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Fluidity and oxidative stress in erythrocytes from very low birth weight infants during their first 7 days of life

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Abstract

Objective: To study the evolution of lipid peroxidation, enzymatic antioxidants response, lipid profile and membrane fluidity in erythrocytes from very low birth weight (VLBW) infants during their first 7 days of extra-uterine life.

Study design: One hundred and twenty infants were selected and divided in two groups according to their weight and gestational age. Hydroperoxides, fatty-acid profile, fluidity (DPH and TMA-DPH) and catalase, SOD and GPx activities were measured in erythrocytes.

Results: VLBW group showed higher concentration of hydroperoxides and lower membrane fluidity during the first 72 h, lower SOD activity during the first 3 h and higher GPx activity during the first 7 days of life. Also, this group showed lower n-3 polyunsaturated fatty-acids percentage with respect to the term group.

Conclusion: Erythrocytes from VLBW infants showed higher oxidative damage and lower fluidity in their membranes, at least during the first 3 days of extra-uterine life, which may cause alterations in their functions and flexibility.

Keywords: *Very low birth weight infants, lipid peroxidation, erythrocyte, enzymatic antioxidant system, fluidity, fatty-acid profile*

Introduction

Birth produces considerable oxidative stress, both because of the rapid change from the relatively hypoxic intra-uterine environment to the extra-uterine one and because of the mediation of the diverse physiological processes involved in the finalization of gestation, followed by delivery [1,2]. In pre-term infants, the effect of this oxidative environment is heightened by various factors which, together with the immaturity of certain organs, means that prematurity in itself may be considered a pathologic state [3–8]. Thus, many pre-term infants require intensive clinical care and more so with lower birth weight and gestational age; due to their vulnerability, these infants are liable to suffer a wide range of disorders such as retinopathy, intraventricular haemorrhage, chronic lung disease, etc., with a significant oxidative component [5,6,9].

Nevertheless, despite the importance of oxidative stress in the pre-term infant, scientific knowledge of the question is still very limited in certain aspects. Few in-depth studies have been made of the evolution of oxidative stress during the neonatal period, with

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most such studies focusing their attention on umbilical cord parameters, on aspects relating to the first hours of life [7,10,11] or on pre-term infants studied on the basis of a specific pathology [5,8,12–15] and not on the prematurity in itself. Moreover, most studies in this field examine plasma parameters, related to oxidative stress, that are readily modified by external factors [15,16], while studies of erythrocytes, which are more stable and precise, are still few and far between. These aspects are of even greater importance as regards very low birth weight (VLBW) infants (< 1500 g, according to the WHO definition), which have been the object of very few studies [3,17,18]. Another aspect that has been largely overlooked in the pre-term infant is that of possible alterations in membrane fluidity of the erythrocyte during the first days of extra-uterine life. Erythrocytes must continually adapt to changes in the circulatory system, such as turbulences and changes in capillary diameter, and so require special characteristics of their membranes that allow them to respond adequately to such variations in their environment [19]. In addition, an essential factor for the maintenance of their functions is that there should be no damage or loss of structural integrity to the membranes [20]. In short, adequate membrane fluidity is necessary to maintain erythrocyte flexibility and correct functioning. Therefore, it is highly important to study the parameters related to changes in erythrocyte fluidity, especially lipid peroxidation and the lipid profile of the membrane [20,21].

For all these reasons, the present paper examines the evolution of lipid peroxidation and the enzymatic antioxidant defence system response, as well as variations in the lipid profile and membrane fluidity in erythrocytes obtained from very low birth weight infants during their first 7 days of extra-uterine life, in comparison with a group of healthy, full-term infants.

Materials and methods

Subjects

A 9-month study was made of 120 infants, grouped in accordance with their birth weight and gestational age. The full-term group (control group) was made up of 63 healthy, full-term infants, after a gestation and delivery with no complications. From this group, we obtained samples of venous cord blood at 0 h and at 3 h of life. A second group (VLBW group) was constituted of 57 pre-term infants, with gestational ages ranging from 26–33 weeks and with a birth weight of less than 1500 g. During the period in which samples were taken, these infants were being treated in the Intensive Care Unit. Cord blood samples were taken at 0 h, 3 h, 72 h and 7 days of extra-uterine life. Table I shows the weight, gestational age and the results of the Apgar test in the infants. The Apgar test is a measurement of a newborn's response to birth and life outside the womb. Ratings are based on the following factors: heart rate, breathing, activity and muscle tone, grimace response and appearance. The high score is 10 and the low end is 1. This test is usually performed at 1 min after birth (Apgar 1) and again at 5 min after birth (Apgar 5).

