Research Article

Analysis of Induced Sputum in Different Forms of Bronchial Asthma

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Abstract

Background: This observational study of induced sputum in asthmatic patients receiving different forms of treatment may provide us with the opportunity to compare sputum cell indices in asthmatic patients and also reveals the different inflammatory subtypes of asthmatic patients. **Patients and methods:** Eighty asthmatic patients were included in this study. They were classified into ٤ subgroups according to the cell type of induced sputum; ١) Neutrophilic group, ٢) Eosinophilic group, ٣) Mixed cellular group and ٤) Paucigranulocytic group. **Results:** Our study showed significant reduction in eosinophils % in asthmatic patients receiving inhaled corticosteroid (ICS) compared to untreated patients (P-value = ٠.٠٠٠١), also, our study reported ١٦ patients with totally controlled asthma, ١٠ of them had eosinophilic inflammation and only ٢ patients with neutrophilic cell type were controlled, however, this difference was not statistically significant (P-value = ٠.٠٦). **Conclusion:** We can use the induced sputum eosinophils count to titrate treatment results which may help in lowering the exacerbation rate with no overall increase in medication.

Keys word: Asthma, Sputum analysis and Flow cytometry.

Introduction

Asthma is characterized by variable airflow limitation, which is validated by spirometry or measurements of airway responsiveness and is treated by bronchodilators (Boulet et al., ١٩٩٩). It is also associated with airway inflammation, this is tradetionally considered to be eosinophilic and is treated by avoidance of any causes and by anti-inflammatory medications, of which, corticosteroids are the most effective. This treatment of the inflammation also reduces variable airflow limitation and airway hyperresponsiveness. The most comprehensive measurement of airway inflammation is spontaneous or induced sputum cell counts (Djukanovic et al., ٢٠٠٢).

This measurement has become established worldwide in research (Parameswaran and Hargreave, ٢٠٠٢). The test is relatively non invasive and has excellent reliability, validity and responsiveness (Djukanovic et al., ٢٠٠٢).

The inflammation (a bronchitis) can be eosinophilic, neutrophilic, both or neither, and its recognition is important in diagnosing and treating the illness. For example, eosinophilic bronchitis, which occurs in patients with or without asthma and in some patients who have chronic obstructive pulmonary disease (COPD), is responsive to corticosteroid treatment, and current evidence suggests that when there is no eosinophilia the condition is not clinically responsive to corticosteroid treatment (Brightling et al., ٢٠٠٠). There is only, at best, a poor correlation between sputum (or bronchial biopsy or lavage) eosinophils and symptoms or physiological abnormalities (Van den Berge et al., ٢٠٠١). As a result, accurate clinical recognition of airway inflammation is poor emphasizing the need to measure it in clinical practice. Support for the use of sputum cell counts to improve treatment was provided by Green et al., (٢٠٠٢).

The aim of the work is to study randomized effect of treatment on sputum cellularity and the ability to tailor therapy according to sputum cellularity for each asthmatic patient.

Patients and methods

Eighty patients with asthma who attended to outpatient clinic at chest department and internal medicine department of El-Minia University Hospital were invited to participate in the study. The study was approved by the hospital's research ethics board. All patients provided written consent.

Asthma was diagnosed according to the criteria recommended by the Global Initiative for asthma (GINA, ٢٠١١). Asthmatic subjects had objective diagnosis of asthma with spirometry.

Inclusion criteria:

١- All asthmatic patients except patients with contra-indication for sputum induction.

Exclusion criteria:

Contra-indications for sputum induction:

١- As hypertonic saline causes bronchoconstriction, the procedure should only be performed after pre-medication with salbutamol and under medical supervision in patients with asthma.

٢- Sputum induction causes severe coughing, the procedure should not be performed in patients, in whom, severe coughing may be harmful. This may include patients with; Haemoptysis of unknown origin, acute respiratory distress, unstable cardio-vascular status (arrhythmias, angina), thoracic or cerebral aneurysms, hypoxia, lung function impairment, pneumothorax, pulmonary emboli, chest trauma and recent eye surgery.

