Oxidative stress and antioxidant status in neonatal hyperbilirubinemia

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ABSTRACT

الأهداف : فحص العلاقات المحتملة بين مستويات البيلروبين البلازما، تركيزات لأكسيد النتريك (NO)، مالونديالدهيد (MDA)، وأنشطة أنزيم مضادات الأكسدة إريثروسايت بين الأطفال الرضع المصابين بفرط البيلروبين في الدم .

الطريقة : تمت معالجة 30 مولود (عمر الحمل 37≥ أسبوعاً) وتقل أعمارهم عن 10 أيام ومصابين بفرط البيلروبين في الدم، وتم ذلك في جامعة كهرمانماراس سوتوك امام – وحدة الأطفال حديثي الولادة – كهرمانماراس – تركيا، خلال الفترة ما بين يناير 2007م وحتى يوليو 2007م. تم اختيار 30 طفلا سليما بشكل عشوائي والذين لديهم نفس العمر ولا يعانون من أي يرقان سريري كمجموعة تحكم. تم قياس مستويات إرتيهاروسايت كاتالاس (CAT)، أكسيد ديسموتاس (SOD)، جلوتاثيون فوق التأكسد (GPx)، جلوكوز ديهايدروجينيس فوسفات–6 (G6PD) والبلازما (MDA) وأكسيد النتريك (NO) .

النتائج : كانت كلتا تركيزات (MDA) و (NO) أعلى في مجموعة الدراسة أكثر من مجموعة التحكم (p=0.000) . تبين أن الأنشطة الفعلية لأنزيمات مضادات الأكسدة إريثروسايت أقل في مجموعة الدراسة مقارنة مع مجموعة التحكم (p=0.000)، إضافة إلى ذلك، أظهر البيلروبين البلازما صلة سالبة ملحوظة مع أنشطة إنزيم مضادات الأكسدة ولكن علاقة إيجابية مع (MDA) و (NO) .

خاتمة : في هذه العينة، تبين وجود ارتفاع في جهد التأكسد لدى الأطفال الذين يعانون من وجود ارتفاع ملحوظ في البيلروبين الدم ونشاط مضطرب لأنزيم مضاد التأكسد . مثل هؤلاء المرضى ينبغي متابعة حالتهم وإخضاعهم للعلاج لمنع الآثار الضارة لارتفاع البيلروبين في الدم . هنالك حاجة لإجراء المزيد من الدراسات لفحص الفائدة الممكنة من مضادات الأكسدة في زيادة البيلروبين في الدم .

Objective: To investigate the possible relationships between plasma bilirubin levels and concentrations of nitric oxide (NO), malondialdehyde (MDA), and erythrocyte antioxidant enzyme activities in newborn infants with hyperbilirubinemia.

Methods: Thirty term (gestational age $\geq$ 37 weeks) newborn infants with indirect hyperbilirubinemia aged less than 10 days were prospectively recruited in the Kahramanmaras Sutcu Imam University Neonatal Unit, Kahramanmaras, Turkey, between January and July 2007. Thirty randomly selected healthy newborns who had similar age and without clinical jaundice comprised the control group. Erythrocyte catalase, superoxide dismutase, glutathione peroxidase (GPx), glucose-6-phosphate dehydrogenase, and plasma MDA and NO concentrations were measured.

Results: Both MDA and NO concentrations were higher in the study group than the controls (p=0.000). The mean activities of erythrocyte antioxidant enzymes were found to be lower in the study group compared with the controls (p=0.000). Furthermore, plasma bilirubin showed significant negative correlations with antioxidant enzyme activities but positive correlations with MDA and NO.

Conclusions: In this sample, infants with significant hyperbilirubinemia had elevated oxidative stress and disturbed antioxidant enzyme activity. Since these states have been shown to cause cellular injury in neonatal patients with indirect hyperbilirubinemia, such patients should be followed-up and undergo therapy to prevent the harmful effects of hyperbilirubinemia. Further studies are needed to investigate possible benefits of antioxidants in hyperbilirubinemia.

