ORIGINAL ARTICLE

Utility of the Sputum Cytology Applying MGG and Pap stains in Monitoring Sudanese Patients Complaining of Bronchial Asthma

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ABSTRACT

Objectives: This is a case control study aimed at detecting cytological changes in diagnosed asthmatic patients, using sputum samples collected and stained by cytological methods.

Materials and methods: The study was carried out on 80 individuals, of whom 30 were normal as controls, and 50 were asthmatic patients. Along with clinical data cytological, patterns in asthmatic patients were mapped using two stains, Papaniclauo (PAP) and May-Grunwald Giemsa (MGG).

Results: Inflammatory response changes were reported in 18 cases (36%) compared with 4 controls (13%). Inflammatory responses with metaplasia and metaplasia changes without inflammatory response were seen within these 18 cases, with asthmatic patients with 27 cases (54%) and 2 cases (4%) respectively, ($p < 0.01$). Inflammatory response, both with and without metaplasia, was reported in 32 (64%;$p < 0.05$) asthmatic patients suffering from environmental allergens with respiratory co-infections. Inflammation with metaplasia was also found to be significantly higher ($p < 0.05$) in females. The PAP stain gave excellent and clear results for screening of sputum.

Conclusion: Sputum cytology is a valuable tool. The PAP stain is suggested to be used as a cytological stain over the MGG stain. Further research is needed to confirm our findings and then to determine whether the assessment of cytological patterns in sputum of asthmatic patients is useful or not for the routine monitoring of asthmatic patients.

Keywords: Metaplasia, Allergy, inflammatory airways, Eosinophilic asthma, environmental allergens.

INTRODUCTION

Asthma is a chronic disease of the respiratory system in which the airway constricts, becomes inflamed and lined with excessive amount of mucus, often in response to one or more triggers such as exposure to allergens, exercise, emotional stress and (commonly in children) viral illness [1]. Globally, more than 300 million patients have asthma with approximately 250,000 asthma-associated deaths [1-3]. The immunopathologic features include denudation of airway epithelium, collagen deposition beneath basement membrane, edema, mast cell activation and inflammatory cells infiltration neutrophils (in sudden onset, fatal asthma exacerbations), eosinophils and lymphocytes [4]. Asthma can be diagnosed based on patient's history and examination. Other methods used are peak flow meter which tests airway restriction, lung function tests, radiological tests such as X-ray or computerized tomography (CT) scan which are required to exclude the possibility of other lung diseases, and blood tests including eosinophil count [5].

Changes in sputum eosinophil count predicts loss of asthma control, suggesting firstly that sputum is a potentially useful marker in predicting loss of asthma control reflected by loss of airway function and secondly that sputum eosinophils seem to be valid markers for detecting asthma in populations of patients with airway [6] . Moreover, the higher diagnostic accuracy of eosinophils in sputum compared to sputum eosinophils cationic protein (ECP) reinforces the role of cytological analysis in diagnosis of chronic stable bronchial asthma [7] . A Japanese study suggested that the participation of damage to the respiratory epithelium in the development of airway hyper-responsiveness in bronchial asthma, and the detection of desquamated respiratory epithelium (creola bodies) in sputum is straightforward for estimation of airway hyper-responsiveness [8]. Sputum sampling is a less invasive method, and most previous studies used sputum to evaluate certain treatment or treatment strategy using sputum eosinophils count only without investigating the cytological changes. To our knowledge there are no previous studies reporting cytological patterns with regard to the cytological assessment of asthma [9].

SUBJECTS AND METHODS

This is a cross-sectional, case-control study which was carried out at Al-shaab chest hospital and Bahri teaching hospital, Khartoum, Sudan. Fifty asthmatic patients and thirty healthy individuals were matched and used as controls were enrolled in this study: a total of eighty participants. Age ranged from 15 to 71 years (mean 37 years). Each case and control were provided with sputum container and asked to collect early morning sputum before food is eaten or toothpaste used to eliminate contamination. Sputum was collected from asthmatic

patients from all age groups, both genders, all being nonsmokers.