Patients were recruited from the outpatient clinic of chest department and internal medicine department of El-Minia University Hospital, if the inclusion criteria were fulfilled. Spirometry and all the measurements of airway inflamemation and sputum induction were done. An x-ray chest, oxygen saturation and electrocardiogram were done, if clinically indicated.

Methods:

Spirometry:

Spirometry was performed using a spirometer (ZAN ٣٠٠, Germany) according to the American Thoracic Standards, before and ١٠ min after inhalation of ٢٠٠ mcg salbutamol**.**

Sputum Induction:

The aim of sputum induction is to collect an adequate sample of secretions from lower airways in subjects who do not produce sputum spontaneously in order to study the features of airway inflammation in asthma and other respiratory disorders**.** Sputum was induced according to the method of Pizzichini et al., (١٩٩٦).

Methods of sputum processing:

Sputum sample homogenization:

Sputum was processed as soon as possible within ٢ hours in order to ensure optimum cell counting and staining**.** Complete homogenization is important and can be achieved by the use of dithiothreitol (DTT) to break the disulphide bonds in mucin molecules, allowing cells to be released (Louis et al., ٢٠٠٢).

The duration and temperature of homogenization time ranging ١٠–٣٠ min and temperature ٤–٣٧°C. It has been demonstrated that different exposure times to DTT at room temperature have no effect on the differential cell count (DCC). The volume of mucolytic used during processing of entire expectorate was fixed at ١:١. Filtration through a ٤٨µm nylon mesh was used to remove mucus and debris (Efthimiadis et al., ٢٠٠٠).

Total cell count and viability

The TCC is performed manually using Neubauer haemocytometer, and cell viability is determined by the trypan blue exclusion method.

Centrifugation, staining and counts

Centrifugation is necessary to separate sputum cells from the fluid phase. The centrifugal force used to date has ranged ٣٠٠–١,٥٠٠×g and the duration of centrifugation ٥–١٠ min. Then sediments were stained using Leishman stain for differential cell count. Care should be taken to ensure that buffers are at the appropriate pH (٧.١–٧.٢) using phosphate buffered saline (PBS). This allows accurate characterization of cells on the basis of their staining and morphology. The differential cell count (DCC) is determined by counting a minimum of ١٠٠ non squamous cells and is reported as the relative numbers of eosinophils, neutrophils,

macrophages, lymphocytes and bronchial epithelial cells.

Flowcytometric enumeration of neutrophils and eosinophils:
Washed cells are incubated with fluorochrome-conjugated mono-clonal antibodies (mAb) (Becton Dickinson Immunocytometry Systems, BDIS, San Jose,CA, USA) for ٣٠ min. at ٤°C.

To identify neutrophils, sputum cells were stained with anti-CD٦٦b (FITC). To identify eosinophils, sputum cells were stained with anti-CD١٢٥ (PE). The analysis windows for neutrophils and eosinophils were established on the basis of their granularity /complexity (side-scatter, SSC).

During analysis granulocytes were gated, excluding mononuclear cells and cell debris.
The samples were analyzed using Argon laser flow cytometry (EPICS XL, Coulter, USA).

Complete blood count (CBC):
Determined by automated cell counter SYSMEX KX-٢١N (TAO Medical Incorpo-ration, Japan).

Renal function tests: Were assayed using fully automated clinical chemistry autoanalyzer system Kone-lab ٢٠i (Thermo-Electron Incorpo-ration, Finland).

C- reactive protein (CRP): Measured by semi-quantitative method

Chest X-ray (CXR): Each patient will undergo posterior- anterior view & lateral view using commercially available radiograph machine model VZW٢٩٣٠RC٢-H٥ (VILLA SISTEMIMEDICALI, CANADA) under standard technique.

Statistical Analysis
The data obtained was analyzed with the SPSS version ١٩ 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. The relationship of the variables with each other was examined using the correlation tests. The significance level was considered as P-value < ٠.٠٥.

Results
Demographic and physiological characteristics:
Eighty patients were included in longitudinal study to investigate induced sputum in asthmatic patients under different forms of treatment. Patient's clinical characterristics are summarized in table I.

