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# Role of bilirubin as antioxidant in neonatal jaundice and effect of ethanolic extract of sweet lime peel on experimentally induced jaundice in rat

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Bilirubin above a threshold level is toxic to human system and is excreted in urinary and through gastrointestinal tract. The role of bilirubin as antioxidant is debatable. This paper aims at elucidating the role of bilirubin as an antioxidant in neonatal jaundice patients. It is observed that bilirubin up to 6 mg/dl in blood acts as an antioxidant and above 12.5 mg/dl is strongly prooxidant. Phototherapy is the accepted therapeutic management of neonatal jaundice and has been shown to enhance the oxidative stress. Approaches have been taken to formulate a herbal medication which will reduce bilirubin level in the neonates without inducing additional damages. The ethanolic extract of sweet lime peel, administered orally at a dose of 72 µg is found to reduce the oxidative stress in erythrocytes of phenylhydrazine-induced jaundiced rats treated with phototherapy.

**Keywords**: Bilirubin, Antioxidant, Neonatal jaundice, Oxidative stress, Phototherapy, Sweet lime peel

Bilirubin, a linear tetrapyrrole is the catabolic product of hemeproteins. The heme ring released from hemeprotein is metabolized by heme oxygenase to produce carbon monoxide, free iron and green biliverdin. Biliverdin is the principal end product of heme metabolism in birds, reptiles and amphibians. In mammals, biliverdin reductase (BVR) reduces biliverdin to yellow bilirubin, utilizing NADPH. The hydrophobic bilirubin is converted to bilirubin-diglucuronide in the liver by uridine diphospho-glucuronate glucuronosyl transferase to facilitate its excretion into the bile. The elevated bilirubin level (>3 mg/dl) i.e. hyperbilirubinemia is evident in the appearance of jaundice, especially in neonates when unconjugated bilirubin concentration is relatively high in the blood[1,2].

The transient neonatal jaundice usually referred as "physiologic jaundice" results from increased lysis of the erythrocytes (red blood cell; RBC) and impaired hepatic uptake, followed by limited ability of conjugation and excretion[3]. At highly elevated level (>12 mg/dl), unconjugated bilirubin impairs the antioxidant system of the RBC, enhances oxidative stress i.e., increases reactive oxygen species (ROS) formation, induces morphological alteration and loss of phospholipids symmetry of RBC membrane[4], impairs the membrane transport systems of RBC[5] and can cross the blood brain barrier causing kernicterus[6]. Phototherapy is used for therapeutic management of neonatal jaundice, although several side effects have been reported[7].

Evidences also suggest that bilirubin scavenges peroxyradicals, protects cell against complement-mediated anaphylaxis, myocardial ischemia, pulmonary fibrosis[8] etc. Antioxidant activity of bilirubin is anticipated by cycling between bilirubin and biliverdin (Fig. 1). Bilirubin interacts with ROS, neutralizes its toxicity and transforms to biliverdin, which is then subsequently reduced by BVR to regenerate bilirubin[9]. However, it is not clear how bilirubin interacts with ROS to reduce its toxicity and on other hand at elevated level increases ROS formation. Increased bio-availability of ROS, in turn, impairs membrane redox potential, lipid and protein oxidation etc[4,5]. NADPH, the major product of pentose phosphate pathway (PPP) of RBC[2] is also expected to play a major role in the bilirubin-ROS interaction.

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*Abbreviations*: BVR, biliverdin reductase; DPPH, 2,2-diphenyl-1-picrylhydrazyl; G6PDH, glucose-6-phosphate dehydrogenase; HNE, 4-hydroxynonenal; LPO, lipid peroxidation; MDA, malondialdehyde; NADPH; reduced nicotinamide adenine dinucleotide phosphate; 6-PGDH, 6-phosphogluconate dehydro-genase; PPP, pentose phosphate pathway; ROS, reactive oxygen species; TK, transketolase.

