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The quality of the antioxidant defence system in term and preterm twin neonates

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Objective: Multiple pregnancy is associated with an enhanced metabolism and demand for O2, which may lead to the overproduction of reactive oxygen species and the development of oxidative stress. The degree of oxidative damage depends on the level of the antioxidant protection system of the foetus. The objective of the study was to identify the relationship between the state of the maturity and the antioxidant status of twin neonates. Investigations of the umbilical cord blood were carried out to detect differences in the antioxidant defence system between mature and premature twin neonates.
Methods: The activities of the superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) enzymes, the levels of reduced glutathione (GSH), protein carbonyls and oxidized lipids and the total antioxidant capacity of the plasma were determined.
Results: The level of lipid peroxidation was significantly higher in the premature neonates. An increase in the total antioxidant capacity was accompanied by a decrease in the damaged protein concentration. Significantly elevated activities of GPx alone were observed in the premature twins, though the GSH content too tended to be increased. The activity of SOD was decreased in the premature neonates.
Discussion: The antioxidant status of twin neonates are mainly influenced by maturity. We suggest that the level of lipid peroxidation might be of clinical value as a marker of pre- and perinatal distress in twins.

Keywords: Antioxidant enzymes, Lipid peroxidation, Oxidative stress, Twin neonates, Umbilical cord blood

Introduction

The number of multiple pregnancies is constantly increasing worldwide, a situation connected with the widespread use of *in vitro* fertilization. Multiple pregnancies are generally endangered pregnancies. The complications that can occur include a low Apgar score, hypoglycaemia, hypocalcaemia, hyperbilirubinaemia, and intrauterine growth retardation (IUGR), when the neonates have birth weight less than the 10th percentile for the gestational age.

The greatest risk factor in multiple pregnancies is prematurity, i.e. delivery before the gestational age of 37 weeks.[1] Premature neonates are usually born with a weight of <2500 g. An elementary cause of premature birth is the situation that the maternal body is fundamentally structured to hold one foetus. Two or more foetuses therefore overstrain the uterus. Unhealthy living conditions or an unhealthy lifestyle also increases the chance of premature delivery, e.g. smoking, alcohol consumption, contact with harmful chemicals, etc. A severe weight discordance can develop between the twins during pregnancy and this is known to be a risk factor for preterm twins in their further life.[2] Premature birth can enhance the likelihood of disorders such as poliomyelitis, blindness, deafness, or immature lungs.

Preterm infants are particularly sensitive to oxidative stress involving an imbalance in the production of oxygen free radicals and the antioxidant defence systems.[3,4] If there is a deficiency of antioxidant molecules and the appropriate enzymes, reactive oxygen species (ROS) can occur in the tissues and can damage the proteins, carbohydrates, lipids, and DNA.[5]

Redox homeostasis is maintained by intra- and extracellular enzymes (superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR), glutathione peroxidase (GPx)), and antioxidant molecules (reduced glutathione (GSH)).[6] The extrauterine oxygen concentration poses a great load on the newborn and may be toxic to foetal tissues.[7] Even though the levels of ROS production and the antioxidant defence are relatively constant in mammals, the expression of these enzymes in high concentrations can take place only at the end of gestation.[6] After a preliminary investigations of antioxidant defence

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Table 1 Data on mature (A) and premature (B) neonates

