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The effect of oral N-acetylcysteine on lung glutathione levels in idiopathic pulmonary fibrosis

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The effect of oral N-acetylcysteine on lung glutathione levels in idiopathic pulmonary fibrosis. A. Meyer, R. Buhl, H. Magnussen. ©ERS Journals Ltd 1994.

ABSTRACT: Idiopathic pulmonary fibrosis (IPF) is characterized by an increased oxidant burden and by a deficiency of glutathione, a major antioxidant, in the lung epithelial lining fluid (ELF). Therefore, a rational therapeutic approach is to reverse the imbalance between oxidants and antioxidants in the lung by enhancing the antioxidant screen. With this background, the aim of our study was to evaluate oral N-acetylcysteine (NAC) as a strategy to augment lung glutathione levels in patients with IPF.

Concentrations of total glutathione in bronchoalveolar lavage fluid (BALF) were quantified spectrophotometrically, before and following oral therapy with 3 × 600 mg NAC per day for 5 days, in 17 nonsmoking patients with biopsy-proven IPF. The volume of ELF recovered by BAL was determined using the urea method.

Pretherapy, total glutathione levels in ELF in IPF patients were significantly less than normal (187±36 *vs* 368±60 µM), in contrast to levels in BALF (0.99±0.12 *vs* 1.18±0.19 µM). Following therapy with oral NAC, glutathione levels in BALF were 1.54±0.24 µM (a significant increase compared to pretherapy), whereas the increase in ELF levels (319±92 µM) did not reach significance. The therapy was well-tolerated, and all routine clinical and bronchoscopic parameters remained unchanged.

It is thus feasible and safe to augment deficient lung glutathione levels in patients with IPF; thereby, potentially augmenting pulmonary antioxidant protection.

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Idiopathic pulmonary fibrosis (IPF) is a chronic inflammatory interstitial lung disease characterized by an accumulation of alveolar macrophages and neutrophils in the lower respiratory tract, parenchymal injury, and fibrosis of the alveolar walls [1–3]. Inflammatory cells in the lung release exaggerated amounts of highly reactive oxygen radicals (oxidants), which mediate the parenchymal cell damage that typifies IPF [4, 5]. This oxidant burden on the respiratory epithelial surface is even more consequential due to a deficiency in the lung epithelial lining fluid (ELF) of glutathione, the major component of the antioxidant defence systems that normally protect the lower respiratory tract against oxidant induced injury [6–8]. In addition, low ELF glutathione levels seem to play a major role in the exaggerated lung fibroblast proliferation seen in IPF [9]. Therefore, a rational therapeutic strategy in IPF is to augment glutathione levels on the respiratory epithelial surface, in an attempt to enhance antioxidant defences and to control fibroblast proliferation.

For two decades, N-acetyl-L-cysteine (NAC) has been widely used as a mucolytic drug in pulmonary medicine [10, 11]. More recently, the antioxidant potential of NAC has been established *in vitro* and *in vivo*. *In vitro*, the antioxidant capacity of NAC is directly related to the inactivation of electrophilic groups of free radicals [12]. *In vivo*, NAC exerts its function as an antioxidant *via* its main metabolite, cysteine, the major precursor in the biosynthesis of glutathione [13–15]. In this respect, in paracetamol poisoning, oral NAC is able to replenish liver glutathione pools and to prevent drug-induced hepatotoxicity [16, 17]. In patients with lung tumours, oral treatment with NAC lead to an increase of glutathione levels in venous plasma and bronchoalveolar lavage fluid (BALF) (for review [11]; and [18]).

Armed with this knowledge, and building on preliminary findings of an increase of BALF glutathione levels following oral NAC in a small group of patients with fibrotic lung disease of various aetiology [19], the aim of our study was to evaluate the effect of oral therapy with NAC on glutathione concentrations in BALF and ELF of patients with IPF.

