# Evaluation of the Possible Antioxidative Role of Bilirubin Protecting from Free Radical Related Illnesses in Neonates

**Seyed Mohammad Fereshtehnejad[1], Karim Poorsattar Bejeh Mir[2], Arash Poorsattar Bejeh Mir[3], and Parisa Mohagheghi[4]**

[1] *Firoozgar Clinical Research Developmental Center, Tehran University of Medical Sciences, Tehran, Iran*

[2] *Department of Pediatrics, Head and Founder, Division of Neonatology, Amir Mazandarani General Hospital, Sari, Mazandaran, Iran*

[3] *Research Committee, Babol University of Medical Sciences and Health Services, Babol, Iran*

[4] *Department of Pediatrics, Division of Neonatology, Rasoul-Akram Hospital, Tehran University of Medical Sciences, Tehran, Iran*

***Received: 20 Sep. 2011; Received in revised form: 7 Nov. 2011; Accepted: 16 Dec. 2011***

**Abstract-** There are disparate data regarding whether bilirubin is protective or toxic during free radical related illness among neonates. Seventy one infants with gestational age (GA) of <32 weeks and/or birth weight (BW) of <1500 *g*, who survived beyond 4 weeks and completed physical examinations were enrolled in this study. The infants were divided into two groups based on the presence or absence of advanced retinopathy of prematurity (ROP), grade III intraventricular hemorrhage (IVH), grade III necrotizing enterocolitis (NEC), respiratory distress syndrome (RDS), bronchopulmonary dysplasia (BPD), sepsis or severe fungal infection (SFI). The mean of total serum bilirubin (TSB) of the first 14 days of life were measured and compared between these two groups. A significant lower TSB were found in severe form of ROP ($P$<0.001), grade III NEC ($P$=0.008), grade III IVH ($P$=0.021), SFI ($P$=0.003) and sepsis ($P$=0.007) in comparison to mild or disease free status. Moreover, the cut-off point of 5.1 mg/dl for the mean of TSB had the sensitivity of 88.1% and specificity of 84.6% to detect severe grades of ROP. Also the cut-off point of 3.25 mg/dl had 97.2% sensitivity and 100% specificity in order to distinguish SFI. It is concluded that bilirubin may play an antioxidant role *in vivo* as *in vitro*; and protect preterm infant against these free radical related disorders. Our findings suggest that not only the upper limits of serum bilirubin, but also the lower limits must be taking into account in order to both preventing from neurotoxic effects and free radical based illnesses, respectively.



*Acta Medica Iranica*, 2012; 50(3): 153-163.

**Keywords:** Bilirubin; Free radical; Preterm; Antioxidant; Retinopathy of prematurity (ROP)

## Introduction

It is known that during delivery, the newborn infant is exposed to considerably higher tissue concentrations of oxygen than during fetal life. Consequently, more than a fivefold increase in the partial pressure of oxygen occurs in the arteries and airways, when the infant starts breathing. Thus, this transition from relative hypoxia to hyperoxia carries a risk of oxidative injury. However, by means of increasing their antioxidant enzyme activities during hyperoxia, healthy term infants tolerate this sudden hyperoxic challenge (1).

Preterm infants are not merely exposed to relative hyperoxia at birth during the transition from fetal life to air breathing, but higher chance for sepsis or undergoing mechanically ventilation with oxygen supplementation as part of the treatment of respiratory distress syndrome (RDS) possesses additional risks meanwhile, previous studies have shown that premature neonates are unable to augment their antioxidant enzyme activities when exposed to hyperoxia (2,3).

On the other hand, there are some evidences that hyperoxia and oxidative stress are implicated in the pathogenesis of disorders associated with prematurity. According to the hypothesis of neonatal oxygen radical disease, bronchopulmonary dysplasia (BPD), retinopathy of prematurity (ROP), intraventricular hemorrhage (IVH), periventricular leucomalacia (PVL), necrotizing enterocolitis (NEC), and patent ductus arteriosus (PDA) all represent different manifestations

**Corresponding Author:** Arash Poorsattar Bejeh Mir
Dentistry School, Babol University of Medical Sciences and Health Services, Ganj Afrooz Ave, Babol, Mazandaran, Iran
Tel/Fax: +98 151 3216383, E-mail: arashpoorsattar@gmail.com

of the same disease caused by attacks of free radicals in premature neonates (4).

Regarding the considerable related mortality and morbidity of these free radical induced disorders, more attention have been paid through the role of antioxidants agents in preterm neonates since recent years. The possible antioxidant role of bilirubin was firstly shown by some *in vitro* studies more than two decades ago (5-7). Further investigations demonstrated that serum bilirubin protects against serum oxidative damage in the first days of life in neonatal Gunn rats exposed to hyperoxia and proposed serum bilirubin as a potent *in vivo* antioxidant (8). However, human studies have resulted in controversial findings. Despite some investigations showed a direct protective relationship between serum level of bilirubin and the potential antioxidant capacity in term neonates (9), some other studies have introduced elevated bilirubin as a considerable risk factor for free radical related illnesses in preterm neonates specially ROP (10,11). In addition to the different gestational age of evaluated neonates and various illnesses, the measured bilirubin was also another source of these controversial results. As in one the recent studies, it was shown that elevated peak bilirubin of two first weeks of life did not protect from and might be a risk for ROP in very low birth weight (VLBW) infants (12). It was also recommended that the mean serum level of bilirubin could be a better index to be evaluated instead of the peak level (12).

Our aim in this study was to evaluate the role of bilirubin as an antioxidant for probable protection from severe ROP, NEC, IVH, respiratory distress syndrome (RDS), BPD, bacterial and fungal infection in preterm neonates measuring the mean serum level of it.