The Institutional Ethical Committee of Ministry of Health approved the study and written consent was obtained from all the participants involved. All the clinical data and history of triggers factors were taken from the patient records

Sample preparation and staining: The clinical specimen was decanted into a Petri dish in which the sputum can be teased out using disposable sticks so that bloodstained, discolored and purulent areas can be selected and then placed on two glass slides. The material was spread evenly over the slides. One smear was fixed in 95% alcohol for 30 min to be stained in PAP stain and the other allowed to air dry followed by fixation in methanol for 10 min.

For PAP staining, the smear was hydrated in 70% alcohol for 2 mins and rinsed in water for 1 min. After that it was stained in Harris' hematoxylin for 5 mins, rinsed in water for 1 min, differentiated in 0.5% aqueous hydrochloric acid for approximately 10 seconds and again rinsed in water for 2 mins. Bluing was the second step, done in Scott's tap water substitute for 2 mins and then rinsed in water. Next, the smear was dehydrated in 70% and two changes of 95% alcohol respectively for 2 mins each. After dehydration it was stained in Orange G 6 dye (OG6) for 2 mins and rinsed in two changes of 95% alcohol for 2 mins each. Finally, it was stained in Eosin Azure 50 (EA50) for 3 mins and rinsed in 95% alcohol for 1 min.

For the MGG staining, the fixed smear was stained in the diluted May-Grunwald solution for 10 mins and then rinsed in pH 6.8 buffer for 10 mins, then rinsed in the diluted Giemsa solution for 30 mins, washed and differentiated in pH 6.8 buffer for 5-20 mins. Finally, the smear was allowed to dry and mounted in DPX [0].

The sputum smears were examined by the investigators, and then by two independent expert cytopathologists to verify the results. The staining results were evaluated, and statistical analyses were carried out using SPSS software.

RESULTS

In this study cytological patterns were categorized into three microscopic findings: inflammatory response smear, inflammatory response with metaplasia changes and metaplasia changes only. When comparing our findings within sputum form the asthmatic patients and matched controls, the inflammatory response profile was reported in 18 cases (36%) compared with 4 controls (13%), while inflammatory response with metaplasia and metaplasia changes only profiles were observed in 27 (54%) asthmatic patients and 2 (4%) matched controls respectively, ($p<0.01$). Within the 18 inflammatory response profile case group smears were mostly eosinophilic in cellularity with considerable smears of eosinophilic / neutrophilic infiltrates and few smears contained a mixed acute and chronic inflammatory cell compared with only neutrophilic inflammatory smear in control group. See Table 1 and Figure 1

Interestingly, the presence of inflammation/metaplasia was found to be significantly higher ($p< 0.05$) in females compared to males. Metaplasia was found with low frequency at 2 (4%) in female asthmatic cases and absent in male patients, although this was not a significant difference (Table 2). 6% of asthmatic cases had no cytological changes

Inflammation and inflammation/metaplasia profiles were significantly more common in 15–45-yearolds (28% and 34% respectively) than in those over 45 (8% and 20% respectively, $p<0.05$). This suggests that the age may play a protective role in reducing asthma in older people. Furthermore, the metaplasia profile was found in 4% of the patients aged 15–45 as opposed to 0% of the over-45 patients. No cytological changes were observed in the older age group with 6% of the 15–45 aged asthmatic patients also having no cytological changes.

Stratifying the cytological changes by occupation shows that this way the housewife group has the highest incidence at (42%), with students and workers (at ~22% and 14% respectively) are slightly more likely to be diagnosed with asthma than doctors, nurses and merchants (Table 3). There is an increased incidence of the inflammation and inflammatory metaplasia profiles in the groups (26% and 14% housewife, 8% and 12% students and 10% and 4% for workers respectively) Other occupations show low but varying incidence of inflammation and inflammatory metaplasia.