Patients and material

Study population

The study population consisted of 17 nonsmoking patients with IPF (mean age 62±2 yrs). The diagnosis was based on history, physical examination, conventional

Table 1. – Patient characteristics

Patient	Age	Sex	VC	FEV_1	DLCO	Pa_{O_2}		Pa_{O_2} exercise	
no.	yrs		%pred	%pred	%pred	torr	kPa	torr	kPa
1	60	F	81	85	ND	68	9.0	48	6.4
2	59	M	58	56	ND	41	5.5	ND	-
3	65	F	47	54	ND	71	9.5	55	7.3
4	62	F	82	75	ND	66	8.8	55	7.3
5	68	M	100	100	60	77	10.3	41	5.5
6	58	F	93	85	45	82	10.9	69	9.2
7	69	M	83	64	51	54	7.2	ND	
8	69	M	100	100	62	72	9.6	77*	10.3*
9	71	M	100	100	51	70	9.3	57	7.6
10	56	F	99	91	53	65	8.7	49	6.5
11	55	M	89	83	89	72	9.6	66	8.8
12	67	F	81	86	76	79	10.5	55	7.3
13	76	F	93	100	ND	70	9.3	53	7.1
14	66	F	65	45	ND	57	7.6	34	4.5
15	72	F	80	70	70	73	9.7	45	6.0
16	40	M	80	80	64	78	10.4	63	8.4
17	64	F	100	100	55	57	7.6	45	6.0

VC: vital capacity; FEV_1: forced expiratory volume in one second; DLCO: diffusing capacity of the lungs for carbon monoxide; Pa_{O_2}: arterial oxygen tension; F: female; M: male; ND: not done. %pred: percentage of predicted value according to Quanjer PH, *Eur Respir J* 1993; 6 (Suppl. 16) 5–40. *: exercise insufficient due to physical handicap.

chest roentgenograms, computed tomography of the chest, pulmonary function tests, and pathomorphology of lung tissue specimens obtained by transbronchial or open lung biopsy (table 1). Eleven patients were still untreated, six were receiving prednisone (28±8 mg·day^{-1}; range 5–60 mg·day^{-1}), and two were also treated with β-acetyldigoxin (200 mg·day^{-1}). The medication was unchanged during the study period.

For comparison, 14 normal individuals (9 males and 5 females; aged 28±1 yrs) were evaluated. All were nonsmokers and free of lung disease, as determined by history, physical examination, chest roentgenogram and pulmonary function tests.

Study design

All patients were admitted to the hospital for diagnostic evaluation of shortness of breath, and/or radiological features of an interstitial lung disease. BAL was performed during fibreoptic bronchoscopy under local anaesthesia as part of the initial work-up to establish the diagnosis. Following the initial BAL, the IPF patients received 1,800 mg NAC per day (600 mg NAC every 8 h; N-acetyl-L-cysteine, Fluimucil long®, Zambon) for 5 days. BAL was repeated 10.5±0.5 h after ingestion of the last dose of NAC. A careful visual examination of the respiratory mucosa was performed as part of the bronchoscopies, to evaluate bronchitic changes due both to NAC or its metabolites and the repeated bronchoalveolar lavages [20].

All patients gave informed consent for the study. The study protocol was approved by the Ethics Committee of the Medical Board in Schleswig-Holstein, Germany.

Biologic samples

Since glutathione levels, at least in venous plasma, show a diurnal variation, venous plasma and BAL fluid were obtained in the morning after overnight fasting by standard techniques. Flexible fibreoptic bronchoscopy and BAL were performed as described previously [21], always by the same investigator (AM). Briefly, following local anaesthesia, the bronchoscope was inserted and gently wedged in a subsegment of the middle lobe. The standard lavage protocol was performed by infusing a total of five 20 ml aliquots (100 ml) of sterile 0.9% saline at body temperature through the aspiration port. After each aliquot, lavage fluid was collected *via* the same port into a plastic trap using wall suction. Portions of the BALF were taken to assay for glutathione (see below), for determination of total cell number, and for cytocentrifuge preparations for differential counts (table 2). The volume of ELF recovered by BAL was quantified using the urea method, based on urea levels in plasma and BALF [22].

