The Use of Hypertonic Saline Challenge and Sputum Induction as an Assessment Tool in Clinical Studies in Paediatric Asthma

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CONTENTS

Abstract

Asthma is characterized by variable airway obstruction, airway hyper-responsiveness and influx of inflammatory cells such as eosinophils into the bronchial mucosa. Recent studies have highlighted the relationship between airway inflammation, airway hyper-responsiveness and the management of childhood asthma. Examination of cellularity in the airways by means of broncho-alveolar lavage or bronchial biopsy is the direct method in assessing airway inflammation. However, their use in paediatric clinical practice is limited because of their invasive nature. Hypertonic saline challenge and sputum induction (HSCSI) has established itself as an easy and reliable method that is able to assess airway inflammation and airway hyper-responsiveness during the same procedure. Examination of the cellular components in sputum could indirectly reflect the inflammatory status of the underlying airways.

In this thesis, studies utilizing HSCSI were carried out to (1) evaluate the factors that predict successful sputum induction in children, (2) evaluate the effectiveness of once-daily fluticasone propionate in children with stable asthma by studying the control of airway inflammation and (3) examine the relationship between cough frequency and airway inflammation in children with stable asthma

In the study of successful sputum induction in children, 130 subjects were

recruited. The median age was 11.25 years (IR: 5) and majority were boys (75%). All except two had normal percent predicted FEV_1 (> 80%). Sputum induction was successful in 93 subjects (74.5%). Sore throat and chest discomfort occurred in 20 (15%) and 8 (6%) subjects respectively and the procedure was prematurely terminated in three cases. Exhaled nitric oxide (eNO) was found to be a significant predictor for successful induction. (Area under ROC curve - 0.634).

In the second study, we were able to show that once-daily fluticasone proprionate were as effective as the twice-daily regimen in controlling airway inflammation. There were, in fact, significant improvements in eNO and sputum eosinophil counts when the corticosteroids therapy was changed from twice-daily to once-daily. In addition, all subjects preferred the once-daily dosing regimen.

We used a validated cough monitor to assess cough frequency in children with mild asthma. They were found to have greater cough frequency compared to normal children - 25.5 (IQR 16 - 42.8) versus (11 episodes per day, $p<0.001$). Cough frequency was found to have a significant positive correlation with sputum neutrophil count (r=0.833, p=0.0001), suggesting that the increased cough may be driven by a neutrophilic pathway.

In conclusion, HSCSI is safe and well tolerated among children. It has its clinical value as a non-invasive assessment tool of airway inflammation.

論文摘要

哮喘患兒的發病症狀中以變異型呼吸道阻塞，氣道高反應性和氣道嗜酸性粒細胞性炎症最為常見。近年來，研究結果表明哮喘的病情控制與哮喘患者氣道的炎症程度和氣道高反應性有密切的關係。高滲鹽水激發試驗是一種可靠和容易測定氣道反應性的方法。

研究哮喘患者氣道炎症的直接方法是透過在支氣管鏡檢查時進行氣管肺泡灌洗及支氣管活組織檢查。但是，這些檢查基於其侵入性本質，在哮喘患兒的日常檢查中並不常用。高滲鹽水激發試驗和誘導痰檢查作為一種氣道炎症檢測技術，其無創傷性及良好反映性的特點，使它在哮喘患兒的氣道炎症檢測中日漸普及。高滲鹽水激發試驗和誘導痰檢查可以同時進行，用以測試氣道高反應性及氣道炎症程度。本篇論文旨在探討高滲鹽水激發試驗和誘導痰試驗在哮喘患兒中使用方法及其應用價值。另外，本文將(1)研究在哮喘患兒成功通過高滲鹽水激發試驗和誘導痰的因素；(2)以氣道炎症程度為指標研究哮喘患兒每天使用一次輔舒酮氣霧劑的可行性及(3)哮喘患兒的咳嗽頻率與其誘導痰中非侵入性指標的相關性。

