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Oxidative Stress in Erythrocytes from Premature and Full-term Infants During their First 72 h of Life

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Objective: The aim of this study was to evaluate the extent of lipid peroxidation and the response of the enzymatic and non-enzymatic antioxidant defence system in erythrocytes from full-term and premature infants at birth, after 3 and after 72 h of life.

Study design: Twenty infants were selected and divided in two groups according to their gestational age. Blood samples were taken at birth, at 3 and at 72 h of life, erythrocytes were isolated and the following parameters were measured: fatty-acid profile, coenzyme Q, α-tocopherol, hydroperoxides and the activity of the antioxidant enzymes catalase, superoxide dismutase (SOD) and cytosolic glutathione peroxidase (cGPx).

Results: For the three studied periods, several differences between full-term and premature infants were found. Premature children showed a higher concentration of hydroperoxides, a lower level of α-tocopherol and lower SOD and cGPx activity (except for cGPx at birth). Moreover, *n*-3 polyunsaturated fatty-acids percentages (essential for good neonatal development) were higher in full term children throughout all the study.

Conclusion: Results suggest a strong imbalance between oxidants and antioxidants in premature infants during their first 72 h of life, a situation which could lead to several pathologies. Therefore, further research is needed, including possible nutritional intervention (with antioxidant therapy, supplementation of essential fatty acids and other dietary constituents) before and after birth.

Keywords: Prematurity; Lipid peroxidation; Antioxidant system; Erythrocyte; Fatty-acid profile

INTRODUCTION

Birth implies a strong oxidative stress, both for the rapid change from relatively hypoxic intra-uterine to the extra-uterine environment, where alveolar pO_2 is almost five times higher, and for the mediation of several physiologic processes involved in the finalization of the gestation and delivery.[1–3] These changes and processes greatly increase the production of free radicals, which must be controlled by the antioxidant defence system, the maturation of which follows the course of the gestation.[4,5] This oxidative environment, which increases in premature infants from birth before the 37th week of gestation, appears to involve an immaturity in enzymatic and non-enzymatic antioxidant mechanisms.[6–11] For this reason, some antioxidants, such as α-tocopherol, ascorbic acid and retinol are given to the foetus during the last period of gestation.[8,12,13] In general, health problems in premature infants increase dramatically with decreased birth weight and gestational age. Therefore, premature birth could itself be considered an illness with a major oxidative component.[14] This means that premature infants require intensive clinical care and, because of their vulnerability, they can suffer a wide range of pathologies such as retinopathy, intraventricular haemorrhage, chronic lung disease, etc.[14–16]

Although these aspects are clinically very important, data are scarce and controversial concerning

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the possible mechanisms of neonatal adaptation to the physiological oxidative stress of delivery and early postnatal life.[2] In the case of prematurity, information is even scarcer and most of the studies have been done only in plasma, focusing on infants with special pathologies,[9,10,14–16] but not on the prematurity itself. Therefore, given that around 5–7% of all infants are born prematurely and that this proportion could increase as a consequence of the rising number of multiple births due to infertility treatments,[16] the need arises to ascertain the current situation of the oxidants/antioxidants balance in premature infants and its evolution through early neonatal life.

Thus, the objective of this study was to evaluate the correlation between lipid peroxidation and the response of the enzymatic and non-enzymatic antioxidant defence system in erythrocytes from full-term and premature infants without any specific intensive reanimation, oxygen therapy or another type of medication, at birth and after 3 and 72 h of life.

MATERIAL AND METHODS

Subjects

Twenty infants were selected and divided in two groups according to their gestational age. The first group (Full-term group), consisted of 10 healthy infants born after a full-term gestation (40.4 ± 0.4 weeks), delivered without complications (eutocic delivery), with an appropriate weight (3273 ± 155 g) and with a normal clinical exploration at birth and over the experimental period. The second group (Premature group), comprised of 10 premature infants with a gestational age of 33.1 ± 1.1 weeks and a birth-weight of 1977 ± 84 g. This second group did not need specific intensive reanimation, oxygen therapy or any other type of medication at birth. The characteristics of the infants are shown in Table I. Both groups were fed with infant formula in accordance with The European Society for Paediatric Gastroenterology and Nutrition (ESPGAN) recommendations. The study was approved by the Bioethical Committee on Research Involving Human Subjects of the University Hospital "Virgen de las Nieves" of Granada, and consent was given by the parents after the nature and purpose of the study was explained and fully understood.

Analytical Methods

All the chemical products and solvents of the highest grade available were acquired from Sigma (St. Louis MO, USA) and Merck (Darmstadt, Germany).