Increased reactive oxygen species (ROS) and its final products malondialdehyde (MDA) and nitric oxide (NO) are responsible from the most important oxidative stress related neonatal diseases including asphyxia, respiratory distress, sepsis, and bronchopulmonary dysplasia.[1-3] However, there is a scavenger anti-oxidant barrier against its effects. The known anti-oxidant barrier consists of major intracellular antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glucose-6-phosphate dehydrogenase (G6PD).[4-7] However, this barrier is not fully developed in newborns. Indirect bilirubin is an important part of the scavenging barrier. It can be hypothesized that this immature oxidant/antioxidant balance is influenced by the plasma indirect bilirubin concentration. Low levels of bilirubin may be able to scavenge single oxygen with high efficiency, to react with superoxide anions and peroxyl radicals, and serve as a reducing substrate for peroxidases.[8,9] However, neonatal hyperbilirubinemia, with very high levels of bilirubin, may be a life-threatening condition when leading to encephalopathy and kernicterus.[10-12] The mechanisms of encephalopathy development is not yet clear. The relationships between bilirubin and oxidant/antioxidant status in vitro, in animal studies and in pre-term infants have been studied previously, however, its role in term infants has not been clarified.[13-15] Therefore, our aim was to investigate the possible correlation between bilirubin levels and oxidative stress markers including plasma NO, MDA, and the activities of erythrocyte antioxidant enzymes (SOD, CAT, GPx, and G6PD) in the hyperbilirubinemic term babies.

Methods. Forty-four term newborn babies who were admitted to the neonatal unit during January 2007 to July 2007 for indirect hyperbilirubinemia were consecutively recruited in the Kahramanmaras Sutcu Imam University Neonatal Unit, Kahramanmaras, Turkey. Fourteen patients who were older than 10 days, less than 37 weeks of gestational age, not breast fed by own mother, who suffered from infections, required respiratory support, suffered from perinatal asphyxia, were clinically non-stable, and having congenital malformations or hemolysis were excluded. The control group consisted of 30 randomly selected healthy newborns from babies in the out-patient clinic with no apparent jaundice. They had similar age features and in hospital for routine control, because all neonates between the first 3-10 days are invited to return to our hospital for Guthrie test control. The same exclusion criteria were applied to the control group. At the beginning of the study, 36 healthy newborns were included as control subjects. During data collection, 6 control subjects were excluded due to acquired infections, deterioration in clinical stability or detection of hemolysis findings, therefore 30 non-jaundiced healthy newborns were included for the final analysis. The study protocol was approved by the University Hospital local ethics committee, and parents of all participating children gave written consent to their child's participation in the study after it had been explained to them verbally and in writing.

For laboratory investigations, 5 ml blood samples were obtained from a forearm vein. The hyperbilirubinemic patients' blood samples were obtained before the therapy and control group's were taken after control examination. The blood samples were divided for complete blood count, C-reactive protein (CRP), biochemical tests, and oxidant/antioxidant studies. The samples for oxidant/antioxidant studies were collected into tubes containing potassium ethylenediaminetetraacetate (EDTA). They were centrifuged at 1000 grams for 10 minutes at 4°C to remove plasma. After plasma remove, the buffy coat on the erythrocyte sediment was separated, and they were washed 3 times with 0.9% sodium chloride solution to remove the plasma remnant. After each procedure, erythrocyte-saline mixture was centrifuged at 1000 gram for 10 minutes at 4°C. The hemolysates were prepared from the washed cells to measure the parameters of biochemical workup. Serum bilirubin level was measured on a Dade Behring RXL, analyzer using the method of Jendrassic, Milano, Italy and results were expressed as mg/dL.[16]

Testing methods. Erythrocyte CAT activity was determined by measuring the decrease in hydrogen peroxide concentration at 230 nm by the method of Beutler, New York, USA.[17] Assay medium consisted of 1-methylimidazole (M) Tris hydrogen chloride (HCl), 5 mM Na_2EDTA buffer solution (pH 8), one M phosphate buffer solution (pH: 7), and 10 mM hydrogen peroxide. The results of CAT enzyme were expressed as U/g hemoglobin (Hb). Erythrocyte SOD activity was measured according to the method described by Fridovich.[18] This method employs xanthine and xanthine oxidase to generate superoxide radicals, which react with p-iodonitrotetrazolium violet (INT) to form a red formazan dye, which was measured at 505 nm. Assay medium consisted of the 0.01 M phosphate buffer, 3-cyclohexilamino-1-propanesulfonicacid (CAPS) buffer solution (50 mM CAPS, 0.94 mM EDTA, saturated sodium hydroxide) with pH 10.2, solution of substrate (0.05 mM xanthine, 0.025 mM INT), and 80 U/L xanthine oxidase. Superoxide dismutase activity was expressed as U/g Hb. Glutathione peroxidase in blood samples were assayed in a 1-mL system containing potassium phosphate buffer (0.1 M, pH: 7.0), nicotinamide adenine dinucleotide phosphate-oxidase (NADPH)

