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Cellularity Pattern in Induced Sputum in School Aged Asthmatics

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Abstract
Background: *Many inflammatory phenotypes previously described in adults with asthma: eosinophilic and non-eosinophilic. Treatment strategies based on these phenotypes have been successful. This study tried to evaluate sputum cytology in school aged asthmatics and to classify sputum inflammatory phenotypes in this age group.*
Methods: *Complete history, physical examination and asthma control test were performed to all children and adolescents. Pulmonary function tests, CBCs, renal function, sputum induction, processing, homogenization, centrifugation and count were done.*
Results: *This study included65 children and adolescents with mean age (11.9) 39 mild to moderate asthma (group1) and 26 with severe asthma (group2) according to pre bronchodilator FEV1% (Forced expiratory volume one). Group 1 mean age of was 12.2 ± 2.9 and was 11.43 ± 3.1 for group 2 (p value >0.05). Our study shows eosinophilic phenotype had more uncontrolled (8 patients) and partially controlled patients (7 patients), while only 4 contolled patients had eosinophilic sputum. In neurtrophilic type, most patients were controlled (9 patients) while 3 patients were partially controlled and 3 patients were uncontrolled. Most patients were controlled (8 patients) in paucigranulocytic type while 3 patients were partially controlled and 2 patients were uncontrolled. The last type is mixed in which patients were controlled (10 patients) while 3 patients were partially controlled and 2 patients were uncontrolled. P value is significant as regard level of control*
Keywords: *asthma phenotypes; induced sputum; children.*

Introduction

Bronchial asthma is considered the most common chronic disease in children leading to school absence, emergency department visit and hospitalization. Up to 50% of adult asthmatics have early childhood onset.[(1,2)]

Assessment of inflammatory cells (eosinophils and neutrophils, and inflammatory mediators in supernatants) can be obtained by analysis of induced sputum cytology whichis a partially noninvasive method.[(3)]

Performance of sputum induction and sample processing have been standardized for use in children and is well described by European respiratory Society (ERS) recommendations[(4)]. Sputum induction has been shown to be safe in asthmatic children, regardless of disease severity, and feasibility is 80–85% in children between 6

and 17 years of age. The methodology has not yet been standardised in preschool children.[5,6]

It is increasingly recognised that a single therapeutic approach for all children with asthma is unlikely to be successful. Phenotyping children with asthma has the potential to provide therapeutic targets and a more individualised management approach.[7,8]

Patients and Methods

Patients

Children and adolescents with bronchial asthma who attended to outpatient clinic at chest department and Pediatric department of El-Minia University Hospital were invited to participate in the study from September 2017 to January 2018. Complete history taking and clinical examination were performed to confirm diagnosis. Asthma was diagnosed according to the criteria recommended by the Global Initiative for asthma (GUINA)[9].

Exclusion criteria

Children who were unable to follow instructions or unable to perform pulmonary function, acute respiratory distress, unstable cardiovascular status, thoracic, abdominal or cerebral aneurysms, hypoxia (SaO2 less than 90% on room air), lung function impairment (FEV1 less than 1.0 Liter), pneumothorax, pulmonary emboli, fractured ribs or other chest trauma, recent eye surgery, currently prescribed an immunomodulatory steroid sparing agent (ciclosporin, methotrexate or azathioprine) or a continuous infusion of subcutaneous terbutaline, or had received intramuscular triamcinolone in the previous 3 months, or had another significant chronic respiratory or medical condition and haemoptysis,.

Methods

1-Pulmonary function tests

Spirometer was performed using a spirometer (BTL-08 Spiro) as the best of three consecutive readings within 100 ml, according to the American Thoracic Standards, before and 10 min after inhalation of 200 Mcg salbutamol[10].

2-Chest x-ray

3-Asthma Control Test (ACT)

Asthma control at the time was assessed using the asthma control test , which assesses control in the preceding 4 weeks. Scores of 5–25 may be attained, with higher scores indicating better control. Scores of 20-25 are well controlled,15-20 are partially controlled and 5-15 poorly controlled.[11]

4-Sputum Induction

Induced sputum aimed to obtain an adequate sample in patients who did not spontaneously produce sputum in order to study the airway inflammation in asthma[12]. Sputum was induced according to the method of Pizzichini et al by using hypertonic saline 4.5% nebulized with ultrasonic nebulizer for 7- min.

