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Effect of Inhaled N-Acetyl-L-Cysteine Treatment on Induced Sputum Glutathione and Nitrite

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ABSTRACT

Objective: An imbalance between oxidants-antioxidants is known to play an important role in the pathogenesis of asthma, especially during exacerbation. The aim of this study was to investigate the effect of N-acetyl-L-cysteine (NAC) treatment on the levels of sputum nitrit (NO_2^-) and reduced glutathione (GSH) contents in patients with asthma during exacerbations.

Materials and Methods: The study had a double blind, placebo controlled design. Induced sputum GSH, NO_2^-, cell counts and plasma NO_2^- contents were evaluated in 11 healthy controls (HCs) and 25 patients with asthma during exacerbation before and after treatment. Fifteen patients with acute asthma attack were treated with inhaled NAC during exacerbation.

Results: Plasma-sputum NO_2^- and sputum GSH contents were significantly higher in subjects with asthma subgroups than HCs ($p<0.01$ for NAC treatment group, $p<0.005$ for placebo treatment group, for plasma NO_2^-; $p<0.001$ for both groups for sputum NO_2^-; $p<0.001$ for both groups for sputum GSH). NAC treatment group had higher GSH contents in sputum samples after treatment which were, however, not significantly different from those before treatment. There was a significant decrease in sputum ($p<0.005$ for NAC treatment group, $p<0.01$ for placebo group) and plasma NO_2^- ($p<0.005$ for NAC treatment group, $p<0.01$ for placebo group) levels in both NAC treatment group and placebo group after treatment when compared with the pretreatment levels.

Conclusion: These findings indicated that addition of NAC to standard attack therapy does not modify the oxidant-antioxidant status in asthma patients during exacerbation.

Key words: *Induced sputum, glutathione, nitrit, exacerbation of asthma, N-acetyl-L-cysteine*

ÖZET

Astım Atağındaki Hastalarda İnhaler N-Asetil-L-Sistein Tedavisinin İndükte Balgamda Glutatyon ve Nitrit Seviyeleri Üzerine Etkisi

Amaç: Oksidanlar ile antioksidanlar arasındaki dengesizlik astım patogenezinde, özellikle de ataklar sırasında önemli rol oynamaktadır. Bu çalışmanın amacı N-asetil-L-sistein (NAC) tedavisinin astım atağındaki hastaların balgam nitrit (NO_2^-) ve redükte glutatyon (GSH) seviyeleri üzerine etkilerini araştırmaktır.

Gereç ve Yöntem: Bu çift-kör plasebo kontrollü bir çalışmadır. Onbir sağlıklı kontrol (SK) ve 25 astım atağındaki hastanın indükte balgamlarında GSH, NO2- seviyeleri, hücre sayımları ve plazma NO_2^- seviyeleri tedavi öncesi ve sonrası dönemde değerlendirildi. Akut astım atağındaki 15 hastaya atak süresince inhaler NAC tedavisi verildi.

Bulgular: Astım subgruplarında plazma ve balgam NO_2^- ve balgam GSH seviyeleri SK grubunda anlamlı derecede yüksek bulundu (plazma NO_2^- seviyesi için; NAC tedavisi alan grupta $p<0.01$, plasebo uygulanan grupta $p<0.005$, balgam GSH ve NO_2^-seviyesi için her iki gruptap<0.001). NAC tedavisi alan grubun balgam GSH seviyesi tedavi sonrasında, tedavi öncesi değere göre anlamlı olmamakla birlikte artış gösterdi. NAC tedavisi ve plasebo uygulanan her iki grupta balgam ve plazma NO_2^- seviyeleri tedavi sonrasında anlamlı derecede azaldı (balgam NO_2^- seviyesi için NAC tedavisi alan grupta $p<0.005$, plasebo uygulanan grupta $p<0.01$; plazma NO_2^- seviyesi için NAC tedavisi alan grupta $p<0.005$, plasebo uygulanan grupta $p<0.01$).

Sonuç: Bu bulgular astım hastalarına atak sırasında standart atak tedavisine NAC eklenmesinin oksidan-antioksidan dengeyi değiştirmediğini göstermektedir.

