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ORIGINAL ARTICLE

Lipid peroxidation, DNA damage and total antioxidant status in neonatal hyperbilirubinemia

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OBJECTIVE: Lipid peroxidation, DNA damage and total antioxidant status (TAS) were assessed in neonates with unconjugated hyperbilirubinemia (UCH).

STUDY DESIGN: Plasma malondialdehyde (MDA), 8-hydroxy-2-deoxy-guanosine (8-OH-dG) and TAS levels were compared between 64 term newborns with idiopathic UCH and 30 age-matched healthy controls.

RESULT: Compared with controls, an overall increase in mean plasma MDA and 8-OH-dG levels and a decrease in TAS level were noted in the UCH group. Within the UCH group, mean plasma MDA level was found to be low in infants with lower bilirubin levels, but a progressive increase was documented above the bilirubin level of 20 mg dl^{-1}. A significant increase in 8-OH-dG level was documented even at lower bilirubin levels, and a decrease in plasma TAS level was found at bilirubin levels above 16 mg dl^{-1}. MDA and 8-OH-dG levels were significantly higher, whereas TAS level was significantly lower in five neonates who developed features of acute bilirubin encephalopathy compared with those with normal outcome. Alteration of MDA, 8-OH-dG and TAS levels showed high predictive accuracy for poor outcome.

CONCLUSION: Moderate-to-severe UCH was associated with higher oxidative stress and lower antioxidant defense. Alteration of oxidative stress parameters may be utilized as early predictors for poor outcome. High DNA damage even at lower bilirubin levels suggests possible genotoxic effect of bilirubin in UCH.

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INTRODUCTION

Unconjugated or indirect hyperbilirubinemia (UCH) remains one of the leading causes of neonatal morbidity and hospitalization. Although in most neonates UCH is physiological and subsides without any adverse effect, in some infants, unconjugated bilirubin (UCB) can rise to a dangerously high level and pose the risk of brain damage. Even today, cases of kernicterus are seen in both developing and developed countries.[1,2] The exact level of bilirubin likely to cause neurotoxicity in a newborn is difficult to predict, and there seems to be a wide variation in susceptibility for a variety of unexplained reasons.

At physiological concentrations, UCB behaves like a strong natural antioxidant and cytoprotectant.[3–5] It scavenges reactive oxygen and nitrogen species with high efficiency.[6] However, at higher concentrations, the same UCB imparts oxidative stress and becomes toxic to the neuronal cells.[7,8] The toxicity is further intensified in neonates for their poor antioxidant defense capacity.[9] The exact level at which UCB imparts oxidative stress is not clear. Various thresholds ranging from 12.5 (Nag *et al.*[10]) to >30 mg dl^{-1} (Mireles *et al.*[11]) have been reported. Several studies have reported possible genotoxicity and DNA damage in UCH after phototherapy, but there is paucity of literature regarding the occurrence of DNA damage by UCB itself.[12]

In a previous study, we had observed decreased oxidative stress in neonatal hyperbilirubinemia.[13] The present study was carried out to determine the extent of lipid peroxidation, DNA damage and antioxidant defense status in newborns with nonhemolytic UCH at increasing bilirubin levels.

METHODS

This prospective observational study was conducted in the neonatal intensive care unit of our University Hospital over a period of 15 months. It was approved by the Institute's Ethics Committee.

Participants

Cases comprised a convenient sample of 64 term (37–41 weeks) neonates of idiopathic UCH with a total plasma bilirubin (TPB) level reaching the threshold of phototherapy requirement as per the hour-specific nomogram of American Academy of Pediatrics (AAP).[14] UCH was defined as indirect hyperbilirubinemia with indirect reacting fraction of bilirubin being more than 80% of TPB. Neonates with ABO/Rh incompatibility, glucose 6-phosphate dehydrogenase (G6PD) deficiency, perinatal asphyxia, systemic or metabolic disorders, shock, sepsis, hypoalbuminemia (serum albumin < 3.0 g dl^{-1}), congenital hypothyroidism or any other malformation were excluded. Both inborn and outborn neonates were included, provided they did not receive phototherapy or any other intervention for UCH before admission. Thirty healthy, postnatal age-matched term neonates without clinical jaundice and bilirubin levels < 5 mg dl^{-1} served as controls. Written informed consents were taken from parents before inclusion.