Table 2. – Bronchoalveolar lavage before and after NAC therapy

	Pretherapy	Post-therapy	Normal
Recovery ml	64±3	64±3	68±1.5
ELF ml	0.72±0.1	0.77±0.18	0.25±0.3
Total cells $10^4 \cdot ml^{-1}$	10±2	11±3	3.5±0.5
Macrophages %	63±4.2	59±6.4	90±1.5
Lymphocytes %	16±3	18±3	6.2±0.8
Neutrophils %	12±3	14±4	0.6±0.1
Eosinophils %	6±2	8±3	0.2±0.05

NAC: N-acetyl-L-cysteine; ELF: epithelial lining fluid.

Glutathione levels

Glutathione in BALF was quantified with minor modifications of standard methods based on the specific reduction of oxidized glutathione (GSSG) by glutathione reductase, as described previously [23, 24]. N-acetylcysteine or cysteine in concentrations in the same order of magnitude as the glutathione concentrations present in BALF after oral administration of NAC do not interfere with the assay. In brief, to determine the total glutathione levels (*i.e*. reduced glutathione + glutathione disulphide (GSSG)), the various fluids were mixed with an equal amount of 10 mM 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) in 0.1 M potassium phosphate, pH 7.5, containing 17.5 mM ethylenediaminetetra-acetic acid (EDTA). The samples were centrifuged (2,000 ×g for 10 min), and aliquots (50 µl) of the supernatants were added to cuvettes containing 0.5 U of GSSG reductase in 0.1 M potassium phosphate, pH 7.5, containing 5 mM EDTA. After incubation for 1 min at 25°C, the assay reaction was started by adding 220 nM of reduced, β-nicotinamide adenine dinucleotide phosphate (NADPH) in 0.1 M potassium phosphate, pH 7.5, containing 5 mM EDTA in a final volume of 1 ml. The rate of reduction of DTNB was recorded spectrophotometrically at a wavelength of 412 nm (Beckman DU-70 spectrophotometer). Determination of the total glutathione concentration was based on standard curves generated from known concentrations of GSSG (0.125 to 4 µM) in phosphate buffered saline, pH 7.4. All measurements were carried out in duplicate.

Statistical evaluation

All data are presented as mean±standard error of the mean (SEM). Statistical comparisons between IPF patients and normal individuals were made using the Mann-Whitney test. The therapeutic response to NAC therapy was analysed using a Wilcoxon matched pairs signed-ranks test. Probability values of less than 0.05 were considered statistically significant.

Results

Bronchoalveolar lavage

The total number of cells, as well as the proportion of lymphocytes, neutrophils, and eosinophils, recovered by BAL in IPF patients was significantly elevated compared to normal ($p<0.05$, all comparisons) (table 2), *i.e*. the IPF patients had a neutrophil-dominated alveolitis, as is typical for this disease [1, 2]. Although the recovery of lavage fluid in IPF patients and normal individuals was comparable ($p>0.2$), the volume of ELF recovered by BAL as determined by the urea method was significantly higher in IPF patients ($p<0.001$) (table 2). Following oral therapy with 9 g of NAC in 5 days, the relative proportions of the bronchoalveolar cells did not change ($p>0.15$, all comparisons). Similarly, the volumes of BALF and ELF remained constant ($p>0.9$) (table 2).

