Pulmonary dysfunction in cystic fibrosis is associated with oxidative stress


ABSTRACT: The aim of this study was to determine whether a relationship exists between the circulating concentration of antioxidants, or markers of oxidative stress, and pulmonary function in cystic fibrosis patients.

Plasma was obtained from 34 patients attending a cystic fibrosis clinic. Oxidative stress was investigated by measuring the concentrations of circulating lipid hydroperoxides and malondialdehyde (lipid peroxidation) and protein carbonyls (protein oxidation). Antioxidant status was determined from the plasma concentrations of α-tocopherol, ascorbic acid, uric acid and total sulphydryls. Forced vital capacity (FVC), forced expiratory volume in one second (FEV1) and forced mid-expiratory flow (FEF25-75) were measured in 25 of the subjects by spirometry, and expressed as percentage predicted for normal height, weight and age.

Lung function decreased significantly with age and was associated with decreased plasma α-tocopherol, ascorbic acid and sulphydryl concentrations. The reduction in pulmonary function correlated with elevated plasma malondialdehyde, but not with lipid hydroperoxide or protein carbonyl concentrations. Patients with severe lung dysfunction (FEV1 <50% predicted) had higher plasma concentrations of lipid hydroperoxides than those with mild-to-moderate lung dysfunction (FEV1 >50% pred).

This study provides evidence that cystic fibrosis patients have inadequate antioxidant defences to cope with the elevated oxidative stress that they regularly experience. We believe that recurring oxidative lung injury contributes to the decline in pulmonary function in these patients.


One important consequence of the repeated cycles of pulmonary infection, which are characteristic of cystic fibrosis (CF), is the deterioration in lung function that occurs in these patients. As a consequence, respiratory disease is responsible for over 90% of the mortality observed [1]. Although the genetic defect responsible for cystic fibrosis is well-characterized, the events leading to the gradual destruction of lung tissue and the consequent decrease in pulmonary function remain unclear.

Lung dysfunction in cystic fibrosis consists of progressive airflow obstruction caused by the accumulation of thick, viscous secretions, and the gradual destruction of the bronchiolar and larger airways [2–4]. Endobronchial infection, particularly in association with colonization by Pseudomonas aeruginosa, leads to damage of the airways and the gradual deterioration of pulmonary function [1, 5, 6].

The role of the immune response in mediating the characteristic pulmonary damage is clearly evident. Several studies have shown that the systemic immune response is increased in patients with cystic fibrosis [7, 8], although paradoxically, high serum concentrations of immunoglobulins have been associated with an unfavourable outcome [9]. Patients with higher titres of serum antibodies specific for P. aeruginosa have poorer pulmonary function and a lower survival rate than those with lower titres [10]. Analysis of bronchoalveolar lavage fluid has revealed a 1,000 fold increase in the numbers of neutrophils recovered from lungs of patients with cystic fibrosis compared with controls [2].

It is well-established that when activated, neutrophils are a major source of free radicals [11], and though considered a potent mechanism for killing organisms, they may also damage the pulmonary epithelium in these patients [12]. The damaging effects of increased free radical production may be exacerbated by a systemic deficiency of the major respiratory antioxidant, glutathione [13] in patients with cystic fibrosis.

Recently, we demonstrated markers of free radical-mediated damage in plasma from cystic fibrosis patients [14]. These products of lipid and protein oxidation were present in patients with normal concentrations of circulating antioxidants, indicating that oxidative damage was most likely the result of elevated rates of free radical production. We believe that the increased oxidative burden, associated with the immune response to infection
in patients with cystic fibrosis, is an important contributing factor to the age-related decrease in pulmonary function seen in this patient group.

The purpose of the present study was to determine the strength of the relationship between the deterioration in pulmonary function and the presence of markers of oxidative stress. In addition, the plasma concentration of four antioxidants, α-tocopherol, ascorbic acid, uric acid and total sulphhydrils, were also measured for comparison with each patient’s lung function.

**Materials and methods**

**Subjects**

The study population comprised 34 patients with cystic fibrosis attending an out-patients clinic at King’s College Hospital. The age of the patients ranged 2–20 yrs, with a mean of 12 (SD 6) yrs. Pulmonary function tests were performed only on patients aged 6 yrs and over (n=25), due to the decreased validity of these tests in younger patients [15]. The study was approved by the Research Ethics Committee for Kings Healthcare and informed consent was obtained from all participants or their legal guardians prior to initiation of the protocol.

