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Treatment of pulmonary exacerbations of cystic fibrosis leads to improved antioxidant status

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Treatment of pulmonary exacerbations of cystic fibrosis leads to improved antioxidant status. S.P. Range, C. Dunster, A.J. Knox, F.J. Kelly. ©ERS Journals Ltd 1999.

ABSTRACT: Many cystic fibrosis (CF) patients have increased circulating levels of oxidation products and/or decreased antioxidant status. This study investigated whether treatment of pulmonary exacerbations decreased oxidative stress in CF patients.

Seventeen adult patients were studied at the beginning and end of treatment with intravenous antibiotics. Plasma concentrations of the antioxidants ascorbic acid, α-tocopherol, uric acid and total reduced thiols, together with plasma retinol, lipid hydroperoxides, malondialdehyde and protein carbonyl levels were determined. Median (interquartile range) pretreatment and post-treatment levels were compared using the Wilcoxon signed rank test.

Clinical resolution was reflected by improved spirometry. Significant increases were observed in plasma ascorbic acid (pre 30.4 (15.7–38.6) μM, post 35.2 (27.3–49.6) μM), α-tocopherol (pre 19.7 (13.6–25.2) μM, post 25.2 (19.3–31.6) μM) and retinol (pre 1.9 (1.5–2.5) μM, post 2.7 (1.7–3.5) μM). No change in plasma total reduced thiols occurred following treatment (pre 409 (366–420) μM, post 392 (366–423) μM), whereas uric acid fell with treatment (pre 307 (274–394) μM, post 260 (216–317) μM). Neither plasma protein carbonyls or malondialdehyde levels altered with treatment (protein carbonyls pre 0.47 (0.28–1.27), post 0.67 (0.42–0.83) nM·mg protein^{-1}; malondialdehyde pre 0.75 (0.53–1.18), post 0.84 (0.65–1.15) μM). Lipid hydroperoxides levels did decrease following treatment (53 (18–85) *versus* 17 (10–55) nM).

This study demonstrated that treatment of infective exacerbations resulted in increased plasma levels of some antioxidant vitamins. No immediate change in plasma protein oxidation was observed, but lipid oxidation was decreased.

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Respiratory failure accounts for the vast majority of deaths in cystic fibrosis (CF). The last two decades have seen major advances in the understanding of the aetiology and pathophysiology of pulmonary disease in CF. In spite of this, the precise mechanism by which the basic underlying genetic defect leads to bacterial colonization, recurrent infection, pulmonary destruction and eventually respiratory failure is not fully understood. One possible mechanism of lung damage in CF is an increased level of oxidative stress resulting in oxidative damage of pulmonary tissues [1–3]. Highly reactive oxygen species (ROS) are produced in large numbers by neutrophils found within the airways of CF patients [4, 5]. Antioxidant defences may also be depleted in CF owing to both the increased utilization of these defences and pancreatic maldigestion leading to reduced levels of blood antioxidants in patients not receiving supplementation [6–8].

Patients with CF have been shown to have higher circulating levels of the products of oxidative damage [7, 9, 10] and an increased excretion of these in their urine [11]. Intervention with antioxidant supplementation has been shown to reduce markers of oxidative stress in CF patients [7, 12]. Recently, the quantity of lipid oxidation products in bronchoalveolar lavage samples has been correlated with the presence of pulmonary inflammation in children with CF [13]. Plasma levels of lipid oxidation products have been shown to be correlated with the severity of pulmonary disease in CF [14].

CF is characterized by a background of chronic pulmonary bacterial colonization with intermittent periods of more severe pulmonary symptoms termed "exacerbations". Pulmonary inflammation increases during these periods and subsides with antibiotic treatment [15]. Alteration in the severity of infection and the inflammation secondary to the host response may modulate the severity of oxidative stress within the CF lung.

The purpose of this study was to investigate possible changes in oxidative stress in patients with CF undergoing treatment for an infective exacerbation with intravenous antibiotic therapy in an inpatient setting. It was hypothesized that treatment of a pulmonary exacerbation would decrease the oxidative burden, leading to a reduction in the level of products of oxidative damage, and an increase in antioxidant capacity. To test this hypothesis, the pretreatment and post-treatment plasma concentration of markers of both protein (protein carbonyls) and lipid oxidation (lipid hydroperoxides and malondialdehyde) were measured, together with plasma antioxidants (ascorbic acid, α-tocopherol, uric acid and total reduced thiols).

