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CORD BLOOD SUPEROXIDE DISMUTASE AND GLUTATHIONE PEROXIDASE ACTIVITY IN PREMATURE INFANTS

AKTIVNOST SUPEROKSID-DISMUTAZE I GLUTATION-PEROKSIDAZE KOD PREVREMENO ROĐENE DECE

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Summary

Background: Birth involves strong oxidative stress for the infant, implying an increased production of free radicals. The aims of this study were to assess the antioxidant response to oxidative insult at birth, by estimating the superoxide dismutase (SOD) and glutathione peroxidase (GPX) activity in umbilical cord blood, and to evaluate their dependency on the degree of maturation of the newborns.
Methods: In the present study, 60 preterm infants (study group) as well as a full-term healthy reference group (A=53) were included. Additionally, the preterms were divided in 3 groups according to their condition at the end of the 1st week of life: preterm control (B=25), on oxygen support (C=18), and ventilated group (D=17).
Results: The obtained results indicate markedly lower antioxidant capacity of the preterm infants: they had significantly lower SOD and GPX activity than the full-term infants ($p<0.001$, for both). Investigated antioxidants also showed significant differences between the groups of preterms. SOD activity was higher in preterms with postnatal respiratory failure compared to preterm control ($p<0.001$). On the contrary, GPX activity was decreased in the oxygen supported group (10%) and even more in the ventilated group (28.5%) ($p<0.001$, for both). The newborns enzyme activities were also profoundly modulated by the gestational age and birth weight, specifically the GPX.

Kratak sadržaj

Uvod: Proces rađanja sam po sebi predstavlja snažan oksidativni stres za novorođeno dete. Cilj ovog rada bio je da se ispita antioksidantni odgovor oksidativnom insultu na rođenju, putem određivanja aktivnosti superoksid-dismutaze (SOD) i glutation-peroksidaze (GPX) u krvi pupčane vrpce, kao i da se utvrdi njihova zavisnost od stepena zrelosti novorođenog deteta.
Metode: U ovu studiju je uključeno 60 prevremeno rođene dece (ispitivana grupa) koja su podeljena u 3 grupe saglasno njihovom stanju krajem prve postnatalne nedelje: prevremena kontrola (n=25), na kiseoničnoj terapiji (n=18) i na mehaničkoj ventilaciji (n=17). Pedeset troje zdrave novorođenčadi činilo je kontrolnu grupu.
Rezultati: Dobijeni rezultati indiciraju slabiji antioksidantni kapacitet kod prevremeno rođene dece: oni su imali znatno smanjenu aktivnost SOD i GPX u odnosu na terminsku decu rođenu u 40. nedelji ($p<0,001$, za oba). Ispitivani antioksidanti su pokazali značajnu razliku među prevremenim grupama. SOD je pokazala znatno povećanu aktivnost u grupama sa postnatalnom respiratornom slabošću u poređenju sa prevremenom kontrolom ($p<0,001$). Suprotno tome, aktivnost GPX je bila znatno smanjena: u grupi na kiseoničnoj terapiji 10% i još više u ventiliranoj grupi (28,5%) ($p<0,001$). Enzimska aktivnost je bila upadljivo modulirana gestacijskom starošću i težinom na rođenju, posebno GPX.

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Conclusions: Because of their deficient and inadequate antioxidant protection, preterm newborns are more susceptible to oxidant injury at birth.

Keywords: glutathione peroxidase, newborns, oxidative stress, preterm infants, superoxide dismutase

Zaključak: Prevremeno rođena deca su izuzetno podložna oksidativnom insultu na rođenju zbog svoje deficitne i neadekvatne antioksidantne zaštite.

Ključne reči: glutation peroksidaza, novorođenčad, oksidativni stres, prevremeno rođena deca, superoksid dismutaza

Introduction

Preterm deliveries, before 37 completed weeks of gestation, account for 10% of all births and yet they account for 75% of neonatal deaths (1). The last trimester of pregnancy is necessary for the maturation of the fetal organs in preparation for extrauterine life. If this process is interrupted by an early delivery, the chances of survival of the newborn are severely decreased (2). Although neonatal intensive care has improved dramatically in the last decades and treatment has become possible for extremely preterm infants, born before the completion of 32 gestational weeks, these infants remain at high risk for potential complications. Neonatal Respiratory Distress Syndrome (RDS) is the leading cause of morbidity in preterms, whose lungs are physiologically and morphologically immature, which renders them vulnerable to injury due to preterm birth, and results in high rates of chronic pulmonary problems (3–5).

