/cm2/nm; n=6), or phototherapy + HSA (2.5 g/kg; n=6) for another 48h. NaCl 0.9% (sham) and HSA were administered as a single *i.p.* injection at T=0h. We determined plasma bilirubin, UCB_{free}, and albumin levels from tail vein blood at T=-24, -12, 0, 12, 24, 36, and 48h under isoflurane anesthesia. Hemoglobin (Hb), reticulocyte count, and hematocrit (Ht), were determined at T=-24h and T=48h. After 48h, all animals were exsanguinated and brain, liver, and visceral fat samples were subsequently harvested for the determination of tissue bilirubin levels, as described above.[19]

Plasma analysis

Blood samples were protected from light, stored at -20 °C under argon directly after collection, and processed within 2 weeks. UCB concentrations were determined by routine spectrophotometry on a P800 unit of a modular analytics serum work area from Roche Diagnostics Ltd. (Basel, Switzerland). Hb, Ht, and reticulocytes were determined on a Sysmex XE-2100 hematology analyzer (Goffin Meyvis, Etten-Leur, The Netherlands). We previously found in Gunn rats that the total bilirubin concentration, measured by spectrophotometry, equals the

total UCB concentration, measured by high-liquid performance chromatography after chloroform extraction (coefficient of variation: ~5%).[22,25] UCB_{free} was determined using a Zone Fluidics system (Global Flopro, Global Fia Inc, WA), as previously described by Ahlfors *et al.*[18]

Tissue bilirubin analysis

Tissue bilirubin content was determined using HPLC with diode array detector (Agilent, Santa Clara, CA, USA) as described earlier. [19] Briefly, 300 pmol of mesobilirubin in dimethyl sulfoxide (used as an internal standard) was added and samples were homogenized on ice. Bile pigments were then extracted into chloroform/hexane (5:1) solution at pH 6.0, and subsequently extracted in a minimum volume of methanol/carbonate buffer (pH=10) to remove contaminants. The resulting polar droplet (extract) was loaded onto C-8 reverse phase column (Phenomenex, Torrance, CA, USA) and separated pigments were detected at 440 nm. The concentration of bilirubin was calculated as nmol/g of wet tissue weight. All steps were performed under dim light in aluminum-wrapped tubes. We did not specifically measure bilirubin deposition in the brain nuclei, but relied on total tissue bilirubin measurements.

Statistical analysis

Normally distributed data that displayed homogeneity of variance (by calculation of Levene's statistic), were expressed as means ± SD, and analyzed with parametric statistical tests. Analysis of variance (ANOVA) with post-hoc Tukey correction was performed for comparisons between groups, and the Student *t* test for comparison of paired data within groups. The level of significance was set at $p<0.05$. Analyses were performed using PASW Statistics 17.0 for Mac (SPSS Inc., Chicago, IL).

6.4 Results

As a model for Crigler-Najjar disease, we first treated permanently jaundiced Gunn rats with routine phototherapy and phototherapy + HSA for 16 days. Figure 1A shows that both treatments decreased plasma UCB levels to a similar extent (46% and 54% at t= 16 days, respectively), compared with untreated controls ($p<0.001$). Figure 1B shows that phototherapy and phototherapy + HSA decreased plasma UCB_{free} levels by 55% and 88%, respectively ($p<0.001$). Finally,