在研究哮喘患兒成功通過高滲鹽水激發試驗和誘導痰的因素時，共有 130 個患兒參與(男:75%人; 女:25%)，年齡中位數為 11.25 歲 (IR: 5) 。所有患兒都有正常基礎 FEV_1 (> 80%) 。eNO 中位數為 48.95 ppb.。的研究結果顯示，誘導痰成功的患兒人數為 93 人(74.5%)。有喉嚨不適徵狀的有 20 人，有胸口不適徵狀

的有 8 人 (6%)，未能完成激發試驗的有 3 人。eNO 是哮喘患兒成功通過高滲鹽水激發試驗和誘導痰的因素(ROC 圖的面積為 0.634) 。

以氣道炎症程度為指標研究哮喘患兒每天使用一次輔舒酮氣霧劑的可行性的研究結果顯示，我們發現 eNO 和誘導痰中的氣道嗜酸性粒細胞在八星期研究中，都有統計上的重要性。所有患兒都喜歡選擇每天使用一次輔舒酮氣霧劑的方案。

哮喘患兒的咳嗽頻率與其誘導痰中非侵入性指標的相關性的研究中，中等程度的哮喘患兒之 FEV_1 中位數及 eNO 中位數分別為 83.3% (IQR 81.1–97.6) 及 56.1 ppb (IQR 37.4–105)。咳嗽頻率中位數為 25.5 (IQR 16–42.8) 與正常兒童之咳嗽頻率(11, $p<0.001$)有統計上重要的分別。有 69%患兒成功誘導痰液，而咳嗽頻率與誘導痰液中嗜中性白血球數目有統計上重要的正關連性 (r=0.833, p=0.0001).

總而言之，高滲鹽水激發試驗和誘導痰檢查是一種安全而受歡迎的氣道炎症檢測技術。

Statement of Originality

This study was carried in the Department of Pediatrics, Princes of Wales Hospital, Chinese University of Hong Kong. The study was designed by Dr Albert Martin Li. The clinical diagnosis of patients was carried out by Dr Albert Martin Li and Dr Dorothy Chan. The skin prick test, spirometry, cough monitoring, hypertonic saline challenge and sputum induction, sputum processing, cytospin staining and the final differential cell counts were performed in Ward 6C, Ward 7A and 7/F Ward AB treatment room by myself in Prince of Wales Hospital. The cough episodes analysis was carried out by me and the final data analysis was performed with assistance of Dr Albert Martin Li and Dr So Hung Kwan.

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Abbreviation

AI	Airway inflammation
AHR	Airway hyper-responsiveness
BAL	Bronchoalveolar Lavage
BTS	British Thoracic Society
DTT	Dithiothreitol
ECP	Eosinophilic cationic protein
eNO	Exhaled Nitric Oxide
FEF_{25-75}	Forced expiratory flow at 25 to 75%
FEV_1	Forced expiratory volume in one second
FVC	Forced vital capacity
GINA	Global Initiative for Asthma
IS	Sputum induction
HSCSI	Hypertonic saline challenge and sputum induction
PBS	Phosphate Buffered Saline
PEFR	Peak flow expiratory rate
NOS	Nitric oxide synthase
SKT	Skin Prick Test

Chapter 1 Objectives of studies

The primary objective of the study was to implement the technique of hypertonic saline challenge with sputum induction (HSCSI) for use in paediatric patients at Prince of Wales Hospital. We then examined the safety and success rate of HSCSI in Hong Kong Chinese children with asthma.

The technique was then used to (1) study the relationship between airway inflammation and cough frequency in children with mild asthma, (2) assess whether the use of inhaled corticosteroids on an once-daily basis was as effective as the traditional well accepted twice-daily regimen, using airway inflammation as our outcome measure. In each study, we used the technique of HSCSI to obtain sputum samples for the assessment of differential cell counts which served as a marker of airway inflammation. We also attempted to find out predictive factors for successful sputum induction in children. .