The process of childbirth is accompanied by an increase in oxidative stress, as birth is, in itself, a hyperoxic challenge. 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) (6–8). Cells normally respond to oxidative stress by upregulating 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 (9–11).

The aims of this study were to assess the antioxidant enzymatic response to oxidative insult at birth, by estimating the superoxide dismutase (SOD; EC 1.15.1.1) and glutathione peroxidase (GPX; EC 1.11.1.9) activities in cord blood of preterm infants in comparison with a group of healthy full-terms, and to evaluate their dependency on the degree of maturation of the newborns.

Material and Methods

Human subjects

All of the infants enrolled in this study were born at the University Clinic of Obstetrics and Gynecology, Skopje, Republic of Macedonia. A control group was established consisting of healthy full-term infants (≥ 38 weeks' gestation). Criteria for enrollment in the study groups included gestational age ≤36 weeks. According to the infants' condition at the end of the 1st week of life, four study groups were established: full-term healthy infants were classified as the »control« group (n=53); preterm infants who did not need specific intensive reanimation, oxygen therapy or any other type of medication at birth were classified as »premature control« group (n=25); premature infants undergoing oxygen therapy because of the risk of RDS, as »O_2 support« group (n=18); premature infants diagnosed with severe RDS who required positive pressure mechanical ventilation as »ventilated« group (n=17). All 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. GPX activity and hemoglobin concentration were determined in whole blood, within 8 h after sampling. 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 on a »Cobas Mira« biochemical analyzer (Hoffmann-La Roche, Diagnostic Systems, Basel, Switzerland). The methods were modified as stated below for the analyzer procedure (12). 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

Determination of superoxide dismutase (SOD; EC 1.15.1.1) activity was performed by using a Ransod kit (Randox Labs. cat. no. SD 125, CrumLin, UK) based on the method developed by McCord and Fridovich (13). This method employs the xanthine/xanthine oxidase reaction to generate superoxide radicals which react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyl tetrazolium chloride (INT) to form a red formazan dye. The superoxide dismutase activity is then measured by the degree of inhibition of this reaction, and was expressed in U/g of Hb, where one unit of SOD is that which causes a 50% inhibition of the rate of reduction of INT. Briefly, at the day of analysis, the hemolysates were thawed and diluted (25 fold dilution; F=100) with 0.01 mol/L 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 H2O) as a start reagent. The final reaction volume was 230 mL.

Assay of glutathione peroxidase activity

Total activity of glutathione peroxidase (GPX; EC 1.11.1.9) was determined by using a Ransel kit (Randox Labs. cat. no. RS505, CrumLin, UK) based on the coupled enzyme procedure developed by Paglia and Valentine (14), 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 nicotinamide adenine dinucleotide (NADPH) oxidized per minute. Briefly, 0.05 mL of heparinized whole blood was 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 hemoglobin (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 at 340 nm, after addition of 0.18 mmol/L cumen hydroperoxide (10 mL plus 20 mL of H_2O) as a start reagent. The final reaction volume was 285 mL.

Assay of hemoglobin (Hb) concentration

The concentration of hemoglobin (g/L), needed for expression of enzymatic activity, was measured on an automated hematological analyzer for *in vitro* diagnostic, Sysmex KX-21N (Sysmex Corporation, Kobe, Japan).

Ethics

The research was conducted in accordance with the Declaration of Helsinki ethical guidelines, and approved by the institution.

Statistical analysis

Statistical data processing was performed using SPSS 13.0 statistical package. Data are presented as means ± SD. Differences between groups with different numbers of infants were tested using a Student 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 listed in *Table I*. There

Table I Perinatal characteristics of healthy full-term infants and preterms: healthy (control), on oxygen therapy (O_2 support), on mechanical ventilation (ventilated).

