NEW DEVELOPMENTS IN THE TREATMENT OF COPD: COMPARING THE EFFECTS OF INHALED CORTICOSTEROIDS AND N-ACETYLCYSTEINE

Inhaled corticosteroids (ICS) are widely used for the treatment of COPD despite of controversial statements concerning their efficacy. The use of N-acetylcysteine (NAC), a mucolytic drug with antioxidant properties, is less clear, but it may counteract the oxidant-antioxidant imbalance in COPD. The aim of this study was to evaluate whether treatment of COPD patients with ICS or NAC is able to improve inflammatory indices and to enhance lung function. ICS treatment enhanced protective markers for oxidative stress such as glutathione peroxidase (GPx) (51.2 ±5.8 vs. 62.2 ±8.6 U/g Hb, P<0.02) and trolox-equivalent antioxidant capacity (TEAC) (1.44 ±0.05 vs. 1.52 ±0.06 mM, P<0.05). NAC decreased sputum eosinophil cationic protein (318 ±73 vs. 163 ±30 ng/ml, P<0.01) and sputum IL-8 (429 ±80 vs. 347 ±70 ng/ml, P<0.05). The increased antioxidant capacity prevented an up-regulation of adhesion molecules, since the levels of intracellular adhesion molecule 1 (ICAM-1) correlated negatively with GPx (P<0.0001) and TEAC (P<0.0001). On the other hand, expression of adhesion molecules was promoted by inflammation, reflected by a positive correlation between the levels of IL-8 and ICAM-1 (P<0.0001). The effects of treatment on lung function were only reflected in the FEV\textsubscript{1} values. The absolute value of FEV\textsubscript{1}, both before and after salbutamol inhalation, increased from 1690 ±98 to 1764 ±110 ml, and 1818 ±106 to 1906 ±116 ml, respectively, after ICS (P<0.05). Ten weeks after treatment, FEV\textsubscript{1} values dropped to 1716 ±120 ml post-salbutamol (P<0.05). When followed by treatment with NAC, these values decreased even further to 1666 ±84 ml. These results suggest that ICS improved lung function in COPD patients with moderate airflow obstruction, beside a minor improvement in the oxidant-antioxidant imbalance leading to a lesser expression of ICAM-1. Treatment with NAC decreased some inflammatory parameters and had indirectly an inhibitory effect on the expression of adhesion molecules.

Key words: chronic obstructive pulmonary disease, fluticasone propionate, inflammation, N-acetylcysteine, oxidative stress
INTRODUCTION

The pathogenesis of COPD is multifactor. It involves neutrophilic airway inflammation (1-3) and the imbalance between oxidants and antioxidants (4). Since these two processes are closely linked to each other, any interference in one of these processes may affect the mechanisms and outcome of other factors. The inflammatory mechanisms in the lungs are complex and the cascade of mediator release depends on the interplay between many stimulants and inhibitors (5). Proinflammatory cytokines and growth factors stimulate the production of reactive oxygen species (ROS) that act as signaling mediators for a variety of signal transduction pathways and gene expression (6, 7). The overproduction of ROS, together with an excessive migration process of inflammatory cells and release of proteolytic enzymes further extends the inflammatory process (8, 9). Normally, this process can be reduced by use of anti-inflammatory drugs, such as inhaled corticosteroids (ICS). Although ICS are widely used for the treatment of COPD, controversial effects concerning their efficacy are reported (10-14). On the other hand, improvement of the antioxidant capacity in COPD patients is an interesting therapeutic approach in view of the fact that ROS play a role in its pathology and in oxidative stress (15). Among the antioxidant enzymes, superoxide dismutase is considered fundamental in the process of eliminating ROS by reducing superoxide to form hydrogen peroxide. Antioxidant enzymes such as glutathione peroxidase (GPx) and catalase are responsible for the reduction of intracellular peroxidases. All these enzyme systems are regulated through a feedback mechanism. Through this system steady low levels of those enzymes and of superoxide and hydrogen peroxide are maintained, which keeps the entire system in a full functioning state. Thus, the ability of an individual to prevent the injurious effects of oxidative stress depends on the antioxidant capacity of tissues and blood (16). In COPD the antioxidant capacity was reported to remain low with a further decrease during exacerbation (17). N-acetylcysteine (NAC), a mucolytic drug with antioxidant properties may counteract the imbalance between oxidants and anti-oxidants in COPD. NAC is a source of sulphydryl groups and it increases the levels of reduced glutathione (GSH). This GSH serves as a reducing agent forming the intermolecular disulfide non-radical end product oxidised glutathione (GSSG). NAC is also a scavenger of free radicals as it interacts with ROS (18).