Chapter 2 Literature Reviews

2.1 Definition of asthma

The term "Asthma" is derived from the Greek word meaning panting. It has been known and described for more than 2,000 years. It is the commonest chronic childhood disease in many areas in the world, including Hong Kong. (Kaur B et al., 1998) Since many different mechanisms and causal pathways are involved in the development of asthma, an exact definition of asthma is seldom defined. However, investigators have devised several working definitions of asthma for epidemiological and research purposes.

Clinical Definition

Asthma is an inflammatory disease of the airways that is associated with abnormal response mechanisms of the airway smooth muscles that lead to episodes of airway narrowing. (Woolcock AJ, 1994)

Pathological Definition

Asthma is a disease of the airways characterized by chronic inflammation with infiltration of lymphocytes, eosinophils and mast cells together with epithelial desquamation, thickening, and disorganization of the tissues of airway wall.

American Thoracic Society Definition

Asthma is a clinical syndrome characterized by increased responsiveness of the tracheo-bronchial tree to a variety of stimuli. The major symptoms of asthma are paroxysms of dyspnoea, wheezing and cough, which may vary from almost undetectable to mild to severe and unremitting. The primary physiological manifestation of this hyper-responsiveness is variable airways obstruction. This can take the form of spontaneous fluctuations in the severity of obstruction following bronchodilators or corticosteroids, or increased obstruction caused by drugs or other stimuli. (American Thoracic Society, 1987)

In the most recent United State asthma guideline, the Expert Panel 2 Report defines asthma as:

"A chronic inflammatory disorder of the airways in which many cells and cellular elements play a role, in particular, mast cells, eosinophils, T lymphocytes, neutrophils, and epithelial cells. In susceptible individuals, this inflammation causes recurrent episodes of wheezing, breathlessness, chest tightness, and cough, particularly at night and in the early morning. These episodes are usually associated with widespread but variable airflow obstruction that is often reversible either spontaneously or with treatment. This inflammation also causes an associated increase in the existing bronchial hyper-responsiveness to a variety of stimuli." (National Asthma Education and Prevention Program Expert Panel Report 2, 1997)

The definition of asthma highlights the importance of exacerbations as a feature of asthma, and emphasizes the fluctuations of the disease. Normally, asthma episodes are referred to as "acute attacks" if they are short-lived and easily reversed with reliever medications. However, if the acute symptom episode lasts longer than one day, the attack is referred to as an "asthma exacerbation".

Definitions for asthma have limitations since they can reflect only current understanding of the diseases, which is quite limited. In addition, clinical symptoms of asthma overlap with other respiratory diseases making a clear distinction difficult. For children, similar symptoms may present with bronchiolitis and wheezy bronchitis, for elderly, chronic bronchitis and emphysema. Asthma will continue to be redefined as our understanding of the condition deepens, and as new effective preventive strategies and treatments become available.

2.2 Diagnosis and classification of asthma in children

The diagnosis of asthma in children often relies heavily on clinical history since many children are too young to do the tests that objectively measure airflow obstruction. Several studies have called attention to significant under-diagnosis and under-treatment of childhood asthma. (Speigth ANP et al., 1983; Clifford RD et al., 1989; Kirshner B et al., 1990) By school-age, children are able to perform simple spirometry and the diagnosis can be supported with more formal evaluation. Therefore, the school-age children who are able to perform spirometry and peak flow measurements are the target for the diagnostic criteria and assessment method suggested in this chapter.

There are two main types of guidelines in classifying asthma severity – the National Institutes of Health Global Initiative for Asthma (GINA) guidelines (National Asthma Education and Prevention Program Expert Panel Report 2, 1997) classify asthma severity based on symptoms and the British Thoracic Society (BTS) guidelines classify (The British Thoracic Society and others, 1997) asthma severity based on the medication needed to control symptoms. However, it is often difficult to apply either system in real life, as many a time's patients are still symptomatic while on treatment and they cannot simply be classified into one or the other system. A general classification of childhood asthma is described as follows.

