Bilirubin isomer distribution in jaundiced neonates during phototherapy with LED light centered at 497 nm (turquoise) vs. 459 nm (blue)

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BACKGROUND: Phototherapy using blue light is the treatment of choice worldwide for neonatal hyperbilirubinemia. However, treatment with turquoise light may be a desirable alternative. Therefore, the aim of this randomized, controlled study was to compare the bilirubin isomer distribution in serum of jaundiced neonates after 24 h of therapy with narrow-band (LED) light centered at 497 nm (turquoise) vs. 459 nm (blue), of essentially equal irradiance.

MATERIALS: Eighty-three neonates (≥33 wk gestational age) with uncomplicated hyperbilirubinemia were included in the study. Forty neonates were exposed to light centered at 497 nm and 43 infants with light centered at 459 nm. Irradiances were 5.2×10^{15} and 5.1×10^{15} photons/cm^2/s, respectively.

RESULTS: After 24 h of treatment no significant differences in serum concentrations of total bilirubin isomers and Z,Z-bilirubin were observed between the 2 groups. Interestingly, concentrations of Z,E-bilirubin, and thus also total bilirubin isomers formed during therapy, were highest for infants receiving light centered at 459 nm, while the concentration of E,Z-bilirubin was highest for those receiving light centered at 497 nm. No significant difference was found between concentrations of E,Z-lumirubin.

CONCLUSION: Therapy with LED light centered at 497 nm vs. 459 nm, applied with equal irradiance on the infants, resulted in a different distribution of bilirubin isomers in serum.

Jaundice occurs in the majority of neonates. The total serum bilirubin concentration (TSB) is a dynamic balance between bilirubin production, distribution, and excretion by the liver and kidneys. In late preterm and term neonates the hyperbilirubinemia is generally harmless and culminates most often from the third to the sixth day of life.

Phototherapy using blue light is the treatment of choice for neonatal hyperbilirubinemia, and it is administered to prevent acute and chronic bilirubin encephalopathy (kernicterus). This condition is rare in the industrialized world, but it is still common in many developing countries (1).

Absorption of light by bilirubin in the skin transforms it from the native nonpolar and toxic Z,Z-bilirubin to more polar bilirubin isomers: the configurational isomers Z,E- and E,Z-bilirubin and structural isomers E,Z- and E,E-lumirubin, which can then be excreted into the bile and urine without conjugation. Configurational isomerization is reversible, whereas formation of lumirubins is irreversible (**Figure 1**) (2). In addition, small amounts of oxidation products are formed (6).

Blue light, matching the absorption spectrum of serum bilirubin *in vitro* with a maximum of 459 nm, is used in standard phototherapy worldwide (7). However, treatment with higher wavelength turquoise light may be a desirable alternative. Using optical models of the skin, Agati *et al.* (8) and Lamola *et al.* (9) suggested that for equal irradiance, the greatest decrease of the Z,Z-bilirubin concentration would occur with exposure of the infants to turquoise light with peak irradiance within the wavelength range of 485 to 505 nm or 475 to 480 nm, respectively. In our first clinical study using broad spectrum fluorescent light with equal irradiance on the infants, the decrease of TSB was 20% greater for turquoise light with peak irradiance at 490 nm than for blue light with peak irradiance at 452 nm in treatment of preterm infants (10). In our subsequent clinical investigation, also with equal irradiance on the infants, we then compared the effects of light emitted from narrow-band light emitting diodes (LEDs) centered at 497 nm (turquoise) and 459 nm (blue), respectively. The same decrease in the TSB concentration was observed in both groups of late preterm and term infants (11). An extension of this randomized, controlled, nonblinded clinical study was done to compare the levels of bilirubin isomers in the infant's serum, the results of which we report below.

RESULTS

Table 1 shows no significant differences in the demographic and clinical data between the infants exposed to LED light centered at 497 nm vs. 459 nm.

As expected, there was a significant decrease in the serum concentration of Z,Z-bilirubin and a significant increase in

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the concentrations of the bilirubin isomers Z,E-bilirubin, E,Z-bilirubin and E,Z-lumirubin, both in infants treated with light centered at 497 nm and 459 nm, respectively ($P < 0.001$, for all statistical analyses). Z,E-bilirubin accounted for the greatest fraction of the bilirubin isomers formed during the therapy (Table 2).