Twin neonates	Weeks of birth	Mode of birth	Birth weight (g)	Apgar scores
(A) *Mature neonates*				
1	39	Vaginal delivery	3590	10;10
2	39	Vaginal delivery	3290	9;10
3	37 + 5	Caesarean section	2870	9;10
4	37 + 5	Caesarean section	2840	9;10
5	38	Caesarean section	2830	9;9;10
6	38	Caesarean section	2560	9;9;10
7	39	Caesarean section	2930	10
8	39	Caesarean section	2750	10
9	39	Caesarean section	2550	10
10	39	Caesarean section	2550	10
11	37 + 3	Caesarean section	2660	10;10;10
12	37 + 3	Caesarean section	2710	10;10;10
13	37 + 3	Caesarean section	2840	10
14	37 + 3	Caesarean section	2590	10
(B) *Premature neonates*				
1	35 + 5	Caesarean section	1520	8;9;10
2	35 + 5	Caesarean section	2330	8;9;9
3	36 + 3	Caesarean section	2480	10
4	36 + 3	Caesarean section	2490	10
5	36 + 2	Vaginal delivery	1940	6;8;9
6	36 + 2	Vaginal delivery	2180	6;8;9
7	34	Caesarean section	2120	6;7;8
8	34	Caesarean section	1980	3;7;7
9	34	Caesarean section	2220	8;10;10
10	34	Caesarean section	1890	6;7;9
11	35	Caesarean section	2380	9;10;10
12	35	Caesarean section	2240	7;9;10
13	33	Caesarean section	2120	2;6;7
14	33	Caesarean section	1900	3;6;8
15	29	Caesarean section	1120	6;7;9
16	29	Caesarean section	1150	6;9;9
17	33 + 4	Caesarean section	2150	8;9;10
18	33 + 4	Caesarean section	2150	8;9;10
19	36	Caesarean section	2470	6;8;8
20	36	Caesarean section	2250	9;10;10
21	35	Caesarean section	2080	4;9;9
22	35	Caesarean section	2160	8;10;10

system in singleton premature neonates, we supposed even more immature enzymatic defence system and enhanced damages of biomolecules in twin premature neonates. Since an assessment of redox homeostasis in twins has not yet been reported, we set out to evaluate the quality of the antioxidant defence system and the oxidative stress parameters (the levels of carbonyl protein and lipid peroxidation (LP)) in the cord blood of twin neonates. Besides the LP levels, we assessed the total antioxidant capacity (FRAP, Ferric Reducing Antioxidant Power) and investigated the activities of the antioxidant enzymes SOD, CAT, and GPx, and measured the level of GSH. We were interested in learning the quality of the antioxidant defence system of twins and in attempting to identify what causes developmental distinction between the twin foetuses.

Materials and methods

The blood samples were obtained from the Department of Obstetrics and Gynaecology at the Medical University of Szeged, Hungary. The Ethics Committee of the Department of Obstetrics and Gynaecology approved the study protocol. Fourteen mature and 22 premature neonates were examined. The neonates were considered preterm if they were born before the gestational age of 37 weeks and full term if the delivery occurred after 37 weeks. The twin neonates were divided into two groups by birth weight and gestational age: mature neonates (born at a gestational age of >37 with a birth weight >2500 g) and premature neonates (born at a gestational age of <37 with a birth weight <2500 g). Data are shown in Table 1.

Blood was taken from the umbilical vein before the birth of the placenta, EDTA being used as anticoagulant. The plasma and red blood cells were separated by centrifugation at 500 *g* for 10 minutes. The samples were stored at –80°C until processing. A Thermo Spectronic Biomate 5 UV-VIS instrument was used for the spectrophotometric measurements (Thermo Spectronic [Europe], Cambridge, UK).

The activity of SOD was determined by the method of Misra and Fridovich.[8] Erythrocyte haemolysates

(10-fold dilution) were treated with ethanol : chloroform (2 : 1) to remove haemoglobin and centrifuged at 12 000 *g*. The supernatants were used for measurements via inhibition of the epinephrine–adrenochrome transformation. The control sample contained 2.9 ml of sodium carbonate buffer (0.05 M, pH 10.2) and 0.1 ml of epinephrine (0.01 M HCl). The rate of autoxidation of epinephrine (ΔA at 480 nm per minute) was determined. SOD activity was measured as the inhibition of the autoxidation of epinephrine. The test mixture contained 2.9 ml of the carbonate buffer, 0.025 ml of supernatant, and 0.1 ml of epinephrine. The blank sample was 3 ml of sodium carbonate buffer. One unit enzyme activity represents 50% inhibition of the epinephrine–adrenochrome transformation. The results were expressed in U/mg protein.

