Treatment of neonatal hyperbilirubinemia

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CHAPTER 5

LED phototherapy in Preterm Infants: Effects on an Oxidative DNA Damage marker

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ABSTRACT

Background Phototherapy is used on the majority of preterm infants with unconjugated hyperbilirubinemia. The use of fluorescent tube-based phototherapy is known to induce oxidative DNA damage in infants, and has largely been replaced by light-emitting diode (LED) phototherapy. To date, it is unknown whether LED phototherapy also induces oxidative DNA damage in preterm infants.

Objective To determine whether LED phototherapy in preterm infants induces oxidative DNA damage as indicated by 8-hydroxy-2'deoxyguanosine (8-OHdG).

Methods Urine samples (n = 512) were collected in a cohort of 42 preterm infants (24-32 weeks' gestational age) during the first week after birth. Urine was analyzed for the oxidative DNA damage marker 8-OHdG and creatinine and the corresponding ratio was calculated. Duration of phototherapy and irradiance were monitored, as well as total plasma bilirubin concentrations.

Results LED phototherapy did not alter the urinary 8-OHdG/creatinine ratio (median; range 20.0; 1.7-288.6 μg/g creatinine) at either low (10 – 30 $\mu W/cm^2/nm$) or high (more than 30 $\mu W/cm^2/nm$) irradiance, at any phototherapy duration (median; range 75.5; 15.6 to 146.3 hours). Postnatal age and birth weight were significant predictors of the 8-OHdG/creatinine ratio, with highest ratios in infants with an extreme low birth weight of less than 1000 g.

Conclusions LED phototherapy at irradiances up to 35 $\mu W/cm^2/nm$ in preterm infants of less than 32 weeks' gestation does not affect 8-OHdG, an oxidative DNA damage marker.

STATEMENTS

What is known

- Phototherapy is the gold standard for treating unconjugated neonatal hyperbilirubinemia and it is used in more than 80% of preterm infants admitted to a NICU.
- Fluorescent tube-based phototherapy is associated with oxidative stress and DNA damage in full-term neonates.
- Fluorescent tube-based phototherapy is being replaced gradually by light-emitting diode (LED) phototherapy, which allows higher intensity phototherapy without significant heat production.

What this study adds

- LED phototherapy up to 35 μW/cm^2/nm is not associated with an increase in the oxidative DNA damage marker 8-hydroxy-2'deoxyguanosine (8-OHdG) in preterm infants of 32 weeks' gestation or younger.
- The ratios of 8-OHdG/creatinine increase during the first week after birth and are highest in infants weighing less than 1000 g.

1. INTRODUCTION

Neonatal jaundice as a result of unconjugated hyperbilirubinemia occurs in up to 80% of preterm infants in the first week after birth.[1] If severe and left untreated, unconjugated hyperbilirubinemia may lead to permanent neurological damage or even death. From 1968 onward, the standard treatment for neonatal hyperbilirubinemia has been phototherapy,[2] and presently more than 80% of preterm infants admitted to a neonatal intensive care unit (NICU) receive phototherapy.[3] Since the introduction of phototherapy exchange transfusions diminished drastically.[4] Nevertheless, conventional phototherapy using fluorescent tubes (FT) phototherapy has several known side effects, including oxidative stress and DNA damage in full-term infants,[5-8] and a tendency towards increased mortality in preterm infants [9, 10]. In the long term, FT phototherapy has been associated with diabetes, asthma, and epilepsy, and a slight increase in the incidence of infant cancer,[11-15] but the mechanisms involved have never been established.

Over the past sixty years, phototherapy has evolved and devices with light-emitting diodes (LEDs) have largely replaced phototherapy devices using FTs, which makes it easier and safer to administer high irradiance phototherapy with less heat production.[16] It remains largely unclear whether using LED phototherapy in preterm infants causes oxidative stress and thereby oxidative DNA damage. Recently, we analyzed effects of LED phototherapy at irradiances up to 100 μW/cm^2/nm in hyperbilirubinemic Gunn rats and found no induction of the oxidative DNA damage as indicated by the markers 8-hydroxy-2'-deoxyguanosine (8-OHdG) or γH2AX. It is unclear, however whether we can reliably extrapolate these results to preterm infants, who are more susceptible to oxidative damage in comparison to full-term infants.[17] In the present study, we aim to determine the effects of LED phototherapy on oxidative DNA damage marker 8-OHdG, in preterm infants of 32 weeks' gestational age or younger.