Precautions of sputum induction: As hypertonic saline causes bronchoconstriction, the procedure should only be performed after pre-medication with salbutamol and under medical supervision. Before sputum induction, all subjects underwent spirometry, with FEV1 and vital capacity measurements. Sputum induction was discontinued if the patient develops bronchoconstriction. Selected sputum plugs from saliva were then analyzed.[13]

5-Sputum processing and sample homogenisation:

Sputum was processed soonly or at least within 2 h to ensure good cell counting and staining[14]. Homogenous sputum can be obtained by the use of dithiothreitol (DTT) allowing cells to be released.[15]

The duration and temperature of homogenization, time ranging 10–30 min and temperature 4–37°C.It has been demonstrated that different exposure times to DTT at room temperature have no effect on the differential cell count (DCC)[16].

6-Sample filtration: Filtration was done through a 48µm nylon mesh to remove mucus and debris. A single filtration step results in a slight reduction in the TCC with improvement of slide quality and the without change in DCC[17].

7-Total cell count (TCC) and viability: The TCC is performed manually using a haemocytometer,

and cell viability is determined by the try pan blue exclusion method[13,14].

8-Centrifugation, staining and counts: Centrifugation was done for 5-10 min by force ranged 300–1,500×g then sediments were stained using Leishmen stain for differential cell count. The DCC is determined by counting a minimum of 400 non squamous cells and is reported as the relative numbers of eosinophils, neutrophils, macrophages, lymphocytes and bronchial epithelial cells, expressed as a percentage of total non squamous cells. The percentage of squamous cells was reported separately[13,18].

Definitions of inflammatory phenotype according to sputum differential eosinophil % and neutrophil % [19]

Phenotype	Sputum eosinophils %	Sputum neutrophils %
Eosinophilic	>2.5	≤54
Neutrophilic	≤2.5	>54
Mixed	>2.5	>54
Paucigranulocytic	≤2.5	≤54

9-Complete blood count (CBC), Renal function and liver function test.

Statistical Analysis

The data obtained was analyzed with the SPSS version 22 statistical program. Data are expressed as means and standard deviation. Continuous variables were compared using the Mann–Whitney U-test and categorical variables using the Chi-squared test or Fisher's exact test, where appropriate. The relationship of the variables with each other was examined using the correlation tests. The significance level was considered as P-value<0.05.

Results

This study included 65 asthmatic school-aged children and adolescents (Mean age 11.9 years - Range 8-15 years old), Asthmatic patients classified according to severity of lung function. The first group of patients had mild to moderate asthma. The second group had severe asthma according to pulmonary function classification of American Thoracic Society (ATS)[10].

Table 1 Demographic data and classification of studied asthmatic children (total number =65)

Item	**Group 1** Mild to Moderate asthma (N=39)	**Group 2** Severe asthma (N=26)	**P value**
Age, (years) mean±SD	12.2 ±2.9	11.43± 3.1	NS
Sex			
Male No (%)	25(64.1)	15(57.7)	0.04*
Female No (%)	14(35.9)	11(42.3)	
PFTs			
FEV1 % (Mean±SD)	72±16.14	43±11.5	0.001
FVC % (Mean±SD)	88± 17.4	84±16.3	NS
PEF (Mean±SD)	79.18±9.58	66±13.13	0.005
Asthma control			
Controlled No(%)	20(51.3)	11(42.3)	0.03
Partially controlled No(%)	14(35.9)	5(19.2)	0.01
Uncontrolled No(%)	5(12.8)	10(38.5)	0.04

FEV_1, forced expiratory volume in 1 s, FVC Forced Expiratory Flow, PEF Peak Expiratory flow.

Table 1 shows demographic characters and classification of 65 asthmatic children with range of age (8-15) years. According to pre-bronchodilator FEV1, patients are classified into two groups; group1patients diagnosed to have mild to moderate asthma and group 2 those with severe asthma. Mean age of group1was 12.2 ±2.9 and was 11.43± 3.1forgroup2 with no significant difference (p value > 0.05). Regarding to sex distribution in studied patients, there were 40 males (25 group1&15 group2) and 25 females (14 group1 &11 Group2) which showed statistically significant difference (p value 0.04). Mean FEV1% was significantly higher in group1 (72%) while it was 43% in group 2 (p value 0.001). FVC was 88% and 84% in group1and 2 respectively.