## Materials and Methods

### Patients

This analytical retrospective longitudinal study was accomplished in the NICU ward of Milad Hospital, Tehran, Iran during June 2006 till May 2007. Medical records of all infants with gestational age (GA) of <32 weeks and/or birth weight (BW) of <1500 g, who survived beyond 4 weeks and completed physical examinations including ophthalmic exams for ROP were recruited. In addition, the infants with incomplete follow up for BPD and ROP, who required exchange transfusion and with G6PD deficiency or other documented hemolytic blood disorders and who died were also excluded. GA was assessed from obstetric ultrasound and confirmed by clinical assessment of infants based on New Ballard Score (NBS). Regarding the presence or absence of advanced or severe ROP, IVH, NEC, RDS, BPD, sepsis or SFI the infants were divided into two groups. This study has been accepted by the ethics committees of Iran University of Medical Sciences and Milad Hospital and all researchers undertook Helsinki's treaty.

### Definitions

Bacterial sepsis was present by either positive blood culture or cerebro-spinal fluid culture for any pathogenic bacteria, severe fungal infection (SFI) as positive blood culture for known fungal species and bronchopulmonary dysplasia (BPD) was defined based upon NIH workshop classification (13). Advanced necrotizing enterocolitis (NEC) was defined as the clinical diagnosis of staff neonatologist with abdominal X-ray consistent with grade III modified Bell's stage (14) and grade III IVH considered as severe form using Papile classification (15). Moreover, we defined advanced ROP or surgical group regarding any location (zone), severity (stage), plus disease, extent and prethreshold or threshold ROP which needs surgical interventions according to the latest AAP guidelines (16).

### Assessments

The first ophthalmologic examination was conducted by expert ophthalmologists at postmenstrual age (PMA) of 31 to 36 weeks by means of indirect fundoscopy. Reports and subsequent examinations were performed at the discretion of ophthalmologist mostly adhere to the International Classifications of Retinopathy of Prematurity (ICROP) and the American Academy of Paediatrics (AAP) guidelines (16,17). Pupil dilation was achieved by twice instilling one drop of phenylephrine 1% and one drop of tropicamide 0.5% within a 5-minute interval. All preterm infants had ultrasound cerebral examinations for IVH at the third and seventh day of life (DOL) and were followed up based on our institutional protocols (18). Severe RDS was our institutional liberal definition for a clinical and radiographic diagnosis of RDS severe enough with at least 3 days of first intubation and mechanical ventilation.

Blood samples were extracted either through umbilical catheters or direct peripheral arteriotomy or venotomy to assess blood stream infections during first 14 days of life. Mean total serum bilirubin (TSB) during first 14 days of life (Pars Azmoon diagnostic kit, Iran) was recorded in all preterm infants with GA of <32 weeks and/or BW of <1500 g. Moreover, other laboratory tests were done if clinically indicated.

**Statistical analysis**

In descriptive analysis, the parameters such as frequency, mean, and standard deviation (SD) were reported. The analytical procedures were performed using statistical tests. To test the differences between parametric variable means in two groups of study, the Independent t-test was used. Receiver operating characteristics (ROC) curve analysis was performed to assess the predictability of either free radical related disorders including advanced ROP, IVH and NEC, RDS, BPD, SFI and sepsis with the mean total serum bilirubin. In addition, the cutoff points were determined in each ROC analysis. The diagnostic values of each cut-off points were calculated including sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV). A One-way ANOVA or Kruskal-Wallis test was used for inter numeric variables comparison, Post-hoc test performed to further analyze and the results were reported with Bonferroni method adjustment where appropriate. In order to evaluate between numeric variables correlation, Pearson or Kendual's tau statistics were used.

Poisson regression analysis was also used to evaluate the predictability of the number of free radical related diseases with the baseline and laboratory variables of the study. A 5 percent probability of a type I error (two-tailed), and a power of 80 percent were considered in the analysis. All reported *P*-values are two-tailed and a *P*-value of <0.05 was considered statistically significant.

## Results

Seventy one neonate infants were enrolled in our study. It was noted that ROP cases were considered the advanced or surgical group (*n*=12) which underwent retinal surgery by either photocoagulation or cryotherapy. Consequently, other group was consisted of non-surgical cases (*n*=59) including the infants without ROP or without ophthalmologic findings strong enough to meet the criteria for retinal surgery.

**Baseline characteristics**

Among a total number of 138 enrolled neonates, 71 were found to be eligible for our assessment. Mean gestational age (± SD) and mean birth weight (± SD) were 30.17 (± 2.47) weeks and 1234 (± 231) g, respectively. There were 12 (16.9%) infants suffered from advanced ROP required surgical intervention, 4 (5.6%) from stage III NEC, 4 (5.6%) from BPD, 2 (2.8%) from SFI, 17 (23.9%) from severe sepsis, 2 (2.8%) from grade III IVH and 21 (29.6%) from severe RDS requiring various degrees of assisted ventilatory support more than three days.

**Total serum bilirubin comparison**

All assumed free radical related illnesses were subjected to be compared splitting severe from mild or disease free situations regarding mean total serum bilirubin (TSB). As shown in table 1, the results of Independent t-test clarified statistically significant lower TSB in severe form of ROP (*P*<0.001), grade III NEC (*P*=0.008), grade III IVH (*P*=0.021), SFI (*P*=0.003) and sepsis (*P*=0.007) in comparison to mild or disease free status. Although the mean of measured TSB in RDS (*P*=0.342) and BPD (*P*=0.236) groups were lower than disease free groups, it didn't reach statistically significant level (Table 2).