Anahtar kelimeler: *İndükte balgam, glutatyon, nitrit, astım atağı, N-asetil-L-sistein*

It has been recognized for many years that patients who die from acute asthma attacks have grossly inflammed airways. As inflammation is often associated with an increased generation of reactive oxygen species (ROS), and the biochemical environment in the asthmatic airways is favourable for free radical mediated reactions, it is rational to surmise that an oxidant stress could be mechanistically important in asthma (1). An imbalance between oxidants and antioxidants, in favor of oxidants leading to oxidative stress, is known to play an important role in the pathogenesis of asthma, and increased oxidative stress is related to disease severity and may amplify the inflammatory response, particularly during exacerbations (2,3). Most studies using blood leukocytes and cells isolated from bronchoalveolar lavage (BAL) of asthmatic patients indicate enhanced ROS generation compared with control subjects (4,5) and decreased antioxidant capacity in plasma of asthmatic patients, both in stable and in acute asthma (6). We have previously demonstrated increased oxidant capacity in

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sputum of both stable and exacerbations of asthma and decreased antioxidant capacity in exacerbations of asthma compared with stable phase (7).

Despite the abundance of evidence indicating elevated oxidative stress and reduced antioxidant defences in asthma, antioxidant supplementation studies to date have been controversial. N/acetyl-L-cysteine (NAC) is a thiol containing compound that is used to reduce viscosity and elasticity of mucus. It is able to scavenge hydrogen peroxide, hydroxyl radicals and hypochlorous acid (8). NAC can be deacetylated to cysteine, an important procursor of cellular GSH sythesis, and thus stimulating the cellular GSH system (9). In view of this, we wondered whether inhaled NAC might also be useful in increasing anti-oxidant capacity during exacerbation. We undertook a double blind, placebo controlled study of inhaled NAC 600 mg bid in addition to standard treatment in patients admitted to hospital with an acute execerbation of asthma.

MATERIALS AND METHODS

Study Design

The study had a double blind, placebo controlled design. The study was performed in asthma patients who were admitted and hospitalized to our clinic for asthma exacerbation between January-April 2005. Patients were immediately evaluated and studied before any asthma treatment. Subjects had mild to moderate exacerbation of acute asthma. Acute asthma patients who had severe exacerbation were excluded from this study. Patients had no systemic diseases, malignancy, vascular disease, thrombosis, alcoholism, renal disease and hepatic disease. Their therapy was done according to the international asthma guidelines (10). Patients who received oral/parenteral corticosteroids, antibiotics, theophylline and antioxidant vitamins were excluded from the study. Hospitalized patients are taken randomly and were separated into two groups. NAC treatment group received nebulized NAC (15 acute asthma patients, twice a day, 300 mg ampuls) and placebo treatment group received nebulized 0.9% NaCl ampul (10 acute asthma patients, twice a day) during exacerbation. The patients were reevaluated 15 days after the therapy.

Patients/Subjects

Acute asthma patients Twenty-five non-smoking atopic asthma patients during exacerbation participated in the study. All subjects were atopic with positive skin prick testing for common aeroallergens from our area (house-dust mites, grass pollen, cat and dog dander, mould mixture). Positive skin prick test was defined when wheal was 3 mm when compared to salin. The diagnosis of asthma was based on international guidelines (10). An exacerbation of asthma was defined by the presence of the dyspnea at rest with wheezing or nocturnal symptoms disturbing sleep. Their current therapy (before the exacerbation) was shown in Table 1.

"Healthy controls" (HCs) Eleven age-matched, non-smoking healthy subjects were included as control subjects. All subjects were randomly selected from hospital staff. Inclusion criteria for non-smokers were; no history of respiratory or allergic disease, normal baseline spirometric parameters as predicted for age, sex and height, no history of upper respiratory tract infection in the preceeding 6 weeks, and no use of any regular medication. The Ethics Committe of Firat University Faculty of Medicine reviewed and approved the protocol, and all subjects gave informed consent to participate in the study.

Pulmonary Function Test

Pulmonary function parameters (FEV_1, FVC, FEF_{25-75}) were measured with a spirometer (SuperSpiro, Micromedical Limited, England).

Sputum Induction

Sputum was induced during the acute exacerbation as previously described (11). Sputum induction was performed by inhalation of 3% NaCl for 20 minutes from nebuliser [Porta-Neb compressor, Medic-Aid Sidestream nebuliser chamber, mass median diameter 3.18 m (Medic-Aid Limited, UK)]. The standard safety precaution was to premedicate with 200 µg of inhaled salbutamol (12). Before and after sputum induction lung function measurements were performed. The safety of sputum induction was monitored by measuring peak expiratory flow rates (PEFR). The procedure would have been stopped if PEFR were decreased by 25%. The sputum induction procedure did not cause troublesome symptoms and the PEFR was not decreased by more than 25% in all "acute asthma patients". Treatment of exacerbation was made according to international guidelines in all acute asthma patients after sputum induction. Expectorated sputum was collected in sterile plastic tubes placed on ice to slow down metabolic processes that might result in loss of GSH.