2. METHODS

2.1 Research population

In this observational cohort study, we enrolled 42 preterm infants with gestational ages between 24 and 32 weeks, and birth weights (BW) between 530 and 1990 g, who had been admitted to the NICU of the Beatrix Children's Hospital, University Medical Center Groningen (UMCG), the Netherlands, between November 2016 and April 2017. Patients with congenital malformations or syndromes were excluded from participation. Parental informed consent was obtained for all participants and the study was approved by the Medical Ethics Committee of the UMCG (METC 2016/437).

2.2 Urine collection

Upon admission immediately after birth, we started collecting urine samples as soon as possible and continued doing so daily during the first week. We collected the urine using specially designed urinary collection pads, so-called PeeSpots (Hessels+Grob, Apeldoorn, the Netherlands).[18] In a pilot study, retrieval from the PeeSpots of both 8-OHdG and creatinine was 100%, whereas retrieval of 8-OHdG from a gauze was only 78% (data not shown). The PeeSpots were placed and collected by the nursing staff upon diaper changing, following standard clinical care. The time of collection was recorded for each urine sample. Upon collection, the PeeSpots were placed in a collection tube and stored at -20°C for at most four weeks until analysis. For analysis, the collection tubes containing the PeeSpots were thawed and centrifuged for 1 minute at 200G to retrieve the urine.

2.3 Urinary analyses of 8-OHdG

We measured the oxidative stress marker 8-OHdG, the breakdown product of guanine, in urine. Guanine is the DNA base that is most susceptible to oxidative stress and 8-OHdG is excreted in the urine completely after oxidative DNA damage.[19] Thus, not only is 8-OHdG obtained in a non-invasive manner, but it

specifically serves as a marker of oxidative stress-induced DNA damage. It is the most commonly used oxidative stress marker in oncology and environmental toxicology, and is very stable: repeated measurements of urinary 8-OHdG in samples stored at -20°C for 15 years did not how any decline [19, 20].

The marker 8-OHdG and creatinine were measured as previously described.[21] In order to obtain the 8-OHdG/creatinine ratio, the concentration of 8-OHdG was divided by the concentration of creatinine to correct for urine osmolality. The 8-OHdG concentration was displayed in ng/mL and the creatinine concentration in mmol/L. The ratio was displayed in µg/g creatinine, which was calculated by dividing the 8-OHdG concentration in ng/mL by the creatinine concentration in g/L (which was obtained by multiplying the creatinine concentration in mmol/L by 0.11312).

2.4 Phototherapy

Phototherapy was administered using either an NeoBLUE mini LED Phototherapy System (Natus Medical Incorporated, San Carlos, CA, USA), or a Mavi LED Phototherapy System (Inspiration Healthcare, Leicester, UK). Both systems emit wavelengths between 450 and 470 nm and were set at varying irradiances ranging from 10 to 30 µW/cm^2/nm. The phototherapy devices were positioned at ~35 cm distance from the infant and all infants were naked except for a diaper and eye protection. We recorded the time of starting and ending a phototherapy session and the irradiance of each phototherapy session. The phototherapy irradiance was measured once a day at the infant's head, trunk, and knees, using a BiliBlanket Light Meter II (GE Healthcare, Madison, WI, USA), and we calculated the mean irradiance based on these three measurements.

For each urine sample, we determined whether it had been collected under phototherapy, defined as collection after at least 2 hours of continuous phototherapy or within 2 hours after phototherapy had stopped. For every urinary "phototherapy sample" thus obtained, we determined the

corresponding irradiance level (μW/cm²/nm) at that time as well as the duration of PT on the days before collection of that particular urine sample. Generally, a distinction is made between high irradiance phototherapy (irradiance equal to or higher than 30 μw/cm²/nm) and 'normal phototherapy' with an irradiance of less than 30 μw/cm²/nm. We therefore categorized all urine samples accordingly.

