

Nitrites in induced sputum as a simple and cheap non-invasive marker of airway inflammation for asthmatic schoolchildren

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To determine if there are differences in the nitric oxide metabolites (nitrites) in sputum of patients with persistent asthma and healthy schoolchildren, we performed a case-control study in a tertiary care hospital in Arequipa, Perú. Nitrites in induced sputum samples were measured using the Griess assay in 30 persistent asthmatics (mean age of 10.1 yr) and 30 controls (mean age of 11.9 yr). The mean $\pm$ s.d. of nitrites among asthmatics was significantly higher than the controls (16.30 ± 8.6 vs. 10.25 ± 4.68 nmol/ml, respectively, $p = 0.001$).

Moreover, the nitrite level in the sputum in children with severe persistent asthma was higher than in the level found in the moderate and mild asthmatics (32.83 ± 9.48 vs. 18.10 ± 1.96 vs. 11.84 ± 4.73 nmol/ml, respectively, $p < 0.01$ for linear trend).

This study showed for the first time in children that asthmatics have significantly higher levels of nitrites in induced sputum than healthy controls and that the level of nitrite correlates with the severity of the asthma. Nitrite levels in sputum, a simple and cheap, non-invasive method, may be a good alternative to measure the severity of inflammation in asthmatic children.

Nitrite levels in sputum, a simple and cheap, non-invasive method, may be a good alternative to measure the severity of inflammation in asthmatic children.

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Most cases of asthma start in the first years of life (1) and some persist into adulthood with both symptoms and lung function tracking (2, 3). The management of asthmatic children depends on monitoring the symptoms and lung function. Asthma guidelines generally recommend the use of forced expiratory volume in the first second [forced expiratory volume in 1 s (FEV₁)] as the most valuable lung function outcome (4), although it has been reported that there is no correlation between FEV₁ and the severity of asthma in children (5). With current treatment, FEV₁ can be normalized and symptoms can be controlled in most asthmatic children (6). However, airway inflammation and even remodeling features can persist in asthmatic children and adults even when they have normal FEV₁ or are clinically asymptomatic (7, 8); suggesting that

these measurements may not reflect the ongoing airway inflammation and hence sub-clinical disease.

Therefore, evaluation of the airway inflammation is crucial for controlling the disease. Direct evaluation using invasive methods like bronchoscopic biopsies and bronchoalveolar lavages is specific and sensitive but too invasive to be used in the routine analysis of airway inflammation in children (9–12). Direct non-invasive methods like cellular quantification in sputum samples (13, 14) and exhaled nitric oxide (eNO) have been studied and are more accurate markers of asthmatic airway inflammation than the proportion of blood eosinophils or serum eosinophil cationic protein (15). eNO was been suggested as a specific, sensitive, repeatable and simple marker reflecting allergic airway inflammation (16);

however, it is an expensive test and not accessible in most of the developing countries. Other direct markers of airway inflammation in induced sputum like nitric oxide (NO) metabolites, e.g., nitrates and nitrites, had been evaluated and high levels have been reported in adult asthmatics (17, 18) and in patients with cystic fibrosis (19), but there is no data available on asthmatic children.

The aims of this study were to assess the differences in nitrite levels in induced sputum of asthmatic and healthy control children and to study the correlation between nitrite level and severity of asthma.

Methods

Thirty schoolchildren (aged 6–18 yr old) with persistent asthma as defined by the WHO guidelines (4), were recruited from the emergency department, outpatient and inpatient clinics at the Honorio Delgado's University Hospital, Arequipa, Peru. They were classified as having mild, moderate or severe persistent asthma (4); they were all on inhaled corticosteroids (beclomethasone-CFC) therapy with doses in the range of 100–300 µg/day for the mild, 300–600 µg/day for the moderate and more than 600 µg/day for the severe cases. Exclusion criteria were cystic fibrosis, bronchopulmonary dysplasia, chronic lung diseases, bronchiectasis and tuberculosis. Thirty control subjects were recruited from non-respiratory outpatient clinics; these subjects had no reported respiratory complaints and had no airway obstruction upon physical examination prior to the study. None of the subjects reported any tobacco consumption.

The sputum induction and processing were performed according to the following standard protocol. All subjects were pre-medicated with inhaled salbutamol two puffs (200 µg). Subjects inhaled 4 ml of normal saline solution aerosol generated by a jet nebulizer (Hudson UP Draft II, model 1730, Hudson Oxygen Sales Co., Temecula, CA, USA) powered by compressed-air and delivered via a face mask for 7 min. A flow rate of 7 l/min was used as measured by a rotameter (Thorpe 1–15 l/min, model 2562, Hudson). Early in the morning, the subjects were asked to rinse the mouth, and blow the nose to minimize contamination with saliva and post-nasal drip. They were encouraged to cough deeply and frequently during normal saline inhalation. The sputum sample was collected in a sterile tube and frozen. Then, the sample was incubated at room temperature. Nitrite levels were measured in the sputum supernatant and