Clinical workup

Baseline details were recorded in all. Before inclusion into the study, cases were subjected to a battery of tests including plasma bilirubin, blood group of the mother and newborn, hemoglobin, peripheral blood smear examination for hemolysis, reticulocyte count, Coomb's test, sepsis screen, free thyroxine (T_4) and thyroid stimulating hormone (TSH) estimation and

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G6PD assay. Samples were protected from light while sending to laboratory and analyzed immediately.

Oxidative stress was estimated by measuring plasma malondialdehyde (MDA; a marker of lipid peroxidation) and 8-hydroxy-2-deoxy-guanosine (8-OH-dG; a marker of DNA damage) in peripheral venous blood in cases before starting phototherapy. Antioxidant defense capacity was assessed by measuring total antioxidant status (TAS). In controls, TPB, MDA, 8-OH-dG and TAS were estimated on days 3–5. To avoid unnecessary pricking, sampling was done along with routine screening for congenital hypothyroidism, which is a part of our hospital protocol.

Cases were closely observed for the development of acute bilirubin encephalopathy, defined as development of abnormal muscle tone, mental status and cry pattern (bilirubin-induced neurologic dysfunction (BIND) score >3).[15] Hyperbilirubinemia was managed as per AAP guidelines.[14] Progress during the hospital stay and outcome was noted.

Sample size calculation

As there was no similar publication in literature, the present study was conducted as a pilot trial with a convenient sample size of 94 (64 cases and 30 controls).

Laboratory analysis

Three milliliters of peripheral venous blood was drawn under aseptic condition in a sterile deionized vial. Plasma was separated immediately by centrifugation at 3000 r.p.m. for 5 min and stored at −20 °C till further analysis. 8-OH-dG was measured by using enzyme immunoassay (EIA) kit (Cayman Chemical Company, Ann Arbor, Michigan, USA, Item Number 589320). MDA was measured by using thiobarbituric acid reactive substances (TBARS) assay as per the manufacturer's instructions.[16,17] TAS was assessed by using TAS assay kit (Cayman Chemical Company, Item Number 709001).

Statistical analysis

The statistical program SPSS version 16.0 (SPSS, Chicago, IL, USA) was used for data entry and analysis. Independent sample *T*-test and χ^2-test were used to compare categorical and continuous variables between two groups. Analysis of variance (ANOVA) along with Bonferroni *post-hoc* analysis was performed to compare variables in more than two groups. Pearson correlation coefficient was calculated. Sensitivity and specificity of different parameters were calculated at different selected cutoff values. Receiver-operating-characteristic curve analyses with measurement of area under the curve were performed to identify the appropriate cutoff values. A *P*-value of < 0.05 was considered statistically significant.

RESULTS

Cases and controls were comparable with respect to their baseline characteristics (Table 1). Among the cases, 22 neonates were delivered outside and were referred to our hospital. All of the controls were inborn infants. Majority of the cases and controls were exclusively breastfed. None of the neonates in the UCH group had hemoglobin $< 14\ g\ dl^{-1}$, received total parenteral nutrition at the time of sampling or had any other morbidity. Five neonates (7.8%) in the UCH group developed features of acute bilirubin encephalopathy, all of them had a TPB >30 mg dl^{-1}.

Plasma MDA, 8-OH-dG and TAS levels had been summarized in Table 2. Overall, mean MDA and 8-OH-dG levels were significantly higher (2.6 ± 1.6 vs $2.0 \pm 0.8\ mmol\ l^{-1}$; $P < 0.05$ for MDA and 342.4 ± 69.9 vs $229.0 \pm 42.5\ pg\ ml^{-1}$ for 8-OH-dG; $P < 0.001$) and TAS was significantly lower (0.13 ± 0.08 vs $0.22 \pm 0.07\ mmol\ l^{-1}$, $P < 0.001$) in cases compared with controls. There was no difference in parameters between male and female infants.