The aim of the present study is to evaluate whether ICS and NAC have any effect on inflammatory and oxidative stress markers and whether any effect could be observed in lung function parameters of COPD patients.

MATERIALS AND METHODS

Patient selection and study design

The study conformed to the Declaration of Helsinki and informed written consent was obtained from each subject. The experimental protocol was approved by the Ethics Committee of the
National Institute of Tuberculosis and Lung Diseases in Warsaw. Twenty patients (F/M - 4/16) with stable COPD were recruited at the above mentioned institute. All subjects had a smoking history of 37 ±4 pack years, 10 were current smokers and the remainder had ceased smoking for at least 2 months. The inclusion criteria were: irreversible airway obstruction (≤10% of baseline), post-bronchodilator FEV$_1$ ≥50–80% predicted, FEV$_1$/VC ≤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, and those who suffered an exacerbation of COPD during the last three months, or used non-steroid anti-inflammatory medication, theophylline, long-acting beta stimulants or oxygen therapy, were excluded from the study. Short acting β$_2$-agonists were allowed during all periods in the study, but only if necessary.

The patients started the study protocol with a 10-week wash-out period to obtain a baseline. This was termed Period 1. During Period 2 and Period 4, patients were treated with either ICS (fluticasone dipropionate, 2 x 500 µg daily) or NAC (600 mg, once a day). Each of the treatment period lasted 10 weeks. Between these two treatment periods another 10-week wash-out period was scheduled, Period 3. The study was performed in a randomised double blind fashion. At the start of each period and at the end of Period 4, induced sputum and blood samples were collected, and spirometry was performed in order to demonstrate clinical stability.

**Sputum induction and handling**

FEV$_1$ measurement was performed before and 10 min after inhalation of 200 µg salbutamol. After spirometry, subjects inhaled 4.5% sterile hypertonic saline aerosol during four consecutive periods of 5 min each. The saline was nebulized with an ultrasonic nebulizer (De Vilbiss, Somerset, USA). Following each inhalation period, subjects were instructed to rinse the mouth, blow the nose, and to cough up and expectorate sputum into a sterile polypropylene container. The procedure was terminated after four periods of 5 min or after a fall in FEV$_1$ of ≥20% from the baseline (19).

The collected sputum was processed as soon as possible, but always within 2 h after expectoration, according to the guidelines of the European Respiratory Society (20). The expectorate was poured into a polypropylene Petri dish and sputum plugs were selected free of salivary contamination. Complete homogenization was achieved by incubation with freshly prepared 0.1% dithiothreitol (Calbiochem, La Jolla, USA) in PBS, equivalent to 4 times the selected sputum weight. The suspension was aspirated, dispensed several times with a plastic pipette, and mixed before placing on a tube rocker for 15 min at 22°C. To remove mucus and debris, filtration through a 48 µm nylon mesh (Becton Dickinson Labware, Franklin Lakes, USA) was performed. After filtration, a manual cell count and viability check was performed before centrifugation at 400 x g for 10 min at 22°C. The obtained sputum sol phase was frozen until use in different assays. Differential cell counts were made on May-Grünwald Giemsa stained cytopspins.