With regards to trigger factors reported by the asthmatic patients, they were categorized into three types: environmental allergens (smoke, pollen, dust, animals, bird remnants and cold) with respiratory system co-infections, environmental with physiological stress, and environmental with food allergens. Inflammatory response profiles, both with and without metaplasia, were reported within 32 patients (64%) and were significantly higher ($p<0.05$) in asthmatic patients suffering from irritation due to the environmental allergens with respiratory co-infections (Table 4). With 40% and 20% showing inflammatory/metaplasia and metaplasia alone profiles respectively versus 10% and 12% in those patients in the environmental with physiological stress group. No cytological change was found in just 6% of all asthmatic patients, with them showing no distinct cytological profile.

The treatment of asthmatic patients can be by inhalation, tablets, injection, or a combination of therapeutic agents. Within this study population, the inhalation route was reported to be the most frequently used by 35 patients (70%), of whom the patients demonstrating inflammatory response with metaplasia, inflammatory response only, and metaplasia changes without inflammation were at 34%, 28% and 2% respectively. The combination treatment with inhalation and tablets was used by 14% asthmatic patients followed by 8% using injections (Table 5).

A high number of cases had a family history of asthma at 29 (58%), while 21 (42%) have no family history. Cases with family history demonstrated a higher significant ($P<0.05$) inflammatory response and inflammatory response with metaplasia. Metaplasia appeared on those who have or have no family history, (see **Table 2)**.

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DISCUSSION

Advances in Knowledge: Most previous studies have used sputum to evaluate asthma status or treatment strategy via eosinophil count without reporting full a cytological profile. To our knowledge there are no previous studies reporting full cytological patterns as reported here

Application to Patients Care: Sputum cytology using Pap and MGG stains are a valuable tool in monitoring Sudanese patients complaining of bronchial asthma. Moreover, the sputum cytology can be introduced as a follow up protocol in asthmatic patient's management and treatment.

Comparing cases and controls, the inflammatory response changes reported mostly in cases, while inflammatory response with metaplasia and metaplasia changes was reported only in the cases. Our findings are supported by Revez, et al. who reported the dramatic increase in sputum inflammatory cells subtype in asthma after exposure to the allergen [11].Another study detected the squamous cell metaplasia changes among asthmatic smokers' group more than non-smokers. Moreover, an interventional study aimed at control and treatment of severe refractory asthma in patients using bronchial thermoplasty showed that the presence of squamous metaplastic epithelium with other histopathologic parameters was significantly improved and its percentage reduced after this type of control [12].

In this study, the younger patient group demonstrated the higher proportion of inflammatory changes (28%, 14 patients), inflammatory with metaplasia (34%, 17) and metaplastic changes (4%, 2) compared with the >45 age group. These findings are in accordance with a study which found severe asthma patients aged ≥65 years had improved symptom control, better asthma quality of life and, in the last year, fewer emergency visits and rescue oral steroid courses [3 (1–6) versus 5 (2–7), $p < 0.001$] than severe asthmatics aged <65 years ref?. Blood eosinophils were also lower in the elderly group. Patients with severe adult-onset asthma had similar symptom control, lung function and health-care utilization compared to severe childhood-onset asthma. Adult-onset asthmatics had higher blood eosinophils and were less atopic [13].Furthermore, there are other studies which report severe outcomes and sputum cytology inflammatory response; which is mostly with eosinophilic and neutrophilic cellular predominates were found in younger patients (< 21 years) and the childhood asthmatic patients group, without comparing their findings with older age group [14-16].

