Nitric Oxide Metabolites in Induced Sputum: A Noninvasive Marker of Airway Inflammation in Asthma

Lata Kumar, Nitin Rajput and Siddhartha Majumdar

From the Departments of Pediatrics and Experimental Medicine and Biotechnology, Postgraduate Institute of Medical Education & Research, Chandigarh160 012, India.*

Correspondence to: Dr Nitin Rajput, 22, Waldren Close, Baiter Park, Poole, BH15 1XS, UK. E-mail: nvraj22@hotmail.com

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Objective: *This study was designed to examine for nitric oxide (NO) metabolites in induced sputum as a marker of airway inflammation in asthmatic children.* ***Design****. Prospective interventional* ***Setting:*** *Pediatric Allergy and Asthma Clinic of a tertiary care referral hospital in Northern India.* ***Subjects:*** *Twenty-one children with asthma who were not receiving corticosteroids for the preceding 3 months and 10 healthy controls were enrolled.* ***Methods:*** *Hypertonic saline-induced sputum was obtained at study entry in controls, and at study entry and after 6 weeks of inhaled corticostered (ICS) therapy in asthmatic children. Fresh expectorated sputum was treated with dithiothreitol and cytospinned for cell count. NO metabolites were measured in the supernatant by the modified Griess reaction.* ***Results:*** *Asthmatic children, compared with controls, had significantly higher concentration of NO metabolites (224.4 ± 209.69 vs. 39.2 ± 15.9 (moL/L, P <0.01) and a higher percentage of eosinophils (15.3 ± 12.0 vs. 0.8 ± 1.1%, P <0.01) in induced sputum. Both NO metabolites and eosinophil percentage declined following treatment with ICS for 6 weeks (P <0.01).* ***Conclusion:*** *The study confirms that the level of NO metabolites is increased in the tracheobronchial secretions of asthmatic children and decreases following ICS therapy. Measurement of NO metabolites in induced sputum may be useful for monitoring airway inflammation in children with asthma.*

Keywords: *Allergic inflammation, Eosinophils.*

NITRIC oxide (NO) plays an important role as a vasodilator, neurotransmitter and inflammatory mediator in the airways(1). NO is synthesized from L-arginine by three isoforms of the enzyme, NO synthase: two constitutive NO synthases (cNOS) are involved in the physiological regulation of airways and an inducible form (iNOS) is involved in inflammatory disease of the airways, and in the host defense against infection. All isoforms of NO synthases-endothelial NOS neuronal NOS, and inducible NOS have been demonstrated in human airways by immunohistological studies(2,3).

NO and its free-radicle products are important mediators of airway inflammation in asthma(4-14). Recent studies have shown increased NO levels in exhaled air in adults and children with asthma using chemiluminiscence analyzers(15-23). High exhaled NO levels in asthmatic patients may reflect induction of iNOS, which is known to be inhibited by corticosteroids. Similarly, a decline in exhaled nitric oxide levels has been demonstrated with oral(15,19,22), inhaled(17,18,20,22,23) and intravenous(21) corticosteroid therapy.

Although, measurement of level of NO in exhaled air is simpler and quicker than measurement in induced sputum, it is much more expensive and requires elaborate equipment(4,24). In future, the products of the

L-arginine-NO pathway, such as nitrite (NO^{2-}), nitrate (NO^{3-}), peroxynitrite ($ONOO^{-}$), nitrosothiols and L-citrulline, in biological fluids may become clinical markers for monitoring of certain pathological conditions and the progress of their treatment(10).

Previous studies have demonstrated a higher concentration of NO metabolites in induced sputum of adult asthmatics as compared to normal subjects(25,26). In one of these studies, NO metabolite levels showed an insignificant decline following 2 weeks of anti-asthma treatment(25). To the best of our knowledge, there have been no published data demonstrating NO metabolites in induced sputum in children with asthma.

The aim of this study was to compare NO metabolites between asthmatic and control children and to evaluate the safety of sputum induction using nebulized hypertonic saline in young children. At the same time we looked into the cytological profile of the induced sputum in control and asthmatic children.

Subjects and Methods

Twenty-one patients with asthma and 10 control children in the age-group 6-16 years (*Table I*) participated in this study after informed consent from the parent/guardian. Control children, who volunteered for this study, had no current or past history of asthma or any other respiratory symptoms. They had forced expiratory volume in one second (FEV_1) > 75%, and a ratio of FEV_1 to forced vital capacity >75%. The patients were selected from the Pediatric Allergy and Asthma Clinic of the Postgraduate Institute of Medical Education and Research, Chandigarh (India) and the physician diagnoses of asthma were based upon their symptoms of recurrent episodic wheezing, cough, and/or dyspnea. No subject had any respiratory infection for 4 weeks prior to the study and none of them had received any form of corticosteroid therapy for the past 3 months. All the subjects had an FEV_1 >60%. The study was approved by the Ethics Committee of our Institute.