(0.2 mM), glutathione reductase (1 U), glutathione (4 mM), sodium azide (4 mM), and an appropriate amount of enzyme GPx (0.02 mL). The reaction mixture was incubated at 37°C for 10 minutes after which 10 mL of 10 mM t-butyl hydroperoxide were added to start the reaction. No t-butyl hydroperoxide was added to the blank cuvette. The rate of reaction was measured at 37°C by following the decrease in the absorbance at 340 nm using a spectrophotometer. Activity was expressed as U/g Hb in blood samples.[17] Erythrocyte G6PD activity was determined at 37°C according to Beutler.[17] The reaction mixture contained one M Tris-HCI pH 8, 6 mM G6PD, 2 mM NADPH, 0.1 M magnesium chloride, and hemolysate in total volume of 3 mL. One unit of enzyme activity is the amount catalyzed by the reduction of 1 mM of NADPH per minute. Results were expressed as U/g Hb. Plasma NO level was measured following reduction of nitrate and nitrite using Greiss method at 540 nm spectrophotometrically.[19] The results of plasma total NO levels were expressed as mmol/L. Plasma MDA was measured according to procedure of Ohkawa et al.[20] The reaction mixture contained 0.1 mL sample, 0.2 mL of 8.1% sodium dodecyl sulphate, 1.5 mL of 20% acetic acid, and 1.5 mL of 0.8% aqueous solution of thiobarbituric acid. The mixture pH was adjusted to 3.5 and the volume was finally made up to 4 mL with distilled water and 5 mL of the mixture of n-butanol and pyridine (15:1, v/v) were added. The mixture was shaken vigorously. After centrifugation at 4000 rpm for 10 minutes, the absorbance of the organic layer was measured at 532 nm. Results were expressed as nmol/mL. The Hb level was measured with Spectronic-UV120 spectrophotometer by the method of cyanomethemoglobin.[14]

Data analysis. Nonparametric statistical methods were applied to continuous variables. Categorical data were compared by the Chi-square test. Mann-Whitney U test were used for between-groups comparisons. The Spearman rank correlation coefficient was used to evaluate the degree of correlation between the parameters. The null hypothesis was rejected for values of $p<0.05$. Analyses were performed by using Statistical Package for Social Science (SPSS Inc., Chicago, IL) software, version 11.0 for Windows.

Results. No significant differences were found in male/female ratio, age, gestational age, birth weight, APGAR score (5th min) and CRP values (existence of infection was an exclusion criteria) between hyperbilirubinemic and the healthy newborns ($p>0.05$). The mean serum bilirubin concentration was significantly higher in the study group than that in the control group ($p=0.000$) (**Table 1**). Erythrocyte antioxidant enzyme activities (CAT, SOD, GPx, and G6PD) were significantly lower in the patient group compared with the control group ($p=0.000$ for each parameter) (**Table 2**). Hyperbilirubinemic newborns had significantly higher plasma MDA concentrations, total plasma NO levels, and serum bilirubin concentrations compared with the control group ($p=0.000$ for each parameter) (**Table 2**).

Table 1 - Baseline characteristics and bilirubin levels of babies in the study and the control groups (N=30).

Characteristics	Study group	Control group	*P*-value
	Mean ± SD		
Male/Female	13/17	16/14	NS
Age (days)	4.4±1.3	4.9±1.7	NS
Gestational age (weeks)	38.1±0.6	38.3±0.7	NS
Birth weight (gram)	2916±499	3105±467	NS
APGAR score (5th min)	9.4±0.4	9.2±0.6	NS
Serum bilirubin (mg/dL)	23.7±5.2	2.6±0.6	0.000
CRP (mg/dl)	3.1±0.2	3.3±0.1	NS
CRP - C-reactive protein, NS - not significant			

Table 2 - Erythrocyte enzymes activities, MDA, and NO levels in the study and the control groups.