Mild asthma: Discrete attacks for no more than 1 – 2 days occurring no more often than once per month with symptom-free intervals or very brief attacks occur no more than twice per week. This group corresponds approximately to GINA steps 1 – 2, BTS step 1.

Moderate asthma: Attacks more often than twice weekly with occasional more prolonged exacerbations and some nocturnal symptoms. This group corresponds approximately to GINA steps 2 – 3, BTS step 2 – 3.

Severe asthma: Continuous or virtually continuous symptoms by day and some nocturnal disturbance with occasional prolonged severe exacerbations. This group corresponds approximately to GINA steps 3 – 4, BTS step 3 – 5.

Mild and moderate asthma may well be seasonal with complete or virtually complete symptoms free intervals once the relevant season is over. Severe asthma is rarely seasonal although the severity of symptoms (and need for treatment) may fluctuate from time to time.

Whatever the overall pattern of asthma takes in a particular individual patient, the severity and frequency of attacks vary from time to time. It is important to note that there is no direct link between the overall severity of asthma and the severity of the individual attacks.

2.3 Assessment of asthma in school-age children

Childhood asthma is a disease that causes much mortality and morbidity (Campbell MJ et al., 1997; Janson C et al., 1997; Dente FL et al., 2004), and emphasis has been given for the close monitoring of one's asthma status. Monitoring the patient's symptoms is the focus of asthma assessment. However, symptom monitoring is subjective and liable to recall bias. In clinical practice, supportive evidence provided by more objective measurements such as spirometry, peak flow expiratory rate (PFER), non-invasive airway inflammatory assessments, such as exhaled nitric oxide measurement and hypertonic saline challenges and induced sputum is often required.

2.3.1 Clinical History

Symptoms associated with asthma include wheeze, dyspnoea, cough and nocturnal waking. For school age children, the diagnosis of asthma is likely given a characteristic history of recurrent wheeze, shortness of breath and cough, or even recurrent cough by itself. An asthmatic child often has a history of cough and / or wheeze with viral illness. Symptoms induced by exercises are also suggestive of an underlying hyperactive airway. There may also be symptoms occurring between respiratory tract infections at night or in the morning on arising and many a times the child would become symptomatic with weather change. However, it is worth noting that clinical history and symptom rely on accurate patient reporting and are difficult to quantify, as individual perception of each is highly subjective.

2.3.2 Atopy

A child with atopy produces specific IgE antibodies after exposure to common environmental allergens and is said to be sensitised to that allergen. The presence of specific IgE antibodies is measured by means of a skin prick test or radioallergosorbent testing (RAST). (Gold MS & Kemp AS, 2005) Allergens were considered as important for the development of asthma but it is reported that the closeness of the association between atopy and asthma remains unknown. (Platts-Mills TA & Chapman MD, 1987) Many individuals who develop asthma in later life and a minority of younger patients fail to show any obvious signs of allergy. On the other hand, whether a non-allergic asthmatic state actually exists has been questioned (Burrows B et al., 1989)

During the last 10 years, there has been substantial effort devoted to genetic studies of asthma and allergic diseases on the basis of candidate gene approaches and genome-wide linkage studies in human and animal models. Up to now, despite significant findings regarding susceptibility regions and genes in some cases, these studies have still resulted in only a very limited understanding of asthma and allergic diseases. Some of the reasons for the failure to replicate the detection of particular loci across studies and for the modest contribution of each of these susceptibility loci might relate to heterogeneity factors between studies that might be difficult to detect and take into account in small-scale studies. Enlarging the number of participants in a given study has thus been proposed to increase the power to detect even small effects. Alternatively, identifying the factor or factors that conceal the underlying effect and thereby unmask the true

relation between genetic susceptibility and development of a certain condition will result in a more profound understanding of the primary biologic mechanisms. (von Mutius E, 2004)

