Oxidative stress in lavage fluid of preterm infants at risk of chronic lung disease

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Schock, Bettina C., David G. Sweet, Henry L. Halliday, Ian S. Young, and Madeleine Ennis. Oxidative stress in lavage fluid of preterm infants at risk of chronic lung disease. Am J Physiol Lung Cell Mol Physiol 281: L1386–L1391, 2001.—There is evidence that oxidative stress plays a role in the development of chronic lung disease (CLD), with immature lungs being particularly sensitive to the injurious effect of oxygen and mechanical ventilation. We analyzed total ascorbate, urate, and protein carbonyls in 102 bronchoalveolar lavage fluid samples from 38 babies (33 preterm, 24–36 wk gestation; 5 term, 37–39 wk gestation). Preterm babies had significantly decreasing concentrations of ascorbate, urate, and protein carbonyls over the first 7–9 days of life (days 1–3, 4–6, and 7–9; Kruskal-Wallis ANOVA: $P = 0.016, P < 0.0001$, and $P = 0.010$, respectively). Preterm babies who subsequently developed CLD. This study has shown the presence of oxidative stress in the lungs of preterm babies during ventilation, especially in those who subsequently developed CLD.

Evidence that oxidative stress plays a role in the development of chronic lung disease (CLD) has accumulated over the last 7–8 yr (26). Preterm infants are often exposed to increased oxidative stress due to exposure to high oxygen concentrations in combination with low surfactant concentrations, lowered antioxidant defenses, and decreased ability to induce antioxidant enzymes (10). Inflammatory cells, in particular neutrophils, are involved in the pathogenesis of bronchopulmonary dysplasia (BPD) (1, 15). The inflammatory response is triggered by proinflammatory cytokines, lipid mediators, and complement activation (22). Additionally, increased protein carbonyls in tracheal aspirates of preterm babies have been shown to correlate with myeloperoxidase activity from neutrophils (5). An imbalance between proteases and anti-protease activity in the respiratory tract has been reported in neonates with BPD (19), which may lead to further lung injury and abnormal remodeling.

The epithelial lining fluid of the lungs contains high concentrations of antioxidants such as ascorbate and urate, providing a first defense against inhaled and endogenous oxidants (8). Few studies have investigated neonatal oxidant defense systems, and reports of antioxidant concentrations are conflicting. Ascorbate concentrations in cord blood have been reported as similar (29) or increased in preterm babies with BPD (3), and plasma concentrations correlated negatively with gestational age (28). The ascorbate-to-dehydroascorbate ratio was found to be higher in tracheal aspirates than in plasma (16). Urate concentrations in plasma and tracheal aspirates did not show any differences in CLD (27), but allantoin concentrations were increased at birth (16). In bronchoalveolar lavage (BAL) fluid, the ratio between urate and allantoin was increased over the first 6 days of life (20) in babies with CLD. Tracheal aspirate concentrations of protein carbonyls, a measure of oxidative damage to proteins, were higher in very-low-birth-weight infants (<1,500 vs. >1,500 g (5)). More recently, Vento et al. (32) reported significant correlations between inspired oxygen concentrations and total antioxidant capacity or uric acid concentrations in tracheal aspirates of preterm babies. The lungs of premature infants are particularly sensitive to the injurious effect of oxygen and mechanical ventilation. The hypothesis of this study is that oxidative stress may decrease antioxidant levels in the lung, leading to higher concentrations of oxidized proteins in the epithelial lining fluid. We therefore aimed to determine the concentrations of the antioxidants ascorbate and urate and the concentration of oxidized proteins in BAL fluid of preterm infants who are at risk of developing CLD and in BAL fluid of ventilated term babies without acute lung disease.
MATERIALS AND METHODS

Subjects

Babies born in the Royal Maternity Hospital in Belfast between March 1998 and April 1999 were recruited into the study. The study was approved by the Research Ethics Committee of The Queen’s University of Belfast, and written parental consent was obtained before babies were enrolled. Babies were eligible for the study if they required intubation and mechanical ventilation within the first 6 days of life. Gestation was estimated by duration of amenorrhea combined with early prenatal ultrasound measurement. Thirty-eight babies were studied. Twenty-one were born before 29 wk gestation, and 12 were 29–36 wk gestation; most had respiratory distress syndrome (RDS) and were treated with surfactant. Five were term babies without significant lung disease who were ventilated for hypoxic ischemic encephalopathy (n = 2) and oversedation and had congenital myopathy or gastrochisis. The severity of respiratory disease at the time of BAL sampling was quantified using the arterial-alveolar oxygen tension ratio (a/A ratio). CLD was defined as oxygen requirement at 36 wk postmenstrual age. The dietary intake of vitamin C was similar in all babies at ~10 mg·kg⁻¹·day⁻¹.