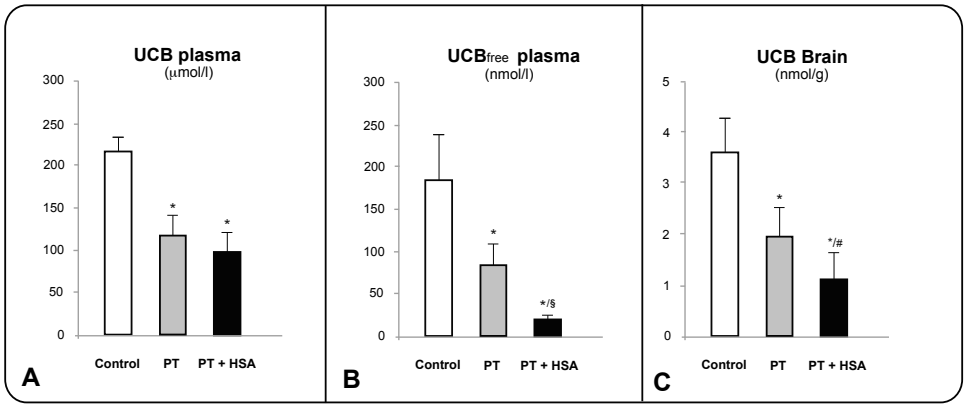

Figure 1. Effects of no treatment (controls), routine phototherapy (PT) or phototherapy + HSA on plasma UCB (panel A), plasma UCB_{free} (panel B), and brain bilirubin levels (panel C) in Gunn rats at t=16 days. Adult Gunn rats were randomized to receive either no treatment or phototherapy (17 μW/cm2/nm) for 16 days. At 14 days, we randomly injected the phototherapy-treated animals with saline (sham) or with HSA (2.5 g/kg). Plasma bilirubin levels were similar in the phototherapy and phototherapy + HSA group at the time of this injection (data not shown). *p<0.001 compared with controls. #p<0.05, §p<0.01 compared with phototherapy alone.

figure 1C shows that phototherapy and phototherapy + HSA decreased brain bilirubin concentrations by 45% and 67%, respectively (p<0.001), compared with untreated controls. Adjunct HSA thus lowered plasma UCB_{free} and brain bilirubin by an additional 33% and 22%, respectively (each p<0.01), compared with phototherapy alone. HSA also decreased hepatic bilirubin levels by an additional 33% (p<0.01), compared with phototherapy alone (figure 2A), but failed to induce a significant additive decrease in visceral fat bilirubin levels (figure 2B). Figure 3A shows that plasma UCB levels correlated well with brain bilirubin levels (y=0.29x-0.60; r^2=0.78; p<0.001). Plasma UCB levels above 200 μmol/L however, appeared relatively poor predictors for individual brain bilirubin concentrations. Figure 3B shows the correlation between UCB_{free} levels and brain bilirubin levels (y=0.24x-0.88; r^2=0.86; p<0.001).

As a model for severe acute jaundice we then treated hemolytic Gunn rats with routine phototherapy and phototherapy + HSA for 48 hours. Figure 4A shows that both treatments decreased the severity of hemolytic unconjugated hyperbilirubinemia, compared with untreated hemolytic controls. Phototherapy, however, only decreased plasma UCB levels by 14% at t=36h (p<0.01), while phototherapy + HSA decreased these levels by at least 29% from 36h onward (p<0.001). Figure 4B shows that phototherapy decreased plasma UCB_{free} levels by

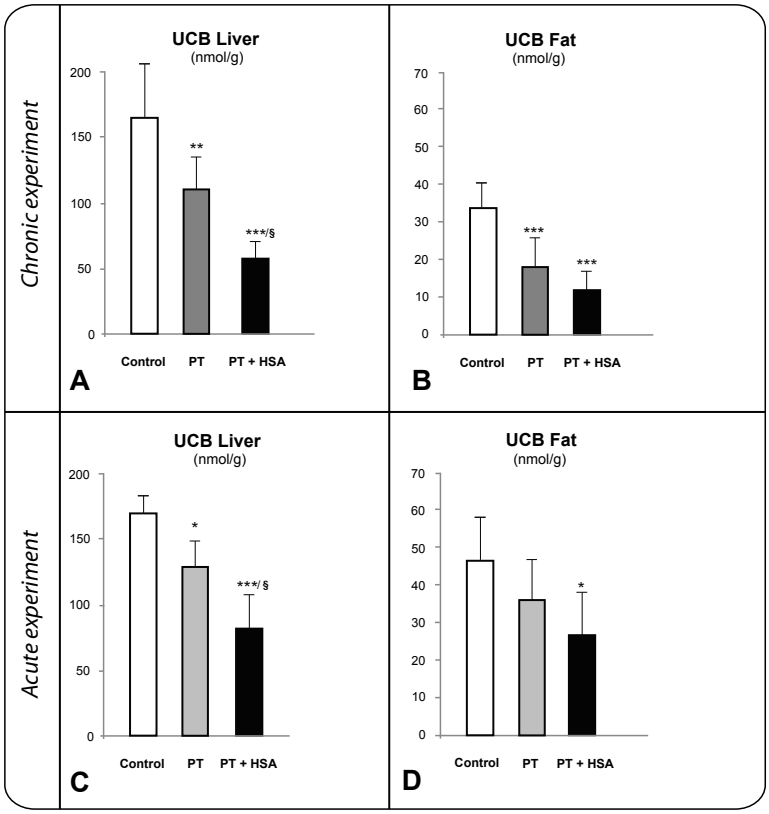

Figure 2. Effects of no treatment (controls), routine phototherapy (PT) or phototherapy + HSA on liver (panel A/C) and visceral fat (panel B/D) bilirubin levels in non-hemolytic (chronic experiment) and hemolytic (acute experiment) Gunn rats. For experimental setup, kindly refer to the Methods section. *p<0.05; **p<0.01; ***p<0.001, compared with controls. §p<0.01, compared with single phototherapy.