Cockroach has been identified as an important source of indoor allergens. The major cockroach allergens are Bla g1 and Bla g2. The presence of cockroach debris in house dust has been shown for a long time. Cockroach infestation is highest in crowded urban areas. It was reported that the increased asthma morbidity and mortality rates in inner cities could be related in part to cockroach allergen exposure (Sarpong SB et al., 1996) In a group of 87 children aged 5 to 17 years with moderate to severe allergic asthma, over 80% of children with bedroom major cockroach allergens (Bla g1 and Bla g2) of 1 U/gm or greater demonstrated skin sensitivity to cockroach allergen. The results has confirmed the high prevalence of sensitization to cockroaches and emphasized the frequency of combined sensitization to house dust mites.

Pollen from wind-pollinated plants such as grass, trees and weeds can trigger both asthma and hayfever. In a study of 5,427 subjects with age of 18 to 65-year-old, prevalence of hay fever symptoms together with a positive skin test to pollen was significantly higher in the exposed (13.6%) vs less exposed community (5.5%) (Charpin D et al., 1993) In contrast, prevalence of asthma with positive skin test was 2.5% and 1.9% respectively. The study, therefore, concluded that high exposure to pollen is a risk factor for developing hay fever but not asthma.

Fungal spores can be found in moist areas such as basements, food storage areas, and waste receptacles; however its triggering power is less than pollen. (Solomon WR et al., 1978)

Household animals especially dogs and cats may release allergen through saliva in hairs. It was reported that that the prevalence rate for asthma in children with animals was twice that of children without animals. (Abdulrazzaq YM et al., 1995)

Fig. 2.1: Skin prick test (SKT). A child was considered atopic if he had at least one skin test result that showed an induration with a diameter of at least 2 mm greater than the negative (normal saline) control.

2.3.3 Lung Function Test

Spirometry and peak flow measurement are useful for the management of asthma. The most common and clinically applicable indices are Forced Expiratory Flow at 1 second (FEV_1), i.e. the maximum volume of air expired in the first second from full inspiration; and Peak Flow Expiratory Rate (PEFR), i.e. the highest flow obtained during a forced expiration starting immediate after a deep inspiration at total lung capacity. The degree of reduction of FEV_1 is used to define the severity of obstruction. Serial measurements of FEV_1 and PEFR are taken in the follow-up of patients to

assess the degree of asthma control. These measurements together with self reported symptoms are then utilized to make therapeutic decisions.

All measurable parameters of pulmonary function may be within normal limits when the patient with asthma is in remission. Typical abnormalities noted with spirometry in an asthmatic patient include a reduction in FEV_1, PEFR, FEV_1/FVC ratio, and an increase of 15% or greater in FEV_1 in response to a bronchodilator. (National Asthma Education and Prevention Program Expert Panel Report 2, 1997) Excessive variability in home peak flow monitoring, either a diurnal variation of 20% or more, or day to day variation of 20% or more, or a 20% response to bronchodilator, would help to confirm the diagnosis of asthma. (Woolcock AJ et al., 2001) Abnormalities in lung volumes include a decreased vital capacity, an increase in functional residual capacity, total lung capacity, and especially in the residual volume are other characteristics of asthma.

Spirometry with flow-volume loops may provide additional information for diagnostic purposes or when assessing the degree of severity of asthma. (Linna OV, 1993) In particular, the maximum expiratory flows from the flow-volume loop are more sensitive to lesser degrees of airway obstructions. (Landau LI, 1993) However, spirometry is only practical in children who can perform a forced vital capacity maneuver, usually those aged over 5 years.