Lung glutathione levels before and after NAC therapy

In IPF patients, pretreatment total glutathione levels in BALF (0.99±0.12 µM) were in the same range as normal glutathione levels in BALF (1.18±0.19 µM; $p>0.5$) (fig. 1a). Following therapy with a total of 15 doses of 600 mg NAC, BALF glutathione levels significantly increased to 1.54±0.24 µM, clearly within the normal range ($p<0.006$, compared to baseline; $p>0.6$, compared to normal values) (fig. 2a). Importantly, glutathione levels were still elevated even 10±0.5 h after the last medication (see study design). Pretreatment total glutathione

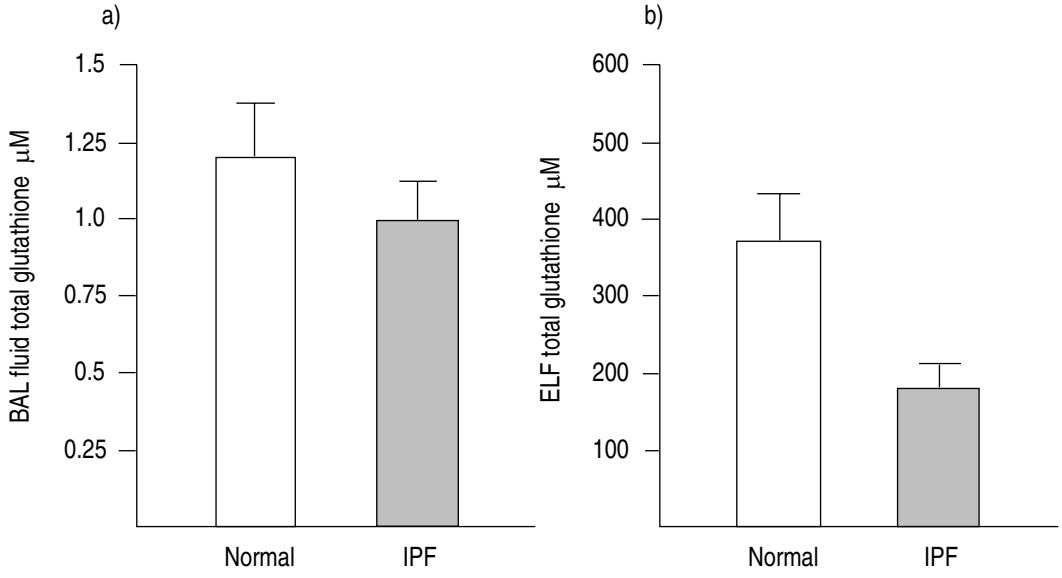

Fig. 1. – Glutathione concentrations in the lower respiratory tract of patients with IPF and normal individuals. a) Concentrations of total glutathione in BAL fluid ($p>0.5$, normal *vs* IPF). b) Concentrations of total glutathione expressed relative to the volume of ELF recovered by BAL ($p<0.015$). IPF: idiopathic pulmonary fibrosis; BAL: bronchoalveolar lavage; ELF: epithelial lining fluid.

