

The effects of N-acetylcysteine and inhaled steroid on inflammatory and oxidative stress markers in chronic obstructive pulmonary disease (COPD)

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Abstract

Introduction: Chronic obstructive pulmonary disease (COPD) is characterised by chronic airway inflammation with increased numbers of neutrophils in the airway lumen. Inhaled corticosteroids are widely used for the treatment of COPD despite of controversial statements concerning their efficiency. N-acetylcysteine is a mucolytic drug with antioxidant properties, counteracting the oxidant/antioxidant imbalance in COPD.

Objectives: The aim of the present study is to evaluate whether treatment of COPD patients with inhaled corticosteroid or N-acetylcysteine will change indices of inflammation and oxidative stress.

Material and Methods: Twenty COPD patients are treated for two times 10 weeks with fluticasone dipropionate (1000 µg/day) or N-acetylcysteine (600 mg/day) in a randomised crossover setting. Induced sputum and blood samples were collected every 10 weeks.

Results: Protective markers for oxidative stress, glutathione peroxidase (47.5 ± 5.3 vs. 57.6 ± 7.8 U/g Hb, $P < 0.05$) and trolox-equivalent antioxidant capacity (1.41 ± 0.05 vs. 1.50 ± 0.06 mM, $P < 0.05$) were increased by steroid treatment. N-acetylcysteine decreased significantly sputum eosinophil cationic protein (266 ± 61 vs. 136 ± 27 ng/ml, $P < 0.02$), sputum interleukin-8 (403 ± 65 vs. 326 ± 62 ng/ml, $P = 0.05$), and sputum tryptase, a serine protease (1.80 ± 0.29 vs. 1.33 ± 0.16 ng/ml, $P < 0.01$).

Conclusions: These results suggest inhaled steroids have no action on inflammatory indices in the treatment of COPD, while N-acetylcysteine has some anti-inflammatory actions. Furthermore, steroids seem to have a positive influence on oxidant/antioxidant imbalance that is thought to be important in the pathogenesis of COPD.

Key words: treatment, induced sputum, inflammatory markers, oxidative stress.

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Introduction

Chronic obstructive pulmonary disease (COPD) has been recognized for many years as a leading cause of mortality and morbidity. There was however a pessimistic approach to the possibilities of effective treatment. Recently

however, some new interest in the pathogenesis and treatment emerged. Obviously the poor outcome of the disease and the lack of progress in the pharmacological therapy have forced many clinicians and scientists to renew their attention. Several consensus statements have been published during the last decade [1-4].

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Damage to lung tissue as a result of an inflammatory response is usually accompanied by an influx of neutrophilic granulocytes. In patients with COPD an enhanced number of neutrophils was found both in the airways and in bronchoalveolar lavage fluid (BAL) [5-7]. The initial step in this process is the expression of adhesion molecules by vascular endothelial cells in the vicinity of the inflammatory spot, followed by an interaction between adhesion molecules of both endothelial cells and neutrophils. Next step is the action of infiltrating neutrophils upon other cells in the lung, with release of toxic metabolites as oxygen radicals and proteolytic enzymes [7, 8] leading to extension of the inflammation [9, 10]. The extent of inflammation may be reduced by use of anti-inflammatory drugs [11, 12]. In a model of extra corporeal circulation during cardiac surgery with activation of neutrophils and complement, it was also shown that administration of steroids or of the radical scavenger N-acetylcysteine (NAC) could inhibit the production of pro-inflammatory cytokines in both peripheral blood and in the lung [10, 13]. It was also reported that soluble intercellular adhesion molecule (sICAM-1) in both serum and BAL fluid of COPD patients was enhanced in comparison with normal individuals, although in serum there existed some overlap with normal values [14]. Recently, elevated levels of cell bound ICAM-1 were reported [15-17]. High concentrations of the cytokines interleukin-8 (IL-8) and tumor necrosis factor α (TNF- α) in induced sputum from patients with COPD have been reported [18], and these cytokines may be involved in the recruitment of neutrophils [19, 20]. Although the role of corticosteroids in the management of COPD is controversial [3, 12, 21, 22] cytokine gene expression is known to be reduced both *in vitro* [23] and *in vivo* [24] in response to corticosteroids.