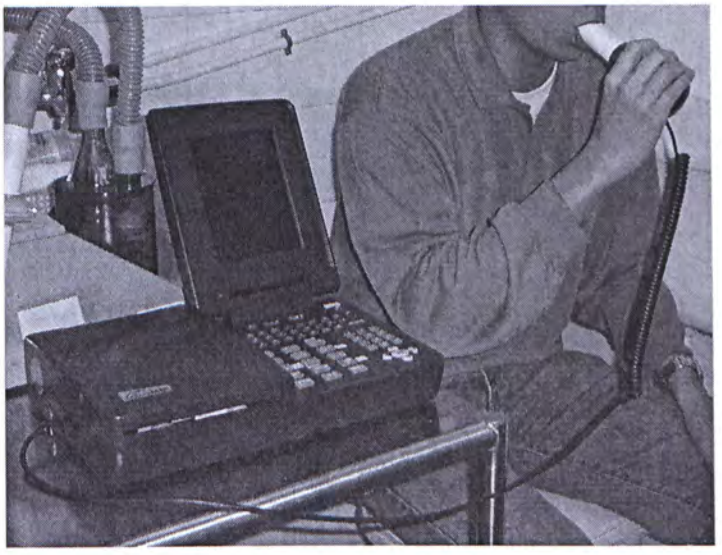


Fig. 2.2 Spirometry

It should be emphasized that the diagnosis of asthma does not rely purely on lung function measurements. (National Asthma Education and Prevention Program Expert Panel Report 2, 1997) Normal expiratory flows do not exclude the diagnosis of asthma because asthma often causes only episodic airway narrowing and signs and symptoms are not always present. Reduced expiratory flow, which improves significantly after inhaled bronchodilator, is also present in other pulmonary disorders, such as obstructive bronchitis, or normal subjects following viral upper respiratory infection.

The use of FEV_1 / PEFR is associated with a variety of limitations:

- Expiratory maneuver is effort dependent, and proper training is required to obtain the best and most reproducible measurements from the individual. (Frischer T et al., 1995)
- PEFR mainly reflects the calibre of large airways and under-estimate the degree of airflow limitation present in

peripheral airways. Therefore, single measurement of PEFR is not recommended in making the first diagnosis of bronchial obstruction. (Sawyer G et al., 1998)

- Although predicted values of PEFR related to gender, race, age and height are available in the literature, (Reddel HK et al., 1998) however, the inter-individual variability is wide and some subjects have PEFR values that are different from the average value of subjects with the same demographic characteristics.
- Some subjects with long-lasting asthma may develop irreversible airflow limitation. Therefore, nowadays, it is more useful to establish the best personal PEFR observed during a period of effective treatment to obtain reference values to use in the assessment of long- term changes. (Reddel HK et al., 2004)
- It has been suggested that post-bronchodilator FEV_1 or FEV_1/FVC ratio are better indicators of airway pathology; however, FEV_1 may not give as reliable guide to the degree of small airway pathology as FEF_{25-75}.

It has to be emphasized that a negative test result of spirometry and PEFR cannot exclude a diagnosis of asthma, as many well-treated asthmatics have normal lung function and simply do not have the capacity to increase their FEV_1 any further. Moreover, the abnormal airway physiology is not often present even in cases with severe asthma. (Enright PL et al., 1994; Hunter CJ et al., 2002; Clancy K., 2004) In addition, these measures do not correlate closely to the underlying eosinophilic airway inflammation, which is a predictor of asthmatic exacerbation and precursor

of airway remodeling. (Crimi E et al., 1998) Furthermore, the lack of a gold standard and the effort dependent nature of the tests might lead to the risk of either over-diagnosis or an under-estimation of asthma. (Britton J & Lewis S., 1998; Miller MR et al., 1998; Sawyer G et al., 1998)

2.3.4 Exhaled Nitric Oxide

Assessment of exhaled nitric oxide (eNO) is an attractive technique in assessing airway inflammation in childhood asthma because of the non-invasive nature of the technique, direct and rapid availability of the results.

eNO is generated by isoforms of nitric oxide synthase (NOS; types I – III) in the airway epithelium and from pulmonary macrophages. Constitutive NOS (Type III) is probably responsible for the basal levels of eNO seen in normal subjects. When the airways are inflamed, inducible NOS (Type II) will be expressed in the epithelium that is responsible for the elevated level of eNO.

