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The Effect of Gestational Age and Labour on Markers of Lipid and Protein Oxidation in Cord Plasma

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There are many potential sources of reactive oxidants around the time of birth and pre-term infants are considered to be particularly vulnerable to oxidative injury. To gain insight into these processes, we have measured biomarkers of lipid and protein oxidation in umbilical cord plasma and related concentrations to mode of delivery and gestational age. Protein carbonyls were measured by ELISA and malondialdehyde (MDA) by HPLC after reaction with thiobarbituric acid, for 54 pre-term (≤36 weeks gestational age) and 43 term infants. Protein carbonyls were significantly lower in pre-term (median for <32 weeks gestational age 0.048 nmol/mg protein) than in term infants (0.105 nmol/mg, $p = 0.004$), and were unrelated to mode of delivery. In contrast, MDA concentrations were higher in the very pre-term (<32 weeks gestation) group (2.47 compared with 1.83 μM for term infants, $p < 0.0001$). MDA concentrations were higher in infants who were born with labour compared with elective caesarean section. Pre-eclampsia in the mother was associated with higher cord blood MDA concentrations. The MDA results are consistent with other studies of this marker and could be interpreted as indicating increased oxidative stress associated with prematurity and labour. However, the lower protein carbonyls in pre-term infants would lead to an opposite interpretation. More information is needed on the source and fate of these and other biomarkers before drawing strong conclusions on how they reflect oxidative stress in this and other clinical situations.

Keywords: Pre-term infants; Pre-eclampsia; Malondialdehyde; Protein carbonyls; Cord plasma

INTRODUCTION

There is strong circumstantial evidence that oxidative stress and perinatal free radical generation contribute to the major diseases of prematurity.[1–3] For the vulnerable premature infant there are many potential sources of oxidative stress around the time of birth. These include hypoxic events, infection, the activation of an inflammatory response and the transition from a low to high oxygen environment. There have been a number of studies showing elevated levels of biomarkers of oxidative injury in infants at risk of developing chronic lung disease, retinopathy of prematurity or periventricular white matter injury.[4–15] Common oxidative markers include the lipid peroxidation product, malondialdehyde (MDA)[16,17] and protein carbonyls, which are formed as a result of protein modification by various oxidants or covalent linkage of aldehyde products of lipid peroxidation.[18] Both have the advantage of being relatively simple and sensitive to measure, although they may in some circumstances arise via mechanisms that do not involve oxidative stress.

In a previous study,[13] we documented that protein carbonyl concentrations were significantly lower in plasma collected at 2 days of age from very low birth weight (<1500 g) infants than in cord plasma from term infants. No significant difference was found for MDA between the groups. Thus, these oxidative markers did not correlate and the trend in protein carbonyls was opposite to what might be expected if the more premature infants were exposed to greater oxidative stress. In other studies, hypoxic events during birth in term infants were associated

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with increased cord blood concentrations of lipid and organic hydroperoxides.[14,19–22] Lower MDA values have also been reported for term infants born by caesarean section than for those born by spontaneous vaginal delivery.[23,24] No information is available on the effects of labour on protein oxidation products. Given the paucity of information relating perinatal variables to protein oxidation markers, and the unexpected results in our previous study,[13] we have investigated whether mode of delivery and gestational age influence protein carbonyl concentrations in cord plasma of pre-term and term infants and whether these influences differ for protein carbonyls and MDA.

MATERIALS AND METHODS

Study Population

An umbilical cord venous blood sample of up to 2 ml was collected from premature (gestational age 25–36 weeks) and full term (37–41 weeks) newborn infants born at Christchurch Women's Hospital. Clinical data was collected on the presence of prolonged spontaneous rupture of membranes (>18 h), pre-eclampsia (defined as a systolic blood pressure ≥140 mmHg or a diastolic ≥90 mmHg on two occasions and in the absence of pre-pregnancy hypertension) and intra-uterine growth retardation (defined as <10th percentile weight for gestational age[25]), mode of delivery and rationale for delivery mode. The cases were divided into delivery by elective caesarean section without labour, and delivery following labour, which included emergency caesarean section and spontaneous vaginal delivery. The study was approved by the Canterbury Ethics Committee and all samples were collected after informed parental consent had been obtained.