This has been postulated as the mechanism of action of corticosteroids in COPD [25]. If steroids do play a role in the treatment of COPD, it is reasonable to suggest that they act through down-regulation of cytokines and adhesion molecules. The latter may also be true for any reducing effect of NAC on cell migration and activation [13, 26-28].

The aim of the present study is to evaluate the mechanisms of inflammation in COPD by studying the release of inflammatory mediators in the airways of patients, and to evaluate the effects of treatment with inhaled corticosteroids or anti-oxidants on the inflammatory pathway.

Material and Methods

Twenty stable patients were recruited (16 males, 4 females) at the out-patient clinic of the National Institute of Tuberculosis and Lung Diseases, Warsaw, Poland. Inclusion criteria for entry were: smoker or ex-smoker, not fully reversible airway obstruction with $50\% < FEV_1 < 80\%$ and chest X-ray compatible with COPD. Patients with any history of atopy, asthma, renal and/or hepatic failure, major cardiac disease, cystic fibrosis, as well as patients who suffered an exacerbation of COPD in the three months preceding inclusion, or used non-steroid anti-inflammatory medication, theophylline, long-acting beta stimulants or oxygen therapy, were excluded from the study. All patients were fully informed about the purpose of the study and gave written informed consent before inclusion. The study protocol was approved by the Ethics Committee of the National Institute of Tuberculosis and Lung Diseases.

Study design

The study consisted of 4 consecutive periods of 10 weeks (Figure 1), and involved at least five visits. At each scheduled visit, induced sputum and blood samples were collected and spirometry was performed. During the first and third period no medication was allowed, except for short-acting beta agonists and/or cholinergics (salbutamol or fenoterol and/or ipratropium bromide) which were allowed to use in case of necessity during the whole study period. During the second and fourth study period, patients were treated with either inhaled FP or oral NAC in a randomized and cross-over setting. A prolongation of the study period by two weeks was allowed in case of an occurring acute exacerbation of COPD which required treatment with antibiotics. Thus the respective study period ended 2 weeks after finishing antibiotic treatment of the patient. Patients were withdrawn from the study if they required treatment with either anti-inflammatory agents, oral steroids, theophylline, and/or oxygen and thus compromising the study protocol.

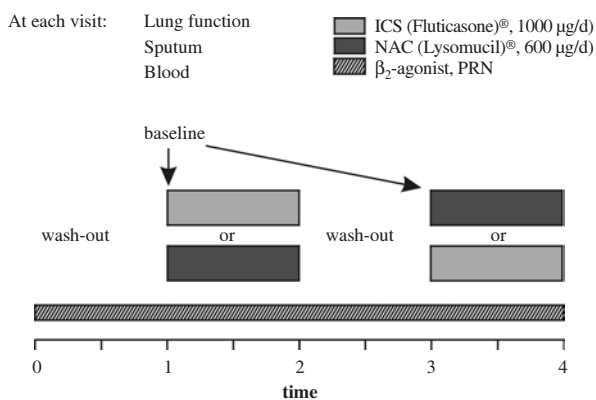


Fig. 1. Study design. At each visit lung function tests were performed and samples of induced sputum and blood were collected. Before and in between the two treatment periods a wash-out was scheduled. Baseline values were obtained after each wash-out. Treatment with β_2 -agonists was continued during the whole study period, but only when it was needed by the patient

Sputum collection and processing

Sputum induction and processing were performed according to the guidelines of the Task Force Induced Sputum of the European Respiratory Society [29, 30]. The

Table 1. Patients' characteristics at visit 1

Characteristics	Mean \pm SEM	Range
number of patients	20	–
age (years)	64.6 \pm 2.0	48-81
sex (male/female)	16/4	–
BMI (kg/m ²)	25.6 \pm 1.1	18.6-38.4
COPD history (months)	54 \pm 13	6-240
current smoker/ex-smoker	10/10	–
smoking (pack years)	36.7 \pm 3.7	15-80
FEV ₁ (% pred.)	60.6 \pm 1.7	46-80
FEV ₁ (% pred.) post salbutamol	65.1 \pm 2.6	47-94
FVC (% pred.)	84.1 \pm 2.7	65-105
FVC (% pred.) post salbutamol	87.9 \pm 2.8	71-110
FEV ₁ /FVC	0.54 \pm 0.02	0.41-0.69
FEV ₁ /FVC post salbutamol	0.55 \pm 0.03	0.40-0.72