	Full-terms (n=53)	Preterms			
		control (n=25)	O_2 support (n=18)	ventilated (n=17)	p*
Gestation (weeks)	39.34 (1.09)	35.04 (0.79)	32.39 (1.38)	29.41 (2.35)	<0.001
Birth weight (g)	3430 (436)	2258 (430)	1736 (375)	1318 (283)	<0.001
Apgar at 1 min	8.13 (0.48)	6.88 (0.83)	6.11 (1.18)	4.06 (1.98)	<0.001
Apgar at 5 min	9.11 (0.51)	7.68 (0.90)	6.89 (1.08)	5.12 (1.65)	<0.001

Values are given as mean (SD) with Student t-test carried out; * indicates significant changes compared with full-terms and between each group ($p<0.001$).

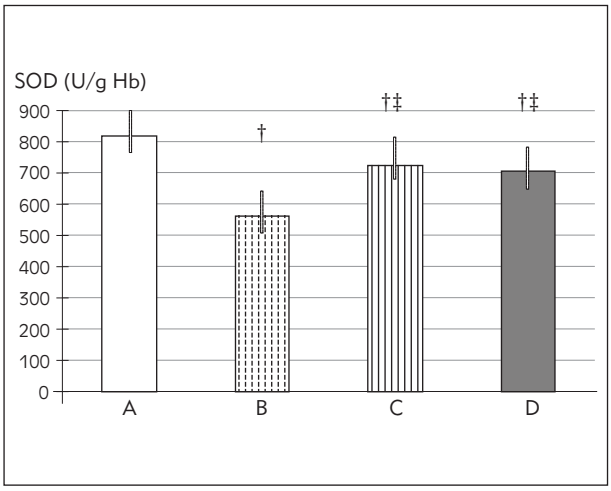

Figure 1 Cord blood, erythrocyte SOD activity in full-term healthy infants (A) and preterms: healthy control (B), on oxygen therapy (C), and ventilated (D), at birth. Values represent mean (SD) with Student t-test carried out; † significant changes compared with full-terms ($p<0.001$), ‡ significant changes compared with preterm controls ($p<0.001$).

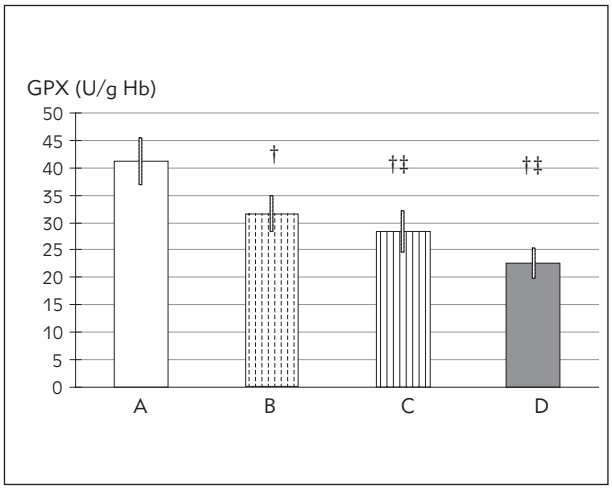

Figure 2 Cord blood GPX activity in full-term healthy infants (A) and preterms: healthy control (B), on oxygen therapy (C), and ventilated (D), at birth. Values represent mean (SD) with Student t-test carried out; † significant changes compared with full-terms ($p<0.001$), ‡ significant changes compared with preterm controls ($p<0.001$).