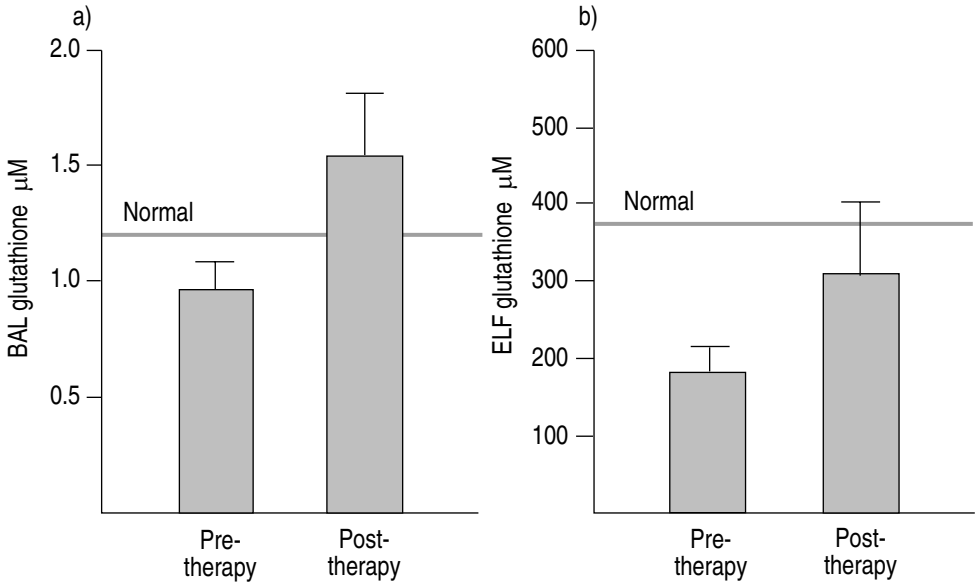

Fig. 2. – Effect of oral N-acetylcysteine on lung glutathione levels of patients with IPF. a) Concentrations of total glutathione in BAL fluid before and after therapy with 3×600 mg NAC per day for 5 days ($p<0.006$). b) Concentrations of total glutathione in ELF before and after NAC therapy ($p>0.2$). NAC: N-acetyl-L-cysteine. For further abbreviations see legend to figure 1.

levels in ELF were 187±36 µM, significantly lower than normal glutathione levels in ELF (368±60; $p<0.015$) (fig. 1b). After therapy with NAC, ELF total glutathione concentrations increased to 319±92 µM, close to normal values. This increase, although impressive, did not reach significance ($p>0.2$, compared to baseline) (fig. 2b).

Safety evaluation

No symptoms or adverse effects were noted referable to the administration of NAC. Physical examination and clinical study remained stable under therapy. NAC therapy did not affect the lower respiratory tract, as judged by visual inspection of the mucous membranes, measurements of ELF volumes, total cell number, and the percentages of alveolar macrophages, neutrophils, eosinophils and lymphocytes recovered by BAL before and after NAC treatment ($p>0.15$, all comparisons to baseline values) (table 2).

Discussion

The present study confirmed previous observations that IPF is characterized by a severe glutathione deficiency in the ELF of the lower respiratory tract [7, 25]. ELF glutathione levels in IPF patients were significantly reduced to about 51% of those seen in normal individuals. In contrast, levels of total glutathione in BALF in both groups were in the same range. This discrepancy is explained by a much greater ELF volume recovered by BAL in IPF patients compared to normal individuals; a consequence of the chronic alveolar inflammation.

While the pathophysiological mechanisms underlying the glutathione deficiency in the lung are still unclear [8], the various functions of glutathione in the lung point to a new therapeutic strategy in IPF: the re-establishment of glutathione levels in the lower respiratory tract by the administration of glutathione or precursor molecules. The feasibility and safety of augmentation of lung glutathione levels has been demonstrated in animal models [26]. In IPF patients, delivery of reduced glutathione directly to the lung by aerosol augmented deficient lung glutathione levels. Importantly, this therapy also resulted in a reduction of the spontaneous release of superoxide anion by alveolar macrophages, a process that plays a major role in the pathogenesis of IPF [25]. Superoxide anion, produced by the membrane-bound enzyme NADPH-oxidase of phagocytic cells in response to various stimuli, is central to pathways leading to highly toxic oxygen radicals, such as the hypohalide anion [4, 27–29]. Thus, therapy with glutathione was able to favourably influence the oxidant-antioxidant imbalance in the lung that is central to the pathogenesis of IPF [4, 7].

Another way to target antioxidants to the lower respiratory tract is oral therapy. Oral administration of glutathione itself is impossible due to gastric degradation by peptidases. However, NAC, a glutathione precursor, administered by the oral route, increased lung glutathione levels in animal models as well as in patients with lung tumours [18]. Our results extend these findings to patients with IPF. Oral therapy with NAC increased the deficient glutathione levels at least in BALF. Importantly, while glutathione aerosol therapy was able to raise lung glutathione only for a few hours [25], the increase of lung glutathione concentrations after oral NAC was still present about 10 h after the last dose of NAC. Although glutathione concentrations in ELF increased in a similar way following NAC therapy, the increase was not significant. This is not surprising, since the variation of the individual response to therapy with NAC between patients, and tolerances introduced by the estimation of ELF, may blur differences between pre- and post-therapy

ELF glutathione levels; especially since, in this case, the ELF volumes pre- and post-therapy were similar and not as clearly different as between IPF patients and normal individuals.