BMI – body mass index, FEV₁ (% pred.) – forced expiratory volume in 1 second as percentage predicted, FVC (% pred.) – forced vital capacity as percentage predicted.

collected sputum was processed as soon as possible, but always within two hours after induction. Total cell count was performed manually using a Bürker counting chamber, and cell viability was determined by trypan blue exclusion method. The sputum supernatant was aspirated and stored at -80°C until assay. The cells were used for cytospin slides.

Laboratory assays

Trolox equivalent antioxidant capacity (TEAC) was measured by use of a spectrophotometrical method [31], glutathione peroxidase (GPx) in full blood was measured based on the method of Paglia and Valentine [32], and superoxide dismutase (SOD) was measured spectrophotometrically using a commercially available kit. Albumin was measured spectrophotometrically as described previously [33]. Alpha-tocopherol and retinol were measured by HPLC. Eosinophil Cationic Protein (ECP) and tryptase were analyzed in serum and in sputum through a fluoroenzyme immunoassay. Free neutrophil elastase activity in serum samples was determined using conversion of a substrate [34]. Soluble ICAM-1 and interleukins were assayed by use of commercially available enzyme-linked immunosorbent assays.

Statistical analysis

All data are presented as mean \pm standard error of the mean (SEM). Assuming Gaussian distribution, data were analyzed for differences by the Student's *t*-test for paired samples. Data were compared between the baselines and the outcome of the treatment after 10 weeks. For non-parametric

Table 2. Total number of sputum cells, and sputum neutrophils (both absolute numbers and percentage) before and after steroid and NAC treatment

Cell count	Baseline	ICS	Baseline	NAC
total sputum cells (10 ⁶ /ml)	5.52 \pm 1.28	4.51 \pm 0.96	6.62 \pm 2.03	3.86 \pm 0.90
n	17	17	19	19
range	0.72-16.45	0.34-13.68	0.21-39.20	0.33-15.00
sputum neutrophils (10 ⁶)	3.66 \pm 0.88	3.18 \pm 0.83	4.72 \pm 1.69	2.82 \pm 0.79
n	16	16	17	17
range	0.42-12.28	0.13-11.24	0.16-29.36	0.14-12.21
sputum neutrophils (%)	60.4 \pm 3.3	56.5 \pm 5.8	63.0 \pm 3.9	63.7 \pm 4.0
n	16	16	17	17
range	41.3-87.2	15.0-89.0	31.1-84.8	27.4-81.4

Data are presented as mean \pm SEM. Baseline values are counted before start of the respective treatment. No statistical significant differences were observed ICS – inhaled corticosteroids, NAC – N-acetylcysteine.

data (IL-10, tryptase, sputum albumin, and neutrophil elastase activity) the Wilcoxon test was used. At a P value <0.05, differences were considered significant.

Results

The results represent an analysis of 20 patients who provided adequate samples from each visit. Two patients dropped out after the fourth visit: one because of protocol violation and the other one due to prolonged exacerbation of COPD. Three patients (included in the analysis) experienced acute exacerbation of COPD during a wash-out period. One of them before FP treatment, the two other patients before NAC treatment. The acute exacerbation of COPD required treatment with antibiotics.

Three patients resumed smoking during the study period. One of them dropped already out because of a prolonged exacerbation of COPD. One patient resumed smoking during the first wash-out period and was considered as active smoker. The third patient resumed smoking during FP treatment in the last study period. Resuming to smoke during FP treatment resulted in a strong enhancement of GPx and TEAC levels. However, the mean values and the resulting statistical outcome were not significantly influenced by this fact.

The characteristics of the patients are given in Table 1. Samples collected after the wash-out period before FP treatment were considered as baseline FP samples. The remaining samples collected during the same visit were considered as baseline NAC samples.