Considering that inflammatory response can be attributed to many factors other than asthma, cases belonging to the age groups between 15 and 45 have more contact with the external environment making them more susceptible to developing recurrent infections; this may lead to metaplasia. Family history is a major predisposing factor to asthma assuming that the same factors may increase the propensity of an asthmatic patient to experience an attack, those concluding that for over 300 million asthmatics worldwide and roughly 250,000 asthma-associated deaths, the family history is one of the major risk factors with other types of allergies, such as allergic rhinitis or eczema [1,3,5].

In regard to the trigger factors and allergens; our findings showed the inflammatory response only and inflammatory response with metaplasia demonstrated mostly in patients suffering from irritation due to environmental factors with respiratory co-infections. This finding in accordance with the review study of Wilmore and David who's concluded that infections as mediated risk factor for induction and exacerbation of asthma [17].Moreover, this finding is reinforced by studies reporting sensitization to staphylococcal enterotoxins associated with an increased subsequent risk of severe asthma and asthma exacerbations [18].Interestingly, regarding use of therapeutics and treatment route in the asthmatic population, our study found patients use inhalation treatment most frequently, and show higher cytological findings as inflammatory response with metaplasia, inflammatory response only, and metaplasia changes. To our knowledge, there is no previous study reporting any cytological changes and the type of therapy used .Finally, what the study showed about the better quality of PAP stain and its more preferable in sputum cytology is reinforced by other studies. These concluded that PAP stain is excellent for the nuclear details. MGG stain showed less air-drying artifacts compared to wet fixed smears and has better optimal cytoplasmic feature [19-21]. However, Arul, *et al.*stated; no differences between the stains used in detecting micronucleus in the exfoliated buccal epithelial Cells [22].

Limitations and Recommendations: This work can act as a pilot study for further research to confirm our findings and determine whether the demonstration of different characteristic cytological patterns in sputum of asthmatic patients is useful or not for the diagnosis of asthma in the first place.

CONCLUSION

Sputum cytology is a valued tool for monitoring, follow-up and diagnosis of asthma. The PAP stain gives excellent and clear results for screening of sputum and we recommend that it be used as a cytological stain over the MGG stain. We recommend researchers be aware of all hazards accompanied by sample collection and preparation; they must follow all safety precaution.

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FIGURE LEGENDS:

Figure 1. Cytological changes in patients with asthma

(A)Pap stain from sputum of asthmatic cases showing eosinophilic type inflammation (X400) , (B) MGG Stain of sputum from asthmatic cases showing mixed inflammatory cells with squamous cells metaplasia (X400), (C) MGG stain from sputum cytological preparation showing squamous cells metaplasia (X400), (D) Pap stain sputum cytological preparation showing metaplastic squamous cells with numerous Inflammatory cells (X400), (E) MGG stain of sputum cytological prep showing mixed acute and chronic inflammatory cells from chronic attack asthma patient (X400).