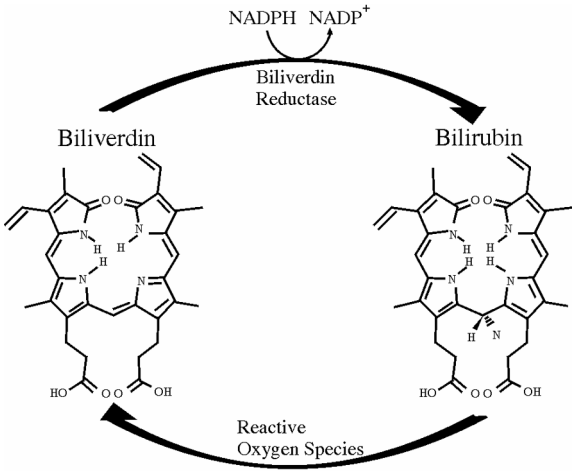


Fig. 1—Bilirubin-biliverdin interconversion

The present study has investigated antioxidative property of bilirubin by assessing the mode of bilirubin-biliverdin conversion and measuring BVR activity under normal and elevated levels of bilirubin in the blood of neonates. Glucose-6-phosphate dehydrogenase (G6PDH) and 6-phosphogluconate dehydrogenase (6-PGDH) of PPP have been assayed in jaundiced neonates, before and after phototherapy to asses the production efficiency of NADPH. The level of transketolase (TK) has been assayed to explore the thiamin status of neonates. The effect sweet lime peel extract to reduce the deleterious side effects of neonatal jaundice treated with phototherapy has also been studied.

## Materials and Methods

### Chemicals

The chemicals used for the study were of analytical grade purchased from E. Merck, SRL, Ranbaxy, Qualigens and the enzymes were purchased from Sigma-Aldrich Chemical Co. USA

### Subjects

The study group included 75 neonates with high bilirubin (19 ± 6.2 mg/dl) and 50 neonates with low bilirubin (5 ± 1 mg/dl) level. As control, 100 age-matched non-jaundiced neonates without hepatic and intestinal diseases were also examined. Whole blood from neonates was obtained by vein puncture. The birth weights of both control and study groups were in the range of 1.8-2.5 kg. Blood was collected from different neonatal cares centers of hospitals of Kolkata. The residual blood left after normal routine clinical examination was used for the investigation, after consulting with the pediatrician. The total case history of the neonates was noted down. The serum was used to measure bilirubin, biliverdin and BVR activity. The hemolysate prepared by osmotic shock method served as a source of enzymes of RBC[4].

### Interaction of bilirubin and biliverdin with superoxide free radical

The interaction of bilirubin and biliverdin with free radical was studied using potassium superoxide ($KO_2$) dissolved in acetonitrile containing dicylohexano-18-crown-6-ether and characterized by UV-Vis spectrophotometer (Spectrascan UV 2600). The superoxide solution-crown ether complex was prepared[10,11] by weighing $KO_2$ and quickly adding to equivalent moles of the crown ether in acetonitrile and stirred in sealed condition for 30 min. In order to minimize the effects of light on these solutions, all experiments were carried out in dark.

### Measurement of bilirubin and biliverdin

The biliverdin concentration in serum was measured by previously described method[12] and bilirubin concentration was estimated by spectrophotometric method[13]. The spectrophotometric method was correlated with Malloy and Evelyn's[14] method and the correlation coefficient was found to be 0.9.

### Measurement of biliverdin reductase

The NADPH-dependent BVR (EC 1.3.1.24) activity was assayed at 37°C for 5 min[15]. The incubation mixture contained 60 μ*M* NADPH dissolved in 10 m*M* potassium phosphate buffer (*p*H 7.4) and 13 μ*M* of biliverdin.

### Measurement of G6PDH, 6-PGDH and TK

G6PDH activity (EC 1.1.1.49) was assayed in the hemolysate utilizing the method of Begrmeyer[16] . The assay mixture contained 0.05 *M* triethanol amine buffer (*p*H 7.5), 0.003 *M* $NADP^+$ and 0.004 *M* glucose-6-phosphate. The activity of 6-PGDH (EC 1.1.1.44) was measured according to the previously described method[17]. The reaction mixture contained 0.04 *M* glycerol glycine buffer (*p*H 9), 0.1 *M* $MgCl_2$, 0.04 *M* 6-phosphogluconate and 0.003 *M* NADPH. The activity of TK (EC 2.2.1.1) was also determined by standard method[18]. The assay mixture contained 0.01 *M* glycyl-glycine buffer (*p*H 7.5), 0.02 *M* ribulose-5-phosphate, 0.02 *M* ribose-5-phosphate, 0.003 *M* NADH, 0.1 ml of α-glycerophosphate dehydrogenase (0.01 mg/ml).

**Measurement of antioxidant potency of the sweet lime peels extract**

Antioxidant potency of the sweet lime peel was measured with DPPH assay[19]. The ethanolic extract of sweet lime peel was prepared by standard method[20]. An ethanolic solution of DPPH (100 μ*M*) was incubated with an ethanolic solution of the sweet lime peel extract (50-400 μg/ml) and the absorbance was monitored spectophotometrically at 517 nm. The concentration ($IC_{0.20}$) of the test compound that induced a decrease of 0.20 in absorbance during a 30 min observation was taken as free radical scavenging potency. It was used as the standard dose for administration to the rats. The herbal extract was administered orally for 3 days.