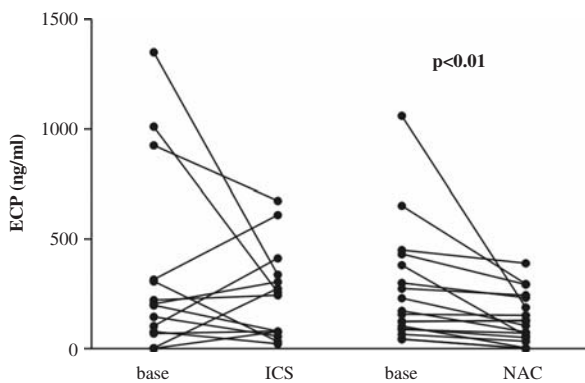


Fig. 2. Levels of sputum ECP measured at baseline and after a 10-weeks treatment period with either fluticasone propionate or N-acetylcysteine (NAC). The levels decreased significantly after NAC treatment, while inhaled steroid treatment led only to a non-significant trend towards decrease

The total number of cells in the sputum was assessed, as was the percentage and absolute numbers of neutrophils. No significant differences in cell numbers were observed between the various visits and treatment modalities (Table 2). Also percentages of sputum macrophages, lymphocytes, and eosinophils did not differ between the sampling points. Differential cell counts and cell numbers in the circulation did not show any significant differences between the subsequent visits.

Inflammatory markers

NAC decreased significantly sputum ECP levels from 266 ± 61 to 136 ± 27 ng/ml ($P < 0.02$, $n = 18$), while FP showed only a non-significant trend towards decrease (352 ± 113 vs. 247 ± 56 ng/ml, $n = 14$) (Figure 2). Treatment with NAC led also significant decreases in the sputum levels of IL-8 (403 ± 65 vs. 326 ± 62 ng/ml, $P < 0.05$, $n = 17$) and tryptase, a mast cell protease (1.80 ± 0.29 vs. 1.33 ± 0.16 ng/ml, $P < 0.01$, $n = 18$). Also here no significant influence of FP treatment was observed. IL-8 changed from 603 ± 225 to 466 ± 64 ng/ml, while tryptase changed from 1.77 ± 0.31 to 1.58 ± 0.37 ng/ml respectively. For all other inflammatory parameters that were measured no significant differences were observed. The results of these measurements are presented in Table 3. The mediator values of the different baselines were never significantly different from each other.

Oxidative stress markers

The only oxidative stress markers that were significantly influenced by treatment were GPx and TEAC. Treatment with FP significantly increased GPx from 47.5 ± 5.3 to 57.6 ± 7.8 U/g Hb ($P < 0.05$, $n = 19$) (Figure 3). Treatment with NAC did not change the GPx levels: 58.1 ± 6.8 vs. 53.6 ± 6.0 U/g Hb ($n = 17$). Although small in increase,

Table 3. Inflammation markers in COPD patients before and after steroid and NAC treatment

Mediator	Baseline	ICS	Baseline	NAC
serum IL-8 (pg/ml)	16.4 ± 0.9	16.9 ± 0.9	16.2 ± 0.9	15.4 ± 0.5
n	19	19	18	18
range	14.0-26.6	14.0-25.7	14.0-27.9	14.0-20.1
serum ECP (ng/ml)	10.4 ± 1.4	10.2 ± 1.5	9.5 ± 1.7	9.0 ± 1.6
n	19	19	18	18
range	2.0-24.7	2.9-23.1	2.0-32.5	2.0-27.5
serum IL-10 (ng/ml)	34.2 ± 6.1	17.7 ± 1.4	40.9 ± 18.6	43.8 ± 18.4
n	7	7	6	6
range	15.0-54.2	15.0-24.3	15.6-133.1	15.0-135.0
serum ICAM-1 (ng/ml)	489 ± 50	496 ± 48	475 ± 38	457 ± 40
n	17	17	17	17
range	262-913	246-929	258-783	213-762
serum SOD (U)	944 ± 95	1147 ± 114	1070 ± 125	1040 ± 96
n	17	17	17	17
range	154-1464	362-2083	290-1977	429-1864
sputum ICAM-1 (ng/ml)	1.40 ± 0.20	2.17 ± 0.47	2.38 ± 0.50	1.76 ± 0.43
n	10	10	14	14
range	0.47-2.80	0.14-4.40	0.49-5.91	0.02-5.44
sputum NE activity (nM)	376 ± 184	187 ± 93	277 ± 123	108 ± 46
n	11	11	13	13
range	0.3-1600	0-820	1.8-1300	0.1-540

Data are presented as mean \pm SEM. Baseline values are collected before start of the respective treatment. No statistical significant differences were observed. ICS – inhaled corticosteroids, NAC – N-acetylcysteine, ECP – eosinophil cationic protein, ICAM – intercellular adhesion molecule, SOD – superoxide dismutase, NE – neutrophil elastase.