BAL

BAL was performed in a standardized way (14). In summary, 1 ml/kg of sterile 0.9% saline was instilled using a syringe via a 5-F gauge feeding catheter that had been placed through the endotracheal tube in the distal right main bronchus. The saline was instilled and immediately reaspirated back in the syringe. The sample was centrifuged at 200 g for 5 min at room temperature, and the supernatant was immediately frozen at −70°C for subsequent analysis. For the analysis of ascorbic acid and uric acid, a 100-µl aliquot of the supernatant was incubated with a final concentration of 10 mmol/l Na₂EDTA and 35 mmol/l DTT for 30 min on ice and then stored at −70°C. In keeping with the recommendations of the European Respiratory Society task force, measurements of noncellular constituents were reported combined with early prenatal ultrasound measurement. Thirty-five babies were studied. Twenty-one were born before 29 wk gestation, and 12 were 29–36 wk gestation; most had respiratory distress syndrome (RDS) and were treated with surfactant. Five were term babies without significant lung disease who were ventilated for hypoxic ischemic encephalopathy (n = 2) and oversedation and had congenital myopathy or gastrochisis. The severity of respiratory disease at the time of BAL sampling was quantified using the arterial-alveolar oxygen tension ratio (a/A ratio). CLD was defined as oxygen requirement at 36 wk postmenstrual age. The dietary intake of vitamin C was similar in all babies at ~10 mg·kg⁻¹·day⁻¹.

Ascorbic Acid and Uric Acid in BAL Fluid

Both antioxidants were analyzed simultaneously using HPLC with electrochemical detection as described by Chevion et al. (6). Standards of both antioxidants (0.25–5 µmol/l) were prepared in 0.9% sodium chloride with a final concentration of 10 mmol/l Na₂EDTA and 35 mmol/l DTT to generate a standard curve. Samples with higher concentrations were diluted in 0.9% sodium chloride before injection. The detection limit was 0.106 µmol/l for ascorbate and 0.030 µmol/l for urate. The coefficients of variation were 5.2% for ascorbic acid and 6.9% for uric acid (intra-assay) and 7.8% for ascorbic acid and 9.1% for uric acid (interassay).

Protein Carboxyls

Carbonyl concentrations were determined using an in-house ELISA as described by Buss et al. (4). Briefly, after derivatization of carbonyl groups with dinitrophenylhydrazine, proteins were adsorbed on 96-well ELISA plates, captured with a commercially available anti-dinitrophenylhydrazine antibody, and detected with a horseradish peroxidase-hydrogen peroxide-phenylenediamine system (4). The limit of detection was 0.28 nmol/mg protein. Coefficients of variation were 8.6% (intraplate) and 9.3% (interplate).

Protein Assay

Total protein concentrations in BAL fluid were quantified using the commercially available Bio-Rad kit (Bio-Rad Laboratories). The detection limit was 2 µg/ml.

Statistical Analysis

From 38 patients, 102 samples were taken between the 1st and the 9th day of life. Nonparametric tests (Mann Whitney U-test and Kruskal-Wallis one-way ANOVA) were used throughout. Results were considered statistically significant at P < 0.05.

RESULTS

Subjects

BAL samples were obtained from 38 babies with gestational ages from 24 to 38 wk at various time points after birth. The main characteristics of the babies are given in Table 1. A total of 102 samples were taken during the first 9 days of life. Samples were taken on average over a similar time period (Table 1). However, preterm babies (29–36 wk) were extubated

<table>
<thead>
<tr>
<th>Table 1. Characteristics of all babies studied</th>
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<tr>
<td>Term Babies</td>
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<tr>
<td>-------------</td>
</tr>
<tr>
<td>No.</td>
</tr>
<tr>
<td>Male/female</td>
</tr>
<tr>
<td>Gestation, wk</td>
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<tr>
<td>Birth weight, g</td>
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<tr>
<td>Maximum FIO₂ (day 1)</td>
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<td>Median a/A ratio (day 1)</td>
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<td>Postnatal age of sampling</td>
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<tr>
<td>Days</td>
</tr>
<tr>
<td>No. of samples</td>
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</table>

Values are given as means ± SD with ranges in parentheses. FIO₂, fraction of inspired oxygen; a/A ratio, arterial-alveolar oxygen tension ratio. The last two columns present data of two subgroups of preterm babies (29–36 wk and 24–28 wk). The days of sampling did not differ significantly between term and all preterm babies (P = 0.053) or preterm and very preterm babies (P = 0.12).