Study methodology

At the first visit, a questionnaire for symptoms and medications was answered by the child and his/her parents and physical examination and spirometry using the Vitalograph (Maids Moreton House, Buckingham, UK) were performed. Clinical severity of asthma was classified according to the

TABLE I–*Clinical and Spirometric Characteristics of Patients and Controls at Study Entry.*

	Study group (n = 21)	Controls (n = 10)	P value
Age (yr)	8.8 ± 1.8	9.7 ± 2.9	NS
Weight (kg)	24.9 ± 5.5	28.1 ± 6.3	NS
Height (cm)	125.7 ±11.2	130.3 ±12.9	NS
PEFR (%)	68.7 ± 9.5	99.4 ± 9.9	<0.001*
FEV_1 (%)	70.9 ± 7.7	93.0 ± 5.2	<0.001*
FVC (%)	68.1 ± 9.1	91.4 ± 6.1	<0.001*

All values are expressed as mean ± SD of the study variable. * NS = statistically not significant.
PEFR : Peak expiratory flow rate.
FEV_1: forced expiratory volume at one second.
FVC: forced vital cpacity.

International Consensus Report(27). Induced sputum was then collected. The patients received regular inhaled corticosteroids (ICS) (budesonide or fluticasone propionate as per the choice of the treating physician) delivered by metered-dose inhalers along with a spacer device and inhaled salbutamol by metered-dose inhaler as needed. Dosage range used for budesonide was 50-200 µg BD and 25-125 µg BD for fluticasone propionate. The patients were followed after 6 weeks of ICS therapy and clinical assessment, spirometry and sputum induction repeated.

Sputum induction and processing

The sputum induction and processing were performed according to the method previously described by Popov, *et al.* and Fahy, *et al.*(28,29). Briefly, PEFR was measured in all children using the mini-Wright peak flow meter. All children were pre-medicated with inhaled salbutamol two puffs (200 µg) using a spacer device. Children were asked to rinse the mouth, and blow the nose to minimize contamination with saliva and post-nasal drip prior to sputum induction. They were then asked to inhale nebulized sterile 3% saline solution for 30 minutes from an ultrasonic nebulizer (ITALIANA AS 1001, Microsonic, Italy) (oscillation frequency 2.35 MHz; mean aerodynamic diameter 0.5-5 µm) at the maximum output. The reservoir of the nebulizer was filled with 3% saline solution (Shree Krishna Keshav Laboratories, Ahmedabad, India). Ten minutes after the start of nebulization and every 5 minutes thereafter children were encouraged to cough sputum into a graduated container. The nebulization was stopped after 30 minutes or earlier if a sample of good quality was obtained. PEFR measurement was repeated after induction. If PEFR had fallen by > 20% the child was required to wait until it returned to the baseline value. An adequate sample was defined as a minimum of 1.5 ml of induced sputum. Previous studies have shown that inhaled hypertonic saline may cause bronchoconstriction(28). None of the children in the current study developed significant symptoms or fall in PEFR >20%.

Sputum cytology

Sputum was treated by adding equal volumes of 0.1% dithiothreitol followed by phosphate-buffered saline. The sample was then mixed gently and subsequently centrifuged at 400g for 10 minutes. The cytospinned specimens were stained with Leishmann and Hansel stains (*Fig. 1*) and differential cell counts of 400 non-squamous cells were then performed and expressed as a percentage of the total non-squamous cell count.

Nitrate and nitrite assay

Nitric oxide metabolites (nitrate + nitrite) were assayed colorimetrically by the Greiss reaction as previously described(30). Two hundred µL of sputum sample or standard was deproteinated by adding 20 µL of NaOH and 30 µL of $ZnSO_4$. Samples were mixed with 5×10^2 units of nitrate reductase, 20 µL of 0.2 mol/L N-trimethyl aminoethane sulfonic acid and 20 µL of 0.5 moL/L of sodium formate. Anerobic incubation was done at room temperature for 20 minutes. One mL of water

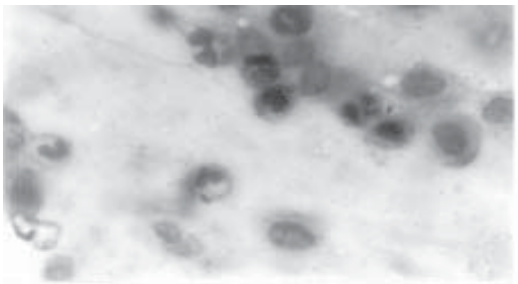

Fig. 1. Induced sputum cytology showing prominent eosinophilia (1000 x).

was added to the samples and nitrite assayed in the supernatant obtained by centri-fugation (5 minutes, 260 g). Deproteinated samples were mixed with 20 µL of 1% sulfanilamide in 15% phosphoric acid. After 10 minutes, 20 µL of 0.1% N-(1-naphthyl) ethylenediamine was added and absorption at 540 nm was determined by colorimetric method. Results were expressed in µmol/L of induced sputum.