2.5 Statistics

For our paired analysis sample size was calculated using the following formula:

N = 2 + C(SD/d)^2. A SD of 15.[22] an expected detected difference (d) of 0.5 SD, C = 7.85, α = 0.05, and β = 0.8, resulted in us requiring a sample of 34 infants. Instead, we recruited 42 infants to ensure a sufficiently large sample in case of unforeseen events.

Changes in the 8-OHdG/creatinine ratio over time were analyzed using generalized estimating equations (GEEs). This technique allows the longitudinal analysis of data sets with different numbers of measurements among the participants. GEEs includes all individual timepoints and takes into consideration that measurements from the same individual are correlated with each other, thereby correcting for this correlation without discarding individual measurements and maximizing the use of available data without artificially increasing the sample size.[23]

Phototherapy parameters were entered as predictors in a GEEs model to assess their influence on the variation in 8-OHdG/creatinine ratios. Potential confounding clinical parameters, eg sepsis, were entered as possible predictors. For all GEEs analyses, an exchangeable working correlation matrix was used, assuming a fixed correlation between measurements within one participant.

Upon entering specific categorical parameters (eg phototherapy versus no phototherapy), the model could predict the 8-OHdG/creatinine ratio for each predictor category: the estimated marginal mean (EMM). These EMMs reflect the 8-OHdG/creatinine ratio more accurately than sample means, because infants with many urine samples will contribute more to sample means than infants with few samples. Therefore, all figures below display EMMs on the y-axes, unless stated otherwise. GEEs analyses were performed on raw 8-OHdG/creatinine ratios, because logarithmical transformation reduced the skewness, but still did not result in a normal distribution (Kolmogorov-Smirnov $P<0.005$), and it did not significantly affect the linearity of the association (homoscedasticity of residuals). In the text, data are displayed as either (median [inter quartile range (IQR]) or (regression coefficient (confidence interval (CI)).

3. RESULTS

Table 1 shows the clinical characteristics of the preterm infants enrolled in the study. Almost all patients received phototherapy (40/42, 95%). On average, infants received 75 hours of phototherapy 16 [15-21] $\mu W/cm^2/nm$.

Table I Clinical characteristics

CLINICAL CHARACTERISTICS	INFANTS (n = 42)
Phototherapy	
Phototherapy	40 (95%)
Phototherapy duration (hours)	71 [40 to 91]
Phototherapy intensity (μW/cm²/nm)* -Head -Trunk -Knees	16 [15 to 21] -16 [13 to 22] -20 [15 to 28] -15 [14 to 21]
Clinical parameters	
Birth weight (g)	1190 [900 to 1550]
Gestational age (weeks)	29 [27 to 30]
Boys/Girls	25(60%) /17 (40%)
Part of twins (yes/no)	16(38%)/26(62%)
Apgar score at 5 min	8 [7 to 9]
Umbilical pH	7.3 [7.2 to 7.3] (n=36)
Umbilical CO_2	7.0 [5.7 to 7.9] (n=35)
Umbilical Base Excess	-4 [-6 to -2] (n=36)
Respiratory support	
Mechanical	29 (69%)
CPAP	40 (95%)
High flow	12 (29%)
Low flow	3 (7%)
Minimal oxygen %	21 [21 to 21]
Maximal oxygen %	22 [21 to 30]
Nutrition	
Breast feeding	42 (100%)
Formula feeding	36 (86%)
Parenteral feeding	42 (100%)
*Sepsis, number **	
Early onset sepsis	2 (5%)
Late onset sepsis	10 (24%)

*Data are presented as numbers (% of total) or medians [interquartile ranges] *Two infants were briefly treated with 50 μW/cm²/nm, but too few samples were collected during this time for reliable interpretations. These samples were excluded from analyses.** Early onset was defined as a positive blood culture during the first 48 hours after birth. Late onset was defined as a positive blood culture after the first 48 hours.*