To identify the changes of MDA, 8-OH-dG and TAS with increasing levels of TPB, cases were divided into four subgroups; subgroup 1 (TPB>12–16 mg dl^{-1}; $n = 18$), subgroup 2 (TPB>16–20 mg dl^{-1}; $n = 23$), subgroup 3 (TPB>20–25 mg dl^{-1}; $n = 15$) and subgroup 4 (TPB>25 mg dl^{-1}; $n = 8$). MDA levels in subgroup 1 were significantly lower than controls, and there was no significant difference between subgroup 2 and controls. However, MDA levels became significantly higher in subgroups 3 and 4 ($P < 0.001$). 8-OH-dG levels were higher in all case subgroups compared with controls, and a progressive rise was noted with increasing TPB. There was no statistically significant difference in TAS levels between subgroup 1 and controls, but the levels in subgroups 2, 3 and 4 were significantly reduced (Table 3, ANOVA and *post-hoc* Bonferroni test).

Pearson correlation coefficient showed significant correlation among the parameters (data not shown). Figures 1, 2 and 3 showed a significant increase in MDA and 8-OH-dG levels and a decrease in TAS levels in UCH with acute bilirubin encephalopathy compared with those with normal outcome. In receiver-operating-characteristic curve analysis, all parameters showed high sensitivity and specificity as predictors for poor outcome (Table 4).

Table 1. Baseline parameters of study neonates

Parameter	*Cases (n = 64)*	*Controls (n = 30)*	P-*value*
Maternal age (years) (mean ± s.d.)	26.5 ± 3.4	26.0 ± 4.3	0.593[a] (NS)
Antenatal care taken, *n* (%)	34 (53.1)	17 (56.6)	0.823[b] (NS)
Gravida, median (range)	2 (1–4)	2 (1–5)	0.584[a] (NS)
Parity, median (range)	1 (0–3)	1 (0–4)	0.548[a] (NS)
Mode of delivery			
SVD, *n* (%)	32 (50.0)	16 (53.3)	0.827[b] (NS)
Cesarean section, *n* (%)	32 (50.0)	14 (46.7)	
Presentation			
Vertex, *n* (%)	57 (89.0)	27 (90.0)	1.000[b] (NS)
Breech, *n* (%)	7 (11)	3 (10.0)	
Birth weight (g)			
Mean ± s.d.	2550 ± 458	2525 ± 325	0.798[a] (NS)
Median (range)	2600 (2250–2850)	2550 (2240–2750)	
Gestational age (weeks)			
Mean ± s.d.	38.8 ± 1.6	39.2 ± 1.7	0.443[a] (NS)
Median (range)	38 (37–39)	38 (37–39)	
Male:female	35:29	18:12	0.662[b] (NS)
Postnatal age at inclusion (days)			
Median (range)	4 (3–5)	4 (3–5)	0.675[a] (NS)
1 min Apgar score, median (range)	8 (7–9)	9 (7–10)	1.000[a] (NS)
Feeding			
Exclusively breastfed, *n* (%)	42 (65.6)	16 (53.3)	0.389[b] (NS)
Topfed, *n* (%)	4 (6.3)	4 (13.3)	
Breastfed+topfed, *n* (%)	18 (28.1)	10 (33.3)	
Weight loss >10%	0	0	—

Abbreviations: NS, not significant; SVD, spontaneous vaginal delivery.
[a]Independent sample *T*- test.
[b]χ^2-test.