Table 2 shows that after 24 h of phototherapy, there was no significant difference in the concentrations of total bilirubin isomers and Z,Z-bilirubin between the two groups. Furthermore, the concentrations of Z,E-bilirubin, and thus also total bilirubin isomers formed during light exposure (Z,E-bilirubin, E,Z-bilirubin, and E,Z-lumirubin), were highest in infants treated with LED light centered at 459 nm, while the concentration of E,Z-bilirubin was highest in infants treated with LED light centered at 497 nm. In addition, no significant difference was found between the concentrations of E,Z-lumirubin.

Table 3 shows the concentrations of bilirubin isomers produced during light exposure in relation to total bilirubin isomers, expressed as percentage. After 24 h of treatment the percentage of Z,E-bilirubin, and thus also total bilirubin isomers formed during therapy, were highest for the group exposed to light centered at 459 nm. In contrast, the percentage of E,Z-bilirubin was highest for the group exposed to light centered at 497 nm. No significant difference was found between the percentages of E,Z-lumirubin.

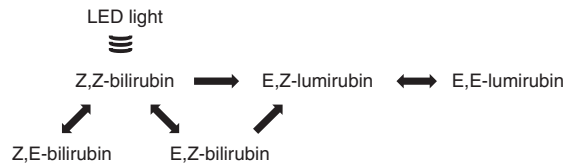

Figure 1. Photochemical reactions of Z,Z-bilirubin in neonates. McDonagh *et al.* (3,4) propose that E,Z-lumirubin is formed from both Z,Z- and E,Z-bilirubin, whereas Onishi *et al.* (5) propose that E,Z-bilirubin is an obligate intermediate in the formation of E,Z-lumirubin. The serum concentrations of E,E-lumirubin were too low to be identified and quantified with certainty and therefore, are not reported.

DISCUSSION

This study shows, that after 24 h of treatment, the serum concentrations of total bilirubin isomers and Z,Z-bilirubin were the same in the infants treated either with LED light centered at 497 nm or 459 nm, applied with equal irradiance on the infants. The concentrations of Z,E-bilirubin, and thus also total bilirubin isomers formed during the treatment, were highest for infants exposed to light centered at 459 nm, while the concentration of E,Z-bilirubin was highest for those exposed to light centered at 497 nm.

Z,E-bilirubin accumulates in plasma (12). The concentration of Z,E-bilirubin increases until equilibrium with Z,Z-bilirubin is reached, which occurs after approximately 4–6 h of exposure to light (13–15). Thereafter, the percentage of Z,E-bilirubin to the sum of Z,Z- and Z,E-bilirubin only depends on the emission spectrum of the light (15). After 24 h of phototherapy, the percentage of Z,E-bilirubin to total bilirubin isomers was higher for infants treated with light centered at 459 nm than for those treated with light centered at 497 nm (23% vs. 14%), and thus also the percentage of total bilirubin isomers formed during treatment to total bilirubin isomers was highest for the infants treated with light centered at 459 nm (26% vs. 18%) (Table 3). This is in agreement with previous *in vitro* studies, which showed that after equilibrium between Z,Z- and Z,E-isomers is reached, the percentage of Z,E-bilirubin decreases with longer wavelengths (4,16).This observation has also been confirmed in earlier clinical studies, where the reported percentages of Z,E-bilirubin to total bilirubin isomers were: 24% to 27% (10,13,14), 16% (10) and 9% (14) in children exposed to light of wavelengths ~460, 490, and 528 nm, respectively.

In this study, we found that the concentrations of E,Z-bilirubin were much lower than those of Z,E-bilirubin, but in contrast to Z,E-bilirubin, it was highest for the group treated

Table 1 Demographic and clinical data of the infants exposed to LED light either centered at 497 or 459 nm