Asthma inflammatory sub-type classification:
Sputum induction was successful in ٧٢ (٩٠٪) subjects. Inflammation was classified into ٤ types neutrophilic if neutrophils ≥ ٦٥٪, eosinophilic if eosinophils ≥ ٢.٤% (Spanevello et al., ٢٠٠٠), mixed (both neutrophilic and eosinophilic) and paucigranulocytic (non neutronphilic and non eosinophilic). Fifty one (٦٣.٨٪) patients had eosinophilic inflammation, ٨(١٠٪) had neutrophilic inflammation, ٥(٦.٢٪) had paucigranulocytic, ٦(٧.٥٪) had both and ١٠ patients were undefined (table II) & (figure ١).

On comparing age, forced expiratory volume in first second (FEV١), FEV١٪, forced vital capacity (FVC), FVC%, peak-flow meters (PEF) and PEF% in asthma inflammatory subtypes, there was no significant differences among the studied groups (Table III).

There was significant correlation between FVC% pre-bronchodilator and neutrophils %, while other parameters showed no significant correlations with both neutrophils % and eosinophils % (Table IV).

There was significant reduction in eosinophils % of asthmatic patients receiving inhaled corticosteroids (ICS) compared to other patients who were untreated (p-value=٠.٠٠٠١) (table V).

Patients with controlled asthma (n=١٦), ten of which showed eosinophilic inflammation, while only ٢ patients with neutrophilic inflammation were controlled. There were no significant differences between partially controlled and uncontrolled patients regarding the inflame-matory cell subtypes (p-value = ٠.٠٦) (table VI).

Table I: Demographic data of asthmatic patients (N=٨٠). Data are presented as number and their percentage from total patient number.

	Number	%
Sex		
Male	٤٣	٥٣.٨
Female	٣٧	٤٦.٢
Residence		
Urban	٢٨	٣٥
Rural	٥٢	٦٥
Smoking		
Smoker	١٧	٢١.٣
Non-smoker	٥٧	٧١.٣
Ex-smoker	٦	٧.٤
Asthma control		
Controlled	١٦	٢٠
Partially controlled	٢٥	٣١.٢
Uncontrolled	٣٩	٤٨.٨

Table II: Sputum cell indices of asthmatic patient.

Sputum cells	Mean±	SD	Minimum	Maximum
TCC/ µl	٣٢٢٥.٢٥	٥٤٧٢.٦٩	١٠٠	٣٥٠٠٠
Neutrophils %	٣٥.٢٢	٢٧.١١	٠.٠	٩٠.٠٠
Eosinophils %	١٧.٨١	١٧.٠٩	٠.٠	٦٠.٠٠
Lymphocytes %	٢٦.٤٨	٢١.٦٣	٠.٠	٩٠.٠٠

TCC= Total cell count

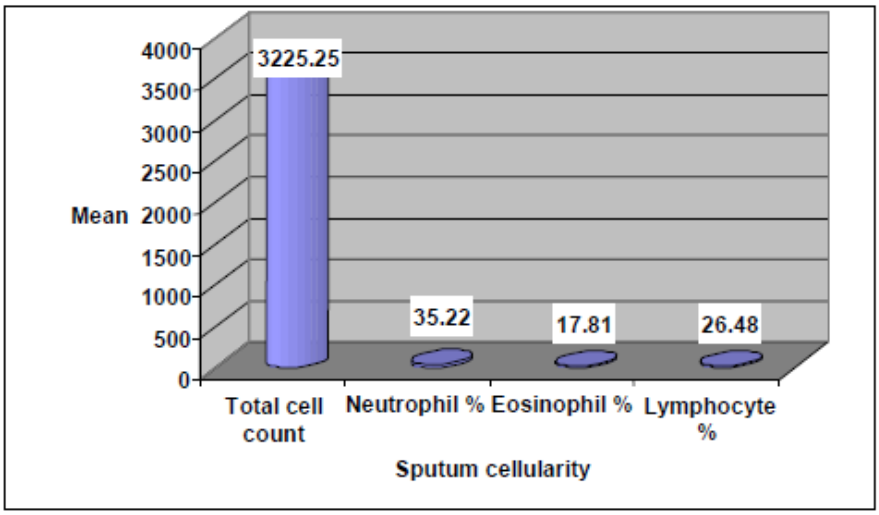

Figure ١: Types of sputum cellularity

Figure ٢: shows different inflammatory subtypes in asthmatic patient which show mean of each type. Mean of neutrophilic % is higher than mean of eosinophilic %.