31% at t=48h (p<0.05), compared with controls, while phototherapy + HSA decreased these levels by at least 41% from 12h onward (p<0.001). Adjunct HSA thereby lowered plasma UCB and plasma UCB_{free} levels by an additional 14-16%, and 25-47%, respectively (each p<0.01), compared with routine phototherapy. Figure 4C shows that phototherapy + HSA decreased brain bilirubin concentrations by 50% (p<0.001). The resulting brain bilirubin levels were comparable to those of untreated non-hemolytic Gunn rats (figure 1C), which demonstrated that phototherapy + HSA completely prevented the increase in brain bilirubin due to hemolytic jaundice. HSA also decreased hepatic bilirubin

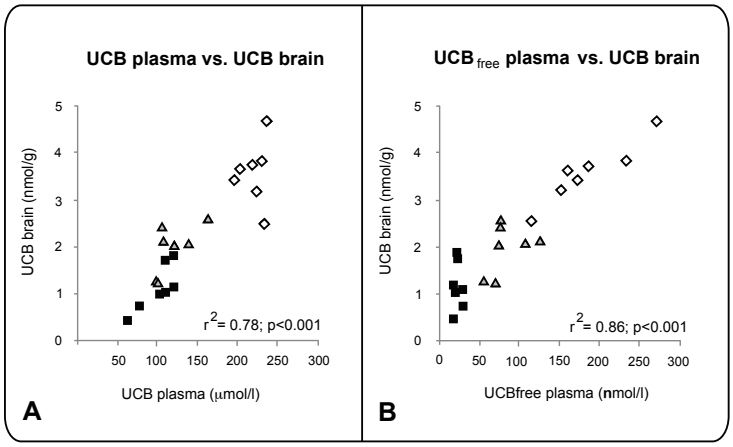

Figure 3. The correlation between plasma UCB and brain bilirubin levels (panel A), and the correlation between plasma UCB_{free} and brain bilirubin levels (panel B) in Gunn rats at t=16 days. Adult Gunn rats were randomized to receive either no treatment or phototherapy (17 μW/cm2/nm) for 16 days. At 14 days, we randomly injected the phototherapy-treated animals with saline (sham) or with HSA (2.5 g/kg). ◇ control, ▲ phototherapy, ■ phototherapy + HSA.

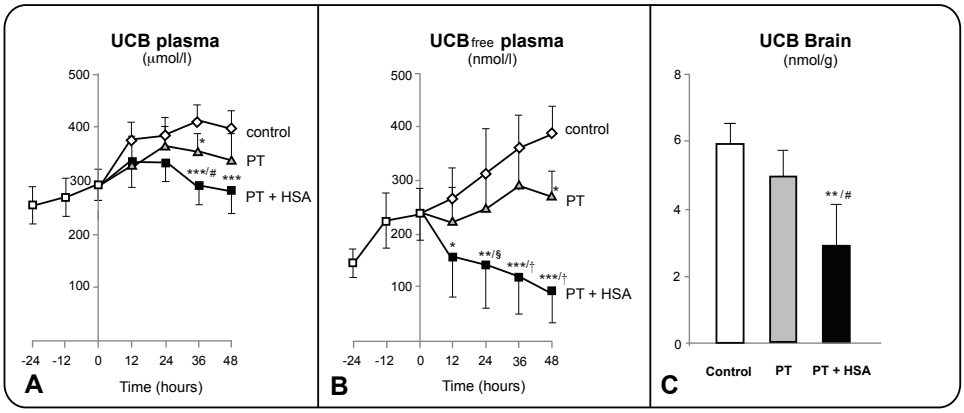

Figure 4. Effects of no treatment (controls), routine phototherapy (PT) or phototherapy + HSA on plasma UCB (panel A), plasma UCB_{free} (panel B), and brain bilirubin levels (panel C) in hemolytic Gunn rats at t= 48h. Adult Gunn rats received APHZ i.p. to induce hemolysis, and were randomized after 24h to receive no treatment, phototherapy (17 μW/cm2/nm), or phototherapy + HSA (2.5 g/kg) for 48h. Plasma bilirubin levels were similar in all groups during the 24h-run in after APHZ injection (data not shown). *p<0.05; **p<0.01; ***p<0.001, compared with controls. #p<0.05; §p<0.01; †p<0.001, compared with single phototherapy.