**Sample collection and treatment**

Samples of venous blood were collected from patients and immediately divided between two heparinized tubes on ice, one of which contained 20 µL of 2 mmol·L⁻¹ butylated hydroxytoluene (BHT) and 20 µL 2 mmol·L⁻¹ Desferal. This step is essential to protect any lipid hydroperoxides present in the blood sample. The blood was then centrifuged at 2,000×g, at 4°C for 10 min, and the plasma stored at -80°C for up to 4 weeks before analysis. Plasma samples protected with BHT and Desferal were used to determine the concentrations of the indices of lipid and protein damage, while those without these synthetic antioxidants were used to determine the concentrations of the individual endogenous antioxidants. All variables measured were found to be stable under the conditions of storage.

**Pulmonary function tests**

Spirometry was performed by means of a computer-assisted spirometer (Compact II Spirometer, Vitalograph Ltd, Buckingham, UK). Forced vital capacity (FVC), forced expiratory volume in one second (FEV1) and forced mid-expiratory flow (FEF25–75) were recorded as the best of two satisfactory respiratory tracings. The results were expressed as a percentage of mean predicted values based on height and sex, as described previously [16].

**Biochemical analysis of plasma antioxidants and indices of oxidative stress**

Determination of the plasma antioxidants α-tocopherol, ascorbic acid, and uric acid were carried out by high performance liquid chromatography (HPLC), as described previously [14], and total plasma sulphhydrals were assayed by the method of ELLMAN [17]. The indices of oxidative stress, lipid hydroperoxides, malondialdehyde and protein carbonyls were determined as described in a earlier study [14].

**Statistical analysis**

The statistical analysis was performed on a PC microcomputer using a statistical package (Statistics©, Blackwell Scientific Publications, Oxford, UK) which included the Mann-Whitney U-test for nonparametric data. The strength of the relationship was assessed using Spearman’s correlation coefficient and significance was determined by linear regression using the least squares method. A p-value of less than 5% was considered significant.

**Results**

Twenty five of the children were older than 6 yrs and took part in the pulmonary function tests. Their mean (SD) age was 13 (5 yrs). The concentration of indices of lipid peroxidation (lipid hydroperoxides and malondialdehyde) and protein oxidation (protein carbonyls) along with plasma antioxidant concentrations are shown in table 1. Markers of oxidative damage were present in concentrations similar to these found in a different cohort of patients with cystic fibrosis reported in a previous study and were elevated compared to reported control values [14, 18].

The plasma concentrations of antioxidants in patients with cystic fibrosis in this study fell within the reported normal ranges for healthy controls, a finding in agreement with the results of our previous studies. Pulmonary function, measured as FVC, FEV1 and FEF25–75 decreased significantly with age (r = -0.56, p<0.01; r = -0.63, p<0.001; r = -0.53, p<0.01, respectively) (fig. 1), a finding in agreement with the results of other studies [19, 20].

In order to investigate the relationship between pulmonary dysfunction and oxidative damage, patients were subdivided into two groups according to their pulmonary function measurements (FEV1 % pred). Patients with an FEV1 of less than 50% pred were classified as having severe pulmonary dysfunction. Those patients with

<table>
<thead>
<tr>
<th>Plasma markers of oxidative stress</th>
<th>Lipid hydroperoxides µmol·L⁻¹</th>
<th>0.044 (0.011–0.454)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Malondialdehydes µmol·L⁻¹</td>
<td>8.92 (5.30–12.20)</td>
<td></td>
</tr>
<tr>
<td>Protein carbonyls mmol·mg⁻¹ protein</td>
<td>1.61 (0.25–4.93)</td>
<td></td>
</tr>
</tbody>
</table>

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<thead>
<tr>
<th>Plasma antioxidants</th>
<th>α-tocopherol µmol·L⁻¹</th>
<th>24.4 (2.9–50.0)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ascorbic acid µmol·L⁻¹</td>
<td>51.8 (3.6–88.9)</td>
<td></td>
</tr>
<tr>
<td>Uric acid µmol·L⁻¹</td>
<td>364.7 (201.9–544.9)</td>
<td></td>
</tr>
<tr>
<td>Total sulphhydrals µmol·L⁻¹</td>
<td>567.7 (385.3–723.5)</td>
<td></td>
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</tbody>
</table>

Values are presented as median, and range in parentheses.

Table 1. Markers of oxidative damage and concentrations of antioxidants in the plasma of patients with cystic fibrosis (n=34)
an FEV₁ of greater than 50% pred were classified as having mild-to-moderate pulmonary dysfunction. From table 2 it can be seen that patients with impaired pulmonary function had significantly elevated plasma concentrations of lipid hydroperoxides. Plasma malondialdehyde concentration, although higher in the patients with severe pulmonary dysfunction, was not significantly different (p=0.074). The plasma protein carbonyl concentration was not significantly different between the two groups.