In children, eNO levels were positively correlated with the absolute level of peripheral blood eosinoiphils (Silvestri M et al., 1999) and sputum eosinophils (Grootendorst DC et al., 1997). Therefore, eNO levels in asthma may be related to the eosinophilic inflammation.

The relationship between eNO and neutrophil in airways is less clear. Studies have shown that eNO is either the same or slightly elevated eNO level was demonstrated in a group of cystic fibrosis children with intense

neutrophils infiltrate compared with normal children. (Ho LP et al., 1998) One possible explanation is that NO is converted to peroxynitrite and nitrate by superoxide in activated neutrophils.

It is reported that eNO levels increased with severity of atopy measured by SKT. (Franklin PJ et al., 1999) The result might imply subclinical inflammation in individuals. However, viral upper respiratory tract infections and influenza vaccination have been associated with elevation of eNO in adults. (Kharitonov SA et al., 1995; Thomas PS et al., 1999)

Fig. 2.3 Sievers 280i NOA analyzer for measuring eNO

The use of eNO measurement in assessing airway inflammation has several limitations:

- There are variations in the methods of collection of samples have been described in literature, including on-line and offline measurements. Guidelines with suggestions for collection and analysis have been published in Europeans and Amercian literatures (Kharitonov S et al., 1997; American Thoracic Society, 1999)

- Lack of universal reference cut off value available for distinguishing normal and asthma children.
- Reported values of eNO in healthy subjects vary from 3 ppb to 88 ppb. (Kharitonov SA et a;l., 1995; Dotsch J et al., 1996; Scollo M et al., 2000; Latzin P et al., 2002; Pijnenburg MW et al., 2002) The variations may be caused by different measurement techniques and low numbers studied.
- Confounding factors in measuring eNO such as age, sex, and atopic symptoms have not always been taken into account in these studies.
- Varieties of commercially available eNO machine are costly, bulky in size, lack of reference value and inter-machine variation studies, which limits its use in clinical setting. (Gutafsson LE et al., 1998)

2.3.5 Airway Hyper-responsiveness measurement

Airway hyperresponsiveness (AHR) is an abnormal increase in airflow limitation following the exposure to a stimulus. The word "abnormal" refers to a comparison with the airway response to the same agonist, using the same method to measure the airflow limitation, in a group of healthy subjects. The wording "airflow limitation" is used because it encompasses the different mechanisms that can lead to a decrease in the parameters of airflow (Pauwels R, 1997). Although AHR is not specific for asthma, nearly all patients with asthma exhibit increased responsiveness. The severity of AHR also predicts the response to inhaled corticosteroids in patients with asthma (Juniper EF et al., 1981)

The stimuli have been divided into direct and indirect so as to highlight the heterogeneity of the airway response to the different stimuli. (Pauwels R, et al., 1988; Rogers DF & O'Connor BJ, 1993; Sterk PJ et al., 1993) Direct challenges with histamine or methacholine have been used by researchers since 1940s for assessment of AHR. They can be used to establish a dose response and time course of the acute bronchoprotective effects of beta-agonists. In addition, they have also been used to assess the potential anti-inflammatory effects of prolonged treatment with new agents. However, inhaled corticosteroids, the current gold standard anti-inflammatory treatment for asthma, reduces AHR to methacholine and histamine to a small degree, which is the limitation for the potential use of direct stimuli in the evaluation of anti-inflammatory drug.