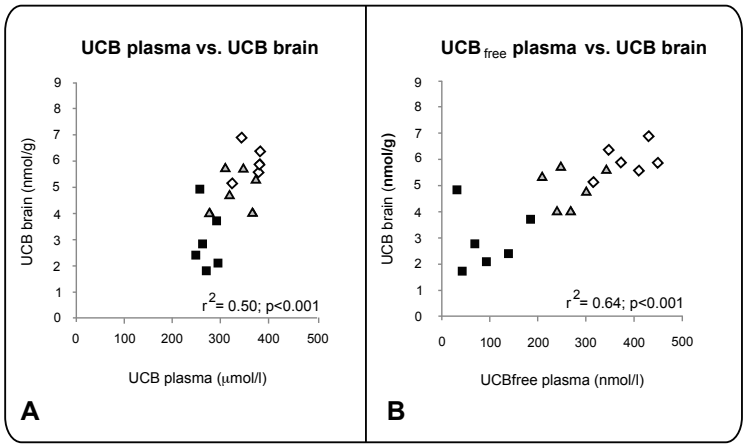

Figure 5. The correlation between plasma UCB and brain bilirubin levels (panel A), and the correlation between plasma UCB_{free} and brain bilirubin levels (panel B) in Gunn rats at t= 48h. Adult Gunn rats received APHZ i.p. to induce hemolysis, and were randomized after 24h to receive no treatment, phototherapy (17 μW/cm2/nm), or phototherapy + HSA (2.5 g/kg) for 48h ◇ control, ▲ phototherapy, ■ phototherapy + HSA.

levels by an additional 36% ($p<0.01$), compared with routine phototherapy (figure 2C). Phototherapy + HSA, but not phototherapy alone, decreased bilirubin levels in visceral fat by 41%, compared with controls ($p<0.05$; figure 2D). Figure 4C shows that phototherapy alone failed to decrease brain bilirubin during hemolytic disease. Again, plasma bilirubin levels above 200 μmol/L appeared relatively poor predictors for individual brain bilirubin concentrations (figure 5A). Figure 5B shows that plasma UCB_{free} levels correlated well with brain bilirubin levels ($y=0.0091x+2.32$; $r^2=0.64$; $p<0.001$).

APHZ administration induced a comparable hemolysis in all groups, as indicated by the similar changes in Hb, Ht, and reticulocyte levels (figure 6A-C). Figure 6D shows that a single *i.p.* HSA injection rapidly increased plasma albumin levels (42% after 12h; $p<0.001$), compared with untreated controls. Mean growth rates did not differ significantly between experimental and control groups in each experiment (data not shown).