The blood samples were taken at birth (from the umbilical cord) and at 3 and 72 h after birth (from a peripheral vein) in heparin-coated tubes. Erythrocytes were isolated by hypotonic haemolysis according to the method of Hanahan and Ekholm,[17] preserving membranes and cytosol fractions for further analyses. Membrane- and cytosol-protein concentrations were determined by the method of Lowry *et al.*,[18] using bovine serum albumin as a standard.

The content of erythrocyte hydroperoxides was determined using the method of Jiang *et al.*[19] Briefly, 100 μg of erythrocyte-membrane protein were incubated for 70 min at room temperature in the dark after adding 900 μl of the following reaction mixture: 100 μM xylenol orange, 250 μM ammonium ferrous sulphate, 25 μM H_2SO_4 and 4 mM butylated hydroxytoluene (BHT) in 90% (v/v) methanol. Under these acidic conditions there, is a rapid oxidation of Fe^{2+}–Fe^{3+} by hydroperoxides. Fe^{3+} forms a chromophore with xylenol orange that strongly absorbs at 560 nm. *Tert*-butyl hydroperoxide was used to prepare a standard curve.

The fatty-acid profile of erythrocyte membranes was measured by gas–liquid chromatography as described by Lepage and Roy.[20] A gas–liquid Chromatograph model HP-5890 Series II

TABLE I Characteristics of full-term and premature infants

Full-term group				Premature group			
Infant	Weight (g)	Gestational age (weeks)	Apgar test	Infant	Weight (g)	Gestational age (weeks)	Apgar test
1	2530.0	39.0	9–9	1	1520.0	32.9	9–9
2	2800.0	40.0	9–9	2	2400.0	34.0	9–9
3	3070.0	41.9	9–9	3	1860.0	34.0	7–9
4	3570.0	40.0	9–9	4	1900.0	31.6	9–9
5	3230.0	38.0	9–9	5	1645.0	34.0	9–9
6	3200.0	41.0	8–9	6	2080.0	34.0	6–8
7	2750.0	41.0	9–9	7	1900.0	35.6	9–9
8	3800.0	40.6	9–9	8	2150.0	35.6	9–9
9	3300.0	41.9	9–9	9	2200.0	35.7	9–9
10	4120.0	40.6	9–9	10	2120.0	34.0	9–9
Mean	3273.0	40.4		Mean	1977.5	33.1	
SEM	155.2	0.4		SEM	83.9	1.1	

(Hewlett Packard, Palo Alto, CA, USA) equipped with a flame ionisation detector was used to analyse the fatty acids as methyl esters. Chromatography was performed using a 60-m-long capillary column; 32 mm id and 20 mm thickness impregnated with Sp 2330™ FS (Supelco Inc., Bellefonte, Palo Alto, CA, USA). The data are expressed as a percentage of the total fatty acids determined.

Coenzyme Q and α-tocopherol in erythrocyte membrane were assayed by high-performance liquid chromatography (HPLC), after extraction with ethanol:petroleum ether (60:40) using the method of Kröger.[21] Briefly, after centrifugation at 2500*g* for 5 min, the upper layer was collected by aspiration and the residue was re-extracted twice with 1 ml of petroleum ether. The dry residue of combined extracts was diluted in the mobile phase (ethanol: water, 97:3). The HPLC system consisted of an apparatus equipped with a Diode Array detector, model 168 (Beckman Instruments, Inc., Fullerton, CA, USA) and the column was a reverse-phase C18 Spherisorb ODS 1 of 25 × 0.46 cm.

Catalase activity was determined following the method described by Aebi,[22] by monitoring at 240 nm the H_2O_2 decomposition, as a consequence of the catalytic activity of catalase. Superoxide dismutase (SOD) was determined by the method of Crapo *et al.*,[23] based on the inhibition by SOD of the reduction of cytochrome *c*, measured by spectrophotometry at 550 nm. Haemoglobin interference is avoided since for this assay haemoglobin was precipitated in the sample by adding chloroform–ethanol, following the recommendations of several authors when this assay is done in erythrocytes.[23] For cytosolic glutathione peroxidase (cGPx), we used the technique of Flohé and Gunzler,[24] a method based on the instantaneous formation of oxidized glutathione during the reaction catalysed by glutathione peroxidase, which is continually reduced by an excess of active glutathione reductase and NADPH present in the cuvette. The subsequent oxidation of NADPH to $NADP^+$ was monitored spectrophotometrically at 340 nm. *Tert*-butyl hydroperoxide was used as substrate.

