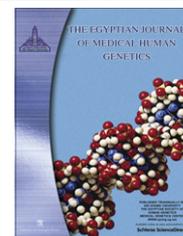




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The Egyptian Journal of Medical Human Genetics

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ORIGINAL ARTICLE

Phototherapy and DNA changes in full term neonates with hyperbilirubinemia

Maha Youssif Zein El-Abdin ^a, Manal Abd El-Salam ^{a,*}, Mona Yousri Ibrahim ^b, Soheir S.M. Koraa ^c, Enas Mahmoud ^a

^a Department of Pediatrics, Faculty of Medicine for Girls, AL-Azhar University, Egypt

^b Department of Clinical Pathology, AL-Azhar University, Egypt

^c National Centre for Radiation Research and Technology, Egypt

Received 20 July 2011; accepted 29 October 2011

Available online 29 November 2011

KEYWORDS

Hyperbilirubinemia;
Phototherapy;
DNA damage;
Apoptosis;
BCL2 gene;
BAX gene

Abstract *Background:* Phototherapy has remained the standard therapeutic approach for neonatal hyperbilirubinemia. Oxidative effects of phototherapy on cell membranes and cell components may have a wide range of potential adverse effects, including enhanced lipid peroxidation and DNA damage. Apoptosis is an indispensable mechanism for maintaining many cellular functions, including cell replication, and removal of damaged cells with high burden of genetic mutations. Many genes function as apoptosis regulatory genes. Examples of these genes include the BCL2 gene which is an anti-apoptotic oncogene, and the BAX gene which acts as a promoter of apoptosis.

Objectives: Assess the effect(s) of phototherapy on DNA and on the rate of apoptosis in full term neonates with hyperbilirubinemia. It comprised 35 neonates with indirect hyperbilirubinemia who received phototherapy for 48 h, and 20 apparently healthy full term neonates with normal serum bilirubin level, as a control group. DNA damage was assessed by DNA fragmentation and micronucleus assay. Determination of the anti-apoptotic effect(s) of BCL2 gene was achieved by quantitative assay of its product, (BCL2) protein, by ELISA and BAX gene expression status was assessed by PCR.

Results: The frequency of micronuclei in circulating lymphocytes of neonates who received phototherapy has significantly increased before and after phototherapy compared to controls, ($p < 0.001$;

* Corresponding author. Tel.: +20 0106337676.

E-mail address: manal_679@yahoo.com (M.A. El-Salam).



$p < 0.00001$), respectively. DNA fragmentation in circulating lymphocytes, was significantly higher among cases before and after phototherapy compared to controls ($p < 0.0001$; $p < 0.00001$). The plasma BCL2 protein was significantly lower in the cases before and after phototherapy compared to controls ($p < 0.01$; $p < 0.01$), respectively, and BAX gene expression was significantly high among cases before and after phototherapy compared to controls.

Conclusions: Geno toxic effects of bilirubin and phototherapy, induce more DNA damage and enhances apoptosis of exposed cells, probably through down regulation of BCL2 expression and upregulation of BAX gene expression in neonates with hyperbilirubinemia.

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1. Introduction

Over 60% of all term newborns develop jaundice in their first days of life [1]. Unconjugated hyperbilirubinemia occurs as a result of excessive bilirubin formation and inability of neonatal liver to clear bilirubin rapidly enough from the blood [2]. The majority of causes of hyperbilirubinemia in the term newborn are benign and reversible [3]. However, considering the potentially irreversible toxicity of bilirubin on the central nervous system, treatment should be carried out to eliminate excessive bilirubin [4].

Since 1950s, phototherapy has been the therapy of choice for newborns with hyperbilirubinemia [5], yet there are controversial results regarding its effects on DNA damage. It has been reported that both conventional and intensive phototherapy cause DNA damage [6], while others found that DNA was not affected with phototherapy [7], so further studies in this issue are needed.

Phototherapy induces the release of cutaneous reactive nitrogen species (RNS) and reactive oxygen species (ROS) [8], and photolysis products are cytotoxic and are associated with the production of free oxygen radicals [9]. Free radicals have been shown to initiate apoptotic cell death in many in-vitro and in-vivo experimental models [10,11]. Two major signaling pathways leading to apoptosis have been identified. One is mitochondria-dependent and is controlled by suppressing or inducing genes, such as BCL2 and BAX, respectively [12]. The other is death receptor dependent, involving the interaction of death receptors involving Fas/FasL interaction receptor-associated death proteases and subsequent activation of downstream effector caspases [13].