Activities	Study group		Control group		*P*-value
	Mean ± SD	(Minimum-Maximum)	Mean ± SD	(Minimum-Maximum)	
Erythrocyte CAT (Ug/Hb)	63.4±14.2	(38-97)	149.1±31.5	(45-213)	0.000
Erythrocyte SOD (Ug/Hb)	772±165	(414-980)	1781±343	(1000-2500)	0.000
Erythrocyte GPx (U/Hb)	672±190	(300-981)	1219±161	(870-1574)	0.000
Erythrocyte G6PD (Ug/Hb)	9.2±2.7	(4.4-14.5)	21.9±4.3	(10-31)	0.000
Plasma MDA (nm/mL)	6.7±1.3	(4.3-9.8)	3.7±0.9	(2-6)	0.000
Total plasma NO (µmol/L)	49.9±14.4	(21-76)	23.4±7.1	(10-40)	0.000

CAT - catalase, SOD - superoxide dismutase, GPx - glutathione peroxidase, G6PD - glucose-6-phosphate dehydrogenase, MDA - malondialdehyde, NO - nitric oxide.
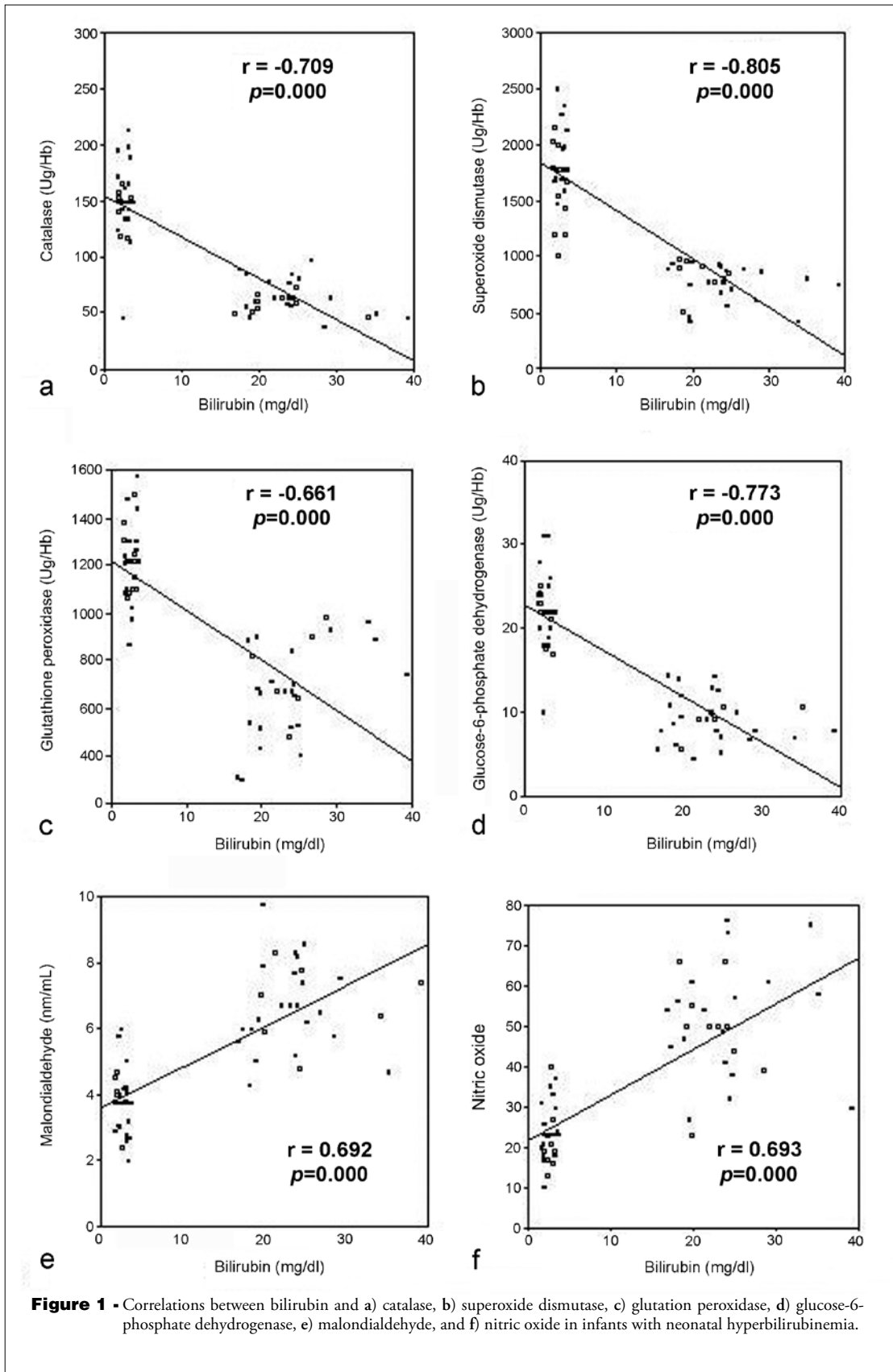
Figure 1 - Correlations between bilirubin and **a**) catalase, **b**) superoxide dismutase, **c**) glutation peroxidase, **d**) glucose-6-phosphate dehydrogenase, **e**) malondialdehyde, and **f**) nitric oxide in infants with neonatal hyperbilirubinemia.