**Table 1.** Comparison of mean total serum bilirubin during first 14 days of life between severe versus mild or disease free status

| Disease | Severe | Mild or disease free | *P* -value |
|---|---|---|---|
| | *Mean ± SD* | *Mean±SD* | |
| ROP | 4.24±1.08 | 6.36±1.18 | <0.001 |
| IVH | 3.75±1.76 | 6.06±1.36 | 0.021 |
| NEC | 3.93±1.90 | 6.09±1.33 | 0.008 |
| SFI | 3.10±0.14 | 6.08±1.33 | 0.003 |
| Sepsis | 5.21±1.57 | 6.25±1.26 | 0.007 |
| RDS | 5.69±1.91 | 6.13±1.12 | 0.342 |
| BPD | 4.20±2.55 | 6.08±1.24 | 0.236 |

Retinopathy of prematurity (ROP), severe fungal infection (SFI), necrotizing enterocolitis (NEC), intraventricular haemorrhage (IVH), respiratory distress syndrome (RDS), bronchopumlonary dysplasia (BPD)

**Table 2.** ROC curve analysis of total serum bilirubin to differentiate severe form from mild or disease free status.

| | RDS | BPD | ROP | SFI | Sepsis | NEC | IVH |
|---|---|---|---|---|---|---|---|
| Cut-off point (mg/dl) | | | 5.1 | 3.25 | 6.05 | 3.25 | 5.1 |
| Sensitivity (%) | | | 88.1 | 97.2 | 56.4 | 97.1 | 77.5 |
| Specificity (%) | | | 84.6 | 100 | 77.8 | 66.7 | 100 |
| AUC | 0.554 | 0.755 | 0.922 | 0.978 | 0.678 | 0.818 | 0.908 |
| PPV (%) | | | 61 | 75 | 34 | 75 | 61 |
| NPV (%) | | | 96 | 85 | 100 | 85 | 96 |
| *P*-value | 0.477 | 0.087 | <0.001 | 0.022 | 0.028 | 0.063 | 0.050 |

Retinopathy of prematurity (ROP), severe fungal infection (SFI), necrotizing enterocolitis (NEC), intraventricular haemorrhage (IVH), respiratory distress syndrome (RDS), bronchopumlonary dysplasia (BPD)

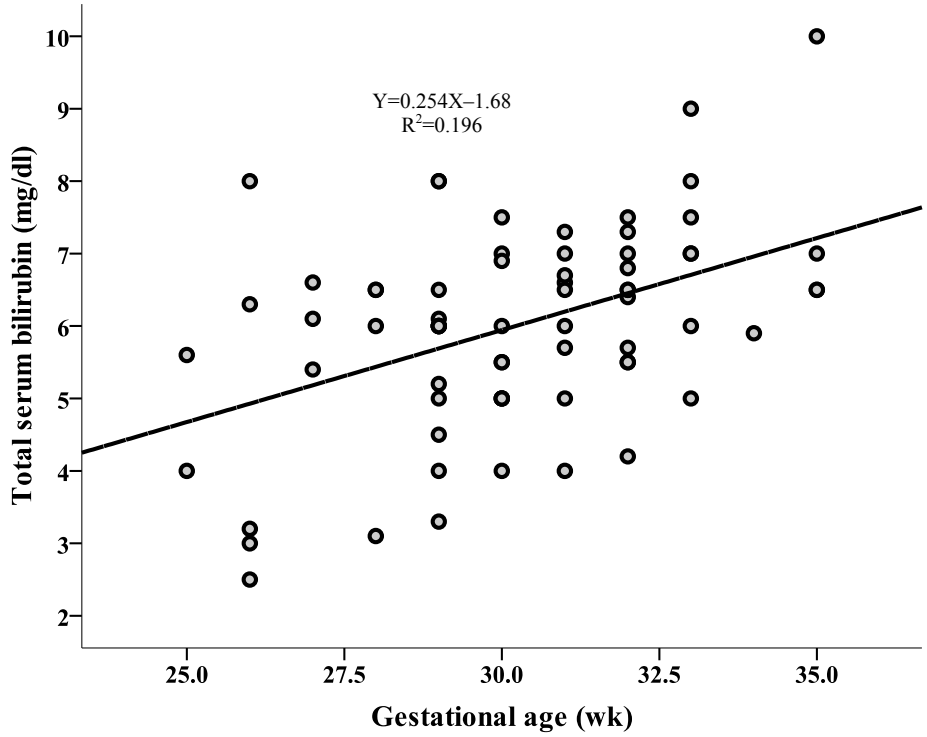


**Figure 1.** Significant direct correlation between mean of TSB and gestational age of the preterm neonates (*P*<0.001)

In regard to the number of free radical related diseases, Kendalls' tau correlation demonstrates a significant reverse association between the mean of TSB and number of diseases (r= -0.275, *P*=0.004). Also, a significant positive correlation was found between gestational age and mean TSB during first 2 weeks of life (Pearson r=0.443, *P*<0.001; Figure 1). Further classification of infants into three groups based on their gestational age (25-27 weeks, 28-32 weeks and ≥33 weeks) revealed significant difference regarding (TSB: F (2,67)=7.13, *P*=0.002). Considerable higher TSB in ≥33 weeks old infants (7.11±1.40) vs. 25-27 weeks (5.07±1.80, *P*=0.001) and 28-32 weeks (5.91±1.15, *P*=0.016) observed. There were no difference considering number of free radical illness diseases between there groups [$X^2$(2)=2.92, *P*=0.232]. Surprisingly, two 26 weeks old newborns were diagnosed with ROP, RDS, BPD, sepsis, NEC and either grade III IVH or SFI.

**ROC curve analysis**

Besides, for each free radical based disease, a ROC curve was graphed, and the level of significance, area under curve (AUC), the best cut-off point and diagnostic values were calculated including specificity (SP), sensitivity (SN), positive predictive value (PPV) and negative predictive value (NPV) (Figure 2 and Table 2).