Table II Generalizing Estimating Equations (GEE) results: individual testing

Parameter*	Categories	Regression coefficient	Standard error	95% CI lower	95% CI Upper
Postnatal day	Day 0	0			
	Day 1	8.34	2.99	2.48	14.20
	Day 2	10.57	3.32	4.06	17.08
	Day 3	9.69	3.17	3.49	15.90
	Day 4	7.36	1.97	3.49	11.23
	Day 5	8.49	2.57	3.45	13.54
	Day 6	11.82	4.38	3.24	20.39
Birth Weight	<1000g	0			
	≥ 1000g	-9.18	3.60	-16.24	-2.12
No phototherapy vs phototherapy samples	No phototherapy	0			
	Phototherapy	0.83	3.06	-5.18	6.83
Low irradiance vs high irradiance phototherapy	No lamp	0			
	Low irradiance	2.86	3.82	-4.62	10.34
	High irradiance	-2.27	4.12	-10.33	5.80
Total phototherapy duration		<-0.01	<0.01	<-0.01	<0.01
Total serum bilirubin		-0.06	0.04	-0.14	<0.01

**All results originated from individual testing of each parameter in a GEE model together with postnatal day in order to correct for the effect of postnatal age.*

3.1 The 8-OHdG/creatinine ratio did not increase after phototherapy

Both the 8-OHdG (2.3 [1.6 to 3.4] µg/L) and creatinine (0.11; [0.08 to 0.16] g/L) concentrations were measured in all urine samples. This allowed us to calculate the 8-OHdG/creatinine ratio (20.2 [14.4 to 28.3] µg/g creatinine). The postnatal day was a significant predictor of 8-OHdG/creatinine (Figure 1A/Table 2). This indicated that the 8-OHdG/creatinine ratio depended on postnatal age.

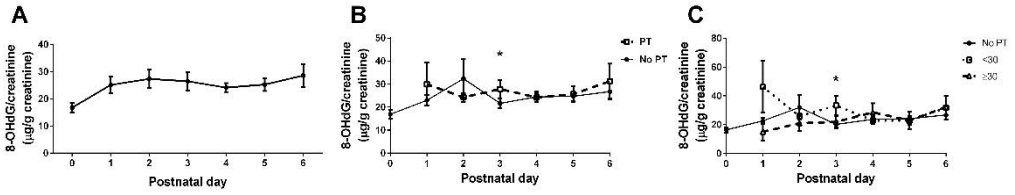

***Figure 1: The 8-OHdG/creatinine ratio during the first week after birth and the effect of phototherapy**. **A)** The postnatal course of the 8-OHdG/creatinine ratio during the first week after birth. **B)** The 8-OHdG/creatinine ratio in samples collected under phototherapy or less than 2 hours before or after phototherapy. **C)** The 8-OHdG/creatinine ratio in samples collected more than 2 hours before or after phototherapy, or under low irradiance (less than 30 nm/µW/cm²) or high irradiance (equal to or higher than 30 µW/cm²/nm) phototherapy. All values on the y-axes represent EMMs.*

With the exception of slightly higher levels on Day 3 (8.4 (2.0 to 14.8), the overall 8-OHdG/creatinine ratio was not significantly different under phototherapy (0.83 (-5.18 to 6.83)) (Figure 1B/Table 2). All but two infants (95%) received phototherapy during the first week after birth. Such a small number rendered it impossible to attempt a reliable statistical comparison with infants who received phototherapy.

Treatment irradiance was not a significant predictor of the 8-OHdG/creatinine ratio. The 8-OHdG/creatinine ratio under normal phototherapy irradiance was similar to that in the high irradiance group (2.86 (-4.62 to 10.34) and (-2.27 (-10.33 to 5.80)) for low and high irradiance, respectively) (Figure 1C).