Ultraviolet-induced DNA damage sets in motion a highly complex well coordinated series of responses. Human cells use many strategies to protect genomic DNA from accumulating such lesions. If the damage is extensive, cell cycle progression is blocked to allow more time for DNA repair. However, if the damage is irreparable the affected cells undergo apoptosis [14].

The BCL2 family of proteins constitutes a critical control point in apoptosis residing immediately upstream of irreversible cellular damage, where family members control the release of apoptogenic factors from the mitochondria. The cardinal member of this family, BCL2, was originally discovered as the defining oncogene in follicular lymphoma [15]. There are two genes (BCL2 and P53) that control the process of apoptosis. BCL2 is an oncogene which blocks apoptosis. It can be called cell death suppressor gene because of its direct regulation of apoptosis. A high concentration of BCL2 protein protects the cell from apoptosis [16].

The aim of the present study was to evaluate outcome markers of DNA damage and apoptosis induced in circulating lymphocytes in neonates suffering from hyperbilirubinemia and receiving phototherapy as the sole treatment. DNA damage was measured in terms of the formation of micronuclei in circulating lymphocytes, while apoptosis was measured in terms of plasma DNA fragmentation and plasma BCL2 level and BAX mRNA expression in circulating lymphocytes.

2. Patients and methods

2.1. Patients

This study was performed on 55 full term neonates in the 1st two weeks of life. 35 of them (23 males and 12 females) with indirect hyperbilirubinemia who were admitted in the neonatal intensive care unit (NICU) and had received phototherapy for 48 h with mean postnatal age 7.4 ± 3.8 days. The other 20 cases (12 males and 8 females) were apparently healthy full term neonates with normal serum bilirubin levels, with mean postnatal age of 8 ± 3.2 days as a control group. The number of cases of physiological jaundice that were included in the present study was 23 and the percentage of them was 65% and the number of cases of pathological jaundice was 12 and the percentage of them was 35%.

All cases were selected from the neonatal intensive care unit (NICU) of El-Zahraa University hospital and Nasr City Insurance hospital. A formal consent letter from the parents of each newborn was obtained after explaining to them the whole procedure. The study was approved by the Ethics Committee of the Hospital.

Exclusion criteria:

- Premature and low birth weight infants.
- Neonates with (asphyxia and sepsis).
- Infants of diabetic mothers.
- Neonates with congenital anomalies.
- Neonates with cholestatic jaundice.

All cases enrolled in the study have been subjected to the following:

Full history taking with special emphasis on:

- Maternal medical history of any acute or chronic illness before or during pregnancy.
- Full postnatal history taking with stress on the onset of jaundice, duration of phototherapy exposure, type of milk (breast, formula or both) and jaundice associated symptoms.

Full general and local examination is done with stress on Neurological examination (neonatal reflexes, state of consciousness and abnormal movement) to detect early CNS damage.

2.2. Routine investigations

Complete Blood Count (C.B.C), total and direct bilirubin, RH and blood group, reticulocyte count, liver enzymes and serum albumin level.

3. Specific investigations

- (A) Determination of DNA damage by: DNA fragmentation assay. Micronucleus assay.
- (B) Determination of the anti-apoptotic protein (BCL2) by Elisa technique.
- (C) BAX gene messenger RNA expression by RT-PCR.

4. Sampling

4.1. Eight millilitre of peripheral blood was taken before phototherapy

3 ml was taken in EDTA vacutainer for C.B.C., Reticulocyte count. 2 ml was taken in plain vacutainer for Serum bilirubin level (total and direct bilirubin), RH and blood group, Liver enzymes and serum albumin level and 3 ml was taken in EDTA vacutainer for DNA fragmentation assay, frequency of micronuclei, plasma BCL2 level and BAX gene messenger RNA expression by RT-PCR.

4.2. Five millilitre of peripheral blood was taken after phototherapy

Three millilitre taken in EDTA vacutainer for:

- DNA fragmentation assay.
- Frequency of micronuclei.
- Plasma BCL2.
- BAX gene messenger RNA expression by RT-PCR.