	497 nm group	459 nm group	*P*-values
Infants, *n*	40	43	
Gender, female/male	13/27	15/28	1.00
Gestational age (d), median (range)	266 (232;294)	268 (238;294)	0.66
Birth weight (g), median (range)	3,260 (1,915;4,450)	3,350 (1,940;4,550)	0.38
Non-caucasian, *n* (%)	2 (4)	2 (4)	1.00
Maternal/gestational diabetes, *n* (%)	4 (10)	3 (7)	0.71
Cephalhaematoma, *n* (%)	1 (3)	1 (2)	1.00
Weight change from birth to phototherapy (%), median (range)	–4.9 (–9.3; –1.0)	–4.7 (–11.2;8.2)	1.00
Age at phototherapy (h), median (range)	73 (38;151)	85 (38;334)	0.38
Weight change during phototherapy (%), median (range)	0.3 (–2.2;4.3)	0.9 (–3.1;8.6)	0.38
Feeding during phototherapy, *n* (%) [a,b]			
Exclusively breast-fed	19 (51)	15 (35)	
Exclusively formula-fed	3 (8)	2 (5)	0.19
Combined	15 (41)	26 (60)	
Infant formula, ml/kg, median (range)	0 (0;144)	35 (0;139)	0.07

LED, light emitting diode. [a]Three values were missing in the 497 nm group. Therefore, the percentages only include 37 infants. [b]The statistical analysis included all three groups.

with light centered at 497 nm. This is in accordance with the formation of E,Z-bilirubin *in vitro,* which increased with longer wavelengths of the light (5,16). E,Z-bilirubin has been studied less than the Z,E-isomer, presumably because configurational isomerization highly favors formation of Z,E-bilirubin.

After 24 h of treatment there was no significant difference in the E,Z-lumirubin concentration between infants exposed to light centered at 497 nm or 459 nm, respectively (**Table 2**). However, it was difficult to detect the presence of a small difference of E,Z-lumirubin between the two treatments, due to the low concentrations. *In vitro* studies have shown, that the formation of lumirubin increases at longer wavelengths of light (3,5,16).

At the initiation of the phototherapy, the sum of Z,E-bilirubin, E,Z-bilirubin, and E,Z-lumirubin comprised ~5% of the total bilirubin isomers (**Table 2**). The reasons might be that the neonates had been exposed to the ambient light and perhaps that the blood accidentally might have been exposed to light during sampling. Thus, the fact that before phototherapy the concentrations of Z,E-bilirubin, and thus also the sum of Z,E-bilirubin, E,Z-bilirubin, and E,Z-lumirubin, were highest in the group of neonates later receiving light centered at 459 nm, was a coincidence.

This study confirms the results of our previous study (10) using fluorescent light, in which the concentration of Z,E-bilirubin was the highest in infants treated with light with peak emission at 452 nm. However, in that study we only found a trend toward a higher E,Z-bilirubin concentration in infants exposed to light with peak emission at 490 nm.

Non-albumin bound Z,Z-bilirubin in plasma is able to cross the blood-brain barrier as it is nonpolar and lipid-soluble (17). Because the bilirubin isomers formed during phototherapy are more polar and less lipid soluble, it was hypothesized that the unbound fraction of these isomers might be less able to pass through the blood-brain barrier (3,18). Furthermore, isolated bilirubin isomers formed *in vitro* during light exposure seem to be biologically inert and do not exert any negative biological

Table 2 Comparison of the serum concentrations of bilirubin isomers in infants exposed to light emitting diode (LED) light centered either at 497 nm or 459 nm

Parameters	497 nm group ($n = 40$) median (95% Confidence interval, CI)	459 nm group ($n = 43$) median (95% CI)	*P*-values
Total bilirubin isomers			
Time: 0 h, μmol/l	294 (272–312)	296 (274–320)	0.66
Time: 24 h, μmol/l	201 (182–214)	201 (183–219)	1.00
Z,Z-bilirubin			
Time: 0 h, μmol/l	279 (256–299)	273 (258–301)	0.51
Time: 24 h, μmol/l	169 (149–178)	148 (134–160)	0.13
Total bilirubin isomers formed during phototherapy			
Time: 0 h, μmol/l	12.4 (10.9–15.5)	17.3 (14.1–20.9)	0.03
Time: 24 h, μmol/l	34.5 (32.1–38.2)	51.7 (45.9–56.4)	<0.001
Z,E-bilirubin			
Time: 0 h, μmol/l	11.1 (9.5–13.6)	15.5 (12.1–19.6)	0.03
Time 24 h, μmol/l	28.4 (25.2–30.1)	46.1 (39.7–50.0)	<0.001
E,Z-bilirubin			
Time 0 h, μmol/l	1.0 (0.7–1.2)	1.1 (0.9–1.4)	0.66
Time 24 h, μmol/l	4.7 (3.9–5.3)	3.4 (3.0–3.8)	<0.001
E,Z-lumirubin			
Time 0 h, μmol/l	0.3 (0.1–0.5)	0.3 (0.2–0.7)	0.66
Time 24 h, μmol/l	2.5 (2.2–2.8)	2.1 (1.6–2.5)	0.19