Table I. Characteristics of full-term infants and very low birth weight infants.

	VLBW group		Term group	
	M	SEM	*M*	SEM
Weight (g)	1053	22.9	2987	75.6
Gestational age (weeks)	28.8	0.2	38.1	0.2
Apgar 1	5.3	0.3	9.5	0.1
Apgar 5	8.3	0.2	9.8	0.1

The study was approved by the Bioethical Committee on Research Involving Human Subjects at the University Hospital 'Virgen de las Nieves' in Granada and consent was obtained from the parents after the nature and purpose of the study had been explained to them and were fully understood.

Analytical methods

All the chemical products and solvents were acquired from Sigma (St. Louis, MO) and Merck (Darmstadt, Germany). The probes 1,6-diphenyl-1,3,5-hexatriene (DPH) and 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene (TMA-DPH) were obtained from Molecular Probe (Eugene, OR).

The blood samples were obtained in heparined tubes. Membrane and cytosol erythrocytes were isolated by hypotonic haemolysis as previously described [10]. Membrane and cytosol protein concentrations were determined by the method of Lowry et al. [22]. The content of erythrocyte hydroperoxides was determined using the method of Jiang et al. [23]. This technique is based on the rapid oxidation of Fe^{2+} to Fe^{3+} by hydroperoxides under acid conditions and on spectrophotometric quantification at 560 nm.

The fatty-acid profile of the erythrocyte membrane was measured by gas-liquid chromatography as previously described [10]. The data are expressed as a percentage of the total fatty acids determined. The determination of the peroxidizability index (PI) was performed as described previously [24]. In brief, the PI = (% dienoic acid × 1) + (% trienoic acid × 2) + (% tetraenoic acid × 3) + (% pentanoic acid × 4) + (% hexaenoic acid × 5).

Catalase activity was determined following the method described by Aebi [25], based on monitoring the H_20_2 decomposition at 240 nm, subsequent to the catalytic activity of the catalase. Superoxide dismutase (SOD) was determined by the method of Fridovich [26], based on the inhibition by SOD

of the reduction of cytochrome C, measured by spectrophotometry at 550 nm. For glutathione peroxidase (GPX), we used the technique of Flohé and Gunzler [27], a method based on the instantaneous formation of oxidized glutathione during the reaction catalysed by glutathione peroxidase.

Membrane fluidity was measured by fluorescence spectroscopy following a technique described previously [20]. Erythrocyte membranes (0.2 mg/ml) were resuspended in Tris-HCl buffer, vortexed for 1 min in the presence of DPH or TMA-DPH and then incubated with shaking at 37°C for 30 min. Fluorescence polarization (P) measurements were performed using a Perkin Elmer LS 50 Luminescence Spectrometer. Excitation and emission wavelengths were 365 nm and 430 nm, respectively. P was obtained from the fluorescence intensities parallel (I_{VV}) and perpendicular (I_{VH}) to the polarization direction of excitation light using the equation

$$P = (I_{VV} - GI_{VH}) - (I_{VV} + GI_{VH}),$$

where G is an instrumental correction factor. In general, an increase in P is related to a decrease in membrane fluidity [20].

Statistical analysis

Results are presented as mean ± standard error of the mean (SEM). One-way analysis of the variance was used to test the time-dependent change in the <1500 g group. Statistically significant differences ($p < 0.05$) in the term group and between groups (0 h vs 0 h and 3 h vs 3 h, 72 h and 7 days) were determined by means of Student's t-test, and Bonferroni correction was used in the <1500 g group. The statistical analysis was carried out using the SPSS package (SSPSS for Windows, 13.0, 2004, SPSS Inc., IL).

Results

Table II shows the erythrocyte membrane lipid profile. The pre-term infants, at 7 days of life, presented a lower percentage of n-3 series polyunsaturated fatty acids (n-3 PUFA) than at the other sample times, these differences being statistically significant, together with a lower peroxidizability index (PI), although the latter only varied with respect to the sample taken at 3 h. The analysis of the inter-group differences that were statistically significant showed that the pre-term infants had a lower percentage of PUFA at 0 h and of n-3 PUFA and PI in all the samples, together with a higher percentage of monounsaturated fatty acids (MUFA) throughout the study period.

Among the full-term group, the levels of membrane hydroperoxides fell significantly by 3 h of life (Figure 1). In the pre-term group, at 7 days of life there was a lower hydroperoxide content, with statistically significant differences, at 7 days of life, with respect to the values measured at 0 and 3 h of life. The inter-group comparison revealed a higher content of hydroperoxides in the pre-term infants at 0, 3 and 72 h of life, with respect to the full-term infants at 0 and 3 h of life.