Significantly higher 8-OHdG/creatinine ratios on Day 3 were present in those collected under normal phototherapy irradiance, in comparison to samples collected under high-irradiance or without phototherapy (8.39 (2.02 to 14.76)). Total phototherapy duration was not a significant predictor of the 8-OHdG/creatinine ratio in the prediction model (< 0.01 (-0.01 to < 0.01)). We also assessed the effect of phototherapy duration by analyzing the correlation between the average 8-OHdG/creatinine ratios on each postnatal day and the total phototherapy duration. We found no significant correlation between phototherapy duration and 8-OHdG/creatinine ratios on any postnatal day (data not shown).

Infants with BWs of less than 1000 g (n = 13) received phototherapy treatment significantly longer than infants weighing 1000 g or more (n = 29) (87.2 [69.7 to 106.8] hours versus 52.9 [35.8 to 79.0] hours, respectively) (-31.8 (-51.4 to -12.2) hours). Although phototherapy duration was not a significant predictor of the 8-OHdG/creatinine ratio, infants weighing less than 1000 g had significantly higher 8-OHdG/creatinine ratios (-9.18 (-16.24 to -2.12)) (Table 2). We therefore assessed the effect of phototherapy duration in these infants of less than 1000 g separately. Phototherapy duration did not predict the 8-OHdG/creatinine ratio (< 0.01 (< -0.01 to < 0.01)) suggesting that not the phototherapy duration, but rather the low BW was a significant predictor of the 8-OHdG/creatinine ratio.

3.2 The 8-OHdG/creatinine ratio was higher in extremely low birth weight infants, irrespective of bilirubin levels

In the GEEs model, BW was a significant predictor of the 8-OHdG/creatinine ratio, whereas phototherapy was not (Table 2). More specifically, there was a significant difference in the 8-OHdG/creatinine ratio between the infants weighing less than 1000 g and those 1000 g or more (Figure 2A/B, Table 2), indicating that the smallest infants might experience more oxidative DNA damage. To make sure this effect could not be explained by the longer phototherapy duration in the group weighing less than 1000 g, we entered BW and phototherapy duration in a GEEs model together, to correct the effect of BW on the potential influence of phototherapy duration. In this model, BW proved to be a significant predictor of the 8-OHdG/creatinine ratio (-9.85 (-2.45 to 6.80)), whereas phototherapy duration was not (< 0.01 (< -0.01 to > 0.01)) (Table S1). When we plotted the 8-OHdG/creatinine ratios for the course of the first week after birth (Figure 2A/B), the difference was seen to occur on the first three postnatal days, although the differences on the individual postnatal days did not reach statistical significance.

We found the highest 8-OHdG/creatinine ratios in preterm infants with the lowest total bilirubin in plasma (TB) (Figure 2A/C). Figure 2C depicts the TB levels in four different BW categories of the Dutch BW-dependent TB-based treatment thresholds. The TB level increased, although not significantly for all groups (data not shown), as BW increased because higher TB levels were allowed as BW increased. Although the trend in Figure 2C shows that TB levels increase along with BW, the 8-OHdG/creatinine ratio did not follow the same pattern (Figure 2A). Moreover, we found no significant correlation between peak 8-OHdG/creatinine ratio values and peak TB values (Figure 2D). When we tested BW and TB in a GEEs model, TB was not a significant predictor (< -0.02 (-0.08 to 0.03)) of the 8-OHdG/creatinine ratio (Table S1).

3.3 Clinical variables did not explain the variations in the 8-OHdG/creatinine ratio

We monitored several clinical parameters (Table 1) as well as medications that could possibly affect oxidative stress or 8-OHdG production. With the exception of BW, none of these parameters or medications were significant predictors of the 8-OHdG/creatinine ratio.