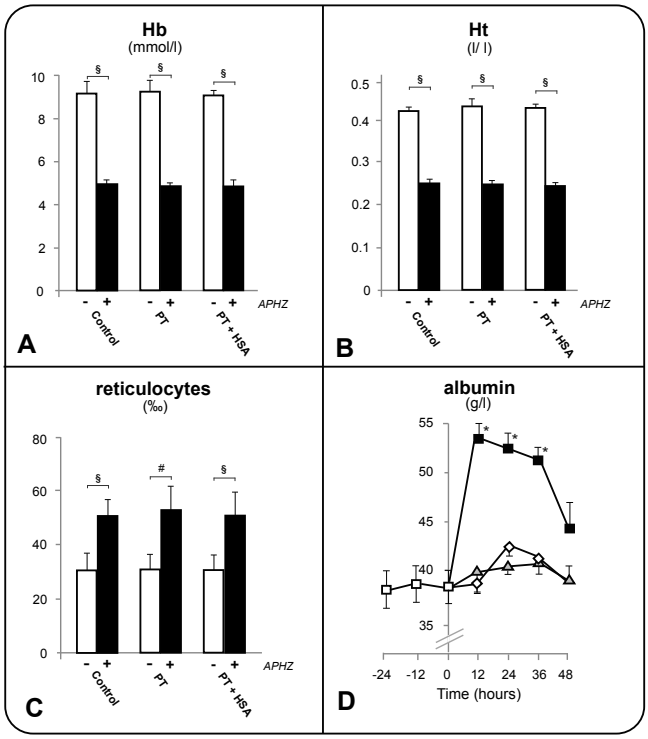

Figure 6. Effects of APHZ injection on Hb (Panel A), Ht (Panel B), and reticulocyte (Panel C) levels, and the effect of human serum albumin injection on plasma albumin levels (Panel D) in Gunn rats. Adult Gunn rats received APHZ i.p. to induce hemolysis, and were randomized after 24h to receive no treatment, phototherapy (17 μW/cm2/nm), or phototherapy + HSA (2.5 g/kg) for 48h. *p<0.001, compared with controls. #p<0.01; §p<0.001, compared with pre-treatment values at T=0h. ◇ control, △ phototherapy, ■ phototherapy + HSA.

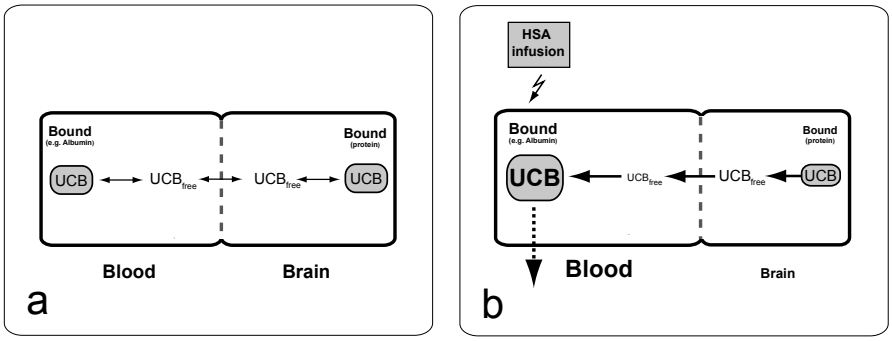

Figure 7. Human serum albumin (HSA) treatment during unconjugated hyperbilirubinemia: (a) Unconjugated hyperbilirubinemia may result in the accumulation of unconjugated bilirubin (UCB) within the brain. Only non-protein bound UCB (UCB_{free}) is able to move between the blood (e.g. vascular compartment) and the brain (e.g. extravascular compartment). (b) Treatment with HSA decreases UCB_{free} levels within the blood. This promotes an UCB_{free}-induced shift of bilirubin from the brain into the circulation. Additional phototherapy subsequently converts this bilirubin into photo-isomers that can readily be excreted with the bile (dashed arrow).

6.5 Discussion

In this study we demonstrate that HSA effectively decreases brain bilirubin levels in phototherapy-treated Gunn rats. The decrease was apparent during both chronic and acute hemolytic jaundice. Our results support the feasibility of adjunct HSA treatment during phototherapy in Crigler-Najjar disease and neonatal jaundice.

The rationale of HSA treatment is based on the premises that UCB_{free} translocates into the brain and, secondly, that i.v. albumin prevents this translocation by binding UCB_{free} within the plasma. The role of UCB_{free} translocation became apparent in the 1950s, when sulfisoxazole-treated neonates developed kernicterus in the presence of unusually low plasma bilirubin levels.[26,27] It was soon discovered that sulfisoxazole displaced UCB from albumin, which first suggested the importance of the non-albumin bound UCB fraction.[28] Since then, many studies have supported the critical role of UCB_{free} in the pathogenesis of bilirubin-induced brain damage.[2,3,5,6] Only recently, Ahlfors et al showed that auditory brainstem response screening, a quantifiable method to evaluate bilirubin-induced neurotoxicity in neonates, correlated with UCB_{free} rather than with total bilirubin levels.[4] The protective role of HSA administration has also been investigated in neonates. Its efficacy, however, has never been established in randomized controlled trials. Two retrospective studies have shown reduced UCB_{free} levels in jaundiced neonates after HSA administration.[15,17] One additional small cohort study has shown some protective effect of HSA administration on the development of brain damage, as measured by auditory brainstem response screening.[16] Other studies, however, failed to demonstrate beneficial effects of HSA treatment.[14] Importantly, most human studies did not assess plasma UCB_{free}, or used methods that seriously underestimate UCB_{free} levels due to a 42-sample dilution.[14-17,29] The absence of reliable data on UCB_{free} levels obviously impeded the interpretability of these studies. In addition, human studies are intrinsically limited by the impossibility of measuring brain bilirubin levels. Taken together, these issues demonstrated the need for a more thorough evaluation of HSA administration. Recently, Ahlfors *et al.* automated and improved the available UCB_{free} test, while Zelenka *et al.* developed a sensitive method for tissue bilirubin determinations.[18,19] These newly developed methods allowed us to reliably measure UCB_{free} and brain bilirubin levels in two well-established animal models. [20,21]