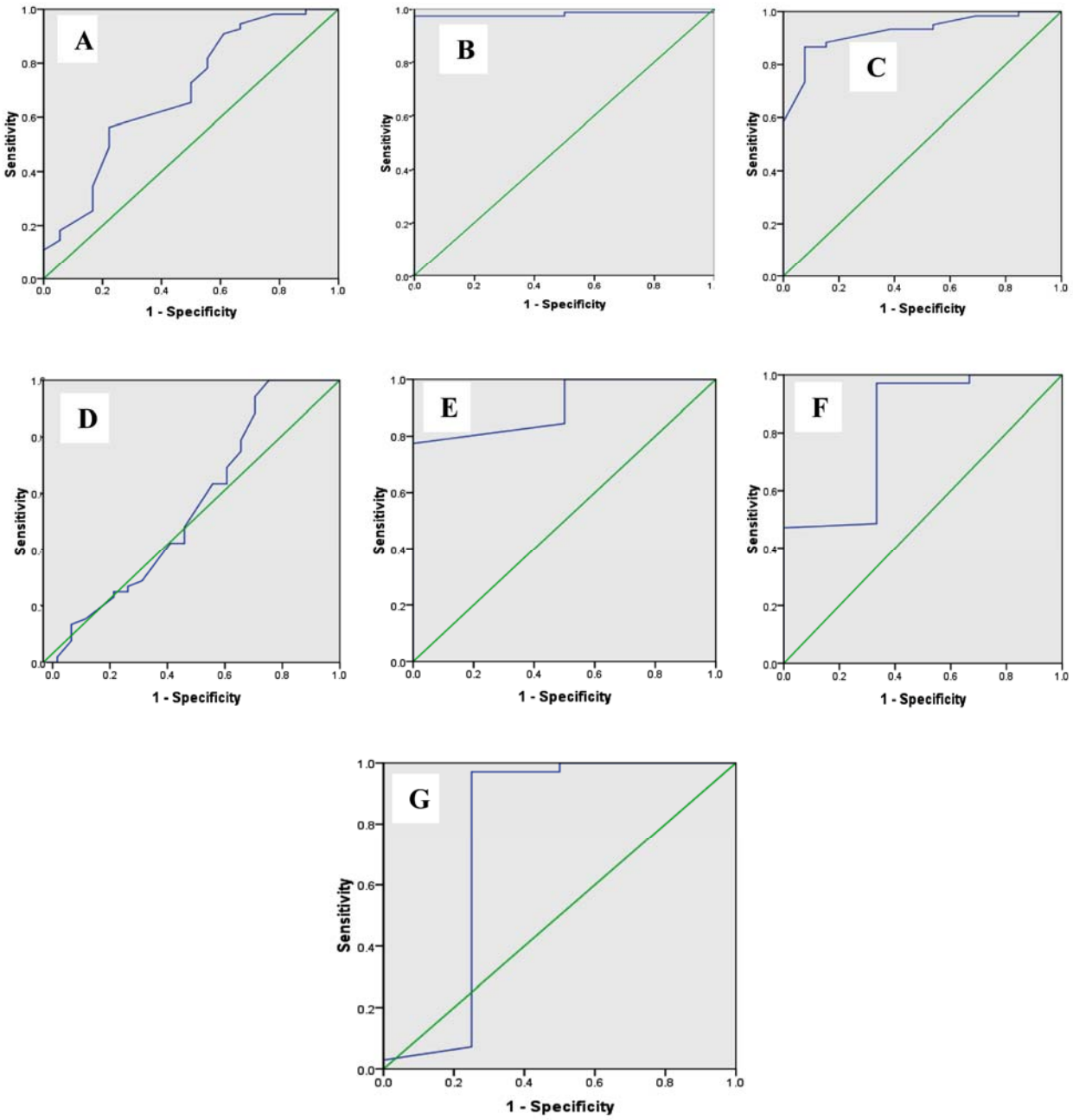


**Figure 2.** Comparison of area under curve (AUC) of bilirubin to differentiate most severe type from mild or disease free status of different diseases derived from ROC curves (A: ROP, B: SFI, C: sepsis, D: NEC, E: IVH, F: RDS, G: BPD)

**Table 3.** Poisson regression analysis of different variables to predict the number of free radical based diseases in preterm neonates ($P$<0.00005)

| Variable | Coefficient | S.E. | 95% CI for Exp (B) | *P*-value |
|---|---|---|---|---|
| Apgar score at 1st minute | -0.157 | 0.075 | (-0.305)-(-0.009) | 0.037* |
| Mean of total serum bilirubin (TSB) | -0.347 | 0.105 | (-0.553)-(-0.141) | 0.001* |
| Constant | 2.591 | 0.486 | (1.638)-(3.544) | <0.0001* |

* Statistically significant

The ROC curve analysis was considerable significant for advanced ROP (AUC=0.922, $P<0.001$), SFI (AUC=0.978, $P=0.022$), sepsis (AUC=0.678, $P=0.028$) and high grade IVH (AUC=0.908, $P=0.050$), while BPD and RDS didn't reach statistically significant level ($P=0.087$ and 0.518, respectively) and advanced NEC displayed a marginal significance (AUC=0.818, $P=0.063$).

As shown in table 3, the cut-off point of 5.1 mg/dl for the mean of TSB had the sensitivity of 88.1% and specificity of 84.6% to detect severe grades of ROP. Also the cut-off point of 3.25 mg/dl had 97.2% sensitivity and 100% specificity in order to distinguish SFI. Other diagnostic values of the cut-off points of mean TSB to detect other free radical based diseases are listed in table 2.

**Poisson regression analysis**

In order to predict the number of free radical related diseases in preterm neonates, Poisson regression analysis was performed. As illustrated in table 3, Apgar score at first minute of life ($P=0.037$) and the mean of TSB ($P=0.001$) were found to be significant predictors in a manner that the lesser the both measurements were the higher number of illnesses were observed ($P<0.0001$).

## Discussion

Comparing the mean of total serum bilirubin (TSB) between the preterm neonates with or without some free radical related illnesses, our findings revealed a significantly lower mean TSB in infants with severe ROP, IVH, NEC, sepsis or SFI during two first weeks of life. In addition, Poisson regression analysis showed that mean TSB is the best variable to predict the number of these free radical based illnesses which would be observed in preterm neonates. It is elucidated that total serum bilirubin during first 2 weeks is correlated with infant GA at which they born. According to our results, it is concluded that bilirubin may play an antioxidant role *in vivo*; and protect preterm infant against these free radical related disorders.