quantified on standard curves using the Griess assay (20, 21). Briefly, 100 µl of induced sputum supernatant was mixed with an equal volume of Griess reagent (1% sulfanilamide dissolved in 3 N of HCl and 0.02% naphthylethylenediamine-dihydrochloride). The nitrite production was measured by spectrophotometry at an absorbance wavelength of 540 nm at room temperature and expressed in nm/ml. The subjects were required to have had no upper or low respiratory infection in the last month and no use of inhaled or systemic corticosteroids in the week prior to the induced sputum collection. Asthmatic children had to be symptom-free during the 2 wk prior to the induced sputum collection. Each nitrite level measurement took approximately 5 min and the materials required cost less than US\$ 1. All measurements in sputum were obtained by a technician who did not know the clinical characteristics of the patients.

This study was approved by the Ethical Committee at the Honorio Delgado's University Hospital and a written consent for participation in the study was obtained from the parents of all children.

Statistical analysis

As the nitrite levels were not normally distributed, the data were analyzed using non-parametric tests (Kruskal–Wallis and Dunn tests for multiple comparison). All tests were calculated in a 2-tailed manner and significance was defined by alfa level of 0.05.

Results

Asthmatic children (56.7%) (mean age 10.1 ± 3.6 yr old) and controls (53.3%) (mean age 11.9 ± 3.9 yr old) were males. The proportion of asthma severity in the asthmatic children was: 60% mild, 26.7% moderate and 13.3% severe persistent asthma. There were no significant differences in gender and age in either group (data not shown).

Sputum could be obtained easily in all children within 15 min. Nitrite levels (mean $\pm$ s.d.) were significantly higher in the asthmatic group than in controls (16.30 ± 8.6 vs. 10.25 ± 4.7 nmol/ml, respectively, $p = 0.001$) (Fig. 1). When we analyzed the asthmatic children according to their severity, the nitrite levels showed a significantly linear trend ($p < 0.01$ for multiple comparison by Dunn test): 11.84 ± 4.7 nmol/ml in mild, 18.10 ± 1.9 nmol/ml in moderate and 32.83 ± 9.5 nmol/ml in severe persistent asthma. Significant differences were found when

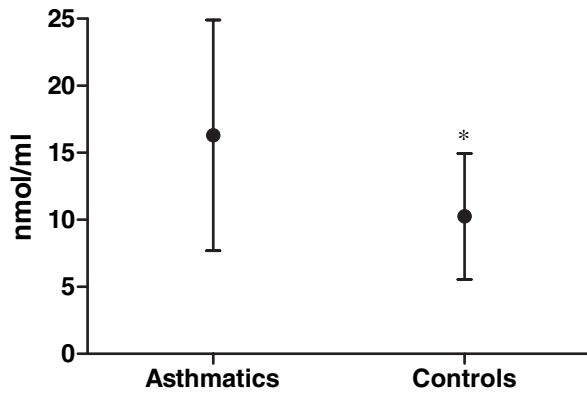


Fig. 1. Levels of nitrites in induced sputum (nmol/ml) in asthmatics and controls. * $p = 0.001$ asthmatics vs. controls.

Table 1. Characteristics of nitrite levels (nmol/ml) in induced sputum in different asthma severity groups and control children

Groups	Nitrites (mean $\pm$ s.d.)
Asthmatics (n = 30)	16.30 $\pm$ 8.6*
Controls (n = 30)	10.25 $\pm$ 4.7
Asthma severity groups**	
Mild (n = 18)	11.84 $\pm$ 4.7***
Moderate (n = 8)	18.10 $\pm$ 1.9****
Severe (n = 4)	32.83 $\pm$ 9.5*****

* $p = 0.001$ between asthmatics vs. controls.

** $p < 0.01$ using multiple comparison Dunn test for trend in different asthma severity groups.

*** $p = 0.0015$ mild vs. moderate asthmatics.

**** $p = 0.0013$ moderate vs. severe asthmatics.

***** $p < 0.0001$ mild vs. severe asthmatics.

comparing mild vs. moderate ($p = 0.0015$), moderate vs. severe ($p = 0.0013$) and mild vs. severe asthmatic children ($p < 0.0001$) (Table 1 and Fig. 2).

Discussion

This study shows that children with persistent asthma have significantly higher levels of nitrites in induced sputum than controls. Moreover, children with severe persistent asthma have significantly higher level of nitrites than moderate and mild asthmatics. To our best knowledge, this is the first report of NO metabolites measured in induced sputum in asthmatic children.

