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Increased levels of Nitric Oxide Metabolites in induced sputum and serum correlates with severity of Bronchial Asthma.

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ABSTRACT

Bronchial Asthma is a chronic relapsing inflammatory disease of the airways characterized by variable and recurring symptoms, reversible airflow obstruction, and bronchospasm. Inflammation is the most critical feature in the pathogenesis of asthma and nitric oxide oxidation product are important inflammatory mediator in asthma. A total of 60 subjects were enrolled in our study. After taking detailed history from their parents, clinical examination ,routine investigation and special investigation like Serum Nitric Oxide(NO) metabolites and sputum Nitric Oxide(NO) metabolites were estimated by using the principle of modified griess reaction. The levels of NO metabolites were correlated with severity of asthma. Both serum and induced sputum nitrite levels showed a statistically significant increasing trend with increasing frequency of exacerbation. We demonstrated a significantly high level of nitric oxide metabolite (nitrite) in serum and sputum of children suffering from bronchial asthma as compared to controls and the high level of nitrites in asthmatics also correlated with the severity of the disease. Thus, measurement of NO metabolites like nitrites in induced sputum can be a non-invasive, simple and useful tool to detect the inflammatory airway disease and it can also indicate the severity of the asthma.

Keywords: Nitric oxide, asthma, metabolites

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INTRODUCTION

Bronchial Asthma is a chronic relapsing inflammatory disease of the airways characterized by variable and recurring symptoms, reversible airflow obstruction, and bronchospasm. Symptoms include wheezing, coughing, chest tightness, and shortness of breath. The morbidity and mortality due to bronchial asthma are on a rise [1]. Most deaths from asthma are preventable with appropriate care [1].

Inflammation is the most critical feature in the pathogenesis of asthma and nitric oxide oxidation product are important inflammatory mediator in asthma [2].Asthmatic patients show an increased expression of inducible Nitric Oxide Synthetase (iNOS) in airway epithelial cells [3].The Nitric Oxide (NO) derived from airway epithelial cells may be a mechanism for amplifying and perpetuating asthmatic inflammation ,through inhibition of Th1 cells and their production of Interferon –γ .This would result in an increase in the number of Th2 cells and the cytokines Interleukin -4 (which is important for Ig E expression) and Interleukin -5 (which plays a critical role in the recruitment of eosinophils into the airways) [4].The role of NO in bronchial asthma may be controversial .NO may have beneficial effects on airway function as a bronchodilator and it acts as a neurotransmitter of bronchodilator nerves in human airways. On the other hand, NO may amplify asthmatic inflammation by generation of toxic hydroxyl radicals and also acts as a vasodilator , increasing plasma exudation and airway edema [5].Long term events related to NO are thickening of the bronchial wall and therefore an irreversible airway hyper reactivity [6].

Exhaled NO measurement can be a non-invasive tool to assess airway inflammation [7]. But, because of the limitations like nasal NO contamination and patients factors like nitrates diet, smoking and circadian rhythm, it may be better to measure NO metabolites like nitrites in induced sputum and serum to assess airway inflammation.

This is a non-invasive, easy and direct method to assess the airway inflammation. Hence, this study was planned to estimate NO metabolites in induced sputum in asthmatic children and to see the correlation with severity of disease, if any.

MATERIALS AND METHODS

The research project was conducted jointly in the Department of Pediatrics, Kalawati Saran Children's Hospital (KSCH) and Department of Biochemistry, Lady Hardinge medical College, New Delhi, India.

A total of 60 subjects (children) were enrolled in our study after informed written consent from their parents. The research project was conducted after Institutional Ethical Clearance.

The subjects were divided into two groups. Group I (Cases) comprises 30 children of 6-12 years with bronchial asthma attending Pediatric Pulmonology clinic at Kalawati Saran Children's Hospital. Group II (controls) comprises 30 children age and sex matched controls.

The inclusion criteria of our study includes (i) all children in the age group 6-12 years with persistent asthma as per National Heart Lung and Blood Institute Guidelines [8]. (ii) Children in whom a diagnosis of asthma has already been established based on a detailed history, clinical examination and the reversibility of airflow limitation by bronchodilators.