Statistical analysis

All data were analyzed using the SPSS version 7.5 for Windows (SPSS. Chicago, Ill, USA). Data are expressed as mean ± SD. Comparison of continuous variables was performed using Student's *t*-test for variables with normal distribution and Mann-Whitney U-test for variables with non-normal distribution; P <0.05 was considered significant.

Results

Asthmatic children had significantly lower PEFR and FEV_1 than control children (*Table I*). Among the 21 patients, 13 had mild persistent symptoms (mean age 9.1 ± 1.7 years; 10 males and 3 females) and 8 had moderate persistent asthma (mean age 8.4 ± 2.0; 6 males and 2 females). The clinical characteristics of patients with mild persistent and moderate persistent asthma are shown in *Table II*. The process of sputum induction was monitored by measuring pre-and post-induction PEFR. Post-induction PEFR was reduced only at initial induction in asthmatics and not in controls or 6 weeks post-treatment in asthmatics (*Table III*). None of the patients suffered any acute symptoms and all patients were able to go home within 30 minutes of sputum induction. Average volume of sputum obtained was 1.8 ± 0.5 mL.

Sputum cytology

There was no difference in squamous cell count of sputum between subjects and controls at baseline (26.0 ± 9.2 vs. 28.7 ± 6.5, P = 0.4) and it did not change with ICS therapy. Differential count was performed as a percentage of non-squamous cells and eosinophil count was noted to be significantly higher in asthmatic children than in control children (15.3 ± 12.0 vs. 0.8 ± 1.1%, P < 0.01), whereas the percentage of macrophages was significantly lower (59.9 ± 10.9 vs. 77.0 ±

TABLE II–*Clinical Characteristics of Patients in Relation to Severity of Asthma at Inclusion.*

	Mild persistent (n =13)	Moderate persistent (n = 8)
PEFR (%)	72.3 ± 8.8	62.7 ± 12.3
FEV_1 (%)	66.4 ± 17.5	57.2 ± 11.4
FVC (%)	78.4 ± 16.5	72.0 ± 21.3

All values are expressed as mean ± SD.

TABLE III–*Decline in Peak Expiratory Flow Rate (PEFR) During Sputum Induction in Asthmatic Subjects and Controls.*

	PEFR (%) pre-induction	PEFR (%) post-induction	P
Study group (n = 21)			
Baseline	68.7 ± 9.5	64.4 ± 9.5	<0.001
6 weeks	78.3 ± 7.1	77.8 ± 7.0	0.09
Controls (n=10)	99.5 ± 9.9	98.9 ± 10.4	>0.5

All values are expressed as mean ± SD.

3.8%, P <0.01) in asthmatic children. The percentage of sputum eosinophils was higher in patients with moderate persistent asthma (n = 8) as compared to those with mild persistent asthma (n = 13) (16.9 ± 14.4 vs. 14.4 ± 10.9%), although the difference was not statistically significant (P >0.1). No significant differences were noted between asthmatic and control children in the percentage of squamous cells, neutrophils and lymphocytes (*Table IV*).

NO metabolites

The level of NO metabolites in the sputum was significantly higher in asthmatic children with a mean value of 224.4 ± 209.6 (mol/L (range 50.9 - 742.6), compared with control children, with a mean level of 39.2 ± 15.9 (mol/L (range 19.3 - 66.1 (mol/L). Children with moderate persistent asthma (n = 8) tended to have significantly higher values of NO metabolites in their sputum in comparison with mild persistent asthmatic children (n = 13) (315.5 ± 238.8 vs. 68.3 ± 136.0 (mol/L, P < 0.05).

Changes in NO metabolites, eosinophils following therapy

On reviewing the patient records at the end of the study period, 12 were taking budesonide (Group B) and 9 fluticasone propionate (Group FP). Nine of the patients on budesonide were classified as mild persistent asthma while 4 patients in the fluticasone group had mild persistent symptoms. There was no statistical difference at study entry in the two treatment groups.