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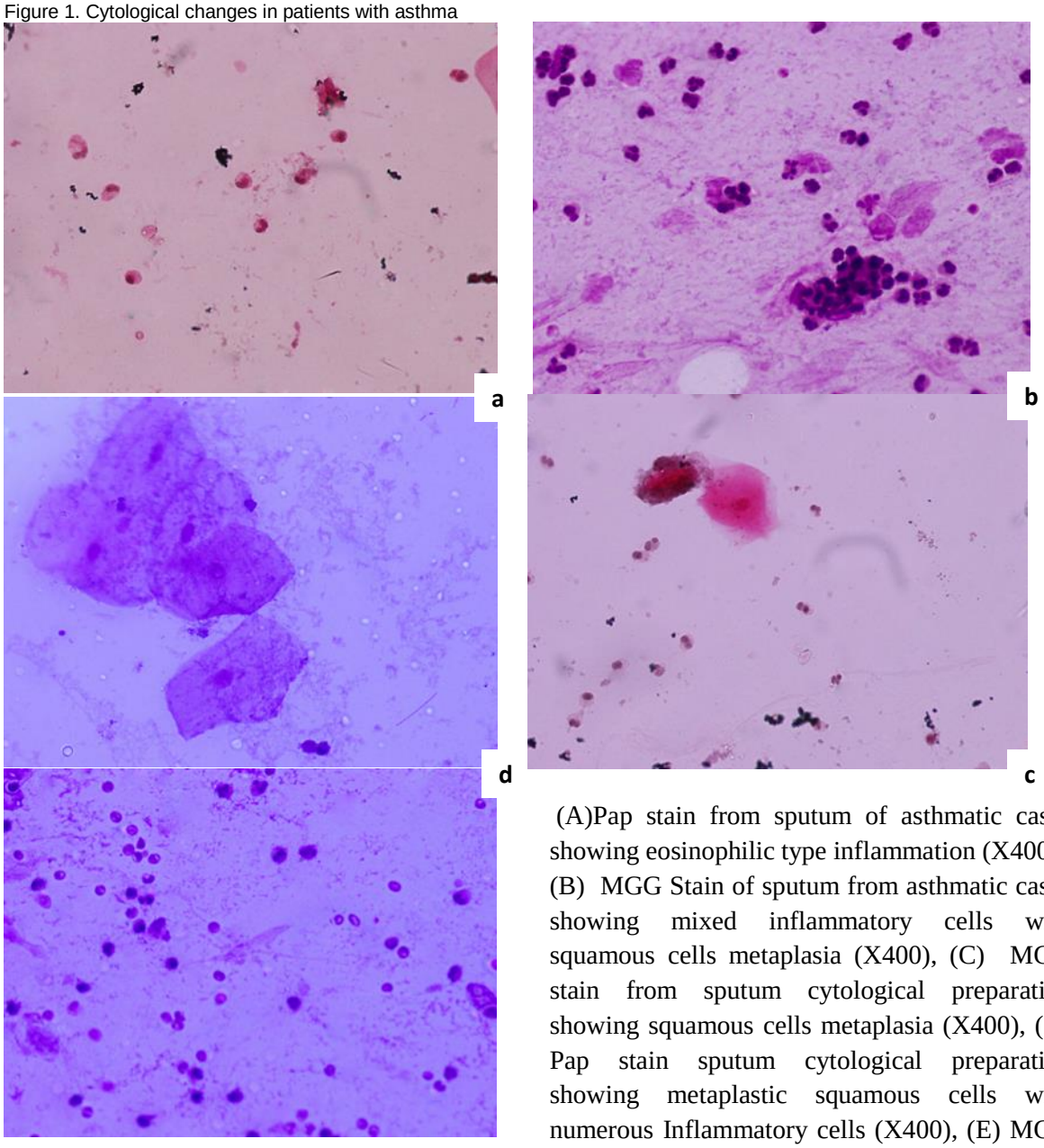

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Table 1. Comparing of the Cytological patterns between cases and control group

Study group	No Cytological changes N (%)	Cytological patterns			Total N (%)
		Inflammatory /metaplasia N (%)	Inflammatory N (%)	Metaplasia N (%)	
Control	26 (86.6) [(a)]	0(0)	4 (13)	0(0)	30(100)
Cases	3 (6)	27(54%) [(a)]	18 (36%)	2 (4%)	50(100)
Total	29	27	22	2	80

Key: Significance determined by comparison between groups. (a) = P<0.05.*Statistical significance shown in parenthesis.