4.3. Two millilitre taken in plain vacutainer for: assay of serum bilirubin level

4.3.1. Assay of the frequency of micronuclei

According to standard methods using the cytokinesis block micronucleus assay, where a duplicate of 1 ml of whole blood was cultured, and proliferation was stimulated by the addition of phytohemagglutinin (5 µg/ml) into 15 ml sterile plastic round bottom tubes containing media 199, 20% fetal calf serum and anti-biotic. Cells were incubated for 72 h adding cytochalasin B 24 h before harvesting. Twenty-four hours after the addition of cytochalasin B, cells were collected and treated with 0.8% sodium citrate for 3–5 min and then fixed in methanol:acetic acid (5:1). Fixed cells were dropped gently onto clean microscope slide, air dried and stained with 4% Giemsa (Sigma) using standard protocols. Slides were scored at 100×

magnification using a Leica Biomed microscope (Germany). The frequency of micronuclei in circulating lymphocytes was calculated according to the standard criteria [17].

4.3.2. Assay of DNA fragmentation

The DNA fragmentation assay was performed as described previously according to the method of [18]. Briefly, plasma leukocyte rich layer was mixed with cell lyses buffer (0.1% Triton X-100, 5 mM Tris-HCl, pH 8, 20 mM EDTA) and centrifuged. Small fragments of DNA were gradient separated from the precipitate with equal volumes of 2 M NaCl and 5% polyethylene glycol 5000 (Sigma) in ethyl ether and were cooled and centrifuged. The supernatants were removed and mixed with an equal volume of Hoechst 33258 in PBS and incubated for 20 min at room temperature. Fluorescence was determined at 360 nm excitation and 460 nm emissions. Total DNA was also determined using Hoechst 33258 as a chromogen

4.3.3. ELISA for BCL2 protein

The amounts of BCL2 in white blood cells homogenate were determined by a sandwich enzyme linked immunosorbent assay (ELISA) using two anti-human BCL2 monoclonal murine antibodies (Quantikine R & D Systems Inc). The measurement was as follows: A 96 well plate pre-coated coated with anti-human BCL2 antibody (10 µg/ml diluted with phosphate buffer saline, PBS) overnight at 4 °C. After washing the plate with PBS containing 0.05% Tween 20 (T-PBS), the wells were blocked by skimmed milk solution for 2 h at 37 °C. White blood cells homogenate diluted twice with 1% bovine serum albumin (BSA)/PBS. Fifty µl of the samples and standard BCL2 (diluted with 1% BSA/PBS) were added to the well and incubated for 1 h at room temperature. After washing with T-PBS, biotinylated NOK-1 anti-human BCL2 antibody (5 µg/ml containing 5% mouse serum) was added and incubated for 1 h at room temperature. The wells were washed five times with T-PBS and incubated with 50 µl tetramethyl benzidine (MO, USA) for 1 h at room temperature. Chromogen activity was detected by absorbance measured at 450 nm using an ELISA reader.

4.3.4. Reverse transcriptase polymerase chain reaction (RT-PCR) assay for BAX mRNA

Total RNA was extracted from lymphocytes separated from the peripheral blood by Ficoll-Hypaque density gradient separation using Histopaque-1077 (Sigma Diagnostics, St Louis, Missouri, USA), using QIAGEN RNeasy extraction Kit (QIAGEN Inc, USA). The RNA samples were reverse transcribed using Superscript reverse transcriptase, using QIAGEN One Step RT-PCR kit (QIAGEN Inc USA, Clini Lab). The thermal cycler was programmed as follows: 30 min 50°, 15 min 95°, 35 cycles of 3-step cycling 1 min at 94°, 1 min at 55° and 1 min at 72 °C with final extension of 72 °C for 10 min. Bax (13) sequences, forward: 5'-CAC CAG CTC TGA-GCA GAT G-3'; reverse: 5'-GCG AGG CGG TGA-GCA CTC C-3'). Primers for β-actin were synthesized simultaneously as an internal reference for all samples (forward: 5'-GTG GGG CGC CCC AGG CAC CA-3'; reverse: 5'-CTC CTT AAT GTC ACG CAC GAT TTC-3'). Bax (13) sequences, forward: 5'-CAC CAG CTC TGA-GCA GAT G-3'; reverse: 5'-GCG AGG CGG TGA-GCA CTC C-3'). Five µl of RT reaction was mixed with different primers together with 25 µl of Ready Mix RedTaq PCR reaction mix