Sample Collection and Analysis

Blood was collected into tubes containing heparin and centrifuged within an hour to separate plasma, which was subsequently stored at −80°C. Samples with a high level of haemolysis (defined as $A_{415} > 1.2$ in a 1:2 dilution) were excluded. Protein carbonyl concentrations were determined by an ELISA, following derivatisation of the plasma with dinitrophenylhydrazine under acid conditions and detection by antibody against dinitrophenylhydrazine (ZenTech Protein Carbonyl Test kit, Dunedin, New Zealand) as previously described.[26] MDA was determined in plasma samples after incubation with thiobarbituric acid at 100°C, using HPLC with fluorescence detection.[27] Protein carbonyls were also examined by Western blotting with the anti-DNP antibody after SDS-PAGE.[28]

RESULTS

Fifty-four pre-term (≤36 weeks gestational age) and 43 term newborn infants were enrolled in the study. Of these, 21 pre-term infants and 1 term infant were the result of multiple (twin or triplet) pregnancies. Infants were grouped according to the gestational age, with the pre-term group further divided into those of <32 weeks and 32–36 weeks gestational

TABLE I Population characteristics and clinical conditions

	Pre-term		Term*
	<32 weeks	32–36 weeks	>36 weeks
Number of infants	30	24	43
GA (weeks)†	27.6 ± 2.1	34.0 ± 1.5	39.2 ± 1.0
Birth weight (g)†	1079 ± 347	2068 ± 421	3344 ± 520
Need for resuscitation	28/30	12/22	
Apgar score 1 min†	5.8 ± 2.1	8.0 ± 1.4	
Apgar score 5 min†	7.7 ± 1.7	9.3 ± 1.5	
Number of infants delivered by			
Spontaneous vaginal delivery	7	7	26
Caesarean section with labour‡	7	3	4
Caesarean section no labour¶	16	14	13
Number of infants with			
Maternal pre-eclampsia	7	5	2
Intra-uterine growth retardation	6	5	5
Prolonged premature rupture of membranes	8	1	0
Twin or triplet	11	9	1

*Pre-term infants were defined as <36 weeks gestation and term infants >36 weeks gestation. †Mean ± SD. ‡Reasons for caesarean section after active labour were delivery complications, foetal distress and antepartum haemorrhage. ¶The reasons for delivery by caesarean section were prolonged premature rupture of membranes, pre-eclampsia, intra-uterine growth retardation, antepartum haemorrhage, fetal distress, death of one twin in utero and hydrops, or elective caesarean section for triplets. For term infants elective caesarean sections were performed for maternal reasons.

TABLE II Oxidative markers in plasma from term and pre-term infants

		Pre-term		
		<32 weeks	32–36 weeks	Term
Protein carbonyls (nmol/mg)	Median	0.048	0.087	0.105
	IQ range	0.026–0.092	0.037–0.158	0.056–0.187
	p^*	0.004	0.284	
MDA (μM)[†]	Median	2.47	1.95	1.83
	IQ range	1.93–3.26	1.61–2.81	1.63-2.14
	p^*	<0.0001	0.234	

Statistical analyses were made using SigmaStat (Jandel Scientific, San Rafael, CA, USA). Initial analysis of group differences was by ANOVA with further analysis by Mann–Whitney rank sum test to assess the difference between groups. *Compared with term infants. †Twenty seven samples from infants <32 weeks and 24 samples from 32 to 36 weeks infants were analysed for MDA after exclusion of haemolysed samples and those where insufficient volume was available.

age. The characteristics of each population are given in Table I.