There were significantly negative correlations between erythrocyte anti-oxidant enzyme activities and bilirubin levels (**Figures 1a-1d**). Correlations of serum bilirubin with anti-oxidant enzyme activities were as follows: with CAT, SOD, GPx, and with G6PD. However, significant positive correlations were found between serum bilirubin levels and MDA and between bilirubin and NO levels (**Figures 1e & 1f**).

Discussion. Increased free radicals may be implicated in the development of various neonatal diseases; such as respiratory distress, circulatory failure, sepsis, aspiration, intraventricular hemorrhage, retinopathy, bronchopulmonary dysplasia, necrotizing enterocolitis and asphyxia.[21] The balance between oxidative stress, and anti-oxidant defence is important. Erythrocyte enzymes, low molecular weight anti-oxidant vitamins such as A, E, and C and trace elements such as selenium are the most important members of anti-oxidant barrier.[3,22,23] Besides to these agents the anti-oxidant role of bilirubin is known and it has been demonstrated that the anti-oxidant effect of bilirubin exceeds that of vitamin E against lipid peroxidation.[8]

We examined the role of bilirubin in the oxidant/antioxidant status of indirect hyperbilirubinemia and found increased oxidative stress and decreased antioxidant enzyme activities in newborns with hyperbilirubinemia. However, positive correlations were found between plasma bilirubin and MDA and between bilirubin and NO concentrations (**Figure 1**). Our results show an increase in serum bilirubin concentration parallel to an increase in oxidants (MDA and NO) levels and decrease in erythrocyte anti-oxidant enzyme activities. These data seem to be discordant with previous studies using in vitro and animal models, because both of those studies showed anti-oxidant effects of bilirubin.[13,14] The latter studies may not exactly present what occurs in human beings especially in neonates.[15] In a study, it has been shown that a bilirubin concentration over 30 mg/dl is associated with an increase in protein oxidation.[13]

Biliverdin and bilirubin are reducing species and hence, potential anti-oxidants formed by the action of heme oxygenase and biliverdin reductase.[24] Heme oxygenase has been reported to have a role as a pro-oxidant agent.[25] Iron is a well-known pro-oxidant, too. Heme oxygenase is the enzyme responsible from heme degradation to carbon monoxide, biliverdin, and iron.[25] Therefore, we speculate that our results could be explained by overexpressed heme oxygenase, which result in a large amount of free iron release due to heme degradation in hyperbilirubinemic term infants. Pro-oxidant activity of iron might have outweighed the anti-oxidant activity of bilirubin in higher bilirubin levels. It can be hypothesized that one cause of high

MDA, and NO levels may be the insufficient anti-oxidant enzyme capacity of term infants against free radicals, because these are the primary enzymes for the protection of erythrocytes against oxidant molecules. The levels of these barriers have been found as low in both cord blood and in the neonatal period.[26] Bracci et al[26] showed similar correlations in newborns with jaundice both at birth and at the fourth day of life. A similar correlation was also found in infants with hemolytic hyperbilirubinemia.[27] One limitation of the present study is the lack of patients' own follow-up oxidant stress data after complete recovery from hyperbilirubinemia. We could not perform oxidative stress studies at the follow-up period due to financial difficulties and insufficient patient compliance.

In conclusion, elevated oxidative stress, and decreased anti-oxidant enzyme activities may lead to cellular injury in patients with neonatal indirect hyperbilirubinemia. Even though bilirubin is known as an anti-oxidative agent the insufficiencies of other anti-oxidants including erythrocyte enzyme activities do contributed to increased oxidative stress of neonatal hyperbilirubinemia. Patients with hyperbilirubinemia should be followed-up closely and undergo therapy in order to avoid its serious consequences. Further studies are required to confirm our results and to investigate possible benefits of antioxidants in hyperbilirubinemia in order to prevent the harmful effects of oxidative stress.

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