(20 mM Tris/HCl, 100 mM KCl, 3 mM MgCl₂, 0.4 mM dNTP), with 1.5 units of Taq DNA polymerase (Sigma), 2 µl of each primer working solution (working concentration of primers: 50 pM/µl), and PCR grade water to a final volume of 50 µl. For FasL thirty eight cycles of amplification were performed in a Perkin Emler thermal cycler After initial denaturation at 95 °C for five minutes, each cycle consisted of denaturation at 94 °C for one minute, annealing at 55 °C for two minutes, and extension at 72 °C for three minutes. A final extension step was performed at 72 °C for 5 min. Ten µL from each PCR reaction product was separated on a 1.2% agarose gel then stained with ethidium bromide The appearance of specific bands (Bax 516 bp and β-actin 540 bp) was evaluated under ultraviolet light and photographed.

4.3.5. Statistical analysis

A. Descriptive statistics (arithmetic mean, standard deviation of the mean) were performed for all cases of the study.

B. The results of the “*t*”-value were checked on student’s “*t*”-table to find out the significance level (*P* value) according to the degree of freedom.

- $p > 0.05$ = Non significant
- $p < 0.05$ = Significant
- $p < 0.001$ = Highly significant

All calculations and data presentations were carried out by the use of the software Microsoft Excel V.5 (2003).

5. Results

The results of the present study were presented in Tables 1–3 and Figs. 1–4. Comparison between cases and controls regarding CBC, liver enzymes, direct bilirubin and serum albumin levels showed no statistical significant differences. On the other hand, reticulocyte count showed high statistical significant differences in the cases before phototherapy compared to controls (Table 1).

Comparison between cases and controls regarding serum bilirubin level, there were significantly higher levels among cases before phototherapy compared to controls. After phototherapy it became significantly lower compared to its level before phototherapy (Table 2).

There is a significant increase in DNA fragmentation in circulating lymphocytes among cases compared to controls. After phototherapy it showed more statistical significant increase in DNA fragmentation compared to its level before phototherapy among cases (Table 2, Fig. 1).

There is significant increase in frequency of micronuclei in circulating lymphocytes among cases compared to controls. After phototherapy the level of the frequency of micronuclei in circulating lymphocytes became highly significant compared to its level before phototherapy (Table 2, Figs. 2 and 3).

Plasma BCL2 protein level was significantly lower among cases before phototherapy compared to controls. After phototherapy it became more significantly lower compared to its level before phototherapy (Table 3, Fig. 4).

BAX messenger RNA expression was significantly higher among cases before phototherapy compared to controls, after phototherapy BAX messenger RNA expression was more significantly higher (Table 3).

6. Discussion

Although adverse effects of phototherapy have been evaluated clinically in several studies, information on the potential cellular effects of phototherapy such as DNA damage is limited [19]. The study of DNA at the chromosome level is an essential part of genetic toxicology, because chromosomal mutations are important events in carcinogenesis. Micronucleus assays have emerged as one of the preferred methods for assessing chromosome damage or loss and chromosome breakage to be measured reliably [20]. Micronuclei are morphologically similar to main nuclei but smaller in size, hence the name [21], in the present study by both micronucleus and DNA fragmentation assays, we had detected the DNA damage.

Although very high levels of bilirubin are known to be toxic, there is continued uncertainty about the risks and benefits of moderate serum bilirubin concentrations [22]. In the present study we found that high level of bilirubin is associated with DNA damage in peripheral lymphocyte as there was statistically significant difference between DNA damage in cases before phototherapy compared to controls. On the other hand Dani et al. described the antioxidant properties of bilirubin. He showed that bilirubin in micromolar concentrations has stronger antioxidant effects than vitamin E [23].

Table 1 Comparison of CBC, reticulocytic count, liver enzymes, direct bilirubin and serum albumin between the cases and the controls.