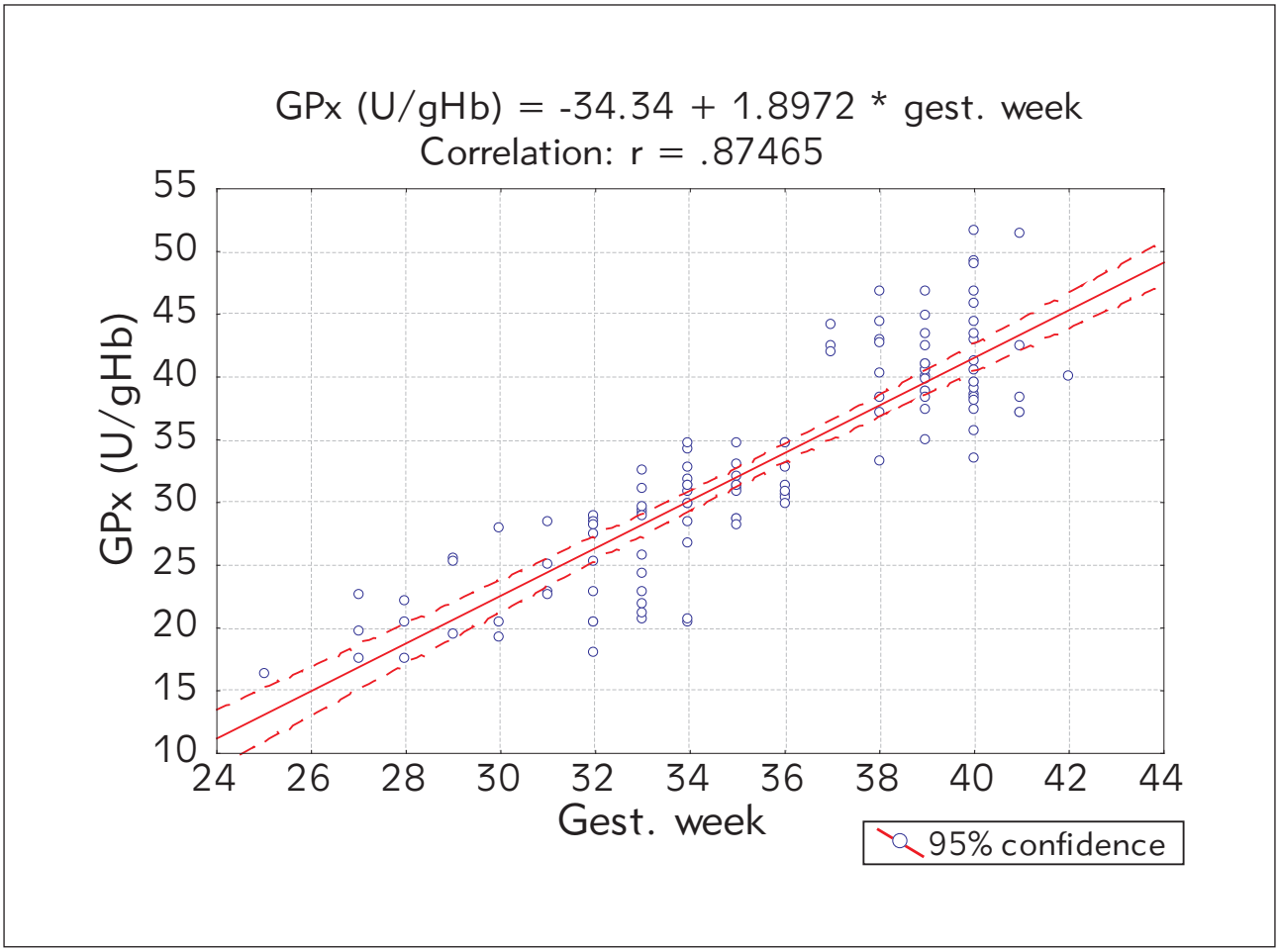

Figure 3 Correlation analysis between cord blood GPX activity at birth and gestation, in newborns ($r=0.875$, $p<0.001$).