**Induction of jaundice and its management by phototherapy and sweet lime peel extract**

Adult albino rats of Wister strain of both sexes (weighing 17.0 ± 5.0 g) were fed with basal diet and water and maintained under standard laboratory conditions. Rats were administered orally with aqueous solution of 1% phenylhydrazine[21] once daily for 3 days. The rats were later given phototherapy (with 420-450 nm lamp) for 1 h with an interval period of 30 min with (group 4) and without herbal extract (group 1) against control rats. Group 2 rats received phenylhydrazine along with ethanol. The group 3 rats were given extract only after phenylhydrazine administration.

**Measurement of malondialdehyde (MDA) and 4-hydroxynonenal-protein (HNE) adducts**

RBC membrane was isolated from the hemolysate, following standard protocol[5] and oxidative damage was assayed by standard markers. Lipid peroxidation (LPO), a marker of cellular damage was assayed by malondialdehyde formation[22] and expressed as nmole MDA formed/mg protein and estimated as described previously[23]. The reaction mixture contained 20% trichloroacetic acid, 0.76% thiobarbituric acid and 0.05 *M* Tris-buffer. Rats were then sacrificed by cervical dislocation and livers were excised and homogenized in 0.25 *M* sucrose containing 1 m*M* EDTA. HNE adducts formed during ROS-mediated damage were also studied by immunoblotting technique in treated and control rats[24]. Protein content was estimated by the method of Lowry *et al*[25]. The study was approved by Institutional Ethical Committee vide registration no. 797CPCSEA.

**Statistical analysis**

Data collected were recorded, analyzed and results expressed as mean ± S.D (standard deviation). Statistical probability was calculated using Student's 't' test and *P* values <0.001 were taken as highly significant.

## Results and Discussion

**Interaction of bilirubin and biliverdin with superoxide free radical**

The interaction of bilirubin and biliverdin with superoxide radical is shown in Figs 2 and 3. Fig. 2 shows bilirubin interacts and can quench the superoxide radical ($O_2^{\cdot-}$), as evidenced by decrease of absorption maxima at 450 nm. A stable product with absorption maxima at 560 nm also appears. After keeping the solution in dark for a 1 day, the product absorbing at 560 nm is diminished and absorption

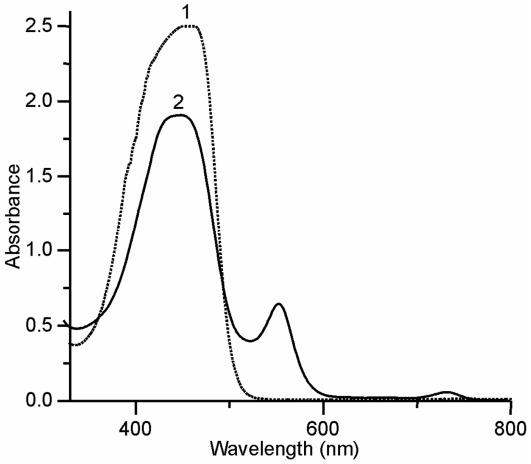


Fig. 2—Absorption curve of bilirubin and its immediate interaction with superoxide radical [Curve 1, bilirubin only; and curve 2, bilirubin + $O_2^{\cdot-}$ immediately]

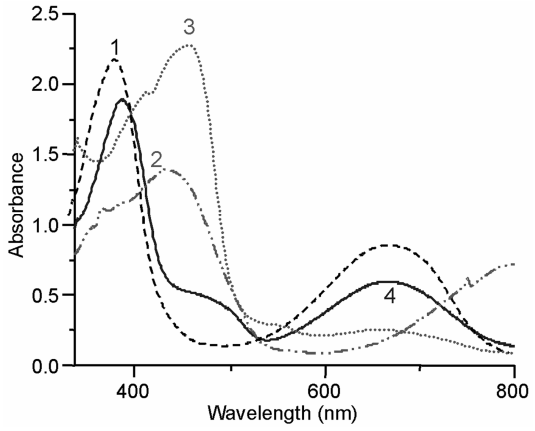


Fig. 3—Absorption spectra of bilirubin and biliverdin interaction with superoxide radical [Curve 1, biliverdin only; curve 2, biliverdin + $O_2^{\cdot-}$ immediately; curve 3, bilirubin + $O_2^{\cdot-}$ after 1 day; and curve 4, biliverdin + $O_2^{\cdot-}$ after 1 day]

around 445 nm is increased (Fig. 3). Along with other products, absorbing in the same region, a part of bilirubin might have been regenerated.