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earlier than very preterm babies (<29 wk), and therefore fewer samples were taken. There was no sampling after day 5.

Markers of Oxidative Stress and Antioxidants in BAL Fluid

Total protein concentrations (µg/ml) were similar in term and preterm babies over the study period (days 1–3: term 327.6 (75.5–1,639) µg/ml vs. preterm 260 (16.1–3,881) µg/ml, P = 0.83; days 4–6: term 239.6 (102.5–509.9) µg/ml vs. preterm 207.3 (17.3–802.4) µg/ml, P = 0.70; days 7–9: term 215.6 (119.7–224.9) µg/ml vs. preterm 393.4 (31.7–895.4) µg/ml, P = 0.23). Protein carbonyls were significantly increased in preterm babies on days 1–3 and 4–6 when compared with term babies (P = 0.005 and P = 0.044, respectively, Fig. 1). Total ascorbate and urate concentrations (µmol/l) did not differ at any time point. There was a trend toward decreased concentrations of ascorbate on days 7–9 (P = 0.127, Fig. 2). Analyses by Kruskal-Wallis one-way ANOVA showed significant decreases with age (days 1–3, 4–6, and 7–9) for the concentrations of protein carbonyls (P = 0.01), total ascorbate (P = 0.016), and urate (P < 0.0001) in preterm babies (Figs. 1–3).

Differences with gestational age. When the group of preterm babies was divided according to their gestational age (preterm: 29–36 wk and very preterm: 24–28 wk), very preterm babies showed higher concentrations of protein carbonyls at days 1–3 (although not significant), which decreased on days 4–6 (P = 0.056) and days 7–9. Total protein concentrations were found to be decreased on days 4–6 only (P = 0.038). Additionally, only in very preterm babies did concentrations of ascorbate decrease significantly at days 4–6 (P = 0.004) and further on days 7–9 (Table 2). Because of small sample numbers from preterm babies, the last time point (days 7–9) could not be statistically analyzed. Analyses by Kruskal-Wallis one-way ANOVA showed significant decreases with postnatal age for the concentrations of protein carbonyls (P = 0.02), total ascorbate (P = 0.017), and urate (P < 0.0001) in very preterm babies only (Table 2).

Differences with outcome. Preterm babies who subsequently developed CLD had a trend toward higher concentrations of protein carbonyls at days 1–3 than babies who did not develop CLD, but this did not achieve significance (P = 0.12, Fig. 4). Urate concentrations were significantly increased at this time point in babies who subsequently developed CLD (P = 0.023, Fig. 5). Ascorbate concentrations did not differ between both groups at different times. Kruskal-Wallis one-way ANOVA test showed significant decreases with postnatal age in babies who subsequently developed CLD for concentrations of protein carbonyls (P = 0.001, Fig. 4) and urate (P < 0.0001, Fig. 5). Although total ascorbate concentrations also decreased with postnatal age in babies who subsequently developed CLD, this did not achieve statistical significance (P = 0.058, Fig. 6).

Correlations. The concentration of protein carbonyls (nmol/mg) in BAL fluid of all babies correlated significantly with gestational age (weeks; r = −0.413, n = 49, P = 0.003) during the first 3 days of life. Urate concentrations (µmol/l) in BAL fluid were significantly correlated with total protein (µg/ml; r = 0.583, n = 51, P < 0.0001) and with total ascorbate (µmol/l; r = 0.449, n =
P 50.001). Urate (μmol/l) also correlated significantly with protein carbonyls (μmol/l; r = 0.508, n = 57, P < 0.0001) during the first 3 days of life. A weak but significant negative correlation was observed between oxidized proteins (μmol/l) and the a/A PO2 ratio (r = -0.319, n = 46, P = 0.031). This correlation was lost when oxidized proteins were expressed as nanomoles per milligram (r = -0.172, n = 46, P = not significant). Concentrations of antioxidants did not correlate significantly with respiratory disease severity (all P > 0.05).