We first investigated the efficacy of HSA treatment in permanently hyperbilirubinemic Gunn rats, as a model for Crigler-Najjar disease. HSA treatment decreased both UCB_{free} and brain bilirubin levels in these animals. HSA also decreased hepatic, and to a lesser extent visceral fat, bilirubin levels in these animals (figure 2). These results support a model in which only UCB_{free} is

able to move between the vascular and extravascular (tissue) compartment of the bilirubin pool (figure 7). This translocation occurs across both the vascular endothelial cells and the BBB. In this model, HSA administration could act *in tandem* with phototherapy. HSA first decreases UCB_{free} within the vascular compartment, and promotes a bilirubin shift from the extravascular pool. The newly recruited intravascular bilirubin is then, after its exposure to photo-isomerization, rapidly transported to the liver and excreted *via* the bile (figure 7).[30] Our results in acutely jaundiced Gunn rats show that HSA completely prevented the deposition of bilirubin in the brain during hemolysis. Routine phototherapy, however, failed to prevent this deposition. Theoretically, this lack of effect may be time-related: phototherapy only decreased UCB_{free} after 48h in our acute experiment, but decreased plasma UCB levels within 36h. We cannot exclude the possibility that non-protein bound bilirubin is less readily converted into photo-isomers than the protein bound fraction. Indeed, long-term phototherapy apparently circumvented this delay and decreased both UCB_{free} and brain bilirubin in permanently jaundiced Gunn rats. Taken together, our results not only demonstrate the benefits of adjunct HSA, but also question the efficacy of phototherapy during acute hemolytic jaundice.

Our present study shows a relatively poor correlation between the higher plasma bilirubin levels (>200 μmol/L) and brain bilirubin levels. This is consistent with clinical evidence that shows a poor predictive value of plasma bilirubin, especially above 300 μmol/L, for neurotoxicity.[6] These results illustrate the limitations of using plasma UCB to assess the individual risk for bilirubin toxicity. UCB_{free} levels correlated well with individual brain bilirubin levels in our experiment. Yet, the r^2-value indicated that even in highly controlled animal studies, a considerable fraction of the variation in brain bilirubin could not be solely related to plasma UCB_{free} levels. Our data therefore confirm that, apart from UCB_{free}, other factors (*e.g.* blood pH, BBB integrity etc.) are also highly important in the pathogenesis of bilirubin-induced neurological damage.[31] It would be interesting to investigate these factors, as well as the accumulation of bilirubin in *specific* brain regions (since bilirubin predominantly accumulates in the deep nuclei of the brain) during HSA treatment in future animal experiments.

In our study we have used a commercially available HSA solution and found clear proof that it enhanced the therapeutic efficacy of routine phototherapy. This albumin solution is currently widely applied in neonates, without serious side effects.[14-17,29] This greatly increases its therapeutic potential and will facilitate the set-up of future clinical trials. These trials should ideally incorporate UCB_{free} measurements and auditory brainstem response screening to monitor the efficacy of treatment. UCB_{free} measurements should be performed according to the recently developed method of Ahlfors *et al.* that enables an automated and reliable measurement of UCB_{free} in a clinical setting.[18]

Taken together, our data show that HSA enhances the efficacy of routine phototherapy in phototherapy-treated Gunn rats, both during permanent and acute jaundice. Our study underlines the need to critically evaluate the use of HSA as adjunct to phototherapy in randomized controlled clinical trials. We expect that a focus on tissue, rather than on plasma bilirubin levels, could induce a paradigm shift that will allow the development of increasingly efficient treatment strategies. These strategies will, hopefully, further decrease the burden of bilirubin-induced brain damage in the near future.

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