In the full-term group, the antioxidant enzymes that were studied (Figure 2) only presented a statistically significant decrease at 3 h of life for the activity of glutathione peroxidase. On the other hand, in the VLBW group (Figure 2), the maximum value for the activity of superoxide dismutase was recorded at 7 days of life, with differences that were statistically significant with respect to the values for the other sample times; the maximum value for the activity of glutathione peroxidase was recorded at 0 h of life, with differences that were statistically significant with respect to the values for the other sample times. As concerns the two groups, the results for the VLBW group revealed a lower level of superoxide dismutase activity at 0 and 3 h of life and a higher degree of glutathione peroxidise in all cases.

Finally, Figure 3 shows the results obtained for the two fluidity probes utilized (DPH and TMA-DPH). Among the pre-term group, the DPH probe revealed a progressive increase in membrane fluidity (a fall in P) during the first 7 days of life, with statistically significant differences at 72 h, with respect to 0 h and 7 days. For the same group, the TMA-DPH probe

Table II. Fatty-acid indices of erythrocyte membranes in full-term infants and very low birth weight infants.

	VLBW group				Term group	
	0 h	3 h	72 h	7 days	0 h	3 h
SFA (%)	53.8 ± 1.1	53.5 ± 0.8	54.9 ± 0.9	54.4 ± 1.1	53.7 ± 0.7	54.5 ± 0.3
MUFA (%)	16.5 ± 0.8†	15.2 ± 0.5‡	15.1 ± 0.3‡	16.1 ± 0.4‡	13.1 ± 0.3	13.4 ± 0.5
PUFA (%)	29.8 ± 1.5†	31.2 ± 1.0	30.0 ± 1.0	29.5 ± 1.4	32.8 ± 0.6	32.1 ± 0.8
n-3 PUFA (%)	4.7 ± 0.2†^{a}	4.5 ± 0.2‡^{a}	4.2 ± 0.2‡^{a}	3.2 ± 0.3‡^{b}	5.5 ± 0.2	5.3 ± 0.2
PI	87.6 ± 4.9†^{ab}	90.3 ± 1.3‡^{b}	88.1 ± 2.8‡^{ab}	78.8 ± 4.2‡^{a}	98.7 ± 2.2	96.1 ± 1.4

Values are expressed as mean ± SEM. Non-coinciding letters (a, b, c) in <1500 g group for each parameter indicate time-dependent significant statistical differences ($p < 0.05$). *Significant differences in term group. †Significant differences between group at 0 h ($p < 0.05$). ‡Significant differences 3 h, 3 days and 7 days in <1500 g group vs 3 h in term group.
SFA: Saturated fatty acids; MUFA: Monounsaturated fatty acids; PUFA: Polyunsaturated fatty acids; PI: Peroxidizability index.

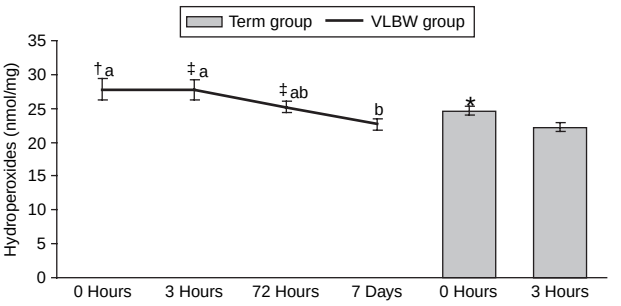

Figure 1. Hydroperoxide content in erythrocyte membrane in full-term infants and very low birth weight infants. Values are expressed as mean ± SEM. Non-coinciding letters (A, B, C) in the VLBW group for each parameter indicate time-dependent significant statistical differences ($p < 0.05$). ⋆ Significant differences in full-term group. † Significant differences between groups at 0 h ($p < 0.05$). ‡ Significant differences at 3 h, 3 days and 7 days in VLBW group vs 3 h in full-term group.

measured a maximum value (lowest degree of fluidity) at 0 h, with statistically significant differences with respect to all the other times. In the inter-group comparison, the DPH revealed a lower level of