Sample Processing

Sputum samples were processed within 30 minutes of collection using the method described by Dauletbaev et al (11). Samples were diluted with three volumes of chilled phosphate buffered saline (PBS: all reagents were purchased from Sigma-Aldrich Chemie GmbH, Steinheim, Germany). Supernatants were obtained by centrifugation (300 g, 15 minutes, 4^oC) and transferred to another vial by filtering through multiple layers of cotton gauze. Additional centrifugation (800 g, 5 minutes, 4^oC) ensured removal of the remaining cell debris and mucus. Aliquots of the supernatants were placed on ice and assayed immediately for reduced glutathione supernatant was waited at -20 ^oC for measuring nitrit (NO_2^-) contents.

GSH Measurement

The sputum GSH was measured using an enzymatic recycling assay (13,14). The standard and sample solutions were added to an equal volume of DTNB and 50 µl of this mixture (final concentration of DTNB 0.25 mM) were pipetted into a 1 ml cuvette followed by glutathione reductase and NADPH (final concentrations 1 U/ml and 0.22 µM, respectively). The reaction mixture was equilibrated and the kinetic reaction was followed for two minutes at 412 nm (Techcomp Ltd., UV-VIS 8500 spectrophotometer, Hong Kong).

NO_2^- Measurement

The measurement of plasma and supernatant NO was difficult because this radical was poorly soluble in water and had a short half-life in tissue (10-60 s), but its half-life might be as long as 4 min in the presence of oxygen. For these reasons, the determination of NO itself was difficult and required the handling of radioisotopes. In spite of this, the end products, nitrate and nitrite, were preferentially used in clinical biochemistry. Nitrite, a stable end -product of NO, was measured in plasma by using the spectrophotometric Griess

reaction (15). One thousand mL experimental samples of deproteinised plasma was reacted with 500 mL N naphthylethylenediamine, 10 g/L sulfanilamide for 45 min at room temperature and analyzed by spectrophotometry at 545 nm. Concentrations were determined by comparison with sodium nitrite. The lower limit of detection was 0.2 mmol/L.

Statistical Analysis

All statistical analyses were done using SPSS v10.0 software. Data were expressed as mean ± standard deviation (SD). Statistical analysis was performed using Kruskal-Wallis test for multiple-group comparisons; Mann-Whitney U test was performed to test any observed differences for significance. Wilcoxon's rank sum test was performed for comparisons before and after treatment values of asthma subgroups. Chi-square test was performed to compare gender distrubion between groups. A p value of <0.05 was considered as statistically significant.

RESULTS

There was no significant difference in mean age and sex between healthy subjects, and both asthma subgroups. The mean FEV_1 levels were significantly lower in both asthma subgroups compared with healthy controls ($p<0.001$ for both group), but there was no significant difference between asthma subgroups. There was no significant difference in mean duration of disease between asthma subgroups. A small decrease in lung function parameters was observed during the sputum induction procedure and there was no significant difference in FEV_1 between the groups. Patients' characteristics, current medications are given in Table I.

Table I. Demographics and lung function datas of all groups, and current medications of asthma subgroups

	Healthy subjects (n=11)	Acute Asthma Patients (n=25)	
		NAC treatment group	Placebo treatment group
Age (yr)	39.64±3.69	40.47±3.72	38.50±3.62
Male/Female	3/8	4/11	3/10
Asthma therapy n (%)			
β_2 agonist only	-	8 (53.4)	7(70.00)
IS and short acting β_2 agonist	-	4 (26.60)	2 (20.00)
IS and long acting β_2 agonist	-	3 (20.00)	1 (20.00)
FEV_1 (L)	3.09±0.73	1.53±0.55[a]	1.72±0.53[a]
ΔFEV_1 (L)	0.017±0.12	-0.009±0.17	-0.061±0.37
Duration of disease (yr)	-	10.60±5.62	11.40±3.37

IS: inhaled steroid.
FEV_1=forced expiratory volume in one second (liter).
ΔFEV_1= change of FEV1 after sputum induction (liter).
[a] *Compared with healthy controls p<0.001*

Table II. Comparison of sputum cell counts, GSH, NO_2^- and plasma NO_2^- levels from asthma patients during exacerbations and healthy control subjects