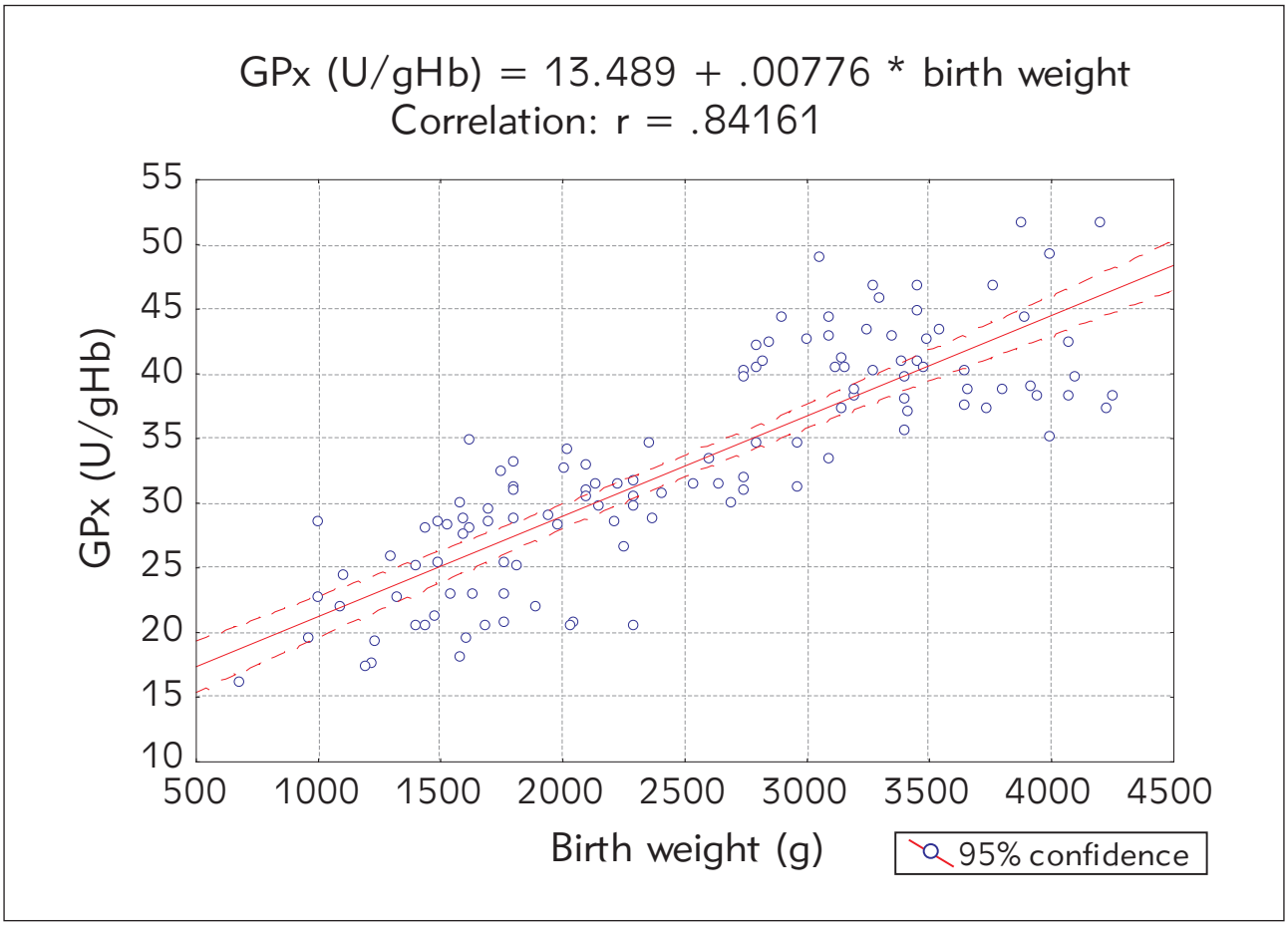

Figure 4 Correlation analysis between cord blood GPX activity at birth and birth weight, in newborns (r=0.842, p<0.001).

was a statistically significant difference between the groups in terms of gestational age and birth weight (p<0.001). The vitality index concerning neonatal adaptation to the physiological oxidative stress of delivery and early postnatal life, Apgar score at 1 and 5 minutes, showed the same significant differences (p<0.001) (*Table I*).

Figures 1–2 represent the antioxidant enzyme activities of each group, at birth. SOD activity in healthy neonates proved to be significantly lower in the preterm healthy population (563.34±62.2) in comparison to full-terms (817.55±80.45 U/g Hb) (p<0.001). GPX activity in preterm control (31.61± 3.01) also showed to be significantly lower (p< 0.001) than in the full-term neonates (41.22±4.09 U/g Hb) (*Figures 1–2*).

Measurement of enzyme activity showed significant differences among the groups of preterm infants. SOD, the primary endogenous protectant against oxygen toxicity (*Figure 1*), was significantly increased in preterms undergoing oxygen treatment (28.8%, p<0.001) and those who required mechanical ventilation (25.7%, p<0.001), in comparison with preterm control. However, this increased activity stays significantly lower than in the full-term group (p<0.001).

On the contrary, GPX, one of the most potent antioxidant enzymes, was significantly decreased in preterms with postnatal respiratory failure. In the oxygen supported group this decrease was 10% (p<0.001) and in the ventilated group even greater (28.5%; p< 0.001), compared to the preterm control (*Figure 2*). The lowest cord blood GPX activity was observed in infants who died at age <7 days (19.04±2.03 U/g Hb). These findings indicate that separating the premature newborns into preterm controls, preterms undergoing oxygen therapy and those requiring mechanical ventilation was statistically justified.