Table 3. Comparison of the serum concentrations of bilirubin isomers formed during phototherapy in relation to the concentration of total bilirubin isomers (%) in infants treated 24 h with light emitting diode (LED) light centered either at 497 or 459 nm

Parameters	497 nm group ($n = 40$) median (95% Confidence interval, CI)	459 nm group ($n = 43$) median (95% CI)	*P*-values
Total bilirubin isomers formed during phototherapy			
Time 24 h, %	17.6 (16.8–18.7)	25.9 (24.2–27.4)	<0.001
Z,E-bilirubin			
Time 24 h, %	14.0 (13.6–14.7)	23.1 (21.9–23.8)	<0.001
E,Z-bilirubin			
Time 24 h, %	2.4 (2.1–2.7)	1.7 (1.5–1.9)	<0.001
E,Z-lumirubin			
Time 24 h, %	1.3 (1.1–1.4)	1.1 (0.9–1.2)	0.05

effects on human neuroblastoma cells (19). If the above was the case, it would be of greatest importance to the very sick extremely preterm infants. Even though their TSB is low, their concentration of unbound Z,Z-bilirubin might be high, which involves a considerable risk of bilirubin encephalopathy (20). Production of Z,E-bilirubin, E,Z-bilirubin, and E,Z-lumirubin start immediately after initiation of phototherapy, and after 60 min of therapy with blue light, ~20% of the serum bilirubin isomers consist of Z,E-bilirubin (13), i.e., a decrease in the risk of neuropathy might occur already short time after start of phototherapy.

It should be pointed out that Z,E-bilirubin and E,Z bilirubin can be converted back to Z,Z-bilirubin in the dark, which is thought to occur in the bile (21). If this also occurs in the intracellular environment, it will reduce the effect of phototherapy.

We know that no other studies of bilirubin isomers during phototherapy with turquoise and blue LED light. This is important, because it is clear that LEDs will be the phototherapy light source of the future, because, in contrast to fluorescent light, they deliver light of narrower wavelength range (fewer noneffective wavelengths), generate significantly less radiant heat, and their irradiance decreases more slowly with time.

Finally, this study in addition with our earlier study (10), are the only investigations exposing infants to light of different peak emission wavelengths at equal irradiances.

We find several strengths of the study: (i) the use of LEDs as the only light source, (ii) the light irradiances were measured by a radiometer with constant sensitivity, which made it possible to expose the two groups of infants to equal irradiance, thereby making the results of the treatments comparable, and (iii) the patient population was homogeneous.

A limitation of the study was that 15% of the randomized infants were either withdrawn or excluded from the investigation. However, as the drop-out was neither related to exposure nor outcome, the risk of selection bias was very small (22).

Conclusion

Distribution of bilirubin isomers in serum was different in neonates treated with LED light centered at 497 nm vs. 459 nm, of essentially equal irradiance.

Perspectives

In future clinical studies comparing the effect of lamps with different emission spectra, the individual bilirubin isomers should be measured. More studies are needed both on the ability of the bilirubin isomers formed during phototherapy to pass through the blood-brain barrier and/or of their toxicity. The first hours after start of phototherapy will be of greatest interest.

METHODS

Study Groups

The study groups and treatments have been described in detail previously (11). Briefly, infants were enrolled in the study at the neonatal intensive care unit at Aalborg University Hospital, Denmark between 1 January 2013 and 31 December 2013. All infants were ≥33 wk gestational age with uncomplicated hyperbilirubinemia. They were randomized either to be exposed to light centered at 497 nm or 459 nm, emitted from LEDs. They were treated for 24 h from above. The irradiances were 5.2×10^{15} and 5.1×10^{15} photons/cm^2/s, respectively. The irradiance was measured using an Ocean Optics spectrometer (Model 2000+, Dunedin, FL), with a constant sensitivity over the wavelength range of 200 to 1100 nm. The irradiance of the light centered at 459 nm was 30 μW/cm^2/nm measured by a handheld clinical radiometer (neoBLUE, Natus Medical, San Carlos, Ca).