	Healthy subjects (n=11)	Acute Asthma Patients (n=25)			
		NAC treatment group (n=15)		*Placebo treatment group*	
		Before treatment	After treatment	Before treatment	After treatment
Sputum GSH	0.34±0.14	2.82±0.69[a]	3.04±0.64[a]	2.94±1.46[a]	2.75±0.90[a]
Sputum NO_2^-	346.67±49.98	485.60±81.48[a]	326.84±41.47[d]	478.97±40.08[a]	305.68±17.74[ef]
Plasma NO_2^-	281.94±43.69	334.27±43.81[b]	280.92±56.01[d]	338.06±24.53[c]	279.79±42.69[e]
FEV_1 (L)	3.09±0.73	1.53±0.55[a]	2.12±0.47[d]	1.72±0.53[a]	2.22±0.33[e]
ΔFEV_1 (L)		0.54±0.42		0.50±0.50	

ΔFEV_1= change of FEV_1 after NAC and placebo treatment (liter).
[a] *Compared with healthy controls p<0.001*
[b] *Compared with healthy controls p<0.01*
[c] *Compared with healthy controls p<0.005*
[d] *Compared with before treatment p<0.005*
[e] *Compared with before treatment p<0.01*

At exacerbation, sputum GSH contents were higher in both acute asthma subgroups before treatment than HCs ($p<0.001$ for both groups), and remained higher also after treatment in both acute asthma groups ($p<0.001$ for both groups). There was no significant difference betweenacute asthma subgroups in sputum GSH levels before and after treatment. There was no significant difference in sputum GSH levels in both acute asthma subgroups after treatment when compared with the pretreatment levels, although there was a trend toward higher levels in patients who received NAC (Table II).

Sputum NO_2^- contents were higher in both acute asthma subgroups before treatment than HCs ($p<0.001$ for both groups). After treatment, sputum NO_2^- contents were significantly lower in placebo group when compared to HCs ($p<0.05$) but there was no significant difference between NAC treatment group and HCs. There was no significant difference between acute asthma subgroups in sputum NO_2^- contents before and after treatment. We observed a significant decrease in sputum NO_2^- levels in both acute asthma subgroups after treatment ($p<0.005$ for NAC treatment group, $p<0.01$ for

placebo treatment group) when compared with the pretreatment levels (Table II).

Before treatment, plasma NO_2^- contents were higher in both acute asthma subgroups than HCs ($p<0.01$ for NAC treatment group, $p<0.005$ for placebo treatment group), but there was no significant difference between acute asthma groups. After therapy, there was no significant difference in plasma NO_2^- contents between all groups. There was a significant difference in plasma NO_2^- contents in both acute asthma subgroups after treatment when compared with the pretreatment levels ($p<0.005$ for NAC treatment group, $p<0.01$ for placebo treatment group) (Table II).

FEV_1 was significantly higher in both asthma subgroups after treatment than those before treatment ($p<0.005$ for NAC treatment group, $p<0.01$ for placebo treatment group). There was no significant difference in FEV_1 between NAC treatment group and placebo treatment group after treatment and the rate of change in FEV_1 was greater with NAC than with placebo but this difference was not statistically significant (Table II).

Sputum GSH, NO_2^- and plasma NO_2^- contents of both asthma subgroups before and after treatment were shown in figure 1 and 2.