Effects of Gestational Age and Birthweight on Cord Plasma Protein Carbonyls and MDA

The cord plasma protein carbonyl concentrations were significantly different between the groups, with the median concentration being twice as high in term compared with <32 weeks infants (Table II). In contrast, MDA concentrations were significantly higher in the <32 weeks infants than in the term infants. Protein carbonyls showed a positive correlation, and MDA a negative correlation, with gestational age (Fig. 1a,b), and birthweight (protein carbonyls CC = 0.244, $p = 0.016$; MDA CC = −0.348, $p = 0.0006$).

Effect of Labour

Infants were categorised on the basis of whether or not they were born following active labour and the data in Fig. 1 are subdivided on that basis. As shown in Fig. 2a, the median protein carbonyl levels in both the pre-term and term groups were not significantly affected by labour. The increase with gestational age was still apparent although significant only in infants that experienced labour. MDA levels were higher in the pre-term infants in comparison to term, regardless of the presence of active labour (Fig. 2b). Labour was associated with a further increase that was significant only for pre-term infants.

These associations were also apparent from multiple regression analysis. Protein carbonyls showed a significant association with gestational age but not labour ($p = 0.040$ and 0.61, respectively) and with birthweight but not labour ($p = 0.030$ and 0.065, respectively). MDA concentrations were most influenced by gestational age ($p < 0.0001$) with labour having an additional significant effect ($p = 0.0081$). MDA was also significantly associated with labour when considered with birthweight using multiple linear regression. Delivery by caesarean section (with or without prior labour), as compared with vaginal delivery, had no significant effect on the levels of oxidative markers when considered alone

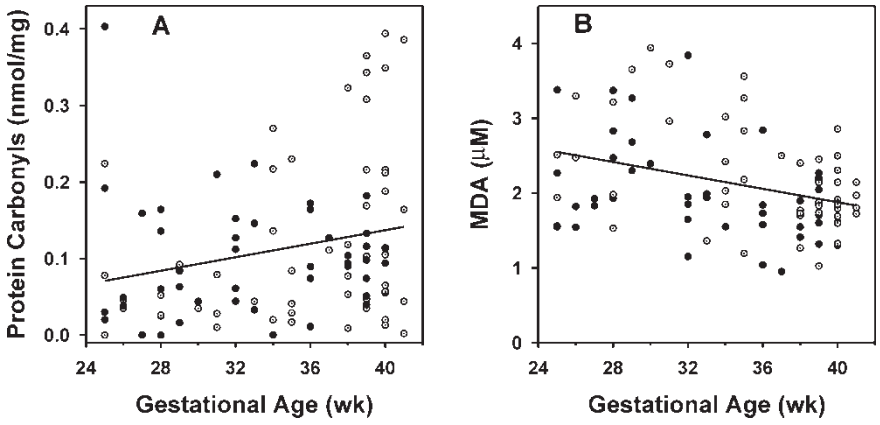

FIGURE 1 Relationships between gestational age and (a) protein carbonyls and (b) MDA concentrations for all infants. ○ infants born with labour; • infants born without labour. The regression lines were drawn for the data for all infants. (Correlation coeffiecent for protein carbonyls CC = 0.232, $p = 0.022$, and MDA, CC = −0.343, $p = 0.0007$ by Pearson's linear regression analysis).