Table III: Clinical characteristics of different inflammatory subtypes

	Eosinophilic (٥١)	Neutrophilic (٨)	Mixed (٦)	Paucigranulocytic (٥)	P-value
Age	٣٢.٠١±١١.١٧٣	٣٥.٧٥±٩.٣٩	٢٧.٢٢±٦.٩٤	٣٤.٠٦±١١.٩١	٠.٤
FEV١	١.٩١±٠.٥١	١.٧٦±٠.٣٩	١.٨٣±٠.٤٨	١.٧١±٠.٦١	٠.٥
FEV١٪	٦٠.٦٩±١٢.٩٨	٥٦.٥٠±٧.٨٩	٥٨.١١±٨.٣٢	٦١.٧٣±١٢.٤١	٠.٨
FVC	٣.٣١±٠.٧٦	٢.٨٧±٠.٥٢	٣.٢٧±٠.٩١	٣.٠٧±٠.٧١	٠.٥
FVC%	٨٥.٧٥±١٠.١٩	٧٦.٥٠±٤.١٢	٨٥.٤٤±٧.١٩	٩٠.٠٦±١٢.٣٦	٠.١
PEF	٢.٦٤±١.٠٣	٣.١٥±١.١٢	٢.٤٦±٠.٥٨	٢.١٥±١.٠٥	٠.٢
PEF%	٣٤.٩٤±١١.٠٧	٤٠.٥٠±٩.٢٥	٣٣.٥٥±٩.٦٥	٣٢.٦٠±١١.٤٦	٠.٦

Data presented as mean and standard deviation.

Table IV: Correlation between pulmonary function and eosinophilic & neutrophilic inflammation.

parameters	Neutrophils %		Eosinophils %	
	r	P	r	P
FVC pre	-٠.١٢	٠.٢	٠.١٣	٠.٢
FVC post	-٠.٠٩	٠.٤	٠.١٦	٠.١
FVC % pre	- ٠.٢٢	٠.٠٤*	٠.٠١	٠.٨
FVC % post	-٠.١٣	٠.٢	٠.٠٢	٠.٨
FEV١ pre	-٠.١٤	٠.٢	٠.٠٨	٠.٥-
Fev١ Post	-٠.٠٩	٠.٤	٠.١٨	٠.١
FEV١ % change	-٠.١١	٠.٣	٠.١٤	٠.١
PEF	٠.١٨	٠.١	-٠.١٥	٠.١
FEV١/FVC pre	٠.١٥	٠.١	-٠.٠٧	٠.٥
FEV١/FVC post	٠.٠٥	٠.٦	٠.٠٤	٠.٧

*P-value <٠.٠٥ is significant.

Table V: Comparison between eosinophils % in asthmatic patients receiving ICS and asthmatic patients not receiving treatment.

Treatment	Eosinophils %	P-value
Inhaled steroid (N=٣٦)	١٨.٨±١٤.٦	٠.٠٠٠١*
No treatment (N=١٦)	٣٧.١±١١.٢	

(*P-value <٠.٠٥ is significant).

Table VI: Inflammatory subtypes versus Asthma control

Sputum cellularity	Asthma control						Total	
	Controlled		Partially controlled		Uncontrolled			
	N.	%	N.	%	N.	%	N.	%
Eosinophilic-neutrophilic	١	١٧	٠	٠.٠	٥	٨٣	٦	١٠٠
Non eosino-non neutrophilic	٣	٦٠	٠	٠.٠	٢	٤٠	٥	١٠٠
Eosinophilic	١٠	١٩.٦	٢٢	٤٣.١	١٩	٣٧.٣	٥١	١٠٠
Neutrophilic	٢	٢٥	٠	٠.٠	٦	٧٥	٨	١٠٠
Total	١٦	٢٢.٨	٢٢	٣١.٢	٣٢	٤٦	٧٠	١٠٠

Fisher's exact= ١١.٦ P-value = ٠.٠٦

Discussion

Asthma is a chronic respiratory disorder characterized by variable airflow obstruction, and bronchial hyper responsiveness. Now it is well recognized that airway inflammation, and its resultant effects on airway structure, are important mechanisms underlying the pathophysiology of asthma. Moreover, these guidelines acknowledge that treatment of this airway inflammation results in better clinical outcomes. However, although airway inflammation is currently considered to be a characteristic feature of asthma, the majority of international asthma guidelines have not yet unequivocally endorsed the use of noninvasive measurements of airway inflammation in the diagnosis and management of the condition (Lougheed et al., ٢٠١٠).