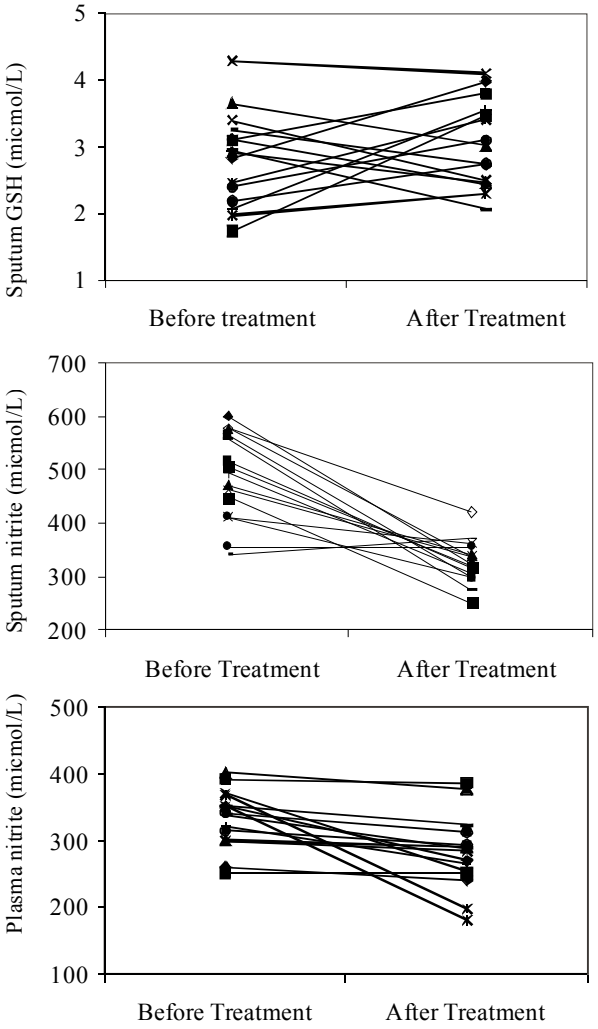

Figure 1. Sputum GSH, NO_2^-, plasma NO_2^- contents in patients who received NAC treatment

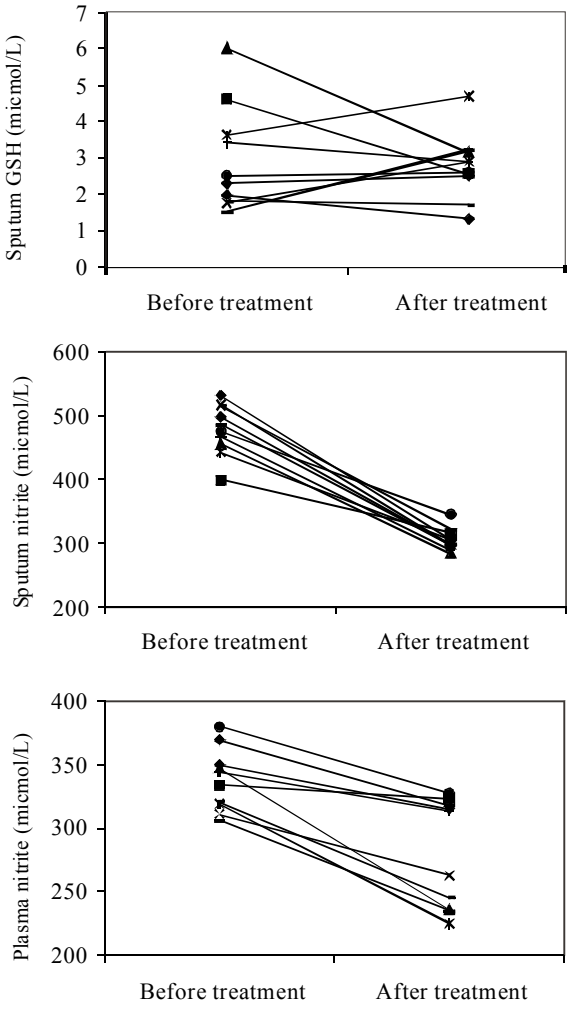

Figure 2. Sputum GSH, NO_2^-, plasma NO_2^- contents in patients who received placebo treatment

DISCUSSION

This study showed that sputum GSH and NO_2^- contents were increased in asthma during exacerbation when compared with HCs and sputum GSH levels were slightly modified during acute asthma attack therapy with inhaled NAC. In addition, sputum and plasma NO_2^- contents were decreased after treatment in all asthma patients.

GSH is the key antioxidant in the respiratory tract lining fluid (RTLF). Disturbed GSH status is reported in asthma patients. Kelly and Smith obtained an increased GSH (total and oxidised) status in BAL fluid in asthma patients (16,17). Doulatbaev et al also demonstrated that stable asthma patients tended to have higher GSH concentrations in sputum samples (11). There are several clinical studies of the oxidant-antioxidant status in asthma. Acute exacerbations of these conditions have received much less attention. We hypothesised that increased numbers of inflammatory leukocytes in the bronchoalveolar space and/or those sequestered in the pulmonary vasculature in acute exacerbations of asthma, may create an increased oxidant burden. Rahman et al showed that decreased antioxidant and increased oxidant capacity in plasma of patients presenting with acute exacerbations of asthma, and antioxidant capacity tended to improve by the time of

discharge (6). We observed increased amount of sputum GSH in patients with asthma during exacerbation. This result suggests that GSH synthesis and/or transport were increased in response to the presence of excess oxidants during acute exacerbation. A compensatory but probably inadequate increase in GSH has been reported in other conditions of oxidative stress (18,19). If ROS and RNS are important in asthma especially during acute exacerbation, enhancement of the antioxidant defences would be expected to have beneficial effects in the disaese. On the contrary, some studies evaluated GSH of exhaled breath condensate in children with asthma during acute exacerbations were found decreased GSH levels compared with control subjects and stable asthma patients (20,21). At present, it is not clear whether altered RTLF GSH status is the cause or a consequence in respiratory disease. Studies carried out with normal volunteers showed that GSH and other antioxidants present in RTLF play an important role in protecting the lung surface from oxidative attack (22).