Significant improvement in symptoms and lung function tests was seen following ICS therapy given for a period of 6 weeks. Peak expiratory flow rate was noted to increase from 68.7 ± 9.5 to 78.3 ± 7.1% at 6 weeks of therapy (P <0.01). This improvement was associated with a significant decline in sputum eosinophil counts (15.3 ± 12.0 to 2.2 ± 1.5% at 6 weeks, P <0.01). Mean reduction in eosinophil count was 85.6% at week 6 of therapy as compared to baseline.

There was a significant decline in level of sputum NO metabolites following ICS therapy (224.4 ± 209.6 to 86.5 ± 53.4 (moL/L at 6 weeks, P <0.01) (*Fig. 2*). Mean reduction in NO metabolites was 61.5% at week 6 of treatment as compared to baseline. However, levels of both eosinophils and NO metabolites in sputum continued to be higher than controls even after 6 weeks of anti-inflammatory therapy (P <0.05 for both).

Discussion

The results of the study indicate that the level of NO metabolites was increased in the

TABLE IV–*Sputum Cytology in Study Group vs Controls at Study Entry and at 6 weeks of ICS Therapy.*

	Controls (n = 10)	Study Group (n = 21)	
		Baseline	After 6 weeks
Eosinophils (%)	0.8 ± 1.1	15.3 ± 12.0 (p = 0.0007)*	2.2 ± 1.5 (p <0.0001)*
Macrophages (%)	77.0 ± 3.8	59.9 ± 10.9 (p <0.0001)*	71.2 ± 6.1 (p = 0.004)*
Lymphocytes (%)	10.2 ± 2.3	9.9 ± 5.9 (p = 0.9)	10.6 ± 5.5 (p = 0.5)
Neutrophils (%)	12.0 ± 3.4	14.9 ± 4.6 (p = 0.08)	16.0 ± 3.0 (p = 0.28)

All values are mean (% of non-squamous cell count) ± SD.
Parentheses indicate P value with controls (for baseline values) and with baseline (for 6 weeks follow up values).

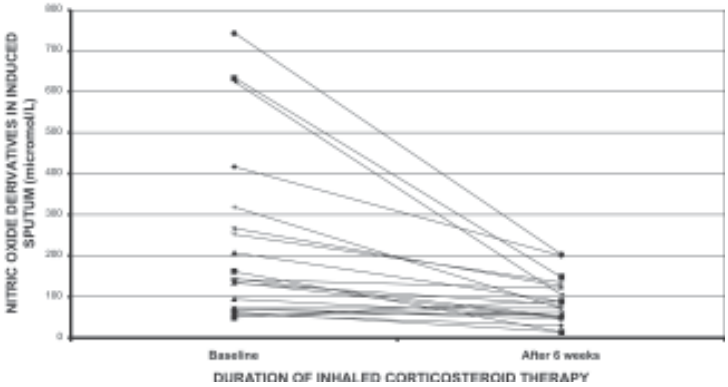

Fig 2. Nitric Oxide metabolities at baseline and following inhaled corticosteroid therapy

tracheobronchial secretion of asthmatic children and reflected the severity of asthma. Induced sputum NO metabolites may be useful in monitoring airway inflammation and anti-inflammatory therapy in bronchial asthma.

Nitric oxide and its free-radicle products are important in the pathogenesis of airway inflammation in asthma(4-7,14,26). Cytokines produced in asthmatic inflammation cause an increased expression of iNOS in airway epithelial cells and an elevation of NO levels(4,6). "Hyper-nitrosopnea" refers to the presence in the lung of an abnormally high concentration of NO(7). This elevation in NO increases in response to allergen challenge and is suppressed after treatment with corticosteroids. Nitric oxide increases airway inflammation by causing airway edema, efflux of eosinophils from airway vessels and formation of reactive nitrogen species(8-10). Nitric oxide also exacerbates the airway inflammation by inducing shedding of epithelial cells and thereby triggering axon reflexes from underlying neurons(13,14). Nitric oxide may amplify allergic inflammation by selective inhibition of T lymphocytes that secrete IFN-γ (TH1 cells), which suppress the proliferation of TH2 cells(6,31). Increased number of TH_2 cells leads to an increase in several cytokines such as IL-10, IL-4 and IL-5 which further increase airway inflammation(6). Inhibitors of NO synthesis may be useful in asthma by decreasing airway inflammation(3,6).