TEAC levels were significantly enhanced by FP treatment (Figure 4). The determined levels rose from 1.41 ± 0.05 to 1.50 ± 0.06 mmol/l ($P < 0.05$, $n = 14$). NAC treatment tended to decrease levels of TEAC, but this effect was not significant (1.55 ± 0.05 vs. 1.46 ± 0.06 mmol/l, $n = 15$). Levels of SOD and albumin did not change during the course of the study, as did not retinol and tocopherol. Also in case of the oxidative stress markers, no differences were observed between the different baseline values.

Discussion

The present study demonstrated that treatment of COPD patients with ICS during a period of 10 weeks did not significantly influence both local and systemic markers of

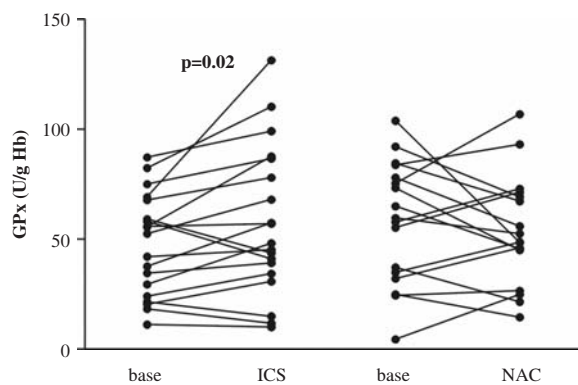


Fig. 3. Levels of glutathione peroxidase (GPx) measured in full blood at baseline and after a 10-weeks treatment period with either fluticasone propionate or N-acetylcysteine (NAC). Treatment with FP significantly increased the blood GPx levels, while a treatment with NAC did not change the levels

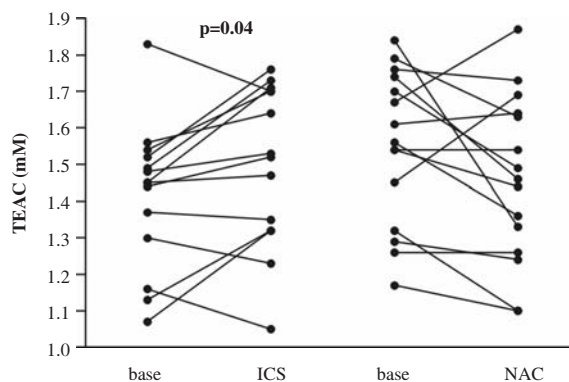


Fig. 4. The trolox equivalent antioxidant capacity (TEAC) of plasma was measured at baseline and after a 10-weeks treatment period with either fluticasone propionate or N-acetylcysteine (NAC). The TEAC value was significantly enhanced by steroid treatment. NAC treatment tended to decrease the value of TEAC, but this effect was not significant

inflammation. However, some enhancing effect of ICS was observed on the levels of GPx and TEAC in the circulation, which suggest a positive influence on antioxidant capacity. Treatment of the same patients with NAC demonstrated that NAC is able to inhibit some local markers of inflammation. Effects on systemic inflammatory markers and on any systemic marker of oxidative stress were not observed. The study was designed as a direct, head-to-head comparison of the effects of a high dose of FP with NAC, with an emphasis on the assessment of inflammatory markers and markers of antioxidant defense.