Sputum cell count analysis is a noninvasive method of objectively assessing the presence and nature of airway inflammation. It has been shown to be a reproducible, valid and responsive clinical outcome measure (Djukanovic et al., ٢٠٠٢).

Regarding the role of induced sputum cell counts in asthma management, the British Thoracic Society Guideline stated, "In (adult) patients with difficult asthma, consider monitoring induced sputum eosino-phils counts to guide steroid treatment" (Lougheed et al., ٢٠١٢).

This study examined the use of sputum cell count in asthmatic patients under different forms of treatment and tried to find out possible correlations between asthmatic patients on treatment and others not treated with sputum inflammatory indices of asthmatic patients.

Our results showed that asthma is heterogeneous disease have various subtypes of airway inflammation and this is in agreement with previous studies that identified different inflammatory phenotypes in subjects with asthma. Lisa et al., (٢٠١٢) reported that Subjects with asthma were classified as having non-neutrophilic asthma or neutrophilic asthma. The asthma (neutrophilic) group had increased systemic inflammation compared with the asthma (non neutronphilic) and healthy control groups.

Green et al., (٢٠٠٢) also demonstrated heterogeneity in induced sputum cell counts of patients with mild to moderate asthma who have predominantly neutrophilic airway inflamemation and who respond less well to treatment with inhaled corticosteroids. Simpson et al., (٢٠٠٦) showed that asthmatic patients had ٤ subtypes of airway inflammation; neutrophilic, eosinophilic, mixed type and paucigranulocytic (non eosinophilic non neutrophilic).

One group of our studied patients had sputum eosinophils (٦٣.٨٪) that, in other studies, has been correlated with responsiveness to inhaled corticosteroids therapy, severity of airflow obstruction and airway hyper responsiveness (Green et al., ٢٠٠٢). Eosinophilic inflammation in the airway mucosa that persists despite the use of high doses of inhaled corticosteroids or oral corticosteroids has been observed in several studies (Benayoun et al., ٢٠٠٣ and Miranda et al., ٢٠٠٤) and has been implicated by Wenzel

and colleagues., (١٩٩٩) as a feature of a separate asthma phenol-type, associated with poor asthma prognosis (Miranda et al., ٢٠٠٤).

Thus, modifying eosinophilic inflammation may be a potential therapeutic target in COPD (Saha and Brightling, ٢٠٠٦). Infiltration of eosinophils to the lung is one of the hallmark characteristics of allergic asthma in humans and animal models alike; however, the function of these cells in asthma has been a mystery. Traditionally, researchers have thought that eosinophils were recruited to the lung by T cells as end stage effector cells. High levels of IL-٥ are found in the blood and bronchoalveolar lavage fluid (BALF) of patients with eosinophilic esophagitis and allergic asthma (Hogan, ٢٠٠٨).

We identified other subgroup of asthmatic patients (١٠٪) that had neutrophilic inflamemation. Subjects with neutrophilic inflammation had more severe airway obstruction and uncontrolled asthma. These results are in agreement with results reported by Shaw and colleagues., (٢٠٠٧) who stated that airway neutrophilia is a characteristic of more severe asthma and suggest a possible mechanistic link between airway neutrophils and chronic airway narrowing in asthma. Li et al., (٢٠٠٦) reported that a significant proportion of asthma and wheezing illness in both adults and children is associated with neutrophilic airway inflamemation and that this pattern is not limited to individuals with severe symptoms. This raises important and interesting questions regarding the mechanisms and consequences of neutronphilic inflammation, as well as presenting a novel and inviting therapeutic target.