In the reaction of biliverdin with superoxide, it is seen that the characteristic absorption of biliverdin at 675 nm diminishes and simultaneously in the lower wavelength of the absorption spectrum, a red shift with a maximum at around 440 nm is observed. The shifting of absorption maxima of biliverdin indicates the formation of bilirubin after immediate interaction with superoxide radical. After keeping the biliverdin-superoxide solution in dark for a day, the absorption due to biliverdin comes back with some signature of presence of low concentration of bilirubin. These findings suggest the both bilirubin and biliverdin can act as a free radical scavenger *in vitro*. Moreover, their interconversion through free radical pathway might be interesting for future studies.

**Measurement of bilirubin, biliverdin and biliverdin reductase**

Table 1 show that biliverdin concentration is higher in jaundiced neonates with low bilirubin concentration. The case study group includes the neonates having mild jaundice (5 ± 1) mg/dl and with elevated bilirubin level (19 ± 6.2) mg/dl. The low biliverdin concentration in age-matched control neonates can be accounted by their high BVR activity. The ROS generated in the system is reduced by the endogenous bilirubin present. So the activity of the redox cycle of bilirubin-biliverdin conversion is found to be normal in the neonates taken as control, maintaining the normal bilirubin level.

Table 1—Bilirubin and biliverdin concentration in serum samples of neonates along with biliverdin reductase activity

| Subject | Bilirubin (mg/dl) | Biliverdin (mg/dl) | Biliverdin reductase (nmole bilirubin/ min/mg protein) |
|---|---|---|---|
| Controls | 1.5 ± 0.7 (100) | 0.471 ± 0.028 | 8.57 ± 0.283 |
| Patients (low bilirubin) | 5 ± 1 (50) | 2.328 ± 0.181* | 0.875 ± 0.15* |
| Patients (high bilirubin) | 19 ± 6.2 (75) | 0.343 ± 0.027 * | _ |

*$P$< 0.0001; Values in parentheses indicate number of individual

At mild elevated level (5 ± 1 mg/dl), BVR activity is found to be low, compared to the control patients. The ROS generated at mild elevated level possibly lowers the BVR activity and lowers bilirubin production. The biliverdin-bilirubin interconversion is thus interrupted in this group. The bilirubin present may not be sufficient to counteract the total ROS generated at that level and gets transformed to biliverdin, resulting in mild elevation of bilirubin level. When the unconjugated bilirubin level is excessively high (19 ± 6.2 mg/dl), biliverdin concentration is found to be very low and no BVR activity is detected. Thus, the bilirubin is incapable to reduce the ROS generated in the patients having excessively high bilirubin level.

Earlier, we have reported from this laboratory that the ROS generated at high bilirubin level enhances morphological changes, cell lysis, and loss of membrane lipids in RBC etc[5]. The increased ROS formation also diminishes the antioxidative functions of the cell[4]. From the results presented in Table 1, it appears that bilirubin can act as an antioxidant in the neonates having serum bilirubin level of 6 mg/dl, above which it becomes a pro-oxidant.

**Measurement of G6PDH, 6-PGDH and TK**

Table 2 shows that G6PDH activity remains unaltered in jaundiced neonates compared to age-

Table 2—Measurement of activity of glucose 6-phosphate dehydrogenase (G6PDH), 6-phosphogluconate dehydrogenase (6-PGDH), transketolase (TK) of pentose phosphate pathway in RBC of jaundiced neonates against age-matched control

| Subject | Serum bilirubin (mg/dl) | G6PDH (nmole of NADPH produced/min/mg protein) | 6-PGDH (nmole of NADPH produced/min/mg protein) | TK (µmole of NADH oxidized/min/mg protein) |
|---|---|---|---|---|
| Controls | 1.5 ± 0.7 (100) | 5.61 ± 0.05 | 0.412 ± 0.028 | 0.265 ± 0.013 |
| Patients | 19 ± 6.2 (75) | 5.58 ± 0.04 | 0.368 ± 0.06* | 0.213 ± 0.025* |
| Patient (during phototherapy) | 5.5 ± 0.13 (75) | 4.31 ± 0.06* | 0.241 ± 0.14* | 0.119 ± 0.012 * |
| Patient (after phototherapy) | 3.5 ± 0.18 (75) | 4.31 ± 0.05 | 0.241 ± 0.10 | 0.119 ± 0.014 |