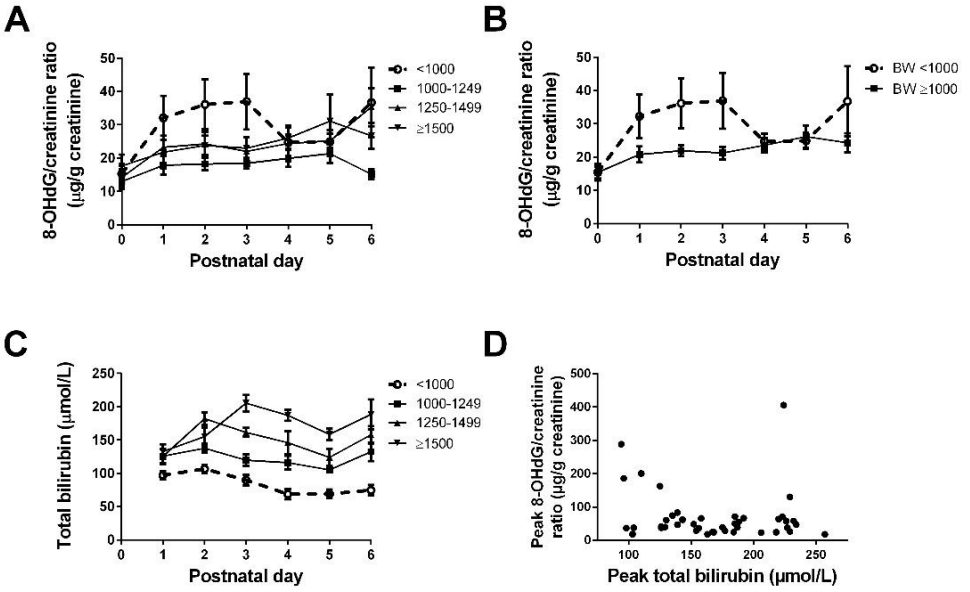

Figure 2: The effect of birth weight on the 8-OHdG/creatinine ratio and total bilirubin. A) *The 8-OHdG/creatinine ratio divided into four birth weight (BW) categories, corresponding to the categories used for the hyperbilirubinemia treatment guidelines.* ***B)*** *The 8-OHdG/creatinine ratio in samples of infants with a BW of less than 1000 g versus a BW of 1000 g or more.* ***C)*** *Total bilirubin levels in four BW categories corresponding to the categories used for hyperbilirubinemia treatment guidelines. Total bilirubin levels were significantly lower in the group weighing less than 1000 g and the group weighing 1000 to 1249 g.* ***D)*** *Scatterplot of the peak-8-OHdG/creatinine ratio in the first week after birth plotted against the peak total bilirubin. In D, both axes represent raw values, no EMMs. For A, B and C, all values on the y-axes represent EMMs.*

4. DISCUSSION

We show that in early preterm infants, LED phototherapy at clinically relevant treatment irradiances of up to 35 μW/cm²/nm is not associated with an increase in the oxidative DNA damage marker 8-OHdG. We found no correlation between the duration of phototherapy or phototherapy irradiance and the 8-OHdG/creatinine ratio. We confirmed that BW was a significant predictor of the 8-OHdG/creatinine ratio, with infants weighing less than 1000 g having the highest levels, predominantly during the first three postnatal days. This suggests that these infants were subjected to higher levels of oxidative DNA damage. Although infants of less than 1000 g received phototherapy for longer periods of time, the differences could only be explained by BW, and not by phototherapy (Table S1). This is in line with previous studies that reported a significant correlation between BW and 8-OHdG. We also showed that, in addition to clinical characteristics, TB was not a significant predictor of the 8-OHdG/creatinine ratio.

Several studies in full-term infants reported an increase in DNA damage after FT-based phototherapy. FT phototherapy could be expected to cause more oxidative stress and DNA damage than LED phototherapy. LED phototherapy allows significantly higher treatment irradiances without producing significant increases in heat. The heat produced by FTs can induce hyperthermia, which is known to enhance oxidative stress.[24, 25] Secondly, FTs are known to emit small amounts of ultraviolet light, especially close to the lamp.[26] This is true particularly when the screens that serve to protect the infant from the ultraviolet light emitted by the FTs become damaged over time or are removed.