Aged RBCs will degrade and resultant heme would be further fragmented into carbon monoxide (CO), iron and biliverdin by means of exclusive heme oxygenase enzymatic systems. Thereafter, biliverdin may reduce to bilirubin with biliverdin reductase (BVR). Hydrophilic biliverdin undergoes reduction and changes into more toxic hydrophobic bilirubin (18). The rationale behind these bizarre energy consuming processing while noticing the early physiologic rises in serum bilirubin during first few days of life, is been a topic of interest since early 1950 to clarify the gain against the cost (19). Bilirubin would be oxidized to biliverdin by intracellular free radicals. Hence BVR is a key enzyme in cellular antioxidant recycling biliverdin into bilirubin, thus enhancing defense mechanism. Since then, various researches attempted to investigate proposed antioxidative role of bilirubin in many oxidative stress related morbidities such as myocardial infarction, coronary heart disease, amyotrophic lateral sclerosis, atopic dermatitis, cancer and peripheral vascular disease (19). Study of the clinical relevance conducted by Benaron *et al.* in terms of circulatory failure, sepsis, aspiration and asphyxia among neonates revealed significant lower initial rise in serum bilirubin than infants which did not suffer from such oxidative diseases (20).

A few *in vivo* investigations have been previously evaluated the association between serum bilirubin and these three free radical based illnesses in one study. We displayed that except for RDS and BPD, there was a significant lower TSB observed in the infants suffer from severe NEC, IVH, sepsis, SFI compared to infants with the milder form of mentioned disease. Hegyi *et al.* found significant lower levels of bilirubin in preterm infants diagnosed with RDS, IVH, BPD and NEC than those without these oxygen-related illnesses (21). Moreover, as mentioned before, in a study by Milner *et al.* (12) with a similar study population in the year 2003, it was indicated that elevated peak bilirubin levels were not protective against severe ROP. Even it was shown that while controlling for weight and IVH, peak bilirubin was indeed a risk factor for severe ROP. However, Milner *et al.* (12) have further declared that peak bilirubin levels were not associated with increased risk for ROP in the subgroup of infants with prolonged oxygen requirement. As an explanation, they added that sicker infants might be less capable of mounting an effective response to free radical insult by upregulating bilirubin production. Another could be increased consumption of bilirubin in this population. They finally concluded the causal relation between oxygen requirement and ROP may obviate variations in bilirubin levels. One of the major differences between the study of Milner *et al.* (12) and ours is the measured index of serum bilirubin; in a manner that we evaluated the mean TSB instead of peak bilirubin. In fact, mean bilirubin levels over time may better correlate with the amount of antioxidant activity that bilirubin is exerting on a particular infant; while,

peak bilirubin levels may not correlate with such antioxidant activity.

Our study supported the beneficial role of bilirubin against ROP which is in agreement with earlier researches (22,23) while opposed by other reports (12,24-27). Total antioxidant capacity was found to be correlated with bilirubin level notably in term infants and to a lesser degree among preterm infants probably due to their immature immune system (9), nevertheless, Hammermann *et al*. showed that total antioxidant activity (TAA) is correlated to serum bilirubin level in preterm infants although not correlated to GA (28). We demonstrated that higher bilirubin level is maintained in first 2 weeks by infants born with higher GA; lacked simultaneous TAA measure, although first day bilirubin and elevation is not considered in this study and may be considered as other limitations of the study.

Other previous studies on the relationship between serum bilirubin and ROP have mostly focused on the population of term infants, adults and animals. In a large study of term infants, neonates who encountered adverse peripartum events had their initial bilirubin levels significantly lower than those who did not (20). Similarly, in another study by Belanger *et al.* (29), the protective antioxidant role of bilirubin was demonstrated in term infants. Moreover, experimental investigations in neonatal Gunn rats exposed to hyperoxia demonstrated that serum bilirubin protects against serum oxidative damage in the first days of life (8).

Other than the study of Milner *et al.* (12), other investigations in premature neonates have been led to various and somehow controversial findings which mostly showed non-protective to slightly antioxidant role of serum bilirubin that was not as strong as its role in term infants or adolescents (10,11,24,30). In contrast, an approximately strong protective role of mean TSB in some free radical related disorders' including ROP was detected in preterm infants.

Regarding the literature review, most studies on the probable antioxidant role of serum bilirubin in free radical based illnesses of neonates have evaluated ROP more than other disorders.

Similar to ROP, our findings show that the mean TSB of preterm neonates are also significantly lower in infants with IVH or NEC in comparison with other groups of no free radical illnesses. In a recent study of 388 newborn infants by Lee *et al.* in 2009 (31), a lower total bilirubin levels was detected in neonates with IVH, identical to our results.

Performing an experimental study on rat intestine, Hammerman *et al.* (32) concluded that hyperbilirubinemia ameliorates the extent of intestinal ischemia-reperfusion injury in animal model (similar to NEC) and appears to act as an antioxidant. This study supports the concept that bilirubin possesses some beneficial antioxidant properties *in vivo*. Interestingly, they observed less histopathologic and biochemical evidence of damage in the intestinal mucosa of hyperbilirubinemic animals. Moreover, like our study, the measured index of bilirubin concentration was the mean TSB in their investigation (32). Also in a human study on extremely low birth weight infants by Oh *et al.* (33), it was shown that NEC was not significant risk factors for neurodevelopmental impairment (NDI); although peak serum bilirubin concentrations during the first 2 weeks of life were directly correlated with death or NDI.

Sepsis and severe fungal infection (SFI) were also the other free radical based conditions which were evaluated in our study. Similar to the others, mean TSB was also significantly lower in preterm infants with severe sepsis or SFI. There are some previous data showing that bile pigment is consumed *in vivo* as an antioxidant in septic infants (20). Furthermore, by an experimental study Wang *et al.* (34) revealed that a single intravenous bolus injection of bilirubin protects against mortality and liver dysfunction induced by *E. coli* endotoxin administration in rats. More recently, in another experimental study by Lanone *et al.* (35), it was shown that a sustained increased level of bilirubin reduces the mortality induced by endotoxin inoculation in rats. Interestingly, increased concentrations of bilirubin oxidative metabolites were also shown in the urine of septic adult patients, suggesting the consuming of bilirubin during the oxidative stress in septic condition (36) which confirms the results of previous animal study (37).