A significant correlation was found between decreasing pulmonary function (FEV₁ and FVC) and increasing plasma malondialdehyde concentration (fig. 2). However, neither the concentration of lipid hydroperoxides nor protein carbonyls showed a similar correlation with pulmonary dysfunction (data not shown).

Table 2 also shows the plasma concentrations of antioxidants measured in the patients with cystic fibrosis with severe and mild-to-moderate pulmonary dysfunction. The

<table>
<thead>
<tr>
<th>Variable</th>
<th>Mild pulmonary dysfunction (FEV₁&gt;50% pred)</th>
<th>Severe pulmonary dysfunction (FEV₁&lt;50% pred)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lipid hydroperoxides μmol·L⁻¹</td>
<td>0.027 (0.011–0.060)</td>
<td>0.054 (0.025–0.454)</td>
<td>0.050</td>
</tr>
<tr>
<td>Malondialdehyde μmol·L⁻¹</td>
<td>8.14 (5.27–11.94)</td>
<td>9.28 (7.93–12.21)</td>
<td>0.074</td>
</tr>
<tr>
<td>Protein carbonyls nmol·mg⁻¹ protein</td>
<td>1.62 (0.25–3.82)</td>
<td>1.52 (0.40–4.93)</td>
<td>0.793</td>
</tr>
<tr>
<td>α-tocopherol μmol·L⁻¹</td>
<td>23.4 (8.7–50.0)</td>
<td>10.7 (2.9–32.6)</td>
<td>0.066</td>
</tr>
<tr>
<td>Ascorbic acid μmol·L⁻¹</td>
<td>56.0 (18.1–76.5)</td>
<td>44.7 (3.6–76.5)</td>
<td>0.240</td>
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<tr>
<td>Uric acid μmol·L⁻¹</td>
<td>355.2 (226.5–532.5)</td>
<td>468.8 (201.9–544.9)</td>
<td>0.279</td>
</tr>
<tr>
<td>Sulphydryls μmol·L⁻¹</td>
<td>582.4 (467.7–673.5)</td>
<td>514.7 (385.3–667.6)</td>
<td>0.013</td>
</tr>
</tbody>
</table>

FEV₁: forced expiratory volume in one second.
concentration of α-tocopherol was lower in the patients with more severely compromised lung function, although the difference observed was not significant (p=0.066). No significant differences were seen between the two groups with regard to the concentrations of ascorbic acid and uric acid; however, plasma sulphydryl concentrations were lowered in the patients with severe pulmonary dysfunction (p=0.013).

Plasma concentrations of α-tocopherol, ascorbic acid and total sulphydryls decreased significantly with age in CF patients (r=-0.68, p<0.001; r=-0.51, p<0.01; r=-0.44 p<0.05, respectively), while the converse was true for plasma uric acid concentration (r=0.38, p<0.05) (fig. 3). Pulmonary function was closely correlated with plasma α-tocopherol, ascorbic acid and sulphhydril status, (r=0.63, p<0.001; r=0.46, p<0.05; r=0.52, p<0.05, respectively)

Fig. 3. – The significant relationships between α-tocopherol, ascorbic acid, uric acid and plasma sulphydryls and age in patients with cystic fibrosis.

Fig. 4. – The relationship between increased concentrations of the plasma antioxidant α-tocopherol and total sulphhydrils and improved pulmonary function (FEV1). FEV1: forced expiratory volume in one second.
but not with plasma concentrations of uric acid (fig. 4).

Classification of the patients with cystic fibrosis into homozygous (+/+) or heterozygous (+/-) for the ΔF508 gene established that there was no significant difference between the two groups for pulmonary function and concentrations of α-tocopherol, ascorbic acid, total sulphhydryls, lipid hydroperoxides, malondialdehyde or protein carbonyls in plasma. The concentration of uric acid was, however, significantly increased (p<0.01) in patients homozygous for ΔF508 (420.2±90.6 μmol·L⁻¹) compared with those who were heterozygous (302.8±51.02 μmol·L⁻¹).

Discussion

It has previously been reported that pulmonary function in cystic fibrosis patients declines with age [3], a finding which was confirmed in the present study. Since pulmonary function also deteriorates during acute respiratory infection [21], we hypothesized that the cumulative effect of repeated episodes of infection is responsible for the gradual decline in lung function in cystic fibrosis patients. In particular, we thought that excess production of oxygen free radicals (probably by activated neutrophils during cycles of infection), overburdens the resident antioxidant defences and oxidises membrane components of lung cells and thus contributes to the decrease in lung function observed. To address this hypothesis, we examined plasma obtained from patients with cystic fibrosis for indices of lipid and protein oxidation to determine whether oxidative stress, when present, was associated with decreased lung function.