**Laboratory assays**

Trolox equivalent anti-oxidant capacity (TEAC) in serum was measured spectrophotometrically at 734 nm using the method of Re et al (21). GPx in full blood was determined by a commercially available kit (Randox Labs Ltd., Antrim, UK), based on the method of Paglia and Valentine (22), and superoxide dismutase in serum was assessed using a commercial kit (Randox Labs Ltd, Antrim, UK). Eosinophil cationic protein (ECP) was measured in serum and in sputum through a fluoroenzyme immunoassay performed on an UniCap 100 instrument (Pharmacia Diagnostics, Uppsala, Sweden). Neutrophil elastase activity present in sputum was determined spectrophotometrically using a synthetic substrate methoxysuccinyl-ala-ala-pro-val-p-nitroanilide (Sigma Chemical, St. Louis, USA). The slow reaction was continued for 20 h at 37°C and the final absorbance measured.
at 405 nm. Soluble intracellular adhesion molecule 1 (ICAM-1) and interleukin-8 both in serum and sputum were measured by a commercial ELISA (Biosource Europe, Nivelles, Belgium).

Statistics

Results are expressed as means ±SE. Data were analyzed for statistical differences by the Student's *t*-test for paired samples. Assuming Gaussian distribution, Pearson's test was used to calculate correlations between different data sets. Differences were considered significant at a *P* value <0.05.

RESULTS

Protective markers for oxidative stress, GPx (51.2 ±5.8 vs. 62.2 ±8.6 U/g Hb, *P*=0.02) and TEAC (1.44 ±0.05 vs. 1.52 ±0.06 mM, *P*=0.04), were increased by ICS treatment. The treatment with NAC demonstrated a significant decrease in sputum ECP (318 ±73 vs. 163 ±30 ng/ml, *P*<0.01) and sputum IL-8 (429 ±80 vs. 347 ±70 ng/ml, *P*=0.05).

The level of serum ICAM-1 correlated negatively with blood GPx (*r*²=0.233, *P*<0.0001) and TEAC (*r*²=0.312, *P*<0.0001) (*Fig. 1*). The expression of adhesion molecules was promoted by inflammation, as reflected by a positive correlation of serum and sputum IL-8 levels with the respective serum and sputum levels of soluble ICAM-1 (*r*²=0.268, *P*<0.0001; and *r*²=0.129, *P*<0.01, respectively) (*Fig. 2*).

Concerning the effects of treatment on lung function parameters, significant changes were but found in the absolute values of FEV₁. As a result of the ICS treatment, FEV₁ increased significantly from 1690 ±98 to 1764 ±110 ml, when measured before salbutamol inhalation (2x100 µg), and from 1818 ±106 to 1906 ±116 ml post-salbutamol (*P*<0.05). In the group of patients who received ICS during Period 2 of the study design, the absolute FEV₁ values at the end of a 10-week long washout period dropped dramatically (nearly by 200 ml) to 1716 ±120 ml post-salbutamol (*P*<0.05). During the subsequent treatment period with NAC, these values decreased even further to 1666 ±84 ml post-salbutamol. Significant changes were not observed when FEV₁ was expressed as the percentage of predicted values.

No effects of ICS on the forced vital capacity (FVC) or FEV₁/FVC were observed. The treatment with NAC never demonstrated any positive effect on lung function parameters and NAC was unable to inhibit deterioration of FEV₁ after stopping ICS treatment.

*Fig. 1.* Correlations between circulating soluble ICAM-1 (Y axes) and plasma levels of glutathione peroxidase (GPx) (Panel A) and trolox equivalent antioxidant capacity (TEAC) (Panel B).
week long washout period dropped dramatically (nearly by 200 ml) to 1716 ±120 ml post-salbutamol (P<0.05). During the subsequent treatment period with NAC, these values decreased even further to 1666 ±84 ml post-salbutamol. Significant changes were not observed when FEV₁ was expressed as the percentage of predicted values. No effects of ICS on the forced vital capacity (FVC) or FEV₁/FVC were observed. The treatment with NAC never demonstrated any positive effect on lung function parameters and NAC was unable to inhibit deterioration of FEV₁ after stopping ICS treatment.