To the best of our knowledge, the present study is the first of its kind in children. Notably, sputum NO metabolites in children in this study were much lower than that reported in adults(25,26). The mean value of sputum NO metabolites in adult asthmatics was reported as 1086 ± 325 µmol/L by Kanazawa, *et al*. and 1252.5 ± 203.3 µmol/L by Jang, *et al.*(26) while we found it to be 224.4 ± 209.9 µmol/L in asthmatic children. The values in adult controls were similarly higher (577±115 µmol/L by Kanazawa, *et al*. and 557.2 ± 101.5 µmol/L by Jang, *et al.*) as compared to the controls in our study (39.2 ± 15.9 µmol/L). The reason for these differences are not entirely clear may be related in part to the lung-size or to environmental factors.

Further, it was observed that an occasional asthmatic child showed very high or low sputum nitrate levels irrespective of the

Key Messages

- Sputum induction can be safely and successfully performed even in young children and easily repeated on multiple occasions.
- Airway inflammation in bronchial asthma may be monitored by measuring nitric oxide (NO) derivatives in induced sputum.
- NO derivatives are important inflammatory markers in asthma and decline with anti-inflammatory therapy.

severity of asthma. This has also been noted in studies done on adults. In a previous study, asthmatic adults (n = 25) had values of sputum NO metabolites ranging from 149.4 to 2232.0 μmol/L(25). The variability was also seen in the control adults with a range from 65.1 to 1107.3 μmol/L. In the present study the range was 50.9 to 742.6 μmol/L for asthmatic children and 19.3 to 66.1 μmol/L for controls. Both these studies were done on a small number of patients. Larger studies are required to find normative data and make the test available for clinical use.

Induced sputum cytology showed a significantly higher eosinophil count in asthmatics as compared to normal children, which reflects the eosinophilic airway inflammation. Sputum eosinophil count declined significantly after 6 weeks of anti-inflammatory therapy with inhaled corticosteroids. Similar elevation in sputum eosinophil counts and decline following anti-inflammatory therapy has been reported previously by other workers(25,26,28,29). Eosinophils play a major role in the inflammation of asthma, primarily by secretary inflammatory mediator products, which cause damage to the airway epithelium(32-35). There was no significant difference in the differential counts of other cells in induced sputum between asthmatic and control children either at baseline or on follow-up. Patients with moderate persistent asthma tended to have higher sputum eosinophil counts (16.9 ± 14.4%) as compared to those with mild persistent asthma (14.4 ± 10.9%), although the difference was not statistically significant (P >0.5).

Corticosteroids are the most effective asthma therapy that suppresses inflammation in asthmatic airways, and they inhibit almost every aspect of the inflammatory process(33). This anti-inflammatory effect is through increased transcription and expression of anti-inflammatory proteins, such as IL-1 receptor antagonist, IL-10, and neutral endopeptidase and through repression of several inflammatory genes(34). Corticosteroids inhibit the activation and recruitment of inflammatory cells, particularly eosinophils, T lymphocytes, macrophages, dendritic cells and mast cells at the airway surface. Corticosteroids also inhibit the release of inflammatory mediators from structural cells in the airways, such as epithelial, smooth muscle and endothelial cells and fibroblasts(33,35). Corticosteroids inhibit the induction of Ca^{2+}-independent NOS without any effect on the activity of either the constitutive or inducible enzymes (35). This may form a major mechanism of action of inhaled corticosteroids in asthma.

The modulation of NO release may lead to application of novel therapies in diseases such as asthma and other inflammatory lung diseases(33). Monitoring of NO levels may also be useful in monitoring compliance in

non-responsive children. Previous studies have shown a decline in exhaled air(15,17-23) and induced sputum NO levels(26) following corticosteroid therapy. In the present study, NO metabolites in induced sputum were higher at baseline and declined significantly following anti-inflammatory therapy, although the levels were still higher as compared to controls. These findings may reflect persistence of low level airway inflammation despite control in symptoms with inhaled corticosteroids.

Induced sputum analysis is a relatively inexpensive test with the only major investment being the ultrasonic nebulizer with a cost range of 10-30,000 INR. The processing cost of sputum was approximately INR 8 per sample. Most samples in this study were processed individually and therefore, the cost should reduce further if the samples are processed in batches. As a comparison, the analyzer for exhaled NO costs to the tune of 500,000 INR. A possible drawback of sputum induction is the requirement of a trained respiratory technician to be present throughout induction of sputum. However, it is a promising new way of monitoring airway inflammation in asthma and this alone should not deter its potential.

Contributors: NR developed the study protocol, and was involved in patient screening, enrolment, outcome assessment, data analysis and writing the manuscript. LK conceptualized the study, participated in development of the protocol and contributed to writing the manuscript. SM was involved in the methodology of measurement of metabolites, contributed to the data analysis and writing of the manuscript.

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