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INADEQUATE ANTIOXIDANT PROTECTION IN PRETERM BABIES: POSSIBLE CAUSE FOR HYPERBILIRUBINEMIA

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Abstract

Many illnesses in pre-term infants are thought to be related to the action of reactive oxygen species and it is conceivable that the oxidants play a certain role in the etiopathogenesis of unconjugated hyperbilirubinemia. We hypothesized that an important factor in the mechanism of oxidative injury in hyperbilirubinemic infants on the first day of life would be increased oxidative stress in relation to antioxidants.

For this aim, 43 pre-term infants as well as full-term healthy reference group (A=50) were subjected in the present study. Additionally, pre-terms were divided in: healthy pre-terms (B=25) and hyperbilirubinemic pre-terms (C=18). Perinatal variables (gestational age, birth weight and Apgar score) were recorded, and erythrocyte superoxide dismutase (SOD) and glutathione peroxidase (GPx) activity and selenium (Se) levels were measured in umbilical cord blood, immediately after the delivery.

The obtained results indicate strikingly lower antioxidant capacity of pre-term infants; they showed significantly lower SOD and GPx activity and Se level, compared to the full-term infants ($p<0,001$, for all). In the hyperbilirubinemic group, GPx activity and Se levels were found to be significantly lower than those in healthy pre-terms ($p<0.001$, for both), while SOD showed highly increased enzyme activity ($p<0.001$). Alterations in enzyme activities were accompanied by a simultaneous significant increase in the bilirubin level ($p<0.001$).

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In conclusion, disequilibrium between SOD and GPx activity ratio may represent a marker of oxidative stress in cells of premature infants. Aditionally, this inadequacy of the protection, may cause erythrocyte haemolysis, resulting with hyperbilirubinaemia.

Key words: *Premature infants, Hyperbilirubinemia, Oxidative stress, Selenium (Se), Glutathione peroxidase (GPx), Superoxide dismutase (SOD)*

Introduction

Birth is an oxidative challenge for the newborn. The fetal to neonatal transition exposes the newborn to a much more oxygen-rich world than the intrauterine environment (Ochoa et al., 2003). The relatively high oxygen concentrations after birth could be toxic to fetal tissues. A potential mechanism of toxicity and pathophysiologic cell alterations is believed to be mediated by increased production of reactive oxygen species (ROS) (Buonocore et al., 2002). Cells normally respond to oxidative stress by up-regulating antioxidant defenses and other protective systems, but overproduction of ROS damages proteins, lipids, and DNA and leads to cell transformation or cell death by apoptotic or necrotic mechanisms (Mishra et al., 1999).

Many illnesses in pre-term infants are thought to be related to the action of ROS and it is, also, conceivable that the oxidants play a certain role in the etiopathogenesis of unconjugated hyperbilirubinemia (Thibeault, 2000). Hyperbilirubinemia is one of the most common diseases encountered in neonates, clinically observed in 60% of term infants and 80% of pre-terms (Newman et al., 1992). This is a condition of major importance and requires urgent clinical attention due to the danger of kernicterus and permanent neurological impairment (Connolly et al., 1990; Turgut et al., 2004; Kaplan et al., 2005).

Reactions of bilirubin involving free radicals have been well documented: unconjugated bilirubin scavenges singlet oxygen with high efficiency, reacts with superoxide anions and peroxyl radicals, and serves as a reducing substrate for peroxidases in the presence of hydrogen peroxide or organic hydroperoxides (Stocker et al., 1987; Aycicek et al., 2007). However, bilirubin is generally regarded as a toxic compound when accumulated at abnormal concentrations (Bracci et al., 1988; Yigit et al., 1999).

We hypothesized that an important factor in the mechanism of oxidative injury in hyperbilirubinemic infants on the first day of life would be increased oxidative stress in relation to antioxidants. Therefore, the current study was designed to measure levels of selenium (Se) and antioxidant enzyme activities of superoxide dismutase (SOD; EC 1.15.1.1) and glutathione peroxidase (GPx; EC 1.11.1.9) in cord blood of newborns**,** and to emphasize the importance of these substances in unconjugated hyperbilirubinemia physiopathology.