Table 2. Cytological patterns among asthmatic patients stratified by gender, age and family history

Gender	No cytological Change No (%)	All changes through gender			Total No (%)
		Inflammatory/metaplasia No (%)	Inflammatory No (%)	Metaplasia No (%)	
Male	2 (4)	10 (20)	10 (20)	0 (0)	22 (44)
Female	1 (2) [(c)]	17 (34) [(a)]	8 (16)	2 (4)	28 (56)[(a)]
Total	3 (6)	27 (54)	18 (36)	2 (4)	50 (100)

Changes	Age Group		Total
	15 – 45 Yrs N (%)	>45 Yrs N (%)	N (%)
Inflammatory	14 (28) [(a)]	4 (8.0)	18 (36)
Inflammatory/metaplasia	17 (34) [(a)]	10 (20.0)	27 (54) [(a)]
Metaplasia	2 (4)	0 (0.0)	2 (4)
No change	3 (6)	0 (0.0)	3 (6)
Total	36 (72)	14 (28.0)	50 (100)

Family history	No Cytological changes N (%)	Cytological patterns with family history cases			Total N (%)
		Inflammatory /metaplasia N (%)	Inflammatory N (%)	Metaplasia N (%)	
Present	1(2%)	15(30%)	12(24%)	1(2%)	29(58%)
Absent	2(4%)	12(24%)	6(12%)	1(2%)	21(42%)
Total	3(6%)	27	18	2	50

Key: Significance determined by comparison between groups. (a) = P<0.05.

Table 3. Relationship between cytological findings and patients' occupation

Occupation		Cytological changes			Total No (%)
	No change No (%)	Inflammatory/metaplasia No (%)	Inflammatory No (%)	Metaplasia No (%)	
Housewife	0 (.0)	13 (26)[(a)]	7 (14)[(a)]	1 (2)	21 (42)
Student	1 (2)	4 (8.0)[(a)]	6 (12)[(a)]	0 (.0)	11 (22)
Doctor	1 (2)	1 (2)	1 (2)	0 (.0)	3 (6)
Nurse	1 (2)	2 (4)	0 (.0)	1 (2)	4 (8)
Worker	0 (.0)	5 (10)[(a)]	2 (4)	0 (.0)	7 (14)
Merchant	0 (.0)	2 (4)	2 (4)	0 (.0)	4 (8)
Total	3 (6)	27 (54)	18 (36)	2 (4.0)	50 (100)

Key: Significance determined by comparison between groups. (a) = P<0.05.

Table 4. Relationship between trigger factors and cytological findings

Triggers	No cytological change N (%)	Cytological changes			Total N (%)
		Inflammatory/metaplasia N (%)	Inflammatory N (%)	Metaplasia N (%)	
Environmental/infections	1 (2.0)	20 (40.0) [(a)]	10 (20.0)[(a)]	1 (2.0)	32 (64.0)
Environmental / physiological stress	1 (2.0)	5 (10.0)[(a)]	6 (12.0)[(a)]	0 (0.0)	12 (24.0)
Environmental/ food type allergen	1 (2.0)	2 (4.0)	2 (4.0)	1 (2.0)	6 (12.0)
Total	3 (6.0)	27 (54.0)	18 (36.0)	2 (4.0)	50 (100)

Key: Significance determined by comparison between groups. (a) = P<0.05. *Statistical significance shown in parenthesis.

Table 5: Relationship between type of treatment route and cytological findings

Treatment	No cytological changes(n%)	Cytological changes			Total (n%)
		Inflammatory/metaplasia(n%)	Inflammatory (n%)	Metaplasia(n%)	
Inhalation	3 (6)	17 (34)[(a)]	14 (28)[(a)]	1 (2)	35 (70)
Tablets	0 (0)	1 (2)	1 (2)	0 (0)	2 (4)
Injection	0 (0)	3 (6)	0(0 .0)	1. (2)	4 (8)
Inhalation/ Tablets	0 (0)	5 (10)	2 (4)	0 (0)	7 (14)
Inhalation/Injection	0 (0)	0 (0)	1 (2)	0 (0)	1 (2)
Inhalation/ Tablets/ Injection	0 (0)	1 (2)	0 (0)	0 (0)	1 (2)
Total	3 (6)	27 (54)	18 (36)	2 (4)	50 (100)

Key: Significance determined by comparison between groups. (a) = P<0.05.*Statistical significance shown in parenthesis.