Variable	Control <i>n</i> = 20	Cases <i>n</i> = 35	<i>t</i> -test	
	Mean ± SD	Mean ± SD	<i>t</i>	<i>P</i> -value
White blood cell count × 10 ⁹ /l	7.900 ± 1.796	7.825 ± 2.111	0.121	0.904*
RBC × 10 ⁶ mm ³	4.43 ± 0.53	3.95 ± 1.09	1.771	0.0846*
HB g/dl	14.550 ± 1.504	13.125 ± 3.149	1.826	0.076*
Hematocrit%	42.650 ± 4.580	38.950 ± 8.532	1.709	0.096*
Platelets × 10 ⁹ /l	266.600 ± 86.408	272.700 ± 91.435	0.217	0.829*
Reticulocytic%	1.000 ± 0.000	2.300 ± 0.470	2.854	0.007*
SGOT U/L	44.500 ± 2.188	43.750 ± 2.531	1.002	0.323*
SGPT U/L	43.900 ± 2.269	44.100 ± 2.174	0.285	0.777*
Direct bilirubin mg/dl	0.7 ± 0.5	0.6 ± 0.4	0.425	0.324*
Serum albumin mg/dl	3.38 ± 0.19	3.38 ± 0.22	0.00	1.00*

* $P > 0.05$ (NS).

Table 2 Comparison of serum bilirubin, DNA fragmentations and frequency of micronuclei in circulating lymphocytes between control and case also among studied cases before and after phototherapy.

		Cases <i>n</i> = 35	Control <i>n</i> = 20	<i>t</i> -test	
		Mean ± SD	Mean ± SD	<i>t</i>	<i>P</i> -value
<i>Serum bilirubin level mg/dl</i>					
Before phototherapy		14.600 ± 4.300	1.300 ± 0.400	13.773	< 0.001*
After phototherapy		6.824 ± 2.099	1.300 ± 0.400	11.561	
Paired <i>t</i> -test	<i>t</i>	9.986			
	<i>P</i> -value	< 0.001*			
<i>DNA fragmentation</i>					
Before phototherapy		1.400 ± 0.400	0.800 ± 0.070	6.6	< 0.0001**
After phototherapy		3.400 ± 0.800	0.800 ± 0.070	4.4	< 0.00001**
Paired <i>t</i> -test	<i>t</i> -test	9.233			
	<i>P</i> -value	< 0.00001**			
<i>Frequency of micronuclei in circulating lymphocytes in full term neonates with hyperbilirubinemia</i>					
Before phototherapy		5.500 ± 2.800	2.800 ± 0.400	4.5	< 0.001**
After phototherapy		7.800 ± 1.200	2.800 ± 0.400	12.5	< 0.00001**
Paired <i>t</i> -test	<i>t</i> -test	6.845			
	<i>P</i> -value	< 0.001**			

* *P* ≤ 0.05 (significant).
 ** *P* ≤ 0.01 (highly significant).

Table 3 Comparison of plasma BCL2 Protein levels and Bax mRNA Expression between cases before and after phototherapy compared to controls.

		Cases <i>n</i> = 35	Control <i>n</i> = 20	<i>t</i> -test	
		Mean ± SD	Mean ± SD	<i>t</i>	<i>P</i> -value
<i>BCL2 Protein ng/ml</i>					
Before phototherapy		8.600 ± 2.400	11.800 ± 4.200	3.1	0.01*
After phototherapy		4.800 ± 1.800	11.800 ± 4.200	5.4	0.01*
Paired <i>t</i> -test	<i>t</i> -test	5.156			
	<i>P</i> -value	< 0.001*			
<i>Bax mRNA expression</i>					
Before phototherapy		0.14 ± 0.1	0.08 ± 0.01	5.3	0.01*
After phototherapy		0.39 ± 0.12	0.08 ± 0.01	6.7	0.01*
Paired <i>t</i> -test	<i>t</i> -test	22.656			
	<i>P</i> -value	< 0.001*			

* *P* ≤ 0.01 (highly significant).