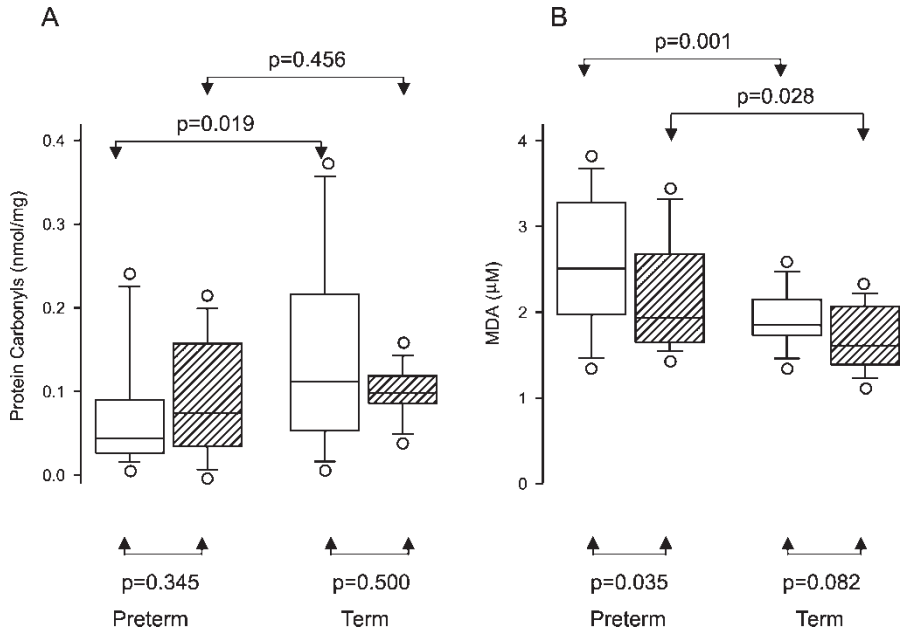

FIGURE 2 Effect of labour on (a) protein carbonyl and (b) MDA concentrations for pre-term and term infants. Box plots show medians plus interquartile ranges for each group with whiskers denoting 5 and 95 percentiles. Open boxes represent samples collected after labour and shaded boxes with no labour. Numbers were not large enough to justify further subdivision of the pre-term infants as in Table I. Initial analysis of group differences was by ANOVA with further analysis by Mann–Whitney rank sum test to assess the difference between groups.

or when controlled for either birthweight or gestational age (data not shown).

Relationships with Clinical Conditions

Fourteen of the infants were born to mothers with pre-eclampsia. This had no effect on protein carbonyls but was associated with higher cord MDA concentrations. When corrected for gestational age or birthweight, pre-eclampsia was associated with an increase of 0.46 or 0.42 μM, respectively ($p = 0.018$ and 0.036, respectively, analysed by multiple linear regression). The 8 infants born following prolonged premature rupture of membranes had significantly lower MDA concentrations (median 2.08 μM) than the other 19 infants in the <32 week group (median 2.72 μM, $p = 0.036$). This difference remained significant when corrected for gestational age. There was no difference in protein carbonyls for this group.

Placentas were examined for chorioamnionitis for only 4 of the initial cohort of pre-term infants. A further group of 22 infants were subsequently studied and had cord plasma protein carbonyls analysed. There was no significant difference between those with evidence of chorioamnionitis (median 0.104 nmol/mg protein, $n = 10$) and those without (median 0.075 nmol/mg protein, $n = 12$). These samples were not available for MDA analysis.

The effect of multiparous pregnancy was only considered for the pre-term group, given that only one term infant was a twin (Table I). There was no significant difference in either oxidative stress marker between infants from multiple or singlet pregnancy. With the proviso that the small sample numbers would only allow detection of substantial differences, the 16 infants with intrauterine growth retardation did not have significantly different protein carbonyl or MDA levels from the rest of the population.

Western blots using anti-DNP antibody were carried out for a selection of plasmas. In all samples, the major carbonyl band was albumin, with several minor bands evident (data not shown). Samples with the higher readings on ELISA showed greater overall antibody staining. No new bands or major differences in relative intensities were apparent between pre-term and term infants.

DISCUSSION

We measured markers of lipid and protein oxidation in cord blood, with the aim of gaining further understanding of oxidative events associated with premature and full term birth. Plasma concentrations of MDA were found to be significantly higher in pre-term than term infants, and were positively associated with labour. These findings are in general agreement with other studies where associations with prematurity[14,29] and labour[23,24] were found. Positive associations between MDA and hypoxia have also been described[14,19–22] and this could be a factor in the relationship with labour.