The activity of CAT was determined by the method of Beers and Sizer.[9] The blank sample was 3 ml of sodium–potassium phosphate buffer (50 mM, pH 7, warmed to 37°C). The reaction mixture contained 2 ml of phosphate buffer, 5 µl of haemolysate (100-fold dilution), and 1 ml of H_2O_2 (30 mM). The change in extinction at 240 nm was recorded for 3 minutes, and was determined as the change per minute. The results were expressed in Bergmeyer units (BU), BU being the amount of CAT that decomposes 1000 mg of H_2O_2 per minute and were expressed in BU/mg protein.

GPx activity was measured spectrophotometrically at 412 nm by using cumene hydroperoxide (3.28 mM in Tris–HCl buffer (0.05 M, pH 7.5)) and GSH (2 mM) as substrates.[10] Every sample had own blank. The blank contained 0.35 ml of Tris–HCl buffer, 0.05 ml of haemolysate (10-fold dilution) and 0.05 ml of GSH. All samples contained 0.35 ml of Tris–HCl buffer, 0.05 ml of haemolysate, 0.05 ml of GSH, and 0.05 ml of cumene hydroperoxide (3.28 mM), and were incubated for 10 minutes at 37°C. After 10 minutes, 0.5 ml of 0.15 g/ml trichloroacetic acid (TCA) was added to all tubes, and 0.05 ml of cumene hydroperoxide solution was added to the blank tubes. All samples were then centrifuged at 12 000 *g* for 10 minutes. One millilitre of Tris–HCl (0.4 M, pH 8.9) and 0.05 ml of 5,5-dithio-bis-nitrobenzoic acid, Ellman's reagent (DTNB) were added to the supernatants. The change in extinction per minute was calculated.[10]

The GSH content of the haemolysate (10-fold dilution) was determined by the method of Sedlak and Lindsay at 412 nm by using Ellman's reagent (5,5-dithio-bis-nitrobenzoic acid). Proteins were precipitated with TCA 0.05 g/ml and were then centrifuged at 12 000 *g* for 10 minutes. The reaction mixture contained 500 µl of Tris buffer (0.4 M, pH 8.9), 300 µl of supernatant, and 30 µl of DTNB. The blank sample was distilled water.[11]

The LP of red blood cells was assayed by the thiobarbituric acid (TBA) method.[12] The TBA reagent was a mixture of 0.15 g/ml TCA, 3.75×10^{-3} g/ml TBA, and 0.25 M HCl. Erythrocyte haemolysates (10-fold dilution) were used, 3.6 ml of TBA reagent solution was added to 0.4 ml of haemolysate. The components were mixed and the mixture was heated for 20 minutes in a boiling water bath. The samples were then cooled on ice, and centrifuged at 12 000 *g* for 5 minutes. Spectrophotometric measurement of the supernatant was carried out at 532 nm. The blank sample contained 0.4 ml of distilled water and 3.9 ml of reagent.[12]

The amount of oxidatively damaged protein was determined in erythrocyte haemolysates (1000-fold dilution) by the carbonyl protein assay.[13] As DNA also contains carbonyl groups, this was removed with 10% streptomycin sulphate. After the addition of 50 µl TCA (0.2 g/ml) to 450 µl haemolysate, the sample was centrifuged at 12 000 *g* for 10 minutes. Five-hundred microlitres of 2,4- dinitrophenylhydrazine (10 mM DNPH) was added to the pellet, for the blank sample 500 µl of 2 M HCl. The mixture was allowed to stand for 1 hour at room temperature, 500 µl TCA (0.2 g/ml) was then added, and the sample was centrifuged at 12 000 *g* for 10 minutes. The pellet was washed twice with 1 ml of methanol : ethylacetate (1 : 1), and the mixture was allowed to stand for 10 minutes, and then centrifuged at 12 000 *g* for 5 minutes. The pellet was next resuspended in 700 µl of guanidine HCl (6 M guanidine in 20 mM KH_2PO_4), and incubated at 37°C for 15 minutes. The absorbance of the supernatants was read at 370 nm.