PEF was 79.18 % and 66% in group1and 2 respectively, the difference was statistically significant (p= 0,005).

According to ACT, 20 of Group1 were controlled on prescribed treatment and 14were partially controlled and 5 were uncontrolled, while in group2 eleven were controlled on prescribed treatment and 5were partially controlled and 10 were uncontrolled. These results carried significant difference (p value < 0.05)

Table 2 Phenotypes of studied asthmatic children

Sputum phenotype (No, %)	Group 1 ((N=39) No	%	Group 2(N=26) No	%	P value
Eosinophilic (19, 29.2%)	4	10.3%	15	57.7%	0.02
Neutrophilic (15,23.1%)	11	28.2%	4	15.4%	0.04
Paucigranulocytic (16, 24.6%)	13	33.3%	3	11.5%	0.03
Mixed (15, 23.1%)	11	28.2%	4	15.4%	0.04

Table 2 shows different phenotypes of asthmatic children classified according to calculated cell type percentage in induced sputum samples**.** Eosinophilic sputum was present in 19 patients (29.2%), 4 cases (10.3%) among group 1 while it was the predominant type among group 2 patients with severe asthma as it was detected in 15 patients (57.7%). The difference was statistically significant (p value= 0.02). Neutrophilic sputum was present in 15 patients (23.1%), 11 patients (28.2%)among group1 and only 4 patients (15.4%) amonggroup2. Paucigranulocytic was present in 16 patients (24.6%), 13 patients (33.3%) among group1 and 3 patients (11.5%) among group 2. Mixed type was present in 15 patients (23.1%), 11 patients (28.2%) among group1 and 4 patients (15.4) among group 2. These results showed that neutrophilic, paucigranulocytic, and mixed types was statistically higher in group 1with mild to moderate asthma than patients with severe asthma (group 2) with p values 0.04, 0.03, 0.04 respectively.

Table 3 Comparison of children with different sputum phenotypes

	Eosinophilic 19(29.2%)	Neutrophilic15 (23.1%)	Paucigranulocytic16 (24.6%)	Mixed15 (23.1%)	P
Age	11.6	12.8	12.9	13.1	NS
Sex male:female	11:8	8:7	9:7	8:7	NS
PFTs					
FEV1% (Mean±SD)	55±12.15	72±14.6	67±14.02	69±14.09	0.02
FVC % (Mean±SD)	86± 15.7	84%±16.3	91± 18.3	90± 19.1	NS
PEF % (Mean±SD)	60.2±8.29	77±17.11	74.18±16.44	75.08±12.8	0.03
Asthma control					
Controlled	4 21.1%	9 60%	8 50%	10 66.7%	0.01
Partially controlled	7 36.8%	3 20%	5 31.2%	3 20%	0.03
Uncontrolled	8 42.1%	3 20%	3 18.8%	2 13.3%	0.04

Table 3 showed there was NS difference regarding age and sex in different phenotypic groups. There was significant difference between the four groups in FEV1 and PEF (p-value .02 , .03 respectively), while difference in FVC was none significant.

Controlled patients were found in only 4 patients (21.1%) in eosinophilic phenotype and in 9 (60%), 8 (50%), 10 (66.7%) in neutrophilic, paucigranulocytic, and mixed respectively. The difference was statistically significant (p value = 0.01).Partially controlled patients were present in 7 (36.8%) and were noticed in3 (20%), 5 (31.2%) and in 3(20%) among neutrophilic, paucigranulocytic, and mixed respectively. Eight

out of 19 esinophilic patients (42.1 %) were uncontrolled, while 3 (20%), 3 (18.8%) and 2(13.3%) of neutrophilic, peucigranulocytic and mixed types were uncontrolled respectively.