Materal and Methods

Human subjects

All of the infants enrolled in this study were born at University Clinic of Obstetrics and Gynecology, Skopje, the Republic of Macedonia. Neonates admitted to the normal nursery were consecutively enrolled, except those with the following exclusion criteria: known intrauterine infection, major malformation, abnormal fetal monitoring, need for resuscitation, evidence of perinatal hypoxia or respiratory distress. Those admitted to the neonatal intensive care unit were also enrolled using the same criteria.

According to the infants condition at the end of the 1st week of life, three groups were established: group **A**, consisted of full-term healthy infants (50 babies with gestational ages ≥ 38 weeks); group **B**, consisted of 25 pre-term infants who did not need specific intensive reanimation, oxygen therapy or any other type of medication at birth, and with bilirubin level below 6 mg/dl; group **C**, consisted of 18 hyperbilirubinemic pre-terms with a bilirubin level of at least 15 mg/dl. All these infants were evaluated by means of a detailed history, physical examination and laboratory findings. For each newborn infant, sex, gestational age, birth weight, type of delivery, Apgar score at 1 and 5 minutes, antenatal steroid treatment, main pathologies, and pregnancy diseases were recorded.

Blood samples

Anticoagulated blood (~3 ml) was obtained from umbilical cord, at birth, from all study subjects. Glutathione peroxidase activity and hemoglobin concentration were determined in whole blood, within 8 h, after sampling. For the measurement of selenium levels, approximately 1.0 ml of whole blood was frozen at −20°C until analyzed. Subsequently, an aliquot (0,5 ml) of the sample was centrifuged at 3000*g* for 10 min to separate the plasma. The buffy coat was removed and the remaining erythrocytes were drawn from the bottom; washed three times in cold saline (9.0 g/l NaCl); made up to 2.0 ml with ice-cold deionized water; mixed and frozen in 500-ml aliquots at −80°C until the measuring of erythrocyte SOD activity. Freezing does not lead to changes in enzyme activity.

Analytical methods

All reagents, except the phosphate buffers, were prepared each day and stored in a refrigerator at 4°C. The reagents were equilibrated at room temperature for 0.5 h before use when the analysis was initiated or reagent containers were refilled. Phosphate buffers were stable at 4°C for 1 month. Both, SOD and GPx enzyme activities were determined with a Cobas Mira autoanalyzer (F. Hoffmann-La Roche, Diagnostic Systems). The methods were modified as stated below for the autoanalyzer procedure. To obtain optimal accuracy in pipetting, small volumes of H_2O or assay buffer were pipetted into the cuvettes together with samples and reagents to rinse the needle. These volumes are included in the final reaction volumes. All measurements were performed in duplicate.

Assay of superoxide dismutase activity (SOD; EC 1.15.1.1)

Determination of SOD activity was performed by using a kit (Ransod; Randox Labs. cat. no. SD 125) based on the method developed by McCord and Fridovich (1969). Superoxide radicals, generated by the xanthine/xanthine oxidase reaction, convert 1-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyl tetrazolium chloride quantitatively to a formazan dye. Conversion of superoxide radicals to hydrogen peroxide by superoxide dismutase inhibits dye formation and serves as a measure of superoxide dismutase activity. The enzyme activity was expressed in U/g of Hb. Briefly, at the day of analysis the hemolysates were thawed and diluted (25 fold dilution; F=100) with 0,01 M phosphate buffer pH 7,0 so that the % inhibition falls between 30% and 60%. The final concentrations of the reagents used in the assay were as recommended by the manufacturer (0.05 mmol/l xanthine and 0.025 mmol/l INT in the main reagent and 80 U/l xanthine oxidase in the start reagent). The diluted hemolysate (5 ml plus 20 ml of H_2O) was added concomitantly with the main reagent (170 ml) to the cuvette. Absorbance was monitored at 500 nm for 150 s after addition of xanthine oxidase (25 ml plus 10 ml of H_2O) as start reagent. The final reaction volume was 230 ml.