The total antioxidant capacity of the plasma was determined with the FRAP assay using 2,4,6- tripyridyltriazine (TPTZ).[14] The ferric complex of TPTZ can be reduced by plasma antioxidants at low pH to form a blue ferrous tripyridyltriazine complex with absorption maximum at 539 nm. The FRAP was calculated via calibration with Fe_2SO_4. The blank sample contained 1.5 ml of FRAP reagent (sodium acetate buffer) (300 mM, pH 3.6), $FeCl_3 \cdot 6H_2O$ (20 mM), HCl (40 mM), and TPTZ). The measured mixtures contained 1.5 ml of reagent and 50 µl of plasma. Measurements were made for 5 minutes.

The total quantity of protein was determined by the method of Lowry *et al.*[15] with the Folin–phenol reagent, bovine serum albumin being used as standard. Erythrocyte haemolysates (1000-fold dilution) and plasma (400-fold dilution) were used. All samples containing 2 ml of solution 'C' (Folin 3), 50 ml of solution 'A'(Folin 1) 0.02 g/ml of Na_2CO_3+ 0.1 ml of NaOH, and 1 ml of solution 'B' (Folin 2) 5.0×10^{-3} g/ml of $CuSO_4 \cdot 5H_2O$ were incubated at 37°C for 10 minutes. After the addition of 200 µl of 'D' (Folin–Ciocalteu

reagent: distilled water (1 : 1)), the reaction mixture was incubated at 37°C for 30 minutes. Spectrophotometric measurements were made at 750 nm.

Statistical analysis was performed with STATISTICA 9 software.

Non-parametric one-way analysis of variance was applied to evaluate whether the tested parameters were influenced by the dates of birth. In order to determine the differences between the dates of the full-term and preterm twin neonates, Mann–Whitney paired *U*-test was utilized.

Spearman's correlation test was used to explore connections between the measured biochemical parameters. Levels of significance is: $*P < 0.05$; $**P < 0.01$, and $***P < 0.001$.

Results

The activities of the red blood cell SOD (Cu, Zn-SOD) was decreased in the premature twin neonates than in the mature twins, although the difference was not significant (Table 2). There was no significant difference in CAT activity between the two groups (Table 2). The activity of GPx was increased in the preterm twins than in the mature ones. The concentration of GSH in the premature twins was likewise higher, though not significantly so (Table 2). These results revealed a weaker antioxidant capacity in premature neonates than in mature ones.

The carbonyl protein content proved to be higher in the premature twin neonates than in the mature ones (Table 2).

The significantly high LP of the red blood cells in the preterm neonates is an indication of severe oxidative damage of the membrane lipids (Table 2).

Spearman's correlation test demonstrated a significant, moderately strong positive correlation between the activities of SOD and CAT ($r = 0.52*$) in the cord blood.

A significant negative correlation was found both between the activities of SOD and GPx ($r = -0.50**$) and between the activities of CAT and GPx ($r = -0.67**$): as the activity of SOD or CAT increased the activity of GPx decreased (Fig. 1). A significant positive correlation ($r = 0.40*$) was found between the GSH concentration and the activity of GPx (Fig. 2). The negative correlation between the total antioxidant capacity and the protein carbonyl content was significant ($r = -0.46*$) (Fig. 3).

Discussion

Our investigations were carried out on cord blood samples, on the assumption that these provide adequate information about the antioxidant status of the neonates at the moment of delivery. We presumed that the activation of the antioxidant defence mechanisms may depend on many factors, e.g. the level of

Table 2 Activities of antioxidant enzymes total antioxidant capacity and parameters of oxidative damage in twin neonates