To determine the maturation dependency of antioxidant enzymes, we evaluated the relationship between investigated antioxidants and perinatal parameters. A positive correlation was found between erythrocyte SOD activity and perinatal variables (SOD: gestation, r=0.543, p=0.038; SOD: birth weight, r= 0.550, p=0.023). Furthermore, a strong positive association with the high correlation coefficients was established between GPX activity and perinatal variables

(GPX: gestation, r=0.875, p<0.001; GPX: birth weight, r=0.842, p<0.001) (*Figure 3–4*). This positive correlation points to the enzymes dependency on the degree of maturation of newborns. No significant variations with respect to sex were detected.

Discussion

The fetal to neonatal transition exposes the newborn to a much more oxygen-rich world than the intrauterine environment. This sudden augmentation in alveolar oxygen concentration and arterial pO_2 after delivery increases the formation of reactive oxygen species (ROS) in the lungs and other organs (15–18). The lungs are the most severely damaged organs by exposure to hyperoxia. Structural immaturity of the lungs, surfactant deficiency and surfactant dysfunction are the main problems of the preterm newborn, leading to respiratory distress (19, 20). Despite of new preventive strategies, neonatal Respiratory Distress Syndrome (RDS) is still the most important cause of mortality and morbidity in neonatal intensive care. The incidence and severity of neonatal RDS have shown an inverse relationship with gestational age. Approximately 50% of infants born after a gestational age of 25–27 weeks develop neonatal RDS, whereas fewer than 20% of preterm infants born at a gestational age of 31 weeks develop the disorder and the incidence decreases to 1% for infants born at term (19).

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 (21, 22). The results of this study indicate markedly lower antioxidant status of preterm newborns at birth, compared with full-terms, resulting in decreased SOD and GPX activities. This finding is in perfect accordance with the previous data published by other investigators (23–27), and 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 (28). Production and activity of antioxidant enzymes increase markedly in the final days before birth, and even more so after birth (29). Therefore, premature birth could itself be considered an illness with a major oxidative component.

Studies of the enzymatic antioxidant system in preterm infants have aroused some controversy, partly because many investigators have drawn conclusions after analyzing the activity of a single enzyme. The present study demonstrates a marked difference in enzyme activities at birth among the groups of preterms. In the case of SOD, the higher cord blood enzyme activity observed in preterms with respiratory difficulties, in comparison with premature controls, probably represents an adaptive response to a higher superoxide ions production. SOD plays a fundamental role in modulating oxygen toxicity and its induction seems related to the extent of the redox abnormality in the cell (30–33). Significantly lower GPX activity, estimated at birth, in preterms receiving supplemented oxygen treatment and even more depressed in those who required mechanical ventilation could be a consequence of a selenium deficiency and, also, a possible glutathione (GSH) deficit in these groups, as has been reported by several authors (34–37). This could lead to an inadequate activity of the glutathione system and therefore less regeneration of glutathione peroxidase, since GPX catalyses the reduction of hydrogen peroxide (H_2O_2) and hydroperoxides originating from polyunsaturated fatty acids at the expense of reduced GSH.

Rise in the SOD activity alone may have unexpected results, because SOD increases the formation of H_2O_2, which if not destroyed could have more detrimental effects than superoxide ions alone (38). The low GPX activity is probably unable to protect from oxidative injury and may contribute to the degree of respiratory distress. Many other authors also drew the same conclusion, showing the preponderant protective role of the glutathione-cycle enzymes, especially GPX, which is postulated to be an etiologic factor in chronic lung disease in preterm infants (38–41). The activity of the first (SOD) and second (GPX) step of antioxidant enzymes must therefore be balanced to prevent oxidative damage in cells. Our study has also shown that the antioxidant enzymes of premature infants are profoundly modulated by the gestational age and the birth weight, specifically the GPX activity.

Based on our measurements, we concluded that preterm babies have deficient and inadequate antioxidant protection against oxidative insult at birth. The immature lung of these newborns, challenged with postnatal therapeutic hyperoxia, may thus be poorly protected biochemically, both from intracellular oxygen free radical toxicity and from extracellularly generated cytotoxic products of activated inflammatory cells that influx into an already injured lung.

Conflict of interest statement

The authors stated that there are no conflicts of interest regarding the publication of this article.

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