The inflammatory processes in the airways may serve as a reason for use of inhaled steroids as anti-inflammatory therapy in COPD. It was suggested, because the inflammation in COPD is neutrophilic, the treatment with steroids would be less successful. Systemic steroids are known to increase peripheral neutrophil counts, which may reflect a delayed apoptosis [35]. Although it has not been investigated if this is also the case for lung neutrophils, we did not observe any reduction in the numbers of neutrophils in sputum. Contrary to Thompson and coworkers, who could also not show any effect on neutrophil numbers, and we did not observe any effect on total cell counts [36], but others did [37]. Although the use of steroid treatment is controversial, even some patients can benefit from it [38]. However, from our study the benefit of steroid treatment was not found on the level of inflammatory markers assessed. One would expect some effect on these inflammatory markers as many *in vitro* studies suggest. Surprisingly, we found an effect on two markers of oxidative stress, albeit only marginal, but significant. However, this effect is limited to systemic members of the anti-oxidant system, GPx and TEAC, the latter a rather unspecific anti-oxidant system. On the other hand, the

observed effects of steroids on the oxidant-anti-oxidant balance are not completely unexpected. A few publications associate estrogen, cortisol and 17β -estradiol with elevated levels of plasma and erythrocyte GPx activity during the menstrual cycle [39, 40]. In acute exacerbation of COPD an enhancement of TEAC and plasma sulfhydryl levels were described after steroid treatment [41]. In asthma it was demonstrated that ICS reduced inflammation, attenuating the release of oxidants by inflammatory cells, and suppressing proinflammatory cytokines production, which may lead to a reduction in lipid peroxidation [42-44]. Typically the inflammatory processes in COPD are dominated by a neutrophilic inflammation, and because lack of suppressive effects of ICS on neutrophils was reported [42], the inability to see inhibitory effects of ICS in COPD could be suspected. This negative observation is supported by studies in COPD patients where exhaled NO and CO, which are in fact indirect markers of oxidative stress, are enhanced even if the patients were taking ICS. This may be explained by a constant activation of NO synthase [45] and therefore reflect airway inflammation and failure of ICS to inhibit neutrophilic inflammation. It was also reported that a potent inhalation corticosteroid (FP) had an inhibitory effect on the production of reactive oxygen species from lavage cells obtained from smoking COPD patients. Heavy smoking impaired the ability of alveolar macrophages to produce reactive oxygen species, which was not further decreased by FP incubation [46]. However, in our study we did not observe any significant different values of oxidative stress related parameters between the current smokers and the ex-smokers. The in the literature described effects of steroid treatment on the level of exhaled gases may be the result of a complex interaction with enzymes and release of

reactive oxygen species by inflammatory cells [47]. The benefit of steroid treatment in certain COPD patients, as reported in literature, may be only applicable to the mixed-type patient with asthma symptoms.

NAC affected some of the inflammatory markers in the sputum, and had no effect on systemic inflammatory markers nor markers of oxidative stress. It was reported that IL-8 levels are elevated in both sputum and lavage fluid from COPD patients as compared to normal individuals [48, 49]. NAC treatment is able to reduce IL-8 levels in plasma, but in the present study no such effect was observed [13]. Our present findings are in accordance with previous reports which showed NAC as an inhibitor of factors with chemotactic and priming activity for neutrophils [13, 50, 51]. It was also reported that NAC is able to inhibit TNF- α -induced NF- κ B activation and reactive oxygen species dependent IL-8 increase [52]. NAC demonstrated a similar potent inhibition on NF- κ B activation in vascular endothelial cells [53]. Therefore, the suppression of NF- κ B activation by NAC, which seems an important property of this antioxidant, may account for the observed decrease in IL-8 levels, and for described reduction in neutrophil influx, although we could not demonstrate this latter effect. Furthermore, the lower levels of IL-8 may lead to lower ECP levels. Despite the fact that ECP is of eosinophilic origin, no relationship was found in some studies between eosinophil number and ECP levels in induced sputum [54]. This fact suggests that ECP sputum concentrations are not merely a function of eosinophil numbers. It has been suggested that ECP can be taken up and transported by neutrophils [55], for which IL-8 is a strong chemoattractant. Therefore, any changes in IL-8 levels would affect neutrophil activation status and thus influence the sputum level of ECP.

In conclusion, we might say that use of inhaled corticosteroids in stable COPD has only minor effects and these effects are probably indirect, leading to an enhancement of the redox balance. Something that was not observed for NAC. On the other hand, NAC demonstrated some anti-inflammatory actions.

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