**DISCUSSION**

This study demonstrates that treatment of COPD patients with ICS during a period of 10 weeks did not significantly influence both local and systemic markers of inflammation. However, minor improvement of the systemic redox balance occurred. Although some studies have shown positive effects of ICS on inflammatory mediators (13, 14, 23), these observations were not supported by our results. Direct antioxidant properties of ICS in humans have not been reported yet. The only reported study in which steroids show antioxidant properties in the lung, as induction of antioxidant enzymes, has been done in adult rats (24). In that study the working mechanism of steroids is more likely to take place on the transcriptional level. This is supported by demonstrating that enzymatic activities do not increase after steroid administration when inhibitors of RNA and protein synthesis are given beforehand (24, 25). Several studies have shown that ICS administered over a period of 3-12 weeks generally do not change the level of airways obstruction, as assessed by FEV₁ and peak expiratory flow (26). Nevertheless, some other studies have shown that some individuals have a

![Fig. 2. Correlations between circulating soluble intracellular adhesion molecule 1 (ICAM-1) (Y axes) and plasma (Panel A) and sputum levels of interleukin-8 (IL-8).](image-url)
substantial improvement in lung function (27, 28). Despite the lack of clear action by ICS in COPD, several studies have described beneficial effects on symptoms and health status (28, 29). The ISOLDE Study Group has shown that the health status improves continuously with inhaled steroids, despite the fact that lung function does not improve further after the first six months of treatment and even deteriorates again (30). Therefore, positive effects of inhaled steroids in COPD may not be completely excluded.

The antioxidant system has many components and a free radical damage to any of them may cause a reduction in the overall antioxidant status of an individual. Thus, the opposite situation, namely the up-regulation of one of the defense mechanisms, for example, GPx, could positively influence the total antioxidant status. The full spectrum of antioxidant activity against various reactive oxygen and nitrogen radicals is reflected by TEAC. In COPD, antioxidant capacity was reported to remain low with a further decrease during exacerbation (17). In addition to the above mentioned antioxidant effects, specific antioxidant therapies were studied. NAC is a glutathione precursor with beneficial properties (31). In the present study, the effects of oral NAC was only seen in induced sputum but not systemically, although it is well known that NAC itself, when administered orally, does not show up in bronchial secretions to any significant amount.

The inhibitory effect of NAC on the migration of neutrophils has earlier been reported in cardiac surgery patients (32). It is now believed the molecular actions of NAC are mainly caused by an influence on the nuclear transcription factor-κB (NF-κB), which when activated by ROS, hypoxia, and proinflammatory cytokines, regulates genes for inflammation promoting cytokines, adhesion molecules, and inflammatory enzymes (33, 34). It has been observed that NAC is able to inhibit NF-κB and IL-8 increase that is ROS-dependent (35). The effects on ECP may be explained by this protein being taken up and transported by neutrophils, although it is an eosinophil product (36). Since IL-8 is a strong chemotactic factor and activator of neutrophils, the decrease of ECP by NAC treatment may have been influenced through this mechanism. Although the mucolytic effects of NAC are not well documented in the literature, NAC may have influenced sputum viscosity, which, in turn, could affect the quality of the induced sputum and consequently the results of sputum analysis.

The coexistence of inflammation with oxidative stress suggests a co-dependent pathway for these processes. Further studies are needed to evaluate possible supplementary effects of inhaled steroids and NAC on reducing inflammation and oxidative stress. More efforts should be directed toward the understanding of the significance of antioxidant enzymes and their potential therapeutic role.

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