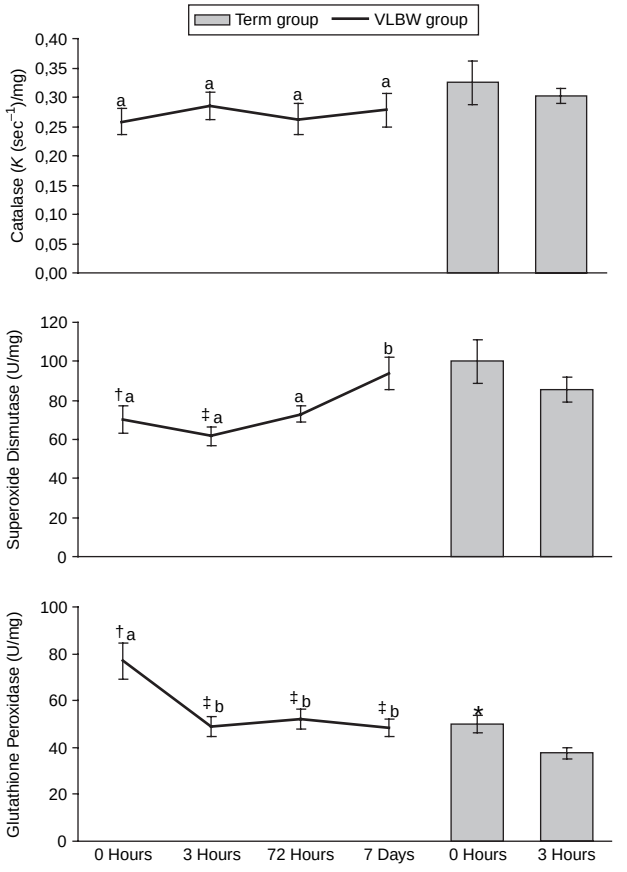

Figure 2. Activity of catalase, superoxide dismutase and glutathione peroxidase enzymes in erythrocytes in full-term infants and very low birth weight infants. Values are expressed as mean ± SEM. Non-coinciding letters (A, B, C) in VLBW group for each parameter indicate time-dependent significant statistical differences ($p < 0.05$). ⋆ Significant differences in full-term group. † Significant differences between groups at 0 h ($p < 0.05$). ‡ Significant differences at 3 h, 3 days and 7 days in < VLBW group vs 3 h in full-term group.

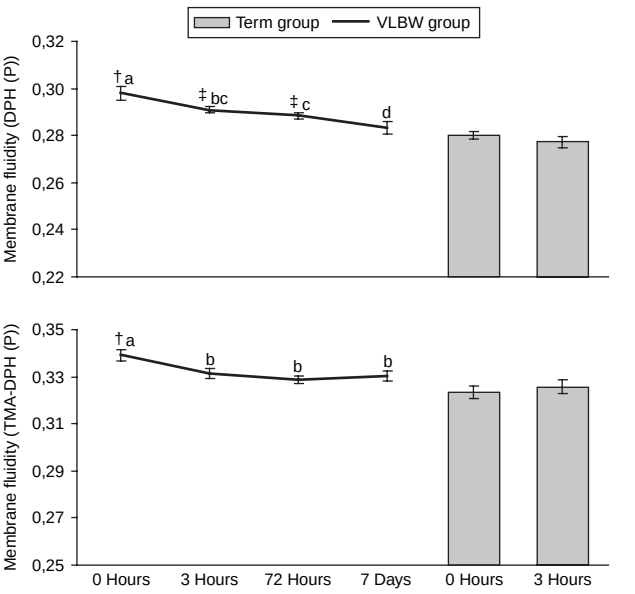

Figure 3. Fluorescent polarization (*P*) of DPH and TMA-DPH in erythrocyte membranes in full-term infants and very low birth weight infants. Values are expressed as mean ± SEM. Non-coinciding letters (A, B, C) in VLBW group for each parameter indicate time-dependent significant statistical differences ($p < 0.05$). ⋆ Significant differences in full-term group. † Significant differences between groups at 0 h ($p < 0.05$). ‡ Significant differences at 3 h, 3 days and 7 days in VLBW group vs 3 h in full-term group.

fluidity in the VLBW group at 0, 3 and 72 h of life, while the TMA-DPH probe only found this difference at 0 h.

Discussion

Few in-depth studies have been made of such important questions as the evolution of oxidative stress or its effect on the membrane fluidity of the erythrocyte during the first days of extra-uterine life in very low birth weight infants. Moreover, taking into consideration the greater susceptibility of erythrocytes to haemolysis in the presence of free radicals [5] and their need for adequate fluidity in order to function correctly [19–21], a factor that is of particular importance given the respiratory problems faced by such infants, the need for such focused studies is evident.

The hydroperoxide content was utilized as an indicator of the degree of oxidative stress in the erythrocyte; in very low birth weight infants there was found to be a higher degree of oxidative damage than in the full-term infant. These data corroborate the observations of other authors [3,5,7]. Furthermore, they reveal that, although the full-term infant needs just 3 h for hydroperoxide levels to fall, the very low birth weight infant, even at 3 days of extra-uterine life, still presents high levels of hydroperoxides.