There are few studies that report on FT phototherapy and LED phototherapy and oxidative stress in full-term neonates. Demirel et al described an increased oxidative stress index after FT phototherapy, but not after LED phototherapy, whereas, Kale et al and El-Farrash et al described increased oxidative stress

after both types of phototherapy. In all these studies, however, the FT and LED irradiances that were being compared were either not in the same range or measured inaccurately. All the studies assessed oxidative stress by measuring total oxidant status (TOS) and/or total antioxidant capacity (TAC) in plasma. The validity of these assays has been questioned, because bilirubin acts as an anti-oxidant and is reduced by phototherapy. Potentially therefore, phototherapy interferes with both TOS and TAC. [27, 28]

In our study, we circumvented these interactions by determining the physiologically relevant effect of oxidative stress by using a marker that is only produced in reaction to DNA damage. This marker, 8-OHdG, is known to be produced in many oxidative stress-induced diseases in preterm infants, such as bronchopulmonary dysplasia, necrotizing enterocolitis, and retinopathy. It has been described to correlate with other frequently used markers of oxidative DNA damage markers in neonates, such as the Comet assay and malondialdehyde. In addition, 8-OHdG is the direct oxidation product of the DNA base guanine, the most oxidation-susceptible DNA base, and is therefore directly correlated with DNA oxidation and DNA damage. Furthermore, using a urinary marker allows for frequent non-invasive sampling.

4.1 Limitations

Although we studied the 8-OHdG/creatinine ratio longitudinally on the basis of several daily urine samples that we collected per child, our study design did not involve continuous measurements. Moreover, on account of our patient characteristics and existing treatment protocols, we did not analyze intensive phototherapy higher than 35 μW/cm^2/nm in this study and the relatively small sample size did not allow us to assess the influence of all potential confounders.

4.2 Strengths

First, our prospective longitudinal design allowed accurate monitoring of phototherapy duration and irradiance, whereas GEEs analysis allowed us to correct for the effect of age during phototherapy. Most clinical studies only determine damage markers twice, that is once before and once after phototherapy. Such a design harbors a methodological risk on account of the fact that certain oxidative stress markers increase with advancing postnatal age. We are therefore cautious about concluding that increased levels of oxidative stress after phototherapy in these studies are caused by phototherapy instead of merely by postnatal age. For 8-OHdG, we and others have shown that the specific postnatal day is a significant predictor of the postnatal course. We therefore corrected for the influence of postnatal age when assessing phototherapy effects. Secondly, in contrast to most existing studies, we chose to test our hypothesis in early preterm infants of 32 weeks' gestation or younger, which is the most relevant patient population, because the vast majority of these infants receive phototherapy and their antioxidative capacities are the weakest.

4.3 Conclusion

LED phototherapy is gradually replacing FT phototherapy and thereby the door is opened toto high-irradiance phototherapy with no heat-induced side effects. Thus far, however, the clinical implementation of LED phototherapy has been impeded by concerns about long-term side effects. We did not find that LED phototherapy resulted in increased levels of the oxidative stress marker 8-OHdG in preterm infants of 32 weeks' gestation or younger. Our data do not exclude other potential harmful effects that could cause previously reported long-term adverse effects. It also remains to be determined whether higher phototherapy irradiances of 50 $\mu W/cm^2/nm$ or more affect oxidative DNA damage in early preterm infants.

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SUPPLEMENTAL MATERIAL

Supplemental Table 1: GEE results corrected for birth weight effect

Parameter*	Categories	Regression coefficient	Standard error	95% CI Lower	95% CI Upper
Postnatal day	Day 0	0			
	Day 1	7.13	2.98	1.28	13.00
	Day 2	9.33	3.30	2.86	15.80
	Day 3	8.81	3.04	2.86	14.76
	Day 4	6.79	2.09	2.69	10.90
	Day 5	7.48	2.84	1.91	13.05
	Day 6	10.92	4.37	2.36	19.48
No phototherapy vs phototherapy samples	No phototherapy	0			
	Phototherapy	0.17	2.96	-5.63	5.97
Low irradiance vs high irradiance phototherapy	No lamp	0			
	Low irradiance	1.16	3.58	-5.86	8.18
	High irradiance	-3.08	4.34	-11.57	5.42
Total phototherapy duration		<-0.01	<0.01	<-0.01	<0.01

All parameters were entered in the GEE model together with birth weight, to correct for the effects of birthweight on these parameters