Statistical Analysis

Results are presented as the mean ± standard error of the mean (SEM). One-way analysis of variance was used to test the time-dependent changes within a group and Bonferroni correction was used in multiple comparisons. Statistically significant differences between groups for each time period were determined with Student's *t*-test. In all cases, statistical differences were considered for $P < 0.05$. The statistical treatments were carried out using the SPSS package (SPSS for Windows, 10.0.6, 1999, SPSS Inc., IL, USA).

RESULTS

Table II show the fatty-acid profile in erythrocyte membranes. Differences between groups were found in the percentage of saturated (SFA) and monounsaturated fatty acids (MUFA) percentage at birth. The full-term group registered the highest SFA percentage and the premature group the highest percentage of MUFA, with significant differences ($P < 0.05$) in both cases. However, after 72 h of life no statistically significant differences in these fatty-acid indices between the groups were found. Total percentage of polyunsaturated fatty acids (PUFA) did not show significant differences between the groups, although there were significant differences ($P < 0.05$) with respect to the percentage of both *n*-6 polyunsaturated fatty acids (*n*-6 PUFA) and *n*-3 polyunsaturated fatty acids (*n*-3 PUFA). At birth, the premature group showed a higher percentage of *n*-6 PUFA with statistically significant differences with respect to the full-term group. By contrast, the full-term group showed a higher percentage of *n*-3 PUFA throughout the study, with significant differences with respect to those of premature group.

Erythrocyte-hydroperoxide content is shown in Fig. 1. The premature group showed higher values during the study. The greatest difference between the two groups ($P < 0.05$) was found at 72 h of life (14.3 ± 1.0 μmol/mg for premature group and

TABLE II Fatty-acid indices of erythrocyte membrane from full-term and premature infants

	Full-term group			Premature group		
	Birth	3 h	72 h	Birth	3 h	72 h
SFA (%)	50.5 ± 0.9*	49.2 ± 0.9	49.1 ± 0.8	47.5 ± 0.3	45.9 ± 1.5	47.2 ± 0.7
MUFA (%)	16.1 ± 0.5[a]*	16.8 ± 0.2[a]*	17.9 ± 0.5[b]	18.6 ± 0.5	19.3 ± 1.5	19.1 ± 0.7
PUFA (%)	35.2 ± 1.5	34.9 ± 1.9	34.2 ± 1.1	34.5 ± 0.4	32.2 ± 2.2	34.2 ± 0.9
n-6 PUFA (%)	23.9 ± 1.3*	22.9 ± 1.2	22.9 ± 1.7	28.7 ± 0.9	25.5 ± 2.6	25.3 ± 1.6
n-3 PUFA (%)	11.8 ± 1.4*	12.1 ± 1.3*	11.5 ± 0.9*	5.3 ± 0.4	5.1 ± 0.5	4.7 ± 0.2

Values are expressed as mean ± SEM. Non-coinciding letters (a, b, c) in each group for each parameter indicate time-dependent significant statistical differences ($P < 0.05$). *Indicates significant differences ($P < 0.05$) between groups for the same period of time. SFA, Saturated fatty acids; MUFA, Monounsaturated fatty acids; PUFA, Polyunsaturated fatty acids.

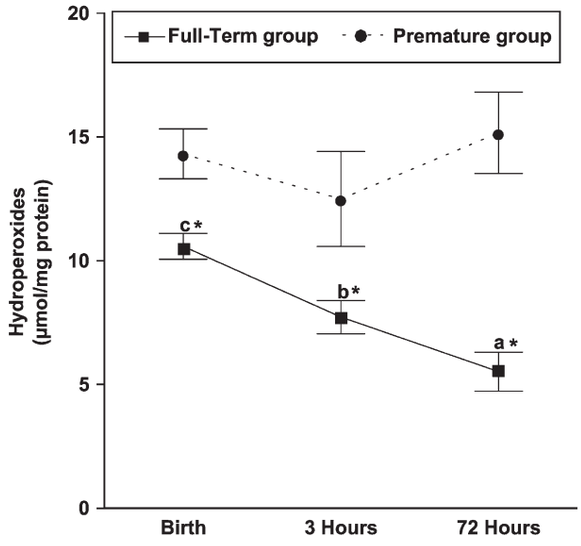

FIGURE 1 Hydroperoxide content in erythrocyte membrane from full-term and premature infants. Values are expressed as the mean ± SEM. Non-coinciding letters (a, b, c) in each group for each parameter indicate time-dependent significantly statistical differences ($P < 0.05$). *indicates significant differences ($P < 0.05$) between groups for the same period of time.