Materials and methods

Study subjects

The study population consisted of 17 patients (seven male) with a mean age of 26±5 yrs admitted to the Nottingham Adult CF Unit. All patients had been diagnosed as having CF on the basis of clinical history, a sweat chloride concentration of >70 mmol·L^{-1}, and in most cases a genotype consistent with CF. The study was approved by Nottingham City Hospital Medical Ethics Committee, and all patients gave written informed consent. The diagnosis of a pulmonary exacerbation was made by the patient's physician based upon clinical symptoms and spirometry. All patients were colonized with either *Burkholeria cepacia*, *Pseudomonas aeruginosa* or a combination of the two organisms. Details of each patient's sputum microbial flora (cultured during the study), intravenous antibiotics received, length of treatment, and concomitant vitamin supplementation are shown in table 1.

Study design

Venous blood samples were collected within 24 h of commencing intravenous antibiotic treatment, and at the end of the antibiotic course. Patients' weight and spirometry were recorded at the time of blood collection. Samples were standardized with respect to time of day and ingestion of vitamin supplements or major meals.

Table 1. – Details of the microbiology, antibiotic treatment and vitamin supplementation of patients enrolled in the study

Patient No.	Sputum microbiology	Antibiotic	Treatment course days	Vitamin supplements
1	PA	AZLO	8	a
2	PA	AZ, G	12	a
3	PA, SA, Coli	I	10	b
4	BC	AZLO, G	14	a
5	PA, HI, SA	TAZ, T, AZ	20	a
6	PA, SA	TAZ, T	14	c
7	PA, SA	CEF, T	14	a
8	PA, BC	CEF, G	14	a
9	PA	CEF, T, DX	15	a
10	BC	CEF, AZ, DX	21	a
11	BC	M, CO	10	d
12	PA	CEF, T	9	c
13	BC	CEF, CO	14	none
14	BC	CEF, T	12	none
15	PA	AZ, T	10	none
16	BC	M, G	12	none
17	PA, HI, SA	CEF, T	13	none

Sputum microbiology: PA: *Pseudomonas aeruginosa*; SA: *Staphylococcus aureus*; Coli: mixed coliforms; BC: *Burkholderia cepacia*; HI: *Haemophilus influenza*. Antibiotics: AZLO: azlocillin; AZ: aztreonam; G: gentamicin; I: imnipenem; TAZ: tazocin; T: tobramycin; CEF: ceftazidime; DX: doxycycline; M: merepenem; CO: cotrimoxazole. Vitamin supplements: a: vitamin A 1,600 IU, vitamin E 220 mg; b: vitamin A 3,200 IU, vitamin E 240 mg; c: vitamin A 8,000 IU, vitamin E 200 mg; d: vitamin A 4,000 IU, vitamin E 150 mg (all supplements are per patient per day).

Methods

Spirometry was measured using a Vitalograph Alpha spirometer (Vitalograph, Buckingham, UK). Blood samples were collected in heparinized tubes and kept on ice in the dark and processed within 30 min. Plasma was extracted by centrifugation at 1,200×*g* at 4°C, and stored at -80°C until assayed. Plasma hydroperoxides were measured in a subset of six patients only as this required a more complex plasma preparation procedure: 3 mL blood was collected in heparinized tubes containing 30 μL of both 2 mM butylated hydroxytoluene (BHT, dissolved in methanol) and 2 mM desferoxamine mesylate. Samples were then centrifuged (1,200×*g*, 10 min, 4°C) to extract the plasma. Plasma (0.5 mL) was mixed with 20 μL 10 mM BHT and 0.25 mL 0.2 M citric acid. The lipid hydroperoxides were then extracted with 6 mL ice cold hexane, and the extract was dried under a stream of nitrogen and stored at -80°C until assayed.

Determination of plasma ascorbic acid and uric acid was carried out by high-performance liquid chromatography (HPLC) with electrochemical detection, as described previously [10]. Total reduced thiols were assayed by the method of ELLMAN [16]. The HPLC determination of α-tocopherol and retinol was based on the method of BIERI *et al.* [17] with the following modifications: 100 μL aliquots of plasma were mixed with 100 μL anhydrous HPLC-grade ethanol containing 5 μg α-tocopherol acetate as an internal standard. This mixture was then extracted with 400 μL HPLC grade hexane. The hexane layer was removed and evaporated to dryness under a stream of nitrogen. The extract was then redissolved in 400 μL HPLC-grade methanol and 100-μL aliquots were analysed. A 7×100 mm, 5-μm C_{18} column (Jones Chromatography, Hengoed, UK) was eluted with methanol–water (39:1 v/v) at a flow rate of 1.0 mL·min^{-1}. α-Tocopherol was detected from its absorbance at 292 nm, and retinol was detected at 325 nm. α-Tocopherol and retinol standards were run routinely for quantification purposes. It was assumed that the recovery of α-tocopherol acetate (generally 70–85%) accurately reflected the recovery of α-tocopherol and retinol.