DISCUSSION

The present study estimated the extent of oxidative stress and antioxidant defense in moderate-to-severe nonhemolytic UCH. Lipid peroxidation, which was less at lower TPB, increased significantly at higher levels (TPB>20 mg dl^{-1}), suggesting the antioxidant property of UCB at lower TPB being converted to a pro-oxidant one at higher levels. DNA damage was significantly higher at all levels, suggesting a possible genotoxic effect of bilirubin on UCH. Finally, although at lower TPB levels TAS level was similar in cases and controls, at TPB>16 mg dl^{-1} TAS was significantly less, indicating poor antioxidant defense at higher

TPB. The alterations in MDA, 8-OH-dG and TAS levels demonstrated high predictive accuracy for poor outcome.

A number of analytes have been studied in literature to estimate oxidative stress in newborns. We have chosen MDA as it is the most commonly studied product of lipid peroxidation, easy to estimate and inexpensive. Reactive oxygen species modifies DNA by inducing lesions on bases and sugars, single- and double-strand DNA breaks and DNA–protein crosslinks.[18] The measurement of 8-OH-dG is considered to be a direct indicator for oxidative DNA damage,[19] as the guanine base, which has the lowest ionization potential of any DNA constituent, is the main target of reactive oxygen species.[20] Different authors have measured various enzymes, such as superoxide dismutase, catalase, glutathione peroxidase, reduced nicotinamide-adenine dinucleotide phosphate, G6PD, and vitamins A, C and E for their antioxidant properties. In the present study, we have measured TAS as it is stable, sensitive, inexpensive and can be measured in automated analyzer without any interference from hemoglobin or bilirubin.[21] We excluded conditions that might induce oxidative stress in neonates. Premature newborns were excluded as they are deficient in antioxidant defense and prone to oxidative stress.

Previous authors had documented increased MDA in neonatal hyperbilirubinemia,[22] which was attributed to free radical formation and abstraction of hydrogen atoms from lipoproteins causing lipid peroxidation.[23] In an earlier study, we have documented decreased MDA levels with increasing bilirubin.[14] This difference in results from the present study could be due to various reasons. First, TBARS assay is not a very sensitive method for estimating lipid peroxidation in UCH because bilirubin interferes with absorbance signal producing erroneous results.[24] Second, in our previous study, we did not include cases with bilirubin levels above 25 mg dl^{-1} where oxidative stress is expected to be more. Moreover, the documented increase in MDA in UCH in the present study was only modest, unlike 8-OH-dG and TAS.

There are limited studies on the effect of hyperbilirubinemia on DNA damage, although several studies have reported phototherapy-mediated DNA strand-breaks, sister chromatid

Table 2. Comparison of oxidative stress markers and TAS between cases and controls (mean ± s.d.)

Parameter	*Cases (n = 64)*	*Controls (n = 30)*	*Independent sample T-test*	
			t-*value*	P-*value*
TPB (mg dl^{-1} (IQR))	19.8 ± 6.5 (15.3–21.2)	3.8 ± 0.6 (3.5–3.7)	13.426	< 0.001
MDA (mmol l^{-1} (IQR))	2.6 ± 1.6 (1.65 ± 3.77)	2.0 ± 0.8 (1.3–2.7)	2.561	< 0.05
8-OH-dG (pg ml^{-1} (IQR))	342.4 ± 69.9 (285.65–378.9)	229.0 ± 42.5 (244.4–258.4)	8.191	< 0.001
TAS (mmol l^{-1} (IQR))	0.13 ± 0.08 (0.07–0.2)	0.22 ± 0.07 (0.16–0.29)	5.167	< 0.001

Abbreviations: IQR, inter quartile range; MDA, malondialdehyde; TAS, total antioxidant status; TPB, total plasma bilirubin; 8-OH-dG, 8-hydroxy-2-deoxy guanosine.