Although the mean of TSB were not significantly different in neonates with and without two other diseases including severe RDS and BPD; however, the lower level of mean bilirubin was also detected in neonates with severe RDS and BPD and the calculated power was achieved as 47.2% and 97.6%, respectively. This shows that a significantly lower mean TSB in neonates with RDS might probably be shown with a higher sample size.

Despite the controversial findings of different studies in this subject, it is now accepted that bilirubin is one of the body's natural antioxidants, contributing up to 10% to 30% of the total antioxidant capacity of premature infants (28,38).

Bilirubin is one the extracellular component contains preventive antioxidants (39) which also can act as preventive antioxidants in plasma as a chain-breaking one (28,40). During the first postnatal days, when general antioxidant defenses are reduced, serum bilirubin is physiologically increased, implying some beneficial role for this physiological hyperbilirubinemia (28) which has been proposed to protect against oxidative injury. Therefore, it is still well accepted that bilirubin is a potent *in vitro* and *in vivo* antioxidant (41,42) and it exerts this properties against lipophilic reactive oxygen species cooperatively with glutathione (GSH) which defend against water soluble oxidants collectively constitute major intracellular antioxidant system (43), although disapproved by one recent study (44). Besides previously mentioned reductase activity of BVR, recently a new feature of this protein evolved in cell growth and apoptosis control and probably in pathogenesis of diabetes and cancer proposed (45). Thus, the complex of BVR, biliverdin as the substrate and bilirubin as the product may be re-testified according its various mentioned capacities. Weather oxidative stresses consume (8,28) antioxidants or induce (10) them is still matter of debate.

Furthermore, bilirubin could be consumed by oxidative stresses that occur early in life and its production may also be upregulated in response to free radicals (46). As a result, the balance between consumption and upregulation may yield some conflicting data. This may explain why some studies have found peak bilirubin to be associated with increased risk of severe ROP. There are also some evidences which propose that the protective effect of bilirubin could be related to the decrease in expression and activity of the inducible NO synthase (NOS2) (34,35). This relationship between the increase in bilirubin levels and the attenuation of the NO pathway is also supported by the strong negative correlation found between plasmatic bilirubin and nitrite/nitrate levels which are a consequence of the potential scavenging activities of bilirubin toward reactive nitrogen species (35).

On one hand, bilirubin is toxic to neurons at high concentrations; on the other hand, it has proven to be neuroprotective against oxidative injury at nanomolar concentrations (47). In the other words, although very high levels of serum bilirubin are known to be toxic, there is uncertainty about the risks and benefits of moderate serum bilirubin values such as physiological hyperbilirubinemia and of the use of phototherapy to reduce the bilirubin values especially in preterm infants.

We support that parallel to physiological bilirubin elevation during early neonatal period probably to compensate for impaired immunity especially among premature infants, free radical related illness may further attenuates antioxidant system by utilizing bilirubin. Our findings adds support to the concept that bilirubin may possess some beneficial as well as toxic properties. Until now most studies tried to determine the appropriate threshold to commence therapy for reducing the level of serum bilirubin; therefore, they have focused on the upper limits of this concentration. Whereas, we have aimed to assess and introduce the lower limits for blood circulating level of billirubin mostly attached to hemoglobin. The founded association between decreased mean level of TSB and the increased incidence of some free radical based illnesses such as ROP, IVH and NEC, RDS, BPD, sepsis and SFI proposed that bilirubin may play an antioxidant role *in vivo* as *in vitro* even in preterm infants. We found out that the mean TSB of more than 5.1 significantly affect the prevalence of ROP, and so our recommendation is to stop lowering TSB whenever reach this cutoff point.

Our study had some limitations including relative small sample size and retrospective designing. Most human studies of bilirubinemia and free radical associated diseases have been designed as retrospective and observational investigations. Therefore, as clinical diseases are multifactorial and potential risk factors cannot be controlled for in retrospective studies, results remain contradictory and inconclusive. Thus, there is a need to perform more prospective studies controlling for the confounders, too. More researches to direct comparison of total and specific antioxidant capacity between preterm and term infants in terms of serum level of antioxidants, fluctuation and detailed total and indirect bilirubin level in early life is still needed. Moreover, precise elucidation of intracellular GSH enhancement with safe and available N-acetylcysteine in aspects of molecular and clinical improvement during free radical illnesses course may be both helpful and interesting. There are controversies about photo oxidation and DNA damage after phototherapy in newborns and whether it is safe or not (48,49). It is postulated that higher bilirubin concentration is correlated with higher antioxidant capacity (29,50). Shahab *et al.* in 2008 (51) described a concentration dependent pattern of antioxidative properties of bilirubin that rationalized earlier study by Dany *et al.* in 2003 (52). They concluded that antioxidant role of bilirubin at higher plasma levels may hampered plausibly by concomitant release of oxidant via heme oxygenase

complex activity. In addition, bilirubin as an antioxidant may attenuate bactericidal activity of neutrophils which is hazardous especially in septic infants (53).

However, our findings suggest that not only the upper limits of serum bilirubin, but also the lower limits must be taking into account in order to both preventing from neurotoxic effects and free radical based illnesses, respectively. According to our study, it is recommended that more judicious lowering of bilirubin in preterm and very low birth weight infants may protect them from free radical based disorders. It seems that continuous transcotaneous checking of end-tidal $CO_2$ concentration may be useful to monitor serum bilirubin more often. Moreover, it is suggested to evaluate the effects of other antioxidant such as vitamins C and E and N-acetylcysteine in preventing the free-radical related diseases of preterm neonates.