This study did not address the question as to the form of glutathione after NAC therapy. Following glutathione aerosol therapy to augment lung glutathione levels, there was an increase in the percentage of total glutathione that was oxidized both in animal studies and in patients with IPF [25, 26]. Since the aerosol process did not alter the glutathione molecule [26], reduced glutathione was probably oxidized in the lower respiratory tract as an antioxidant [25]. Further studies are necessary to determine whether the increase in the levels of total glutathione observed in this study is due to increased concentrations of reduced glutathione, of oxidized glutathione, or both. Similarly, the question of a potential influence of age on glutathione levels needs to be addressed, especially since IPF patients and normal individuals differ in their mean age.

Several lines of evidence suggest that augmentation of glutathione levels in lung ELF may be beneficial in IPF. Firstly, the glutathione system functions in ELF as a major component of the antioxidant screen that protects the pulmonary epithelium from oxidants [6, 8, 30]. In the context of an alveolar macrophage generated exaggerated oxidant burden and of the severe lung glutathione deficiency in IPF, the fragile alveolar structures of these patients are continuously exposed to increased amounts of toxic oxygen radicals without adequate protection, *i.e.* the reactive oxygen metabolites cause sufficient tissue damage culminating in interstitial lung disease [4, 5, 7]. Augmentation of the antioxidant protective screen of the lung is a therapeutic strategy to counterbalance this oxidant burden [26]. It remains to be seen if the increase in lung glutathione levels following oral therapy with NAC influences potential markers of disease activity in IPF, such as oxidant release by bronchoalveolar inflammatory cells, similar to glutathione aerosol therapy [25].

Secondly, patients with IPF have exaggerated numbers of fibroblasts in their lower respiratory tract. While physiological concentrations of glutathione are necessary to control human lung fibroblast proliferation, a decrease of extracellular glutathione levels resulted in an increase of fibroblast proliferation [9]. Current concepts of the pathogenesis of IPF include fibroblast proliferation as a major component of the interstitial changes leading to pulmonary fibrosis [3, 31]. Re-establishment of normal glutathione levels normalized fibroblast metabolism *in vitro* [9], further arguing in favour of augmentation of lung glutathione levels in IPF.

Thirdly, oral NAC therapy not only increased lung glutathione levels, but it did so with no short-term adverse effects. The therapy was safe, as judged by all routine clinical and bronchoscopic parameters evaluated. Importantly, there were no signs of an increased permeability of the alveolar-capillary barrier consequent to an increased inflammatory activity following NAC therapy. The volume of ELF recovered by BAL, a good indicator of influx both of plasmatic and interstitial fluid [22], remained constant (table 2). In contrast, in animal models NAC therapy increased albumin transudation into the airway lumen, a point that was not addressed in this study [32].

Up to now, nearly all evidence in favour of an antioxidant therapy in diseases like IPF is based on either *in vitro* experiments or on animal models. It remains to be seen if the "biochemical efficacy" of therapeutic strategies such as augmentation of lung glutathione levels by NAC translates into a more favourable course of the disease. We, therefore, suggest that oral therapy with NAC may be a rational approach to reverse the glutathione deficiency in the lower respiratory tract of patients with IPF in order to restore, at least partially, the diminished antioxidant capacity in this compartment and to control fibroblast proliferation. Since long-term NAC therapy has little adverse effects [33], since current therapeutic regimens often only marginally influence the fatal course of the disease [34], and since the glutathione deficiency may play a central role among the pathophysiological processes in IPF, a long-term therapeutic trial of NAC in IPF should be considered.

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