Malondialdehyde was assayed by HPLC with fluorescence detection as described previously [18]. Lipid hydroperoxides were assayed by HPLC with chemiluminescence detection as also described previously [10]. Plasma protein carbonyl content was determined by a colorimetric assay according to LEVINE *et al.* [19], but with the following modifications. Briefly, each sample was divided into two 100-μL aliquots which were run in parallel throughout the assay. Four-hundred microlitres 10 mM 2,4-dinitrophenylhydrazine (DNPH) in 2.5 M HCl was added to one of the 100-μL aliquots (+DNPH sample), and 400 μL 2.5 M HCl to the other (-DNPH). The samples were incubated for 15 min at room temperature, with continual mixing. Protein was then precipitated with 500 μL 20% (w/v) trichloroacetic acid (TCA), and the samples were centrifuged (5 min at 14,000×*g*, 4°C). Supernatants were then decanted and discarded before the addition of 400 μL 10% TCA (w/v) to both the "+DNPH" and "-DNPH" samples, followed by mechanical disruption of the protein pellet with mild sonication (14 μm wavelength for 10 s), and then further centrifugation (conditions as above). Samples were washed three times in a 1:1 (v/v) absolute ethanol–ethyl acetate solution. On each occasion, samples were mixed

and allowed to stand for 5 min in the wash solution before centrifugation and aspiration of the supernatant. The final pellet produced from these washings was redissolved in 1.0 mL 6 M guanidine–HCl in 20 mM potassium phosphate (pH adjusted to 2.3 using concentrated HCl). After centrifugation, the absorbance of each supernatant, both "+DNPH" and "-DNPH" samples, was read against a 6 M guanidine–HCl solution blank using an LKB Ultraspec II (Cambridge, UK) at a wavelength of 370 nM. The carbonyl content for each sample was calculated from the Beer–Lambent law, using the molar extinction coefficient of DNPH, 22,000 $M \cdot cm^{-1}$, and corrected for nonspecific absorbance at 370 nm by subtraction of the absorbance associated with the "-DNPH" sample. In order to compare the carbonyl content of different samples, protein assays were performed on the "-DNPH" samples. Results are expressed as $nmols \cdot mg\ protein^{-1}$. The intra-assay variation associated with this assay was <5%, and the inter-assay variation was corrected for with aliquots of a positive control sample (a highly oxidized plasma sample of known carbonyl concentration) in each assay. The detection limit of sensitivity was determined as 2.5 $nmol \cdot mg\ protein^{-1}$.

Analysis

Statistical analysis was performed using Unistat (Unistat, London, UK). Within-subject differences were not normally distributed (Shapiro-Wilk normality test); therefore, the data are expressed as the median (interquartile range) for each group pre and post antibiotic treatment. Differences between groups were analysed using the Wilcoxon signed rank test.

Results

Of the 20 patients that entered, 17 patients completed the study (three patients did not undergo a post-treatment assessment at their own request). The clinical resolution of exacerbation was reflected by improved spirometry (pre-treatment forced expiratory volume in one second (FEV_1) 0.91 (0.59–1.57) L, post-treatment 1.09 (0.83–1.77) L, p=0.004).

The plasma ascorbic acid concentration increased following treatment (pre 30.4 (15.7–38.6) μM, post 35.2 (27.3–49.6) μM, p=0.012, fig. 1). Similar increases in the plasma concentration of α-tocopherol (pre 19.7 (13.6–25.2) μM, post 25.2 (19.3–31.6) μM, p=0.012, fig. 1) and retinol (pre 1.9 (1.5–2.5) μM, post 2.7 (1.7–3.5) μM, p=0.02, fig. 1) were also observed. No significant change in the plasma concentration of total reduced thiols occurred following treatment (pre 409 (366–420) μM, post 392 (366–423) μM, p=0.37). The plasma uric acid concentration fell with treatment (pre 307 (274–394) μM, post 260 (216–317) μM, p<0.001, fig. 1).