Discussion

Bronchial asthma is the most common incommunicable respiratory disease that affecting children all over the world[20]. It has genetic base [21] although nongenetic (nonatopic) types are present[22]. Asthma has multiple phenotypes, which may differ as regard age of onset, trigger factors, and patterns of severity, as reflected by variably reversible loss of pulmonary function[23].

Sputum cell counts are able to identify eosinophilic, neutrophilic, paucigranulocytic and mixed patterns of airway inflammation in asthma [13]. This is an important point as different types of cellular mediated inflammation may respond differently to asthma treatments[24]. Interestingly, the documentation of the heterogeneity of airway inflammation in children is scarce[25,26].

Our study tried to evaluate sputum phenotypes in school aged asthmatics hoping in changing treatment modalities. As regard sex of studied groups number of males is significantly increased than females in both groups. In childhood, approximately 2/3 of the subjects with asthma or wheezing are males and 1/3 are females, giving odds ratios (ORs) of 1.4–1.6 or above on comparing males to females[27,28,29,30,31,32]. In adolescence, the pattern changes and adolescent-onset wheeze or asthma is more prevalent in females than males[33,34,35,36,37].

It has been argued [38] that male predominance in childhood asthma is attributed to boys having smaller airway diameters relative to lung volume [39] and more allergen sensitivities.

Our study showed that eosinophilic inflammation was present in 29.2% of the patients. Wang et al study differed with our study. Their study showed that asthma phenotype was predominantly eosinophilic in children with acute asthma (about 50% of cases).[40] Nevertheless, for severe asthma, the recognized inflammatory phenotypes are eosinophilic, noneosinophilic and paucigranulocytic asthma[41]

Our study showed that eosiphilic type of sputum is more severe type as regard FEV1 and more uncontrolled patients according asthma control test. Our results are in line with results of Drews etal, 2009 in Australia. Drews et al studied asthmatic children aged 6–17 years and followed them for 5 years and classified their patients at the start of the study as eosinophilic or noneosinophilic asthma based on their IS cell profile. Children with eosinophilic asthma were more symptomatic, used more beta-2 agonists, presented lower FEV1/FVC ratio and were more allergic to pollens when compared with noneosinophilic asthmatics[42]. Mucus plugging and epithelial denudation in the large-airway wall and inside lumen could explain that asthmatic children with eosinophilic sputum develop more severe and uncontrolled asthma. It is associated with increased expression of Tumour Growth Factor β (TGFβ) and reticular basement membrane (RBM) thickness[43]. The RBM is formed by the fusion of alveolar basal lamina and pulmonary capillaries, and constitutes the interface for oxygen and carbon dioxide diffusion. Active recruitment of eosinophils is largely mediated by proteins secreted by the epithelia: the most potent chemo attractant for eosinophils being eotaxin 1 (CCL11), which is responsible for 80% of TGFβ expression in asthma[44].

Zacharasiewicz *et al* observed that children who remained stable during ICS reduction maintained low levels of eosinophils and those who experienced a loss of asthma control had an increase in sputum eosinophil level.[45]

This study concluded that neutrophilic inflammation had more mild and controlled asthmatic children on treatment. Study of Cowan et al also surprised thatthe mild to moderate group had higher sputum neutrophil levels at baseline despite significantly lower ICS doses. These finding were in contrary to their previously discussed findings.[46]

Conclusion

The majority of international asthma guidelines have not yet unequivocally endorsed the use of induced sputum as noninvasive measurements of airway inflammation in the diagnosis and management of asthma. Induced sputum analysis is recommended for every patient diagnosed as bronchial asthma before starting asthma management and for asthmatic patients who are not controlled on full asthma management to understand type of airway inflammation in all guidelines. There is an obvious need for a non-invasive means of assessing airway inflammation, particularly in children in whom direct methods of assessment are rarely possible.

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Abbreviations

Forced expiratory volume one (FEV1), European respiratory Society (ERS), asthma control test (ACT), Global Initiative for asthma (GUINA), dithiothreitol (DTT), differential cell count (DCC), Total cell count (TCC), American Thoracic Society(ATS), FVC Forced Expiratory Flow, PEF Peak Expiratory flow, Tumour Growth Factor β (TGFβ) and reticular basement membrane (RBM), chemoattractant for eosinophils being eotaxin 1 (CCL11).