In our study, all asthmatic patients underwent spirometry with fulfilled reversibility criteria post-bronchodilator, weak positive correlation between eosinophils and (FEV١ and FVC) which was not significant, also negative correlation with PEF which was not significant. These results disagree with results reported by Gorska and colleague., (٢٠٠٨) who showed a negative correlation between the FEV١ increase in the bronchial obstruction reversibility test and the sputum eosinophils count ($r=-٠.٥$, $P<٠.٠٥$), and also disagree with results reported by Elbehidy and colleague., (٢٠١٠) who found significant negative correlation between changes in FEV١ and change in Eosinophils.

Sputum induction is noninvasive method useful for monitoring asthma and COPD management (Chaudhuri et al., ٢٠٠٦). Our study showed that asthmatic patients (٦٨.٢٪) who did not receive treatment have increase eosinophils percent. Normal sputum cell count reported by Belda and colleague., (٢٠٠٠) stated that macrophages account for about ٦٠-٧٠٪ of all cells and represent the predominant sputum cell type in healthy individuals. Neutrophils are the second most common cells (٣٠-٤٠٪ of all cells), whereas the relative eosinophils count does not exceed ٢٪. These results in agreement with result reported by Godon and colleague, (٢٠٠٢) who revealed that more than ٥٠٪ of asthmatic patients receiving no anti-inflammatory treatment have an increased induced sputum eosinophils count.

Asthmatic patients, in our study, who received ICS showed reduction in eosinophilic count when compared with patient who did not receive treatment which is significant. This is in agreement with Bacci et al., (٢٠٠٦). Also Elbehidy and colleague., (٢٠١٠) showed that sputum eosinophils is the best predictor of steroid response in asthmatics and support the use of sputum cell counts to guide steroid treatment.

Godon et al., (٢٠٠٢) suggested that sputum eosinophils levels predict the response to treatment with inhaled corticosteroids, while, Bacci et al., (٢٠٠٦) reported that patients with low sputum eosinophils showed no significant improvement in most clinical and functional outcomes, whereas others found no difference in the response to inhaled corticosteroid treatment between patients with high and patients with low sputum eosinophils (Meijer et al., ٢٠٠٢).

In our study, we assessed patients with asthma control which ٢٠٪ were controlled, ٣١.٢٪ partially controlled and ٤٩.٨٪ uncontrolled. Patients who were uncontrolled showed inflammatory subtypes of eosinophilic bronchitis. Eosinophilic inflammation in the airway mucosa that persists despite the use of high doses of inhaled corticosteroids or oral corticosteroids has been observed in several studies (Chakir et al., ٢٠٠٢, Benayoun et al., ٢٠٠٣ and Miranda et al., ٢٠٠٤).

In our study, patients with neutrophilic bronchitis were eight, two of them were controlled and six of them were uncontrolled, however, this difference was not significant. This result was in agreement with results reported by Simpson and colleague., (٢٠٠٦) who showed that in adults with stable asthma treated with inhaled corticosteroids~ ٤٠٪ of them have eosinophilic asthma, whereas ٢٥٪ have neutrophilic asthma. In the most severe forms of asthma, sputum neutronphils levels are elevated (Ordoñez et al., ٢٠٠٠) and negatively correlate with lung function and airflow obstruction (Simpson et al., ٢٠٠٦). Lisa and colleague., (٢٠١٢) have observed that systemic inflammation is increased in neutrophilic asthma, in addition, plasma IL-٦ may be more clinically relevant than plasma CRP and may provide a useful therapeutic target in neutrophilic asthma as systemic inflammation was increased in the asthma (neutrophilic) group.

Thus, the observational study of induced sputum in asthmatic patients receiving different forms of treatment provided us with the opportunity to compare sputum cell indices in asthmatic patients under different forms of treatment and others who are not treated which revealed that patients without treatment were not controlled and patients on treatment vary in the degree of control according to proper treatment, also, showed different inflammatory subtypes of asthmatic patients. Induced sputum examination for inflammatory markers holds much promise in the evaluation of the anti-inflammatory properties of asthma medications and identifying the appropriate medication to control symptoms. They may be useful to compare the relative potencies of different anti-inflammatory drugs.

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