The exclusion criteria includes the following conditions which could possibly affect the nitric oxide levels like children with acute respiratory tract infections, children who have taken oral glucocorticoids within last four weeks, children with major systemic illness, tuberculosis, pyrexia due to infection, children with immunodeficiency disorders and children with chronic exposure to smoke.

After taking a detailed history from the parents, clinical examination of the subjects were done. The following investigations were done in these subjects like routine biochemical investigations (Glucose, Liver function tests like total and direct bilirubin, Alanine aminotransferase, Aspartate aminotransferases, Alkaline phosphatase, Kidney function tests like urea, creatinine, uric acid, Electrolytes like sodium, potassium, calcium and phosphate, Total protein and albumin and lipid profile like cholesterol ,triglyceride) and hematological investigations (Hemoglobin, Total Leukocyte Count, Differential Leukocyte Count and Absolute Eosinophil Count) .Special tests like Spirometry and serum Nitric Oxide(NO) metabolites and sputum Nitric Oxide(NO) metabolites were also done.

Sample collection

Dietary moderation was done in all the patients before sample collection. The subjects were instructed to take a low nitrite/nitrate diet for a minimum of 4 days, as per published guidelines [9]. Under all aseptic precautions, 5 ml of fasting venous blood was collected. One milliliter was transferred to an EDTA vial for hematological investigations and 4 ml blood was collected in a plain serum tube and was allowed to clot. Serum thus separated was stored at -20 ^{0} C until further use.

Collection of sputum samples

Subjects inhaled 3 % saline solution at room temperature by a nebulizer. Throughout the nebulization, subjects were encouraged to cough and to expectorate in to a container. The sputum samples were stored at 4 ^{0} C for not more than 2 hours before further processing.

Processing of sputum samples

Sputum sample was diluted with equal volume of phosphate buffer saline containing 10 mmol/l Dithiothreitol and gently vortexed at room temperature. The sample was then centrifuged for 10 minutes. The supernatant was stored at – 70 ^{0} C for subsequent assay.

Nitrite assay by Modified Griess Reaction [2,10,11]

Principle

The Griess reaction relies on a simple colorimetric reaction between nitrite, sulphanilamide and N-(1-Naphthyl) ethylene diamide to produce a pink/magenta azo product with a maximum absorbance at 543 nm.

To 500 µl of sample, equal volume of freshly prepared Griess reagent was added. It was prepared by mixing equal volumes of 0.2 % (w/v) of naphthylene ethylene diamine dihydrochloride and 0.2 % (w/v) of sulphanilamide in 5 % (v/v) phosphoric acid. The mixture was incubated at room temperature for 10 minutes. After 10 minutes of color development, absorbance was measured at 340 nm in a semi autoanlyser.

Sodium nitrite ($NaNO_2$) standards were prepared by serial dilutions. The concentration of serum/sputum samples was determined on the basis of sample absorbance and calculated from standard curve.

Statistical analysis

The data obtained from our study was analyzed by using SPSS version 12 software. Results were expressed as mean ± standard deviation. Correlation analysis was also done. P value < 0.05 was considered as significant.

RESULTS

According to the severity, bronchial asthma is classified into four grades like grade I (mild intermittent), grade II (moderate intermittent),grade III (moderate persistent) and grade IV (severe persistent) [1].

All the children enrolled for the study belonged to Grade II (19 cases) and Grade III (11 cases) asthma. Among the grade II cases, 6 were within the age group of 6-8 years,5 were within 8-10 years and 8 cases were within 10-12 years. Among the grade III cases , 3 were within the age group of 6-8 years,4 were within 8-10 years and 4 cases were within 10-12 years.

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Table I. Comparison of serum nitric oxide metabolite levels in study & control group

Samples	Nitrites NO_2^- (µmol/l) Mean ±SD		
	Cases	Controls	p value*
Serum	23.051±6.82	11.40±2.02	<0.01
Sputum	18.10±3.80		

*p value <0.05 is considered as statistically significant
Sputum nitrite levels were not done in controls because sputum could not be induced in the control group.