Parameters	Mature newborn twins (*n*=14)	Premature newborn twins (*n*=22)
	Mean ± SD	
Superoxide dismutase (U/mg protein)	2.42 ± 0.32	2.19 ± 0.59
Catalase (BU/mg protein)	$5.2 \times 10^{-3} \pm 1.3 \times 10^{-3}$	$5.3 \times 10^{-3} \pm 1.4 \times 10^{-4}$
Glutathione peroxidase (U/mg protein)	$3.5 \times 10^{-3} \pm 0.6 \times 10^{-3}$	$4.5 \times 10^{-3} \pm 1.3 \times 10^{-3}$*
Reduced glutathione (μmol/mg protein)	$5.2 \times 10^{-3} \pm 1.9 \times 10^{-3}$	$6.1 \times 10^{-3} \pm 0.20 \times 10^{-3}$
Total antioxidant capacity (μmol/l)	1028.4 ± 251.7	921.9 ± 241.9
Carbonyl protein (mmol/mg protein)	$5.2 \times 10^{-5} \pm 1.4 \times 10^{-5}$	$0.6 \times 10^{-5} \pm 1.7 \times 10^{-5}$
Lipid peroxidation (nmol/mg protein)	0.422 ± 0.082	0.518 ± 0.083**

*P<0.05; **P<0.01.

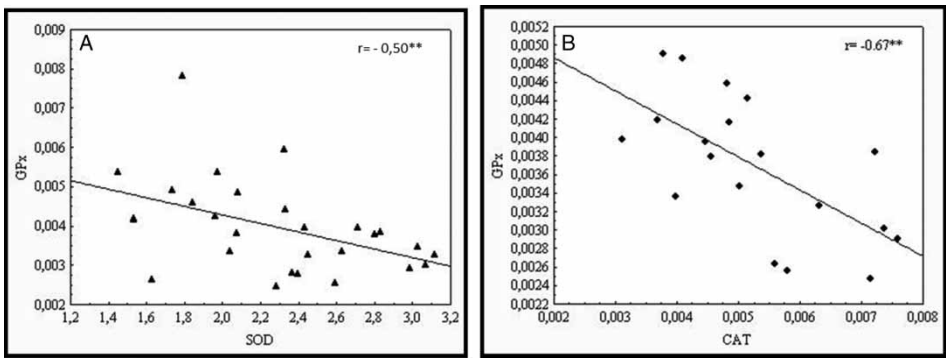

Figure 1 (A) Spearman's rank correlation between the activities of GPx and SOD. Spearman's rank correlation coefficient, $r = -0.50$; $P < 0.01$. (B) Spearman's rank correlation between the activities of GPx and CAT. Spearman's rank correlation coefficient, $r = -0.67$; $P < 0.01$.

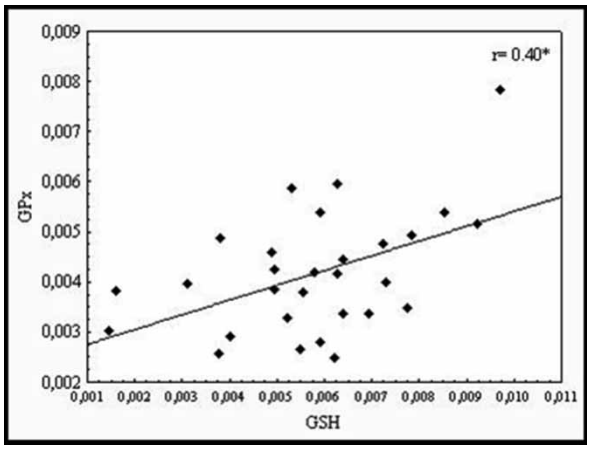

Figure 2 Spearman's rank correlation between the activities of GPx and the level of GSH. Spearman's rank correlation coefficient, $r = 0.40$; $P < 0.05$.