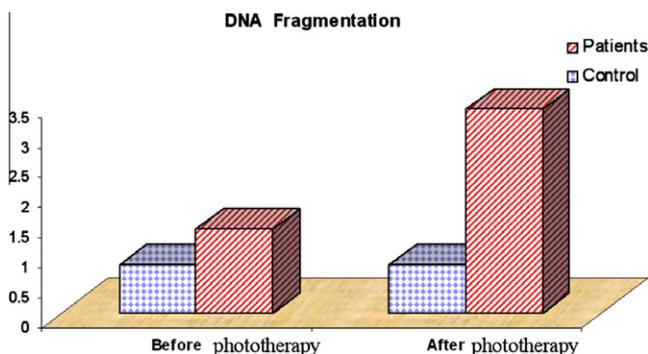


Figure 1 DNA fragmentation in the study group before and after phototherapy compared to controls.

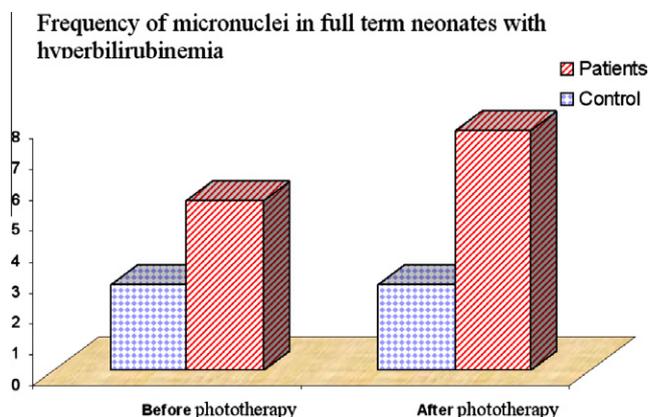


Figure 2 Frequency of micronuclei among cases before and after phototherapy compared to controls.

In the present study we had found that both phototherapy and high serum bilirubin levels can lead to DNA damage as there was a highly significant difference between cases before

phototherapy and controls. After phototherapy exposure DNA damage had significantly increased in the cases. Our findings are in agreement with Krakukcu et al. who showed

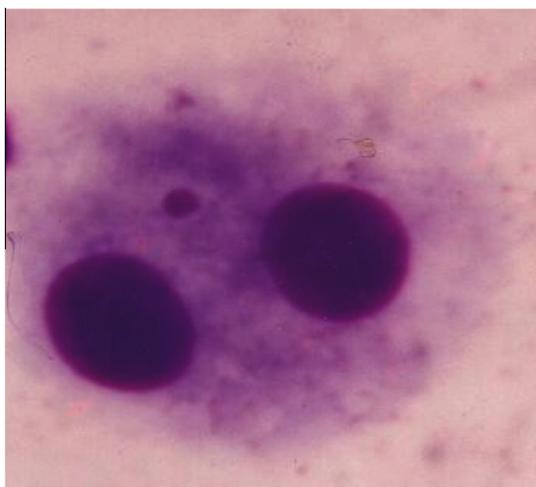


Figure 3 Binucleated cell showing one micronucleus.

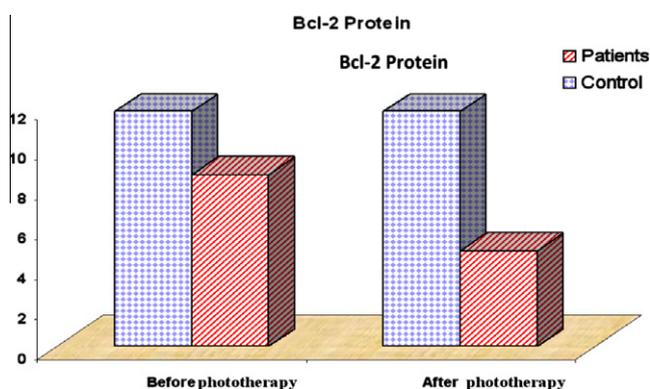


Figure 4 BCL2 Protein level in the cases before and after phototherapy compared to the controls.

that high level of bilirubin may lead to oxidative damage in newborns as photochemical reactions may produce toxic photoproducts, probably peroxides [4]. Similarly Dani et al. reported that membrane bound bilirubin is a photo sensitizer as it captures light energy and becomes energized to its triplet state. This photo-excited molecule can transfer its energy to molecular oxygen, singlet oxygen and becomes a highly reactive radical that can oxidize tissues at the molecular level [24].