No change in the plasma concentrations of malondialdehyde or protein carbonyls was observed. The plasma protein carbonyl concentration pretreatment was 0.47 (0.28–1.17) $nmol \cdot mg\ protein^{-1}$, post-treatment 0.67 (0.42–0-83) $nmol \cdot mg\ protein^{-1}$ (p=0.54, whereas malondialdehyde was 0.75 (0.53–1.18) pretreatment *versus* 0.84 (0.65–1.15) μM post-treatment (p=0.71). Pretreatment and post-treatment lipid hydroperoxide measurements were made in six patients. Of these, three subjects had detectable circulating lipid hydroperoxides that all decreased following treatment (pre 53 (18–85) *versus* post 17 (10–55) nM).

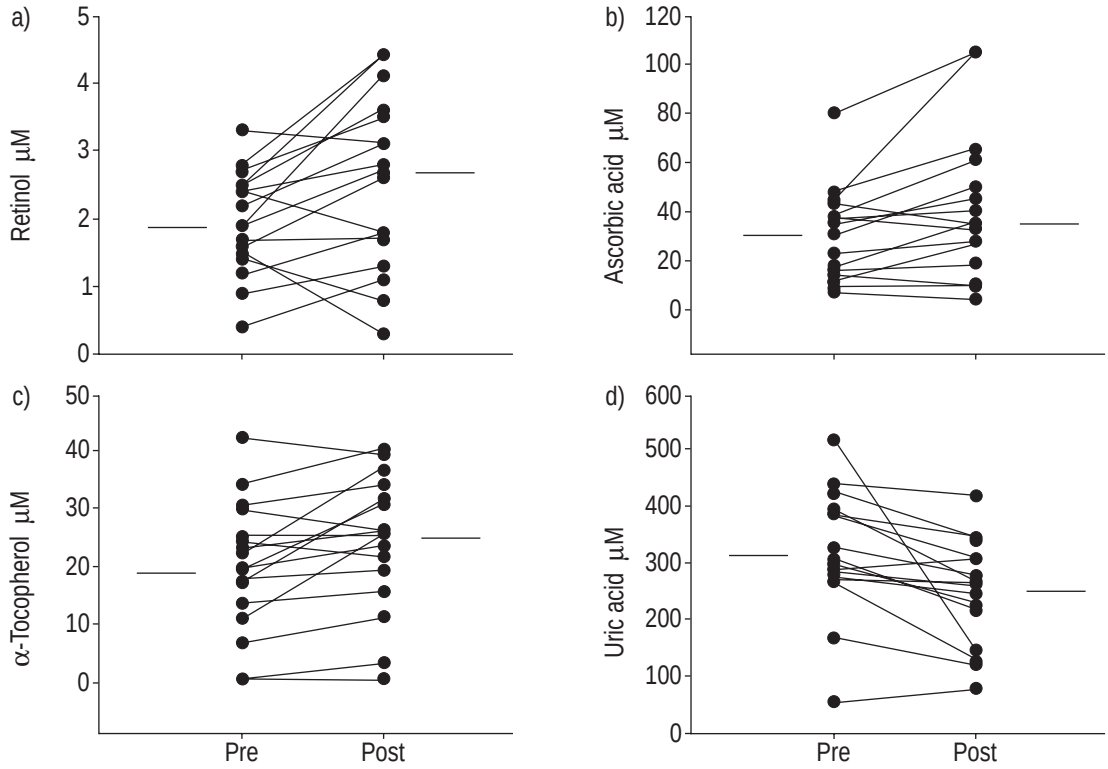

Fig. 1. – Effect of treatment on plasma antioxidants in 17 cystic fibrosis (CF) patients during an infective exacerbation. ●: individual patient values; ——: group medians. a) Retinol; difference between medians p=0.02; normal plasma range 1.3–2.8 μM [20]. b) Ascorbic acid; difference between medians p<0.01; normal plasma range 11–66 μM [20, 21]. c) α-Tocopherol; difference between medians p<0.01; normal plasma range 14.5–33.2 μM [20]. d) Uric acid; difference between means p<0.01; normal plasma range 50–900 μM [22].

Discussion

In this study, the hypothesis that the level of oxidative stress would be decreased following treatment of pulmonary exacerbations in CF was examined. This would be reflected as an increase in plasma concentrations of antioxidants and/or a decrease in plasma concentrations of markers of oxidative damage. Partial evidence was found in support of both proposals. Plasma concentrations of ascorbic acid and α-tocopherol increased, whereas no change in the concentration of plasma total reduced thiols was seen with treatment. In addition, although the plasma concentrations of the end products of oxidative stress, malondialdehyde and protein carbonyls, did not change over the treatment period, lipid hydroperoxide levels, an early product in lipid peroxidation chain of events, did fall in patients who had these oxidation products prior to treatment.