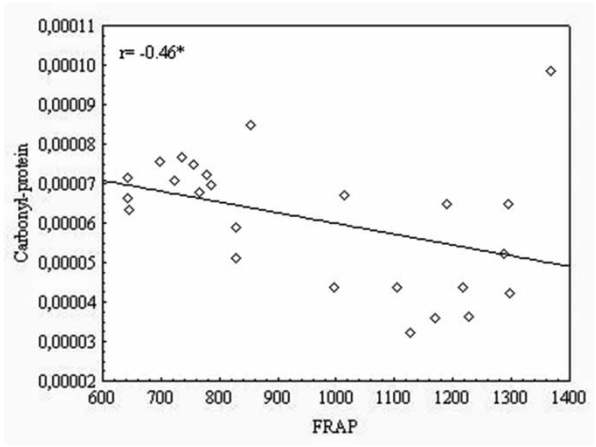

Figure 3 Spearman's rank correlation between the level of FRAP and the carbonyl protein. Spearman's rank correlation coefficient, $r = -0.46$; $P < 0.05$.

development of the foetus, the duration of labour, and the analgesia used.

Under normal conditions, there is a balance in the organism between the presence of free radicals and antioxidants, but in the event of a disturbance of this balance, oxidative stress can arise. A high level of ROS occurring for any reason can particularly affect newborn infants.[16] Although oxidative stress is a physiological event in the foetal-to-neonatal transition, many neonatal diseases are closely connected with oxidative stress. Premature infants are especially susceptible to ROS-induced damage and preterm birth is a well-known complication of a multiple pregnancy.[17] Multiple pregnancies are more exposed than single pregnancies to complications, such as gestational diabetes, IUGR, malformations, the twin–twin transfusion syndrome,[18] or preterm birth, which seems to be the greatest risk. A low birth weight and prematurity are major risk factors for perinatal mortality and morbidity in multiple pregnancies.[2] In singleton pregnancies during the third trimester, there are linear increases in growth and weight increase up to week 37 of gestation, whereas in twin pregnancies this linear growth stops at week 32 of gestation.[18]

The expression of antioxidant enzymes reaches the appropriate level only at the end of gestation, and preterm infants are therefore vulnerable to oxidative stress.[6]

An early sign of oxidative stress is the appearance of LP products. Although LP is a part of the normal metabolism, and this polyunsaturated fatty acids can be damaged significantly by free radicals as a result of oxidative stress, can ultimately lead to cell death. Significantly higher levels of LP have been reported in twin neonates than in singleton neonates, and in premature twins than in mature twin neonates.[4] Minghetti *et al.* measured 15-F_{2t}-isoprostane in the cord plasma as a marker of LP, which correlated negatively with the birth weight. We measured the levels of Malonyl-dialdehyde (MDA) in the cord blood, another index of LP. Premature twins exhibited significantly higher MDA concentrations. Both methods therefore demonstrated oxidative damage to the lipids in premature twin neonates.

The appearance of protein carbonyls is a further sign of oxidative stress. Hracsko *et al.*[19] did not detect any basic difference. Karowicz-Bilinska *et al.*[20] reported a significant difference in protein carbonyl level between normal pregnancies and those involving IUGR. Our investigations too revealed a negative correlation between the protein carbonyl concentration and the total antioxidant activity (FRAP).

The ROS and the antioxidant enzyme systems play important roles in the process of pregnancy.[21] In mammals, the enzymes SOD, GPx, and CAT are at the focus of attentions which serve to minimize the damaging effects of ROS. In our own investigation only the activity of GPx was enhanced significantly in the cord blood in premature twins, probably because of the high level of oxidized lipids. The GSH content was non-significantly increased, and the activity of SOD was decreased in the preterms, but again not significantly so (Table 2). The immaturity of SOD can result in increased damage to lipids and proteins. The Spearman correlation test demonstrated significant negative correlations between the activities of SOD and GPx, and between those of CAT and GPx (Fig. 1), and a strong positive correlation between the GPx activity and GSH content (Fig. 2).

Similar results were earlier published on placental and foetal tissues.[22–24]

Conclusions

From the results of our investigation we can conclude that the level of LP might be of clinical value as an early marker of pre- and perinatal distress in twins.

Disclaimer statements

Contributors All authors contributed equally.

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Conflicts of interest None.

Ethics approval The number of ethical approval of Ethics Committee of the Department of Obstetrics and Gynecology, Faculty of Medicine, University of Szeged is 149/2012.

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