Nitric oxide is endogenously derived from the amino acid L-arginine using three forms of the NO synthetase (NOS); two constitutive NOS (cNOS) are involved in physiological regulation of airway function, and inducible NOS (iNOS) is involved in inflammatory disease of the airways (22). iNOS generates much greater amounts of NO than cNOS. NO can be measured in exhaled air (eNO) and many studies reported that eNO is

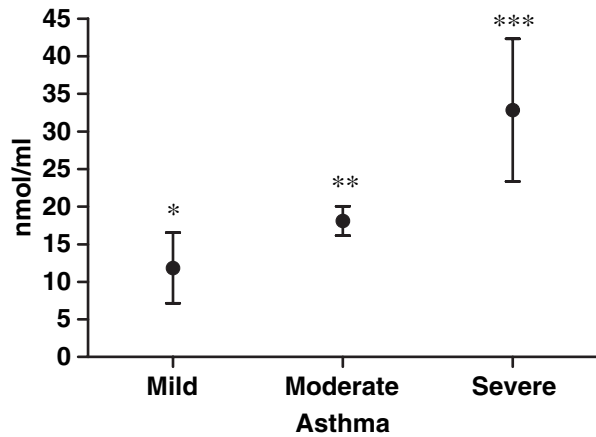


Fig. 2. Levels of nitrites in induced sputum (nmol/ml) in different grades of severity of asthma. * $p = 0.0015$ mild vs. moderate; ** $p = 0.0013$ moderate vs. severe; *** $p < 0.0001$ mild vs. severe.

increased in adults and children with asthma (23–25). eNO in asthmatic patients is mainly produced by iNOS expressed in bronchial epithelial cells and some inflammatory cells and it is considered to reflect airway inflammation (26, 27). However, it has been shown that eNO does not correlate with disease severity in asthmatic children (28). Recently, Pijnenburg et al. (29) showed a significant diurnal variation in eNO with geometric mean morning levels 14% higher than evening levels and with a mean of 40% intra-subject coefficient of variation for the morning and 36% for the evening values; and with no correlation between FENO and cumulative symptom scores. Consequently, the use of eNO as a parameter to manage therapy is still controversial (30, 31). Moreover, eNO is an expensive method to measure inflammation, as it is not easy to measure because it is unstable, volatile, needs to be produced by a constant flow and it is unlikely to be a cost-effective option in developing countries. Therefore, a cheap and simple method like NO metabolites (nitrites, nitrates, peroxynitrite and possible L-citrulline) measurements could be a good alternative (32).

Formanek et al. (33) reported that symptomatic asthmatic children tend to have higher concentrations of nitrites in the exhaled condensates when compared with stable asthmatics independent of atopy, gender or age; he also found that inhaled corticosteroids reduce the level of nitrites. Another study from the same group found that nitrites in exhaled condensates correlated well with maximum expiratory force, symptoms and airway hyper-responsiveness (34). Exhaled condensates have the advantage in that they can be collected from children as young as

3 yrs old; however, it is important to mention that this method is still not easily available worldwide. There are also some unanswered questions regarding the method of the measurement of nitrites in breath condensate (35): for example, the importance of rolling out salivary contamination using equipment that includes a saliva trap, the potential influences of high ambient NO in breath condensate. There are also no data in children on collection time and flow dependence.

On the contrary, it has been reported that NO metabolites measured in induced sputum are more valuable indicators for monitoring asthmatic airway inflammation than those in serum (36). For example, Kanazawa et al. (17) reported an increase of NO metabolites (nitrate + nitrite) levels in induced sputum in adults with asthma, compared with normal subjects. The NO levels correlated with the severity of asthma, with the percentage of eosinophils, eosinophil cationic protein and epithelial shedding cells in sputum and with the degree of airway limitation [FEV₁/forced vital capacity (FVC)] in asthmatics, but not with airway hyper-responsiveness. Another study performed on adult asthmatics (18) found a significant correlation between NO metabolites (nitrate + nitrite) levels in induced sputum and eosinophils and eosinophil cationic protein in sputum, but not with epithelial cells percentage nor with parameters representing airway obstruction (e.g., FEV₁, FEV₁/FVC) nor with hyper-responsiveness. Although tobacco consumption can increase NO production, the authors found no influence of smoking history on NO metabolites levels (18). Our results suggest that nitrite levels are a simple and cheap non-invasive method for the evaluation of airway inflammation in children with asthma and may offer a good alternative for monitoring their severity, especially in developing countries where eNO and exhaled condensates measurements are still expensive and not available to everybody.

The present study has some limitations; we did not measure inflammatory cells count in the sputum samples, atopy condition, pulmonary function test or airway hyper-responsiveness, and it could be interesting to correlate those with the level of nitrites in induced sputum. Therefore, more studies of NO metabolites in induced sputum need to be performed in children, including these variables and with more number of patients in each asthma severity group. In addition, further studies need to be carried out to assess the role of nitrites in induced sputum in the monitoring of the treatment in asthmatic children.

In conclusion, as in previous reports in asthmatic adults (17, 18), we found for the first time in children that the level of nitrites in induced sputum in asthmatics was higher than controls and that the nitrite level is proportional to the severity of asthma. Nitrite levels in induced sputum, a simple and cheap, non-invasive method, may be a good alternative to measure the severity of inflammation in asthmatic children.

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