## References


1. Frank L. Developmental aspects of experimental pulmonary oxygen toxicity. Free Radic Biol Med 1991;11(5):463-94.
2. Frank L, Sosenko IR. Failure of premature rabbits to increase antioxidant enzymes during hyperoxic exposure: increased susceptibility to pulmonary oxygen toxicity compared with term rabbits. Pediatr Res 1991;29(3):292-6.
3. Morton RL, Das KC, Guo XL, Iklé DN, White CW. Effect of oxygen on lung superoxide dismutase activities in premature baboons with bronchopulmonary dysplasia. Am J Physiol 1999;276(1 Pt 1):L64-74.
4. Saugstadt OD. Oxygen radical disease in neonatology. Semin. Neonatol 1998;3:239-44.
5. Stocker R, Yamamoto Y, McDonagh AF, Glazer AN, Ames BN. Bilirubin is an antioxidant of possible physiological importance. Science 1987;235(4792):1043-6.
6. Stocker R, Ames BN. Potential role of conjugated bilirubin and copper in the metabolism of lipid peroxides in bile. Proc Natl Acad Sci U S A 1987;84(22):8130-4.
7. Stocker R, Glazer AN, Ames BN. Antioxidant activity of albumin-bound bilirubin. Proc Natl Acad Sci U S A 1987;84(16):5918-22.
8. Dennery PA, McDonagh AF, Spitz DR, Rodgers PA, Stevenson DK. Hyperbilirubinemia results in reduced oxidative injury in neonatal Gunn rats exposed to hyperoxia. Free Radic Biol Med 1995;19(4):395-404.
9. Gopinathan V, Miller NJ, Milner AD, Rice-Evans CA. Bilirubin and ascorbate antioxidant activity in neonatal plasma. FEBS Lett 1994;349(2):197-200.
10. Romeo MG, Tina LG, Scuderi A, Di Pietro M, Caracciolo M, Distefano G. Variations of blood bilirubin levels in the newborn with and without retinopathy of prematurity (ROP). Pediatr Med Chir 1994;16(1):59-62.
11. Teng RJ, Wu TJ, Yau KI. Retinopathy of prematurity in very-low-birthweight neonates: epidemiology and risk factors. Zhonghua Min Guo Xiao Er Ke Yi Xue Hui Za Zhi 1997;38(5):370-4.
12. Milner JD, Aly HZ, Ward LB, El-Mohandes A. Does elevated peak bilirubin protect from retinopathy of prematurity in very low birthweight infants. J Perinatol 2003;23(3):208-11.
13. Jobe AH, Bancalari E. Bronchopulmonary dysplasia. Am J Respir Crit Care Med 2001;163(7):1723-9.
14. Walsh MC, Kliegman RM. Necrotizing enterocolitis: treatment based on staging criteria. Pediatr Clin North Am 1986;33(1):179-201.
15. Papile LA, Burstein J, Burstein R, Koffler H. Incidence and evolution of subependymal and intraventricular hemorrhage: a study of infants with birth weights less than 1,500 gm. J Pediatr 1978;92(4):529-34.
16. Section on Ophthalmology American Academy of Pediatrics; American Academy of Ophthalmology; American Association for Pediatric Ophthalmology and Strabismus. Screening examination of premature infants for retinopathy of prematurity. Pediatrics 2006;117(2):572-6.
17. International Committee for the Classification of Retinopathy of Prematurity. The International Classification of Retinopathy of Prematurity revisited. Arch Ophthalmol 2005;123(7):991-9.
18. Maines MD. The heme oxygenase system: a regulator of second messenger gases. Annu Rev Pharmacol Toxicol 1997;37:517-54.
19. Sedlak TW, Snyder SH. Bilirubin benefits: cellular protection by a biliverdin reductase antioxidant cycle. Pediatrics 2004;113(6):1776-82.
20. Benaron DA, Bowen FW. Variation of initial serum bilirubin rise in newborn infants with type of illness. Lancet 1991;338(8759):78-81.
21. Hegyi T, Goldie E, Hiatt M. The protective role of bilirubin in oxygen-radical diseases of the preterm infant. J Perinatol 1994;14(4):296-300.
22. Yeo KL, Perlman M, Hao Y, Mullaney P. Outcomes of extremely premature infants related to their peak serum bilirubin concentrations and exposure to phototherapy. Pediatrics 1998;102(6):1426-31.
23. Boynton BR, Boynton CA. Retinopathy of prematurity and bilirubin. N Engl J Med 1989;321(3):193-4.