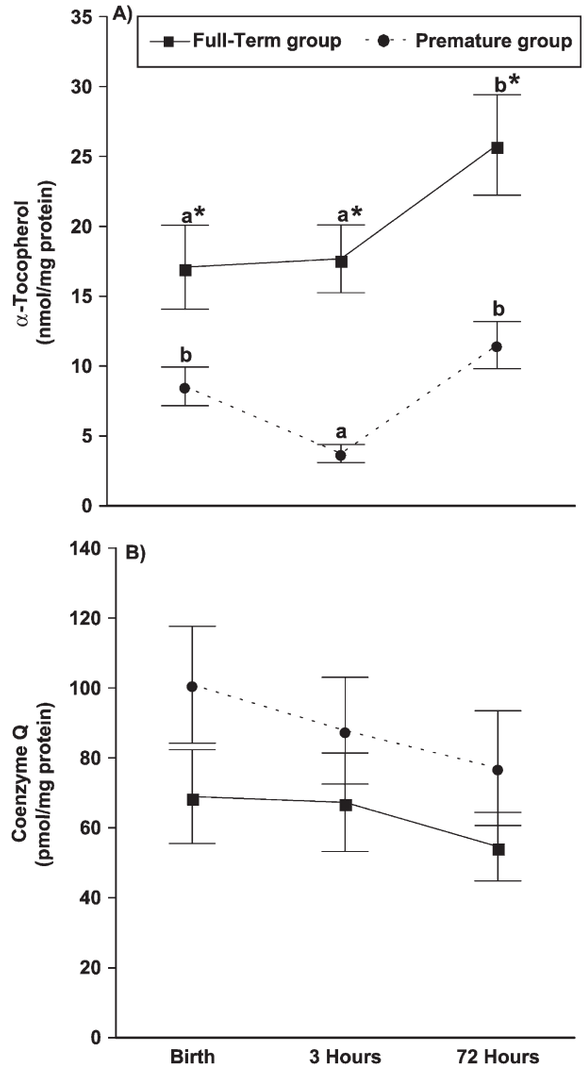

FIGURE 2 Content of α-tocopherol (A) and Coenzyme Q (B) in erythrocyte membrane from full-term and premature infants. Values are expressed as the mean ± SEM. Non-coinciding letters (a, b, c) in each group for each parameter indicate time-dependent significantly statistical differences ($P < 0.05$). *indicates significant differences ($P < 0.05$) between groups for the same period of time.

5.5 ± 0.8 µmol/mg for full-term group). There were no difference among the hydroperoxide values for premature group at the different time periods. However, the full-term group decreased in the hydroperoxide content during the study with the lowest value at 72 h.

The erythrocyte-membrane non-enzymatic antioxidants studied (α-tocopherol and Coenzyme Q) are shown in Fig. 2. With respect to α-tocopherol (Fig. 2A), the full-term group registered significantly higher values ($P < 0.05$) for all periods of time than did the premature group, with the maximum at 72 h of life (25.8 ± 3.6 vs. 11.5 ± 1.7 mmol/mg). The full-term group increased in α-tocopherol values during the study, with statistically significant differences between the values at birth and at 72 h (17.1 ± 3.0 and 25.8 ± 3.6 nmol/mg, respectively). However, the premature group significantly decreased at 3 h compared to birth (3.7 ± 0.6 and 8.5 ± 11.4 nmol/mg, respectively) ($P < 0.05$). Coenzyme Q content (Fig. 2B) did not show significant differences between groups and among time periods for each group.

The activity of antioxidant enzymes catalase, SOD and cGPx are shown in Table III. Catalase activity was similar for both groups at the different time periods. SOD activity proved significantly higher in the full-term group, in all samples, than in the premature group. cGPx activity was similar between groups at birth. However, at 3 and at 72 h of life these values were significantly higher in the full-term group than in the premature group. In addition, cGPx activity sharply decreased at 3 and 72 h in the premature group, significantly differing with regard to the cGPx activity at birth.

DISCUSSION

Birth involves strong physiological stress for the infant, implying an increased production of free radicals that must be controlled by the infant's antioxidant system.[1–5] This could lead to several functional alternations with important repercussions for the organism.[1–3] In the case of premature infants, these repercussions are more acute.[6,11,14–16]

TABLE III Activity of catalase, superoxide dismutase (SOD) and cytosolic glutahione peroxidase (cGPx) enzymes in erythrocytes from full-term and premature infants

	Full-term group			Premature group		
	Birth	3 h	72 h	Birth	3 h	72 h
Catalase (k (s^{-1})/mg protein)	0.38 ± 0.09	0.36 ± 0.09	0.23 ± 0.04	0.37 ± 0.07	0.22 ± 0.01	0.27 ± 0.04
SOD (U/mg protein)	10.79 ± 1.59*	10.13 ± 1.34*	9.45 ± 0.97*	5.22 ± 0.77	3.77 ± 0.40	4.03 ± 0.45
CGPx (U/mg protein)	45.45 ± 5.95*	38.61 ± 3.90*	43.84 ± 5.35*	45.76 ± 8.00[b]	28.85 ± 2.85[a]	28.54 ± 3.21[a]

Values are expressed as mean ± SEM. Non-coinciding letters (a, b, c) in each group for each parameter indicate time-dependent significant statistical differences ($P < 0.05$). *indicates significant differences ($P < 0.05$) between groups for the same period of time.