**DISCUSSION**

This is the first study to report concentrations of ascorbate in BAL fluid from ventilated newborn babies. Additionally, concentrations of urate and oxidized proteins are also reported. We investigated the concentrations of the water-soluble antioxidants ascorbate and urate and markers of protein oxidation in BAL fluid of 38 babies with gestational ages from 24 to 39 wk. From these babies, 102 samples were taken over a time period of 9 days. Samples in the group of preterm babies were taken over a shorter time compared with very preterm babies, as they inevitably required ventilation for a shorter period.

Prematurely born babies showed high concentrations of protein carbonyls, ascorbate, and urate in the first 72 h of life, which fell progressively over the next 6 days. A similar observation has been made for protein carbonyls in tracheal aspirates (5) and for ascorbate in plasma of very-low-birth-weight infants (28). Very few studies have investigated tracheal aspirates or BAL fluid to determine oxidative stress. Moison et al. (16) quoted the ratios of dehydroascorbate to ascorbate and allantoin to urate in a mixed group of babies with RDS or CLD. Ogihara et al. (21) used the same ratio of allantoin to urate to show increased oxidative stress in babies with CLD. In both studies, only the ratios were given, so a comparison with results obtained in our study is not possible since we did not measure oxidation products of ascorbate or urate. Schrod et al. (27) standardized uric acid to the secretory component of IgA. In babies with severe CLD, this babies were taken over a shorter time compared with very preterm babies, as they inevitably required ventilation for a shorter period.

Table 2. Antioxidant and protein carbonyl concentrations in BAL fluid of preterm and very preterm babies

<table>
<thead>
<tr>
<th>Day of Life</th>
<th>Preterm Babies (29–36 wk)</th>
<th>Significance (P*)</th>
<th>Very Preterm Babies (24–28 wk)</th>
<th>Significance (P†)</th>
<th>Significance (P†)</th>
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<tr>
<td>Total protein, μg/ml</td>
<td></td>
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<tr>
<td>1–3</td>
<td>462.6(16.1–3,881)</td>
<td>0.58</td>
<td>207.4(69.5–1,610)</td>
<td>0.072</td>
<td>0.10</td>
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<tr>
<td>4–6</td>
<td>417.9(201.5–681.5)</td>
<td>NA</td>
<td>140.8(17.3–802.4)</td>
<td>0.036</td>
<td>0.038</td>
</tr>
<tr>
<td>Protein carbonyls, nmol/mg</td>
<td></td>
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<tr>
<td>1–3</td>
<td>2.84(1.16–7.97)</td>
<td>0.89</td>
<td>3.20(1.70–6.57)</td>
<td>0.002</td>
<td>0.97</td>
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<tr>
<td>4–6</td>
<td>3.10(0.89–5.05)</td>
<td>NA</td>
<td>2.28(1.08–3.85)</td>
<td>0.056</td>
<td>0.056</td>
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<tr>
<td>Total ascorbate, μmol/l</td>
<td></td>
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<tr>
<td>1–3</td>
<td>3.42(0.16–13.92)</td>
<td>0.58</td>
<td>3.42(0.33–10.49)</td>
<td>0.017</td>
<td>0.66</td>
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<tr>
<td>4–6</td>
<td>3.88(2.34–8.15)</td>
<td>NA</td>
<td>2.13(0.11–23.85)</td>
<td>0.004</td>
<td>0.004</td>
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<tr>
<td>Urate, μmol/l</td>
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<tr>
<td>1–3</td>
<td>14.26(3.70–57.74)</td>
<td>0.69</td>
<td>13.77(1.14–40.33)</td>
<td>&lt;0.0001</td>
<td>0.32</td>
</tr>
<tr>
<td>4–6</td>
<td>14.57(6.66–17.07)</td>
<td>NA</td>
<td>3.62(1.14–16.61)</td>
<td>0.56</td>
<td>0.56</td>
</tr>
<tr>
<td>7–9</td>
<td>1.95(1.25–28.39)</td>
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</table>

Total protein, protein carbonyls, total ascorbate, and urate concentrations in bronchoalveolar lavage (BAL) fluid of preterm (29–36 wk) and very preterm (24–28 wk) babies. Results are given as medians and ranges. Comparisons between two groups were performed using the Mann-Whitney U-test (†). Kruskal-Wallis one-way ANOVA (*) was used to determine changes with time (day of life). A P value of <0.05 was considered statistically significant. NA, no samples available.
developed CLD. Mann-Whitney U test showed no significant differences with time (\( P = 0.058 \)) for preterm babies who subsequently developed CLD. Mann-Whitney U-test showed no significant differences between groups.