Unfortunately, we were unable to obtain lung function measurements and plasma from age-matched control children for direct comparison. However, both quantitatively and qualitatively, the indices of free radical-mediated damage measured in the present study (table 1) were comparable to those reported previously for a different group of patients with cystic fibrosis [14], and are significantly greater than those reported for reference adult populations [22–24], and control children [14].

Separation of the patients with cystic fibrosis into two subgroups according to pulmonary function (assessed by FEV₁), resulted in the observation that patients with severely impaired pulmonary function had significantly elevated plasma concentrations of lipid hydroperoxides. Neither malondialdehyde or protein carbonyls exhibited this relationship, although malondialdehyde approached significance (p=0.074) and given greater patient numbers may have reached significance. Indeed, plasma malondialdehyde concentration was inversely related to pulmonary function when all patients were considered (fig. 2). These findings suggest that lipid peroxidation, rather than protein oxidation, is closely associated with the decrease in pulmonary function seen in CF. The cytotoxic effects of lipid peroxidation products have been described previously [25], and malondialdehyde has been shown to affect cell morphology and deoxyribonucleic acid (DNA), ribonucleic acid (RNA) and protein synthesis in vitro. It is, therefore, conceivable that malondialdehyde may have some direct effect on pulmonary function.

The upregulation of the inflammatory response in cystic fibrosis and the link between elevated markers of inflammation and decreased pulmonary function is well-established [26], and the results of the present study provide evidence for a free radical-mediated component of this. Thus, lipid peroxidation, whilst leading to structural damage to membranes (such as changes in lipid hydrophobicity and integrity) due to the formation of hydroperoxides affecting lung viability, may also promote increased inflammation and oedema formation amplifying the disruption caused to pulmonary function.

The plasma antioxidant concentrations of patients enrolled in the present study were within the reported normal ranges for healthy control subjects, although interestingly, α-tocopherol, ascorbic acid and total sulphhydryls all showed a significant decrease with age (fig. 3). This occurred even though patients received regular multivitamin supplements with additional α-tocopherol prescribed when their blood chemistries proved unsatisfactory. Although reduced compliance in taking the supplements by older patients may partially explain this observation, following discussion with our patients, we are reasonably confident that this was not the case in this study.

Other workers have linked decreased antioxidant concentrations with an elevated oxidative load both in cystic fibrosis and other diseases [27, 28]. Such a possibility is supported by the positive correlation's observed between pulmonary function and plasma concentrations of α-tocopherol, ascorbic acid and total sulphhydryls (fig. 4). The reason why these plasma antioxidants show positive correlation's with lung function is almost certainly related to the antioxidant milieu present in the respiratory tract. ROUM et al. [13] reported decreased pulmonary concentrations of glutathione in patients with cystic fibrosis, and the positive relationship between total plasma sulphhydryls and pulmonary function that we observed in this study may reflect increased oxidation of glutathione in the lung during periods of pulmonary exacerbation. The significantly lower concentration of plasma sulphhydryl groups in patients with the more severe pulmonary dysfunction (table 2) supports this premise.

The lack of a significant correlation between plasma uric acid concentrations and pulmonary function may arise from the effect that the high purine content of pancreatic enzyme supplements have on plasma concentrations [29]. The older patients in this study had markedly worse pancreatic insufficiency and were receiving larger doses of pancreatic enzymes. This may have masked any decrease in uric acid brought about by an increased pancreatic oxidative burden. We observed no relationship between genotype and phenotype with regard to pulmonary function, antioxidants and oxidative stress. The significant difference found between ΔF508 homozygous and heterozygous groups with regard to plasma uric acid may reflect increased pancreatic insufficiency in the homozygous patients, who consequently have a greater need for supplements of pancreatic enzymes [29, 30] as mentioned above.
In summary, the findings of this study support the assertion that free radical-mediated damage may play a role in the increase in pulmonary dysfunction in patients with cystic fibrosis [12, 31]. Importantly, markers of oxidative stress were present in many patients, even though they had normal concentrations of circulating antioxidants, although those patients with plasma concentrations at the higher end of the normal range had better pulmonary function. We conclude that normal levels of antioxidant defences in cystic fibrosis patients are insufficient to protect against the oxidative stress they experience from repeated episodes of infection. As a result, cumulative oxidative lung damage contributes to the progressive decrease in pulmonary function observed in these patients. Based on our understanding of free radical generation, additional antioxidant supplementation, as originally proposed by Udén et al. [32], may prove beneficial in slowing the rate of deterioration in pulmonary function when combined with current therapies. We suggest that the time has come to address this question directly through a controlled antioxidant supplementation trial.

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References