A possible marker for the lipid-peroxidation susceptibility of cell membranes is their fatty-acid profile, since it is well known that an increase in the number of double bonds in a fatty acid augments its oxidative potential.[25] In our study, erythrocyte membranes from both groups had similar percentages of total saturated and monounsaturated fatty-acid indices. This situation is similar to those reported in other studies conducted with premature and full-term neonates.[26,27] With respect to total polyunsaturated fatty acids, the two experimental groups did not differ, as in other studies.[28,29]

However, despite of this similar content in total polyunsaturated fatty acids in the infants, differences were found between the two groups for *n*-6 PUFA and *n*-3 PUFA indices. These results might have minor consequences in terms of lipid peroxidation, but could be crucial for correct neonate development.[28,29] The biggest difference has been found for *n*-3 PUFA, which remained at low levels in the premature group even after 72 h of life. This is noteworthy because this fatty-acid fraction represents essential fatty acids with tremendous influence on the normal development of the eyes and the brain in the child.[28,29]

From the standpoint of lipid peroxidation, a similar PUFA content between the groups should lead to erythrocyte membranes with a similar susceptibility to lipid peroxidation.[25] However, the hydroperoxide content found in erythrocytes showed a higher degree of lipid peroxidation in premature infants, these levels remaining higher throughout the study. In accordance with our results, others studies have also reported a high oxidative stress in premature newborns.[9,10] The explanation for this higher peroxidation in premature infants may reside in the concentration and activity of different members of the antioxidant system. In fact, the very important membrane antioxidant α-tocopherol was found in a lower concentration in the premature group (around 50%) compared to full term-infants. This low α-tocopherol level could be a consequence of a low contribution of this vitamin to the foetus from the mother, being a direct consequence of the prematurity itself, because it is well-known that α-tocopherol is supplied to the foetus during the last period of gestation.[8,13] Other data concerning α-tocopherol is that this antioxidant remained low during the first 72 h of life, this being noteworthy because the content of α-tocopherol in the nutritional formula for premature infants was higher than in the formula for full-term infants (13.0 and 6.2 μg/100 g, respectively). This could indicate some problems with the intestinal absorption in premature infants (it has been documented that gestational age is critical for the intestinal absorption of this vitamin[30]) or just a higher demand. In any case, this is a risk situation for the integrity of the erythrocyte membranes and should considered. No differences were found concerning the other lipid-soluble antioxidant studied, coenzyme Q, although we cannot rule out a possible role for this antioxidant molecule during the neonatal life.

The study of antioxidant enzymes showed, in general, a low activity of these enzymes in premature infants. SOD activity was lower for all periods (around a 50%, compared with full-term infants). This situation is not desirable, because premature infants show a high activity of the xanthine/xanthine oxidase system and therefore a high production of superoxide anion.[31] The low activity of this antioxidant enzyme at birth in premature infants has been previously described,[32] and could be responsible (at least in part) for the lower activity that this enzyme shown at 3 and 72 h, together with possible inadequate mechanisms of support of enzyme activity in this group such as a possible nutrient deficit in zinc or copper.[32]

Similar cGPx activity was found in both groups at birth. However, activity fell sharply at 3 and 72 h in premature infants. The decrease in the cGPx activity could be a consequence of its use together with a possible glutathione deficit in this group, as has been reported by several authors.[33] This could lead to an inadequate activity of the glutathione system and therefore less regeneration of glutathione peroxidase.

In summary, our data show that despite the improvement in the acute management of premature infants that has led to less severe pathologies, there is a strong imbalance between free radicals production and antioxidant-defence system in these infants.

This unbalance is not restricted only to the birth (as described in other studies) but remains even after 72 h of life, a situation which could lead to several functional repercussions. In addition, inadequate content in some essential fatty acids is observed in premature infants. Therefore, further research in this matter is needed, including possible nutritional intervention (with antioxidant therapy, supplementation in essential fatty acids and other dietary constituents) before and after birth.

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