In a previous study, Wilson et al. (33) have indicated a higher utilization as a result of increased vitamin C absorption must be considered, a decrease in ascorbate concentrations may indicate excess oxidative stress. Endogenous antioxidant defense mechanisms are poorly developed in the preterm baby and may be overwhelmed by the generation of excessive reactive oxygen species (10, 26). This may lead to a heavier burden on exogenous antioxidants during ventilation. Therefore, fetal nutritional state and level of antioxidant defense at birth might be of particular importance. In a previous study, Wilson et al. (33) have shown that intrauterine growth restriction was a high risk factor for CLD. Additionally, free iron has been detected in both plasma (2) and tracheal aspirates (12) of preterm babies and may act as a prooxidant, increasing oxidative stress via the Fenton reaction. Moreover, activated neutrophils, the predominant inflammatory cells in preterm babies, also contribute to oxidative stress (5) as they release not only reactive oxygen species but also proteolytic enzymes. Neutrophil-derived proteases, such as matrix metalloproteinases, are upregulated by oxidative stress (30) and have been implicated in hyperoxic lung damage (23).

In respiratory lining fluids, urate and ascorbate are key antioxidants, although there is little available information about the levels or function of these antioxidants in respiratory lining fluids from young children. Uric acid is the major low-molecular-weight antioxidant in upper respiratory tract fluids (24). It is cosecreted with lactoferrin in the upper airways and is closely associated with mucin. In plasma, urate is the most potent scavenger of oxygen, and it seems likely that it is similarly effective in the respiratory tract (7). In addition to its direct antioxidant effect, urate can also chelate transition metals, which may also contribute to its antioxidant activity. Ascorbate is the most important aqueous-phase chain-breaking antioxidant in plasma (11) and makes a major contribution to antioxidant protection in the lung, particularly the lower respiratory tract. This is likely to be mainly due to the direct radical scavenging properties of ascorbate but also to its capacity to recycle vitamin E (18).

Protein carbonyls have been determined in two studies of ventilated neonates using mixed groups of babies [RDS/CLD (31) and RDS/CLD/sepsis (13)]. Recently Buss et al. (5) reported similar levels of protein carbonyls to those measured in preterm babies in our study. However, concentrations of protein carbonyls of preterm babies were not compared with those of term babies with normal lungs, and no association between carbonyl concentration and outcome was found (5).

When investigating the first 72 h only, protein carbonyl concentrations in BAL fluid correlated negatively and significantly with gestational age, in agreement with a previous report (31). The observed correlation of protein carbonyls with urate may be explained by the association with total proteins. Inflammation and hypoxia/reoxygenation injury cause an increase in vascular permeability that leads to protein leakage and edema (22). Increased vascular permeability may also explain the presence of ascorbate in BAL fluid and its correlation with urate. In all babies, increased oxygen requirement expressed by a low a/A PO2 ratio is associated with increased oxidation of proteins. This is in agreement with the findings of Vento et al. (32) who described a positive association between uric acid concentrations and inspired oxygen concentration.

However, although protein carbonyl concentrations decrease with postnatal age in very preterm babies (but not in preterm babies), the parallel dramatic decrease in ascorbate concentrations may indicate exces-
sive oxidative stress, which cannot be attenuated by the antioxidant defense system in the lung lining fluid.

Our study indicated the presence of oxidative stress in preterm babies with CLD. We have observed substantial changes in ascorbate and urate concentrations in BAL with time. Oxidative damage may occur primarily in proteins (13) although increased malondialdehyde concentrations have also been found (5, 31). However, this is the first study to report a decrease in ascorbate concentrations with postnatal age. Additionally, a decrease in urate concentrations in preterm babies has also been shown. This suggests that oxidative stress may overwhelm the antioxidant defenses, which may contribute to the development of CLD.

Strategies to prevent oxidative stress in very-low-preterm infants may reduce the incidence of CLD, but further studies are necessary to show a potential clinical utility of these observations. We believe that the observed changes are likely to be biologically and statistically significant, as suggested by the relationship observed changes are likely to be biologically and statistically significant. In vitro studies assessing the effects of antioxidants on cell function and in vivo studies in which antioxidant concentrations are augmented.

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REFERENCES