24. Gaton DD, Gold J, Axer-Siegel R, Wielunsky E, Naor N, Nissenkorn I. Evaluation of bilirubin as possible protective factor in the prevention of retinopathy of prematurity. Br J Ophthalmol 1991;75:532-4.
25. Fauchère JC, Meier-Gibbons FE, Koerner F, Bossi E. Retinopathy of prematurity and bilirubin--no clinical evidence for a beneficial role of bilirubin as a physiological anti-oxidant. Eur J Pediat 1994;153(5):358-62.
26. Hosono S, Ohno T, Kimoto H, Shimizu M, Nozawa M, Genkawa R, Yoshida T, Wada S, Harada K. No clinical correlation between bilirubin levels and severity of retinopathy of prematurity. J Pediatr Ophthalmol Strabismus 2002;39(3):151-6.
27. DeJonge MH, Khuntia A, Maisels MJ, Bandagi A. Bilirubin levels and severe retinopathy of prematurity in infants with estimated gestational ages of 23 to 26 weeks. J Pediatr 1999;135(1):102-4.
28. Hammerman C, Goldstein R, Kaplan M, Eran M, Goldschmidt D, Eidelman AI, Gartner LM. Bilirubin in the premature: toxic waste or natural defense? Clin Chem 1998;44(12):2551-3.
29. Bélanger S, Lavoie JC, Chessex P. Influence of bilirubin on the antioxidant capacity of plasma in newborn infants. Biol Neonate 1997;71(4):233-8.
30. Wayner DD, Burton GW, Ingold KU, Barclay LR, Locke SJ. The relative contributions of vitamin E, urate, ascorbate and proteins to the total peroxyl radical-trapping antioxidant activity of human blood plasma. Biochim Biophys Acta 1987;924(3):408-19.
31. Lee YK, Daito Y, Katayama Y, Minami H, Negishi H. The significance of measurement of serum unbound bilirubin concentrations in high-risk infants. Pediatr Int 2009;51(6):795-9.
32. Hammerman C, Goldschmidt D, Caplan MS, Kaplan M, Bromiker R, Eidelman AI, Gartner LM, Hochman A. Protective effect of bilirubin in ischemia-reperfusion injury in the rat intestine. J Pediatr Gastroenterol Nutr 2002;35(3):344-9.
33. Oh W, Tyson JE, Fanaroff AA, Vohr BR, Perritt R, Stoll BJ, Ehrenkranz RA, Carlo WA, Shankaran S, Poole K, Wright LL; National Institute of Child Health and Human Development Neonatal Research Network. Association between peak serum bilirubin and neurodevelopmental outcomes in extremely low birth weight infants. Pediatrics 2003;112(4):773-9.
34. Wang WW, Smith DL, Zucker SD. Bilirubin inhibits iNOS expression and NO production in response to endotoxin in rats. Hepatology 2004;40(2):424-33.
35. Lanone S, Bloc S, Foresti R, Almolki A, Taillé C, Callebert J, Conti M, Goven D, Aubier M, Dureuil B, El-Benna J, Motterlini R, Boczkowski J. Bilirubin decreases nos2 expression via inhibition of NAD(P)H oxidase: implications for protection against endotoxic shock in rats. FASEB J 2005;19(13):1890-2.
36. Otani K, Shimizu S, Chijiiwa K, Yamaguchi K, Kuroki S, Tanaka M. Increased urinary excretion of bilirubin oxidative metabolites in septic patients: a new marker for oxidative stress in vivo. J Surg Res 2001;96(1):44-9.
37. Yamaguchi T, Horio F, Hashizume T, Tanaka M, Ikeda S, Kakinuma A, Nakajima H. Bilirubin is oxidized in rats treated with endotoxin and acts as a physiological antioxidant synergistically with ascorbic acid in vivo. Biochem Biophys Res Commun 1995;214(1):11-9.
38. Frei B, Stocker R, Ames BN. Antioxidant defenses and lipid peroxidation in human blood plasma. Proc Natl Acad Sci USA 1988;85(24):9748-52.
39. Berger HM, Molicki JS, Moison RM, Van Zoeren-. Grobben D. Extracellular defence against oxidative stress in the newborn. Semin Neonatol 1998;3:183-90.
40. Lindeman JH, Lentjes EG, Berger HM. Diminished protection against copper-induced lipid peroxidation by cord blood plasma of preterm and term infants. JPEN J Parenter Enteral Nutr 1995;19(5):373-5.
41. Shukla SP, Sarkar A, Adhikari S, Joshi R, Ghanty TK, Mukherjee T. Experimental results and theoretical validation for the antioxidant mechanism of bilirubin. J Indian Chem Soc 2010;87:139-46.
42. Neimark E, LeLeiko NS. Antioxidant effect of bilirubin and pediatric nonalcoholic fatty liver disease. Pediatrics 2009;124(6):e1240-1.
43. Sedlak TW, Saleh M, Higginson DS, Paul BD, Juluri KR, Snyder SH. Bilirubin and glutathione have complementary antioxidant and cytoprotective roles. Proc Natl Acad Sci U S A 2009;106(13):5171-6.
44. Maghzal GJ, Leck MC, Collinson E, Li C, Stocker R. Limited role for the bilirubin-biliverdin redox amplification cycle in the cellular antioxidant protection by biliverdin reductase. J Biol Chem 2009;284(43):29251-9.
45. Florczyk UM, Jozkowicz A, Dulak J. Biliverdin reductase: new features of an old enzyme and its potential therapeutic significance. Pharmacol Rep 2008;60(1):38-48.
46. Dennery PA, Rodgers PA, Lum MA, Jennings BC, Shokoohi V. Hyperoxic regulation of lung heme oxygenase in neonatal rats. Pediatr Res 1996;40(6):815-21.
47. Doré S, Takahashi M, Ferris CD, Zakhary R, Hester LD, Guastella D, Snyder SH. Bilirubin, formed by activation of heme oxygenase-2, protects neurons against oxidative stress injury. Proc Natl Acad Sci U S A 1999;96(5):2445-50.

48. Aycicek A, Erel O. Total oxidant/antioxidant status in jaundiced newborns before and after phototherapy. J Pediatr (Rio J) 2007;83(4):319-22.
49. Aycicek A, Kocyigit A, Erel O, Senturk H. Phototherapy causes DNA damage in peripheral mononuclear leukocytes in term infants. J Pediatr (Rio J) 2008;84(2):141-6.
50. Wiedemann M, Kontush A, Finckh B, Hellwege HH, Kohlschütter A. Neonatal blood plasma is less susceptible to oxidation than adult plasma owing to its higher content of bilirubin and lower content of oxidizable Fatty acids. Pediatr Res 2003;53(5):843-9.
51. Shekeeb Shahab M, Kumar P, Sharma N, Narang A, Prasad R. Evaluation of oxidant and antioxidant status in term neonates: a plausible protective role of bilirubin. Mol Cell Biochem 2008;317(1-2):51-9.
52. Dani C, Martelli E, Bertini G, Pezzati M, Filippi L, Rossetti M, Rizzuti G, Rubaltelli FF. Plasma bilirubin level and oxidative stress in preterm infants. Arch Dis Child Fetal Neonatal Ed 2003;88(2):F119-23.
53. Arai T, Yoshikai Y, Kamiya J, Nagino M, Uesaka K, Yuasa N, Oda K, Sano T, Nimura Y. Bilirubin impairs bactericidal activity of neutrophils through an antioxidant mechanism in vitro. J Surg Res 2001;96(1):107-13.