The evidence of increased lipid peroxidation in our population could be taken as support for the prevailing concept that pre-term infants are more vulnerable and experience more oxidative stress than infants born at term.[1,5] However, we are presented with a dilemma because protein carbonyl concentrations showed an opposite trend to MDA. These were higher in plasma from full term compared with pre-term infants, with the increase being seen over the range of gestational age or birthweight. There was no additional effect of labour. Protein carbonyls in plasma collected at 2 days from pre-term infants have been found previously to be lower than in cord plasma from term infants.[13] The present results show that this difference is not related to the time after birth when the blood was collected. Protein carbonyls are less in pre-term infants at birth and this is not because of events associated with labour in the term group.

It is not obvious why the two markers showed opposite trends, but the results do sound a note of caution for interpreting the data in terms of oxidative damage. One possible explanation is that oxidative stress occurs at different times or different sites, with some selectivity towards proteins or lipids. MDA will diffuse out of tissues more readily than protein carbonyls, so plasma MDA may be a better reflection of intracellular oxidative stress. Protein carbonyls are more likely to be confined to their site of generation. As a small molecule, MDA may also be more transient. However, factors such as specificity, sensitivity and metabolism of the marker must be considered. MDA, especially as measured using thiobarbituric acid, is not specific for lipid peroxidation.[16,17] Many of the limitations are overcome by using an HPLC assay, but there are other sources of MDA including activated platelets.[30] Platelet activation, for example in association with labour or pre-eclampsia, might be responsible for increased MDA levels. Carbonyl groups are readily generated on proteins by various oxidants, and protein carbonyls are widely regarded as useful biomarkers of oxidative stress.[18,31,32] They can be measured colorimetrically or by ELISA. The two assays correlate although absolute values differ. ELISA requires fewer samples and is more sensitive. Western blotting showed that most of the carbonyl groups were associated with the albumin band, with no obvious increase in staining of other bands that could account for the higher levels in some samples. In particular, the lower levels of proteins such as transferrin or immunoglobulins in pre-term plasma could not explain their lower carbonyls. The fate of protein carbonyls in circulation is not well understood, but it is most likely that they are removed via protein turnover.[33] Differences in turnover rates could, therefore, contribute to variation between patient groups.

In other clinical studies where more than one oxidative marker has been measured, relationships have not always been straightforward. Elevated concentrations of both protein carbonyls and MDA and have been measured in the lung aspirates from infants with hyaline membrane disease, but they were only weakly correlated and plasma levels were not increased.[9,11,13,34] Adults with respiratory distress syndrome had very high protein carbonyls in both lung lavage and plasma, but MDA concentrations were in the normal range.[35]

Although our population size was small for relating oxidative markers to clinical conditions, several trends are apparent. Our findings of increased MDA concentrations in cord blood when mothers had pre-eclampsia are consistent with a link between pre-eclampsia and oxidative stress[36] and suggest that oxidation is also detectable in the infant. In several studies, concentrations of protein carbonyls, MDA, and the more specific lipid peroxidation marker, 8-isoprostane, in mothers' plasma and placental tissue were higher in pre-eclampsia compared with normal pregnancy[37–41] and antioxidant supplementation during pregnancy was found to reduce risk.[42] In other studies, however, increases in 8-isoprostane in plasma or urine from pre-eclamptic mothers have not been observed[40,43,44] and no difference seen in cord blood MDA between normal and pre-eclamptic pregnancy.[45] Although intrauterine inflammation might be expected to increase oxidative marker levels, chorioamnionitis was not associated with higher protein carbonyls and prolonged premature rupture of membranes was in fact linked to lower MDA levels.

In conclusion, we have observed that cord blood MDA concentrations increase with prematurity and in association with labour, but protein carbonyl concentrations are lower in the more premature infants. The different clinical patterns seen for these two established biomarkers highlight uncertainties about interpreting these data and the need for greater understanding of how and where they are formed and the processes responsible for removal from circulation. Until we have this information for these and other biomarkers, uncertainties about relationships between oxidative stress and prematurity and other clinical conditions will remain.

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