Table II. Changes in serum and induced sputum nitrite levels with respect to severity of asthma

	NO_2^- (µmol/l)		
Severity	Grade II (n=19)	Grade III (n=11)	p value*
Serum	18.87±3.23	30.25±5.10	<0.01
Induced sputum	16.33±3.21	21.16±2.64	<0.01

*p value <0.05 is considered as statistically significant

Table III. Comparison of Nitrites in serum and induced sputum with frequency of exacerbations

NO_2^- (µmol/l)	≤ 12 attacks /yr (n=26)	≥12 attacks /yr (n=4)	p value*
Serum	21.92±6.57	30.39±2.74	0.0178
Induced sputum	17.55±3.78	21.69±0.59	0.0401

*p value <0.05 is considered as statistically significant
Both serum and induced sputum nitrite levels showed a statistically significant increasing trend with increasing frequency of exacerbation.

DISCUSSION

Bronchial asthma is an inflammatory disease of airways and there is increasing evidence that endogenous NO plays a key role in physiological regulation of the airways and is implicated in the pathophysiology of airway diseases [2, 10, and 11].

Thus, to study about the airways inflammation in asthma we have to assess the NO status in exhaled air .Several researchers have assessed the role of NO in airways diseases by measuring NO in exhaled air of asthmatics [10, 11, 12, 13].The use of expired NO although a good indicator of NO production, its measurement has certain drawbacks which include nasal NO contamination and contamination with ambient NO. Exhaled NO concentration exhibits significant expiratory flow dependence. Another method of determining NO status in airway disease is by estimation of its stable end products 'nitrites'. Direct measurement of endogenous NO has been difficult and therefore nitrites being the stable end product has been used as an index of NO generation. Therefore, we studied the nitric oxide metabolite in induced sputum and serum in asthmatics patients and also correlated with the disease severity.

In our study, we found that the children with asthma had higher levels of serum nitrites as compared to the demographically (age, sex) matched controls. The serum and induced sputum nitrite levels in asthmatics are 23.05±6.82 µmol/l and 18.10±3.80 µmol/l. Several researchers had demonstrated increased sputum concentration of nitrites in patients of asthma. Zetterquist W et al in 2008 demonstrated increased levels of nitrites in exhaled breath condensate of children of asthma [11]. Fitzpatrick AM et al in 2009 mentioned an significantly increased level of nitric oxide oxidation products in the epithelial lining fluid of children with persistent asthma (2). Malinovschi A et al in 2011 demonstrated an increased NO metabolites in exhaled breath condensate of children of asthma [7].

In the present study, we found that both serum and induced sputum nitrite levels showed a statistically significant increasing trend with increasing frequency of exacerbation (Table III & IV).Children with mild persistent asthma have serum nitrite levels of 18.87±3.23 µmol/l and sputum nitrite levels of 16.33±3.21 µmol/l whereas children with moderate persistent asthma have serum nitrite levels of 30.25±5.10 µmol/l and sputum nitrite levels of 21.16±2.64 µmol/l.

Thus there appears to be a correlation between NO metabolite levels in serum and sputum with severity of disease implying an indirect correlation of NO metabolites in serum and sputum with the underlying inflammation of the airways. Our study substantiates the observation that an iNOS may be involved in inflammatory disease of the airways like bronchial asthma. The increased levels of NO derivatives in induced sputum and serum of patients with asthma may be due to induction of iNOS by inflammatory mediators released into asthmatic airways. Measurement of NO derivatives in serum and sputum may be useful for assessing inflammation.

To conclude, we demonstrated a significantly high level of nitric oxide metabolite (nitrite) in serum and sputum of children suffering from bronchial asthma as compared to controls and the high level of nitrites in asthmatics also correlated with the severity of the disease. Thus, measurement of NO metabolites like nitrites in induced sputum can be a non-invasive, simple and useful tool to detect the inflammatory airway disease and it can also indicate the severity of the asthma.

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