Aycicek et al studied DNA-damaging effects of intensive and conventional phototherapy by the comet assay and compared results with the control group [6]. The main outcome of this study was that endogenous mononuclear leukocyte DNA strand-breaks had significantly increased in both conventional and intensive phototherapy-treated infants when compared with controls that did not receive phototherapy. He believed that bilirubin is more active as an oxidant than its photo-excited form and the waste products are formed by phototherapy. In contrast to the present results Krakukcu et al. found that phototherapy does not stimulate or enhance the DNA-damaging effects of bilirubin as their results indicate that the DNA damage in infants receiving phototherapy was not different from that in the control group [4]. As in vivo conditions, phototherapy does not cause an increase in DNA oxidation by itself, in contrast to its effects in vitro as the situation in vivo is different from that in vitro, as the phototherapy light

is filtered through tissues. Although DNA damage is induced in human cells through exposure to phototherapy light, it is clear that these alterations do not result in immediate or widespread harmful consequences because thousands of babies have been treated with phototherapy since 1958 and no serious side effects have been noticed [25].

In spite of this observation it is wise to avoid unnecessary use of neonatal phototherapy as unwanted effects on DNA have been demonstrated in both peripheral lymphocytes and mononuclear leukocytes in term infant [6,26]. And also our study showed that phototherapy causes unfortunate effects on DNA, and also long term follow up studies are recommended. Interestingly, a recent study including patients with more than a 30-year follow up period has shown that neonatal phototherapy is associated with allergic rhinitis and conjunctivitis [27]. In this study, we have assessed the role of BCL2 protein as an anti-apoptotic protein and assessed bax gene expression in peripheral blood lymphocytes induced by phototherapy exposure in newborn with hyperbilirubinemia. We found that our findings are in agreement with [28] who reported that among the effects of phototherapy noted was increase in apoptosis of polymorphonuclear cells which leads to decreased number of neutrophils in areas of inflammation and neutrophil anti-apoptotic factors.

In the present study, we found that the level of BCL2 Protein was lower among the cases compared to controls. After phototherapy it became significantly lower compared to its level before phototherapy in the cases. El-Haddidi et al who studied the effect of PUVA phototherapy on BCL2 level in dermal lymphocytes, found that PUVA phototherapy induces evident down regulation of BCL2 level and causes early significant depletion of epidermal and dermal T cells in psoriatic tissues via the induction of apoptosis [19]. Finally, the hematopoietic system, particularly the lymphoid lineage, is highly susceptible to DNA damage and organisms rely on apoptosis for the removal of damaged cells. Excess BCL2 overcomes the apoptosis inducing the effect of P53 protein, so the alleviation of cell cycle arrest by a decreased level of BCL2, potentiates P53 induced apoptosis [29].

Members of the Bcl2 family of proteins are important regulators of programmed cell death pathways with individual members that can suppress (e.g., BCL2, BCL-X_L) or promote (e.g., BAX, BAD) apoptosis [30]. The BAX and BCL2 are the key players of this family [31]. We evaluated the effect of phototherapy treatment on the levels of BAX protein expression and we found that BAX gene expression in peripheral lymphocytes was significantly higher compared to controls before phototherapy. After phototherapy, it showed more significant increase, suggesting an apoptotic role through a concomitant decrease in BCL2 levels.

We reported increased DNA damage and a concomitant increase of rate of apoptosis. This might be explained by BCL2 down regulation and increased BAX gene expression. However, this does not guarantee that all damaged cells will undergo apoptosis. The concept of survival at the expense of proliferation awaits further validation as more mechanistic data come to light.

7. Conclusions

This study is another proof of the geno toxic effects of both bilirubin and phototherapy. High bilirubin level is toxic in neonates as it induces DNA damage. Phototherapy was found to

induce more DNA damage than that was induced by high bilirubin level. Phototherapy seems to induce an evident down regulation of BCL2 level in newborn with hyperbilirubinemia as BCL2 and upregulation of BAX gene expression in neonates with hyperbilirubinemia

8. Recommendations

Restriction of the use of phototherapy only in serious cases of hyperbilirubinemia considering risks and benefits is according to the guidelines of American Academy of Pediatrics. Long term follow up studies are recommended for the jaundiced babies who were exposed to phototherapy.

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