The reason for this could be the greater production of free radicals in the pre-term infant [3,5,6],

although we should also consider the possibility of the greater susceptibility of such infants' membranes to lipid peroxidation, as well as the fact that they have fewer antioxidant defences.

The best indicator of the susceptibility of membranes to oxidative damage is the fatty-acids composition and especially the peroxidizability index (PI) [24], as the greater number of double bonds increases their oxidative potential [28]. However, this index shows that the membranes of VLBW infants are, in fact, less susceptible, as their measured PI is lower than that recorded for full-term infants. This lower index value is due to the presence in the VLWB group of a lower percentage of highly unsaturated fatty acids (n-3 PUFA), which are more vulnerable to attack by free radicals [28], while there is a higher proportion of monounsaturated fatty acids, which are less susceptible to oxidative damage [29]. With respect to membrane fluidity, the monounsaturated fatty acids effect is closer to that of polyunsaturated fatty acids than to saturated fatty acids. Nevertheless, it should be noted that, although from the oxidative standpoint a smaller proportion of n-3 PUFA is beneficial, from that of normal development, especially that of the eyes and the brain, it is not so beneficial for the infant, given the great importance of these essential fatty acids for these organs [30,31].

Studies of the enzymatic antioxidant system in the pre-term infant have aroused some controversy, partly because many studies have drawn conclusions after analysing the activity of a single enzyme [14,18] and partly because of the selection criteria utilized or the gestational age of the infants in the study [4,6,7,9,16]. In the case of catalase, no differences were observed, either between the groups or as regards its evolution, this finding corroborating that of a previous study [7]. On the contrary, glutathione peroxidase (GPx), for all sample times, presented a higher level of activity in the very low birth weight infants, although this fell after 0 h. This higher level of activity contradicts the results reported in other studies [7]. A possible explanation for this activity could be found in studies of the development of the activity of this enzyme during gestation, with maximum activity between weeks 26–35, these levels being even higher than those recorded at the moment of delivery for the full-term infant [32]. On the other hand, superoxide dismutase activity in the VLBW group was lower, at least during the first 3 days of life, which is in accordance with the findings of other studies [7,18]. Taking into account that this antioxidant enzyme plays a crucial role in antioxidant defence [14] and that pre-term infants present a high level of activity of the xanthine/xanthine oxidase system and, therefore, a high production of the superoxide anion [7], this situation is not a desirable one for the infant. Indeed, this enzyme is the only one to show an inverse correlation with the hydroperoxide content ($r = -255$, $p < 0.01$). Thus, although we cannot categorically speak of a weaker antioxidant enzyme defence system in the very low birth weight infant, it is definitely one that is less efficient and especially so because of its lower level of SOD activity.

Finally, while it is important to study the evolution of lipoperoxidation in the erythrocytes of the very low birth weight infant and some of its causes, it is even more important to study the possible repercussions on the functionality of these cells. One of these possible repercussions can be found in the membrane fluidity, bearing in mind the importance of this factor for the correct functioning of the erythrocyte [19–21]. To study membrane fluidity, we used two different fluorescent probes; DPH, which is preferentially situated in the inner apolar core at different positions along the normal membrane and TMA-DPH, which is a cationic derivate of DPH that remains anchored at the lipid–water interface of the membrane, with the hydrocarbon chain entering the lipid part of the membrane [33]. The results obtained show that at birth there is an increase in polarization or membrane rigidity with both probes revealing a lower percentage of polyunsaturated fatty acids and a higher proportion of hydroperoxides. Both parameters are two of the most important causes of alterations in membrane fluidity [19–21,33], together with cholesterol levels. These latter did not present any differences in the present study (data not shown). However, after 0 h, the increase in membrane rigidity was only observed with the DPH probe, which is more sensitive to oxidative damage than is TMA-DPH [19,33]. Logically, the effects of lipid peroxidation are more apparent in the hydrophobic centre of the membrane, which is where part of the double bonds of fatty acids in the membrane is situated and thus where oxidative reactions take place.

In conclusion, despite presenting membranes that are less susceptible to oxidative damage, pre-term infants weighing < 1500 g at birth have a higher hydroperoxide content, perhaps because their enzymatic antioxidant system is less efficient, and in consequence the membranes are more rigid, at least during the first 3 days of extra-uterine life. This greater rigidity may cause alterations in erythrocyte functions [20,33], contributing to reducing their flexibility and thus increasing blood viscosity [19], aspects which are not at all desirable for such infants.

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