*$P$< 0.0001, Values in parentheses indicate number of individual

Table 3—Percentage of DPPH scavenged by ethanolic extract of sweet lime peel

| No. of obs. | Sweet lime peel extract conc. (μg/ml) | DPPH scavenged (%) |
|---|---|---|
| 1 | 50 | 2.2 |
| 2 | 100 | 10.9 |
| 3 | 150 | 30.8 |
| 4 | 200 | 33.5 |
| 5 | 250 | 40.6 |
| 6 | 300 | 41.4 |
| 7 | 350 | 48.9 |
| 8 | 400 | 52.2 |

matched control, suggesting the supply of NADPH remains unaltered before phototherapy. However, activity of 6-PGDH and TK is low in jaundiced neonates. The activity of the G6PDH, 6-PGDH and TK are markedly affected during and after phototherapy. Absorption maxima of bilirubin and riboflavin are nearly identical (i.e. in the range of 445-540 nm). Phototherapy causes photoisomerisation[7] of bilirubin, accompanied by photodegradation of riboflavin. Loss of activity of G6PDH can be related to the indirect effect of reduction of glutathione reductase (GR) activity, an FAD-containing enzyme[4]. The GR recycles NADPH to $NADP^+$, providing the substrate for G6PDH. This recycling is hampered during phototherapy as NADPH cannot be recycled to $NADP^+$, and thus G6PDH activity is reduced.

During phototherapy, the rate of conversion of glucose-6-phosphate to 6-phosphogluconolactone, and then to 6-phosphogluconic acid is reduced. The dearth of metabolites also hampers the activity of 6-PGDH, along with TK. The low activity of TK reflects the thiamin deficiency of the neonates. G6PDH deficient cells are in a greater risk for glycation of protein which, in turn, enhances cellular damage[4,5,26]. Thus, an inverse correlation has been found between the activity of G6PDH and the fragility of RBC. Pentose phosphate pathway along with glutathione and its related enzymes protect RBC against hemolysis[2,4]. Thus, phototherapy affects the enzymes of PPP and increases the susceptibility of RBC to hemolysis, further enhancing oxidative stress in the neonates.

**Measurement of antioxidant potency of the sweet lime peels extract**

The antioxidant activity of alcoholic extract of sweet lime peel has been tested *in vitro* by using DPPH and results given in Table 3 show that it is an effective free radical scavenger. The $IC_{0.20}$ value of the extract is found to 136 μg/ml. The 500 μl of the extract of concentration 136 μg/ml has been administered to the rats.

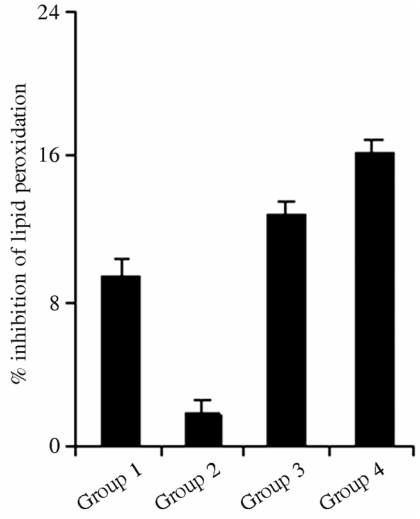


Fig. 4—Percent of LPO inhibition in the RBC membrane of experimental rats by sweet lime peel extract [The extent of LPO was plotted against LPO phenylhydrazine-treated rats. Groups of rats receiving different treatment: Group 1: administered with phenylhydrazine and then treated by phototherapy; Group 2: administered with phenylhydrazine and ethanol supplement; Group 3: administered with phenylhydrazine and crude extract of sweet lime peel; and Group 4: administered with phenylhydrazine and crude extract of sweet lime peel along with phototherapy treatment. $P = 0.004$]

**Measurement of MDA and HNE adducts**

The percent inhibition of LPO (MDA formation) in RBC membrane of the rats receiving treatment is shown in Fig. 4. The group of rats receiving herbal supplement after jaundice induction have shown lower extent of LPO, compared to the rats receiving phototherapy only. The HNE-protein adduct formation in the liver is also found to be lower in the herbal extract-treated rats compared to the untreated rats (photograph of the gel not shown). The herbal extract probably scavenges the ROS generated at the high bilirubin level, reduces its deleterious effect and induces the antioxidant activity of the bilirubin itself.

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