The LED light centered at 497 nm had bandwidth of 484–511 nm and emission range 450–575 nm; and the LED light centered at 459 nm had bandwidth of 449–469 nm and emission range 416–524 nm, respectively.

Of the 104 infants who were eligible for the study, parents of 6 infants refused participation. As a result, a total of 98 infants were randomized to receive either LED light centered at 497 nm ($n = 50$) or 459 nm ($n = 48$). In the 497 nm group 2, infants were withdrawn from the study, 1 infant because of a high initial TSB and suspicion of blood type ABO immune hemolytic disease, the other infant due to communication issues with the mother. Furthermore, 8 infants were excluded because of liver disease ($n = 1$) or due to problems either with blood sampling or transportation of the samples from the neonatal intensive care unit to the Department of Clinical Biochemistry during evening and night ($n = 7$). In the 459 nm group, 1 infant was withdrawn from the study because of a high initial TSB and suspicion of blood type ABO immune hemolytic disease, and 4 infants were excluded due to either problems with blood sampling or sample transportation. Thus, a total of 40 infants receiving LED light centered at 497 nm and 43 infants light centered at 459 nm, were included in the study.

Measurements

The concentrations of bilirubin isomers were measured in serum from capillary blood drawn by a heel stick at initiation of phototherapy (time, 0 h) and 24 h after start of the phototherapy (time, 24 h). Sampling occurred under subdued light and the blood was drawn into tubes wrapped with aluminum foil to avoid light exposure. The blood samples were immediately centrifuged and the serum stored at −20°C in the dark. The bilirubin isomers were identified and quantitated using isocratic reversed phase high performance liquid chromatography (23). The imprecision of the method expressed as coefficients of variation was 2.4% for the concentration of total bilirubin isomers; 3.3% for total bilirubin isomers formed during phototherapy; Z,Z-bilirubin and Z,E-bilirubin, 3.5% for E,Z-bilirubin; and 5.1% for E,Z-lumirubin.

The total bilirubin isomers are defined as the sum of the following bilirubin isomers shown in **Figure 1**: Z,Z-bilirubin, Z,E-bilirubin, E,Z-bilirubin, and E,Z-lumirubin. Likewise, the total bilirubin isomers formed during phototherapy is defined as the sum of bilirubin isomers Z,E-bilirubin, E,Z-bilirubin, and E,Z-lumirubin.

The TSB is the sum of all bilirubin derivatives in serum, both unconjugated and conjugated. It was determined by a diazo-method (24). This method is used routinely in our department, and the inclusion of infants in the study was based on such a determination of TSB according to our guidelines for phototherapy.

Ethics

This study was approved by the Committee for Biomedical Research Ethics in Region North Jutland, Denmark. Verbal and written informed consents were obtained from the parents.

The study was registered with number NCT 02154165 in the Clinical Trial Registry.

Data Analysis

Fisher's exact test for contingency tables was used to calculate significant differences between medians of the two groups. A two-by-two table was obtained by computing the overall median for the entire study group and then for each treatment group counting the number of infants with a value lower or higher than the overall median value. To calculate confidence intervals (CI) for the medians of each group, we applied an approximation relying on the quantiles of the binomial distribution as implemented in the *ci.median* function from the R-package *asbio*. Comparisons of categorical variables between

the groups were also conducted using Fisher's exact test. For all analyses the statistical software program R (version 3.2.1) was used. Significance level was set at $P < 0.05$.

Presentation of Data

The content in serum of Z,E-bilirubin, E,Z-bilirubin, and E,Z-lumirubin is not only presented in absolute values (μmol/l) (**Table 2**), but also as a fraction of total bilirubin isomers (**Table 3**). This is due to two reasons: (i) After 24 h of treatment equilibrium exists between Z,E- and Z,Z-bilirubin (13–15). Perhaps an equilibrium also exists between E,Z- and Z,Z-bilirubin, but this has not been investigated and (ii) To compare our results with earlier studies, in which the results most often are presented as fractions (10,13,14).

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