Assay of glutathione peroxidase activity (GPx; EC 1.11.1.9)

Total activity of glutathione peroxidase was determined by using a kit (Ransel; Randox Labs. cat. no. RS 505) based on the coupled enzyme procedure developed by Paglia and Valentine (1967), with cymene hydroperoxide as substrate. Enzyme activity was expressed as units per gram of hemoglobin (U/g Hb), where units are the μmols of reduced nictotinamide adenine dinucleotide (NADPH) oxidized per minute. Briefly, 0,05 ml of heparinized whole blood has been diluted with 1 ml diluting agent, then incubated for 5 minutes and added 1 ml of double strength Drabkin's reagent to inhibit the peroxidase activity of haemoglobin (dil.factor = 41). The main reagent consisted of: 0,05 mmol/l phosphate buffer (pH 7.2); 4,3 mmol EDTA/l; 4.0 mmol/l GSH; 0,5 U/l GR and 0,34 mmol/l NADPH. The main reagent (220 ml) and the sample (5 ml hemolysate plus 30 ml of H_2O) were added to the cuvette and the change in absorbance was monitored, after addition of 0,18 mmol/l cumen hydroperoxide (10 ml plus 20 ml of H_2O) as start reagent. The final reaction volume was 285 ml.

Assay of selenium concentration (Se)

Whole blood selenium was measured by electrothermal atomic-absorption spectrometry, using Zeeman background correction. This method relies upon the reduction of the palladium modifier (on Varian SpectrAA 640Z). The results were expressed in micrograms per liter (g/l).

Assay of total bilirubin (TBil) and hemoglobin (Hb) concentration

Plasma bilirubin was determined with TBil-Vitros Clinical Chemistry Slide (Johnson and Johnson Clinical Diagnostics, Inc), a dry, multilayered, analytical element coated on a clear polyester support. The analysis is based on a modification of the classical diazo reaction and results were expressed in

mg/dl. Hb concentration was measured to obtain erythrocyte enzymatic activity in U/gHb, on hematological analizator for in vitro diagnostic (Sysmex K-1000).

Statistical analysis

Statistical data processing was performed using SPSS 13.0 softwere. The data are presented as means ± SD. Differences between groups with different numbers of infants were tested using a **t**-test ($p<0.05$ value was considered significant). Pearson's correlation coefficient was used as a measure of linear association between two variables.

Results

The short- and long-term prognostic clinical markers of newborn infants are presented in Table 1. There was statistically significant difference between groups in terms of gestational age ($p<0.01$) and birth weight ($p<0.02$). By contraries, the vitality index concerning neonatal adaptation to the physiological oxidative stress of delivery and early postnatal life, was similar in both groups (Apgar score at 5 minutes, $p>0,05$). The plasma bilirubin levels were found to be 297% higher in the jaundiced babies compared to the healthy pre-terms ($p<0.001$).

Measurement of SOD activity showed significant differences among the groups (Fig. 1). The primary endogenous protectant against oxygen toxicity, was significantly lower in cord blood of healthy pre-terms vs. full-terms (31,1%, $p<0.001$), and highly increased in hiperbilirubinemic group, with respect to healthy pre-terms (25,7%, $p<0.001$). However, this increased activity stays significantly lower than in full-terms ($p<0.001$).

Table 1. Clinical characteristics of the newborns: full-terms (A), healthy pre-terms (B) and hyperbilirubinemic pre-terms (C).

	full-terms A	**pre-terms**		
		B healthy	**C hyperbilirubinemic**	***p*****
gestation (weeks)	39,34±1,09	35,04±0,79 *	32,80±0,91**	<0,01
birth weight (g)	3430±435,6	2258±430,4 *	1901±229,3**	<0,02
Apgar at 5 min.	9,11±0,51	7,68±0,90 *	7,1±1,2	>0,05
TBil (mg/dl)	–	4,83+0,82	19,02+3,61**	<0,001

Note: Average Values ± SD of all determination carried out. *Statistical significance as compared with full-terms ($p<0,001$); ** Statistical significance vs. healthy pre-terms.