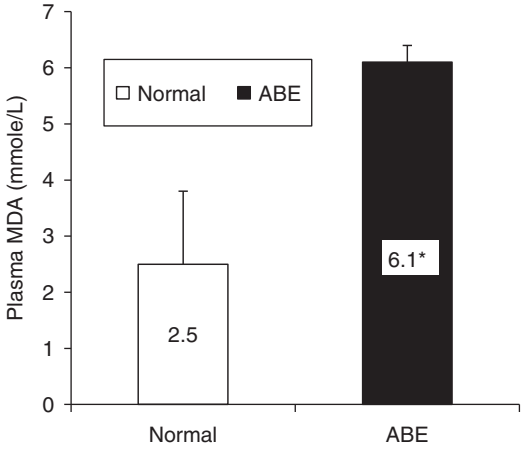

Figure 1. Comparison of plasma malondialdehyde levels between cases with normal outcome ($n = 59$) and those with acute bilirubin encephalopathy (ABE) ($n = 5$). □, normal outcome; ■, ABE; *$P < 0.001$.

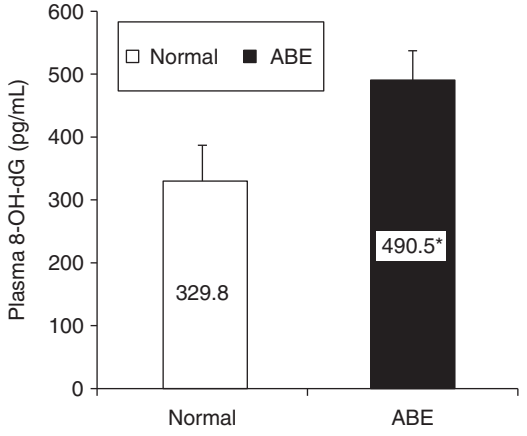

Figure 2. Comparison of plasma 8-hydroxy-2-deoxy guanosine (8-OH-dG) levels between cases with normal outcome ($n = 59$) and those with acute bilirubin encephalopathy (ABE) ($n = 5$). □, normal outcome; ■, ABE; *$P < 0.001$.

Table 3. Comparison of oxidative stress markers and TAS among subgroups of cases and controls (mean ± s.d.)

Parameter	*TPB (mg dl^{-1})*	*MDA (mmol l^{-1})*	*8-OH-dG (pg ml^{-1})*	*Total antioxidant status (mmol l^{-1})*
Subgroups of cases				
Subgroup 1 (TPB >12–16) ($n = 18$)	13.8 ± 1.1	1.3 ± 0.3	261.8 ± 19.6	0.21 ± 0.07
Subgroup 2 (TPB >16–20) ($n = 23$)	18.1 ± 1.0	2.2 ± 0.3	332.9 ± 17.9	0.14 ± 0.06
Subgroup 3 (TPB >20–25) ($n = 15$)	21.6 ± 1.1	3.9 ± 0.6	382.0 ± 14.4	0.07 ± 0.01
Subgroup 4 (TPB >25–40) ($n = 8$)	34.4 ± 4.3	6.0 ± 0.3	476.6 ± 26.4	0.03 ± 0.01
Controls				
(TPB 2.4-4.8) ($n = 30$)	3.8 ± 0.6	2.0 ± 0.8	229.0 ± 42.5	0.22 ± 0.07
ANOVA	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$

Abbreviations: MDA, malondialdehyde; TAS, total antioxidant status; TPB, total plasma bilirubin; 8-OH-dG, 8-hydroxy-2-deoxy guanosine.

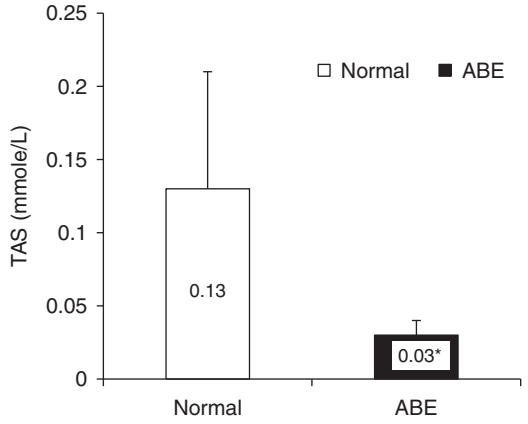

Figure 3. Comparison of plasma total antioxidant status (TAS) levels between cases with normal outcome ($n = 59$) and those with acute bilirubin encephalopathy (ABE) ($n = 5$). □, normal outcome; ■, ABE; *$P < 0.001$.