The data presented in this paper were derived from measurements made in plasma samples. A major limitation to the interpretation of these data is that the majority of oxidation products probably arise within the respiratory tract. Hence, the relationship between plasma and lung levels of these oxidation products, and indeed antioxidants, may be crucial. Ideally the level of antioxidants and markers of oxidative damage in lung lining fluid would have been measured, but such highly invasive sampling in a comparatively sick group of patients is difficult to justify. Sampling of plasma antioxidants and markers of oxidative damage have been used previously to investigate oxidative stress in inflammatory lung disease [7, 9, 10, 12, 14], where again its limitations were recognized.

In agreement with studies carried out in two other UK CF units [10, 14] these patients were not, as a group, antioxidant-deficient. Several individuals were, however, well outside the accepted normal range for several antioxidants. Following treatment, there were marked improvements in the antioxidant status of some, but not all subjects. Moreover, those patients that did respond were not necessarily those with an initially poor antioxidant status (fig. 1). As a group, however, the changes were significant. Several factors may be responsible for the observed increase in plasma ascorbic acid, retinol and α-tocopherol seen with treatment: the reduced infective burden following treatment leads to a reduction in neutrophil number and a reduction in neutrophil secretory products [15]. An associated reduction in neutrophil-derived ROS would thus be expected, which in turn would decrease the consumption of antioxidant vitamins such as ascorbic acid and α-tocopherol.

Alternatively, the increase in antioxidant vitamin concentrations may be explained by an improvement in compliance with vitamin supplements during an inpatient stay. Compliance with vitamin supplementation in CF is often poor owing to the large therapeutic burden of these patients. This cannot, however, explain the increase in ascorbic acid concentration observed in 13 of the 17 patients as no patient received ascorbic acid as a supplement. In addition, in the five patients not receiving vitamin supplementation, an increase in retinol was observed in all following treatment (pretreatment 1.2 (0.65–2.15) μm, post-treatment 1.8 (1.2–3.1) μm, p=0.043). Plasma levels of α-tocopherol increased in three unsupplemented patients, remained unaltered in one, and decreased in one (pre-treatment 19.7 (10.1–24.7) μm, post-treatment 21.6 (13.4–24.4) μm, p=0.27).

A further explanation for the observed increases in plasma antioxidants seen in this study may be improved nutrition during the treatment period. Although the dietary intake was not formally quantified in this study, it would be expected that as the patients′ infections resolve, nutritional intake would increase as they begin to feel better, with a corresponding increased intake of antioxidant vitamins.

Plasma uric acid concentration fell with treatment. Patients with CF often have a raised plasma uric acid, and this has been attributed to the high concentration of purines present in pancreatic enzyme supplements [23, 24]. A further study in CF patients suggested that increased catabolism was the underlying explanation and that uric acid secretion was correlated with disease severity [25]. In the present study, the decrease in uric acid concentration observed following treatment is probably most likely due to reduced catabolism subsequent to the resolving infection.

Oxidative stress is generally considered to represent the imbalance between oxygen radical generation and available antioxidant defences. Although marked improvements in antioxidant status occurred in the majority of patients, these changes were not accompanied by any decrease in two primary end products of oxidative stress, malondialdehyde and protein carbonyls. This apparent lack of effect is puzzling given previous findings that demonstrate decreased malondialdehyde following treatment of exacerbations of chronic obstructive pulmonary disease [26]. Possible explanations for the present finding include: 1) the improvement in antioxidant status achieved in most patients was still not sufficient to overcome their oxidative burden; 2) sufficient time in the presence of the increased antioxidant status had not elapsed to have an impact on the circulating levels of these markers of oxidative stress; or 3) there is no direct correlation between antioxidant status and oxidant stress in CF patients. Some evidence to refute partially the second and third of these possibilities was found in those patients in which lipid hydroperoxides were found before treatment, as these fell following treatment. As lipid hydroperoxides are amongst the first products in the lipid peroxidation cascade, this finding suggests that the improved antioxidant status may have benefited these patients.

In conclusion, the present study has demonstrated that plasma ascorbic acid and α-tocopherol concentrations increase following the treatment of infective exacerbations of cystic fibrosis. However, in the short term this improvement in antioxidant status does not appear to decrease the oxidative burden in these patients substantially.

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