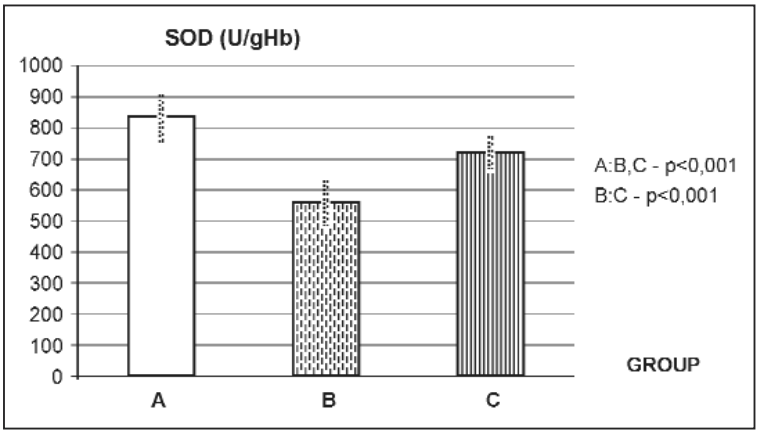

Figure 1. Erythrocyte superoxide dismutase (SOD) activity in cord blood of: full-term newborns (A), healthy pre-terms (B) and hiperbilirubinemic pre-terms (C). Results are expressed in terms of U/g of Hb and represent means ± SD.Note: Average Values ± SD of all determination carried out. *Statistical significance as compared with full-terms (p<0,001); ** Statistical significance vs. healthy pre-terms.

The activity of GPx (Fig. 2), one of the most potent antioxidant enzymes crucial to cell survival, was significantly decreased in healthy pre-terms (23,3% vs. full-terms, p<0,001) and even more depressed in hiperbilirubinemic babies (27,6% vs. healthy pre-terms, p<0.001).

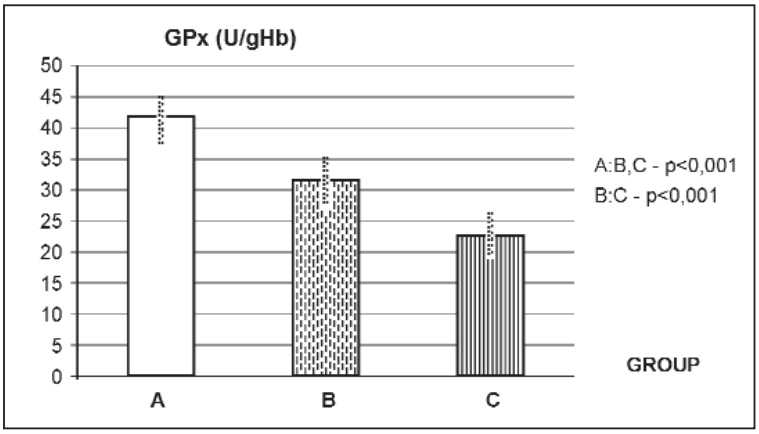

Figure 2. Glutathione peroxidase (GPx) activity in cord blood of: full-term newborns (A), healthy pre-terms (B) and hyperbilirubinemic pre-terms (C). Results are expressed in terms of U/g of Hb and represent means ± SD.

With respect to Se content (Fig. 3), dramatically decreased value of this essential trace element was estimated in healthy pre-terms than the one achieved in full-term infants (30,4 %, $p<0.001$) and an 18% lower value in hyperbilirubinemic group vs. healthy pre-terms ($p<0,001$).

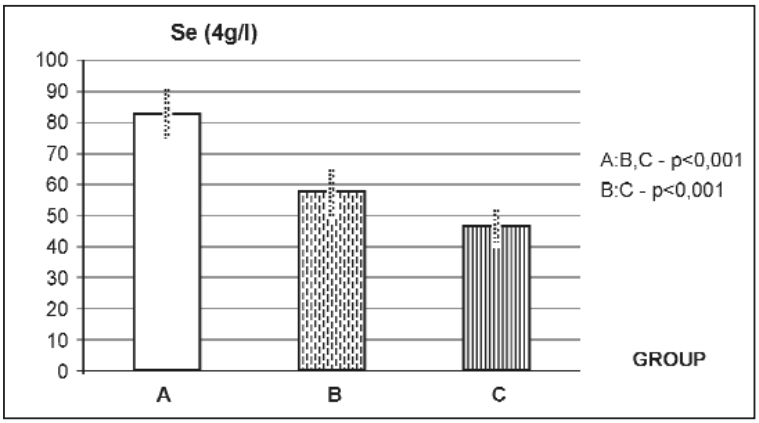

Figure 3. Whole blood selenium (Se) content in cord blood of newborns, at birth: full-terms (A), healthy pre-terms (B) and hiperbilirubinemic pre-terms (C). Results are expressed in terms of g/l and represent means ± SD.

There was no significant relationship between any of the investigated antioxidants and either, perinatal variables or infants' sex.