Sputum concentrations of NO_2^- and nitrate, which are stable metabolites of NO have been shown to be elevated in asthma (23,24). NO can be measured in the exhaled air of asthmatic patients (25,26), and it is thought to reflect the inflammatory state of the airways. In the current study, we demonstrated that sputum NO_2^- content was higher in acute asthma exacerbation when compared with the healthy subjects and their levels were significantly decreased after treatment. To our knowledge, this is the first study in which sputum NO_2^- contents in acute asthma exacerbation were evaluated, therefore the data cannot be compared with previous findings. Recently, Hunt et al showed that the pH in the airways drops dramatically during acute asthma attack, which facilitates the conversion of nitrite to NO. Hence, increased NO concentrations in the exhaled air of asthmatic patients may reflect nitrite conversion rather than NOS activity. Acidosis at levels seen in subjects with acute asthma causes both necrosis of human eosinophils and conversion of airway NO_2^- to NO effects relevant to asthma pathophysiology (27). In addition, increased sputum NO_2^- contents may develop due to increased numbers of inflammatory leukocytes in the bronchoalveolar space during acute exacerbations of asthma. High concentrations of NO released in an inflammatory context, it has putatively detrimental effects via the formation of ROS (28).

Despite the abundance of evidence indicating elevated oxidative stress and reduced antioxidant defences in asthma, antioxidant supplementation studies to date have been limited. NAC is one of the most widely investigated antioxidants having shown beneficial effects in disease states. In contrast with the activity found different pulmonary disease, the influence of NAC in clinical asthma remains uncertain (29,30) but has not been recently evaluated. We found that short-term administration of inhaled 600 mg/day NAC increased sputum GSH contents in acute asthma patients, but this increase was not significant according to before treatment. When compared with placebo group, NAC group had increased sputum GSH levels after treatment but it was not significant. Administration of NAC had no significant effect on FEV1, serum and plasma NO_2^-, and sputum GSH levels in asthma patients during exacerbations. Improvement in postreatment levels of FEV1 could be attributed to the use of standard attack therapy (β2 agonists and/or inhaled steroids). NAC can easily be deacetylated to cysteine, an important precursor of cellular GSH synthesis, and thus stimulate the cellular GSH system (9). The upper level of cellular GSH is regulated by feedback inhibition, but cellular levels also depend upon availability of the substrates, especially cysteine (31). In several studies, it was shown that intracellular and plasma cysteine levels were increased following NAC administration (32,33). Meyer et al found that GSH levels increased in BAL fluid after the oral NAC (3x600 mg NAC per day for 5 days) augmentation compared with pretherapy (34). On the contrary, Cotgreave et al demonstrated that the cysteine and GSH content in the lavage fluid were unaltered during NAC (600 mg, daily for 2 weeks) administration in healthy subjects (35). NAC in a daily dose up to 1800 mg administered for 5 days did not significantly increase the concentration of GSH or cysteine in BAL fluid (36). Oral administration of NAC for several days did not elevate the concentration of GSH in the airways. There has been many studies about oral administration of NAC but a few studies is present with nebulized NAC administration. In these studies it is shown that inhaled NAC has anti- or pro-oxidant action (37) and it is reported that nebulized NAC transiently increases exhaled H_2O_2 level, whereas it has no effect on other oxidative parameters (38). In our study, sputum GSH contents were not increased significantly in patients with acute asthma attacks. This may be due to both short-term and nebulized administration of NAC during exacerbation.

In conclusion, the results of this study demonstrate that short-term inhaled treatment with NAC produced insufficient increase in sputum GSH contents in the airways in patients with asthma during exacerbation. In addition, this therapy had no effect on sputum NO_2^- content. For this reason, our study does not suggest that NAC supplementation therapy is effective during asthma exacerbations. Further studies can be made by using higher doses of NAC to mild to moderate asthma patients.

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