Table 4. Sensitivity and specificity of different parameters as predictors of poor outcome

Parameters	*Area under the curve*	*Cutoff*	*Sensitivity (%)*	*Specificity (%)*
MDA (mmol l^{-1})	97.1	⩾5.6	100	95.0
8-OH-dG (pg ml^{-1})	97.5	⩾459.8	100	96.7
TAS (mmol l^{-1})	0.972	⩽0.05	100	93.3

Abbreviations: MDA, malondialdehyde; TAS, total antioxidant status; 8-OH-dG, 8-hydroxy-2-deoxy guanosine.

exchange and mutations.[13,25] Karakukcu, *et al.*[26] have demonstrated increased DNA strand-break frequency by using comet assay in peripheral lymphocytes of newborns with moderate hyperbilirubinemia (mean bilirubin level 19.3 ± 3.22 mg dl^{-1}).

The neonatal period is a vulnerable time for free radical damage, and the antioxidant defense systems are not fully mature.[27] Free radicals are continuously generated in newborns even in the absence of any pathology. Furthermore, newborns are exposed to a relatively hyperoxic environment at birth, mediated by an increased oxygen bioavailability leading to the generation of free radicals.[28] Human plasma has an antioxidant capacity of 0.5–2 mM (mmol l^{-1}), but in neonates the values are much less and variable,[29,30] which may lead to a further increase in bilirubin-induced neurotoxicity.

Several mechanisms have been put forward to explain the pro-oxidant effects of bilirubin at high concentrations, such as over-stimulation of glutamate receptors,[31] release of pro-inflammatory cytokines from astrocytes and microglia,[32,33] inhibition of multi-drug resistance-associated protein-1[34] and cytochrome c oxidase activity, thereby inducing ATP release and disrupting glutathione redox status in immature neurons.[35] Moreover, exposure to high UCB is associated with an increased expression of neuronal nitric oxide synthase and production of nitric oxide, cyclic guanosine 3′,5′-monophosphate and reactive oxygen species.[31,36] Reactive oxygen species in turn leads to cytotoxicity by DNA damage, protein oxidation, lipid peroxidation, depletion of glutathione and impairment of mitochondrial oxidative phosphorylation causing cell necrosis and apoptosis.[9,31]

Our study had several limitations. Number of controls was less compared with cases. First, we tried to avoid unnecessary sampling of healthy neonates only for research purpose. Second, the biochemical markers were estimated only once; serial measurements in same neonates could have been more informative. Because of financial constraints, we could use only one EIA/ELISA kit for each analyte, and one kit allowed the analysis of a maximum of 96 samples. Finally, use of more definitive tests for BIND, such as acoustic-evoked responses, could have been a better marker to assess the statistical validity of the biochemical parameters.

Before 1980s, physicians used to manage neonatal hyperbilirubinemia with early phototherapy and exchange transfusion. With the passage of time, the aggressive management approach has been relaxed and the antioxidant role of bilirubin is appreciated more. Unfortunately, this change in attitude has led to a resurgence of bilirubin-induced neurotoxicity. Concern has also been expressed that the increase in the number of early hospital discharges, coupled with a rise in breastfeeding, might have led to a rise in the rate of preventable kernicterus.[37]

In this study, we have included term otherwise healthy neonates with UCH, as this group is usually discharged early from the hospital and is rarely followed up for developmental delay, especially in India where general follow-up is poor and there is no tracking system. Although there are no definite data, one might speculate that increased oxidative stress from otherwise harmless UCH in newborn period might be the cause of scholastic failure in later life.

To conclude, moderate-to-severe UCH is associated with higher oxidative stress and lower antioxidant defense, which might be a cause of concern in present day relaxed management approach of UCH. Future studies may be carried out to explore the possibilities of earlier therapeutic intervention or a role of antioxidant supplementation in UCH.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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