Discussion

Oxidative reactions form an essential part of all biological systems, but toxic effects of the derivatives of these reactions depend on a critical balance between the oxidative stimulus and the antioxidant defense mechanisms available. The fetal tissues, which are in a permanent state of low oxygenation during intrauterine life, are subjected to oxidative damage, by the increase of free radicals, soon after birth (Stocker et al., 1987; Stahl and Sies, 1997; Huertas et al., 1998). With the present study, we wanted to test the validity of hypothesis that inadequate antioxidant defense in pre-term babies may predispose them to increased oxidative stress and cause hyperbilirubinemia, by determining the Se levels and activity of antioxidant enzymes such as SOD and GPx, immediately after birth.

The obtained results, indicate strikingly lower antioxidant status of pre-term newborns compared with full-terms, resulting in decreased SOD and GPx activities, and lower Se levels. This finding is in perfect accordance with the previous data published by other investigators (Georgeson et al, 2002; Buhimschi et al., 2003) and it could be explained by the inadequate supply of specific cofactors, essential for the proper functioning of these enzymes: copper, zinc and selenium, respectively; since placental maternal-to-fetal passage is very limited before (the latter part of) the third trimester (Frank, 1996; Falciglia et al., 2003; Ochoa et al., 2003).

Measurement of antioxidant enzyme activities in pre-terms, suggests that hyperbilirubinemia, which is considered to be a significant problem in neonates, should be evaluated from a different perspective. We marked significant increase in SOD activity in hyperbilirubinemic group vs. preterm control; however, it was insufficient to confront oxidative insult at birth (its stays significantly lower in comparison with full-term infants). SOD is the primary endogenous protectant of cells against oxygen toxicity and increases the formation of hydrogen peroxide (H_2O_2), which if not destroyed could have more detrimental effects than superoxide ions alone (Michels et al., 1994). By the contrary, the activity of GPx was furthermore decreased in hyperbilirubinemic group. GPx, catalyzes the reduction of lipid peroxides in addition to the destruction of H_2O_2, thus acting on more sensitive cellular targets, and protects biological membranes by preventing lipid peroxidation propagation (McCay et al., 1976). Alterations in enzyme activities in the hyperbilirubinemic group were accompanied by a significant increase in the bilirubin level.

In the light of this information, it may be assumed that oxidative injury occurs because of the cytotoxic effect of the high level of H_2O_2 on erythrocytes and the low GPx activity are unable to protect neonatal erythrocytes from this oxidation. Hemolysis, as a result of these changes, leads to an increase in the serum bilirubin level. The activity of first (SOD) and second (GPx) step antioxidant enzymes must, therefore, be balanced to prevent oxidative damage in cells. Namely, GPx protects hydrophobic membrane compartment of the cell; on the other hand, SOD principally acts in hydrophilic regions (Michels et al., 1994). Consequently, when SOD is adequately combined with GPx, the protection of hydrophobic and hydrophilic compartments is complementary, and such a combination better maintains the cellular integrity against the free radical attacks.

Does bilirubin increase as a result of deficient antioxidant enzymes or to compensate this deficiency? Bilirubin has been postulated to be an antioxidant. It has an extended system of conjugated double bonds and reactive hydrogen, and can act as an antioxidant (Stocker et al., 1987). However, it is generally regarded as a toxic compound when accumulated at abnormal concentrations. Bracci et al. (1988) showed that erythrocyte antioxidant enzymes were significantly lower in cord blood and on the 4th day of life in babies with high bilirubinemia compared to less jaundiced babies. Studies on this topic have found higher incidence of hyperbilirubinemia with lower GPx levels (Pati et al., 1990; Aydın et al., 1997) and also showed that hemolysis which developed in erythrocytes was inhibited by antioxidant substances (Niki et al., 1988; Kondo et al., 1997). Recently Dani et al. (2003) showed a decrease in plasma bilirubin concomitant with an increase in plasma antioxidant capacity and decrease in oxidative stress in preterm infants.

In our study, only several important elements (Se, SOD, GPx) of the human antioxidant system were investigated. Since antioxidants form a fairly large group, more comprehensive studies are needed, in order to elucidate the role of antioxidants in unconjugated hyperbilirubinemia. In addition, most of the factors that could lead to erythrocyte hemolysis should be recognized and premature infants having these pathologies should be included in future studies.

Conclusion

Disequilibrium between SOD and GPx activity ratio may represent a marker of oxidative stress in cells of premature infants. Aditionally, this inadequacy of the protection, may cause erythrocyte haemolysis, resulting with hyperbilirubinaemia.

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