The proposed mechanism for the airflow limitation caused by methacholine and histamine challenge (direct stimuli) in adult involve the direct action on the effector cells, such as airway smooth muscle cells, bronchial vascular endothelial cells and mucus producing cells. For indirect stimuli such as 4.5% hypertonic saline, it caused airflow limitation by an action on cells other than the effector cells; these cells then interact with these effector cells. However, whether the same mechanism will apply to children is still under investigation. (Joos GF et al., 2003)

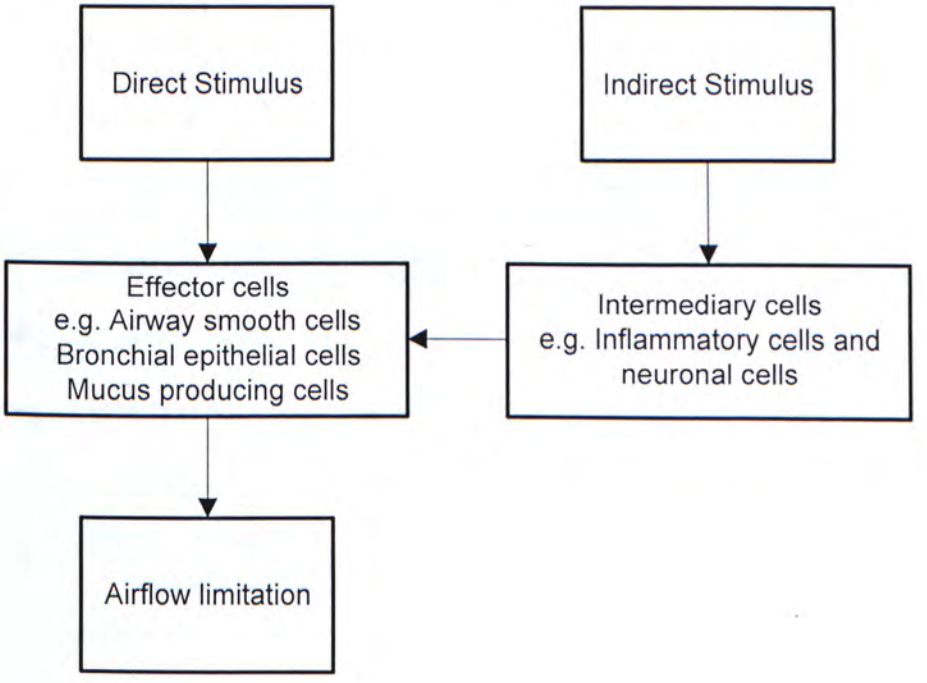


Fig. 2.4 Direct and indirect stimulus lead to airflow limitation

(Joos GF et al., 2003)

In recent years an increasing number of studies have investigated the relative usefulness of indirect airway challenges in monitoring anti-inflammatory treatment in asthma. The airways of asthmatics, but not healthy persons, narrow in response to "indirect" stimuli. For this reason, airway inflammation and the number of inflammatory cells such as mast cells and eosinophils are likely to be the important determinants of the abnormal airway response to these challenges. Treatment with inhaled steroids significantly reduces airway responsiveness to indirect stimuli in adult studies (Vathenen AS et al., 1991; Anderson SD et al., 1994; Pedersen S et al., 1995; du Toit JI et al., 1997; Doull J et al., 1997; Brannan JD et al., 2002) and mast cell release of mediators is associated with hyper-responsiveness to indirect stimuli. (Polosa R et al., 1995; O'Sullivan S et al., 1998)

The pattern of indirect stimuli differs from direct stimuli is shown by the following evidence.

- Airway responsiveness to direct and indirect challenges are rather poorly correlated with each other (Pauwels et al., 1988)
- A wide array of mediators including histamine, leukotrienes, prostaglandins, acetylcholine, neuropeptides are involved in the airway narrowing induced by the indirect stimuli. (Van Schoor J et al., 2000)
- The airway narrowing caused by an indirect, but not a direct challenge, can be prevented by acute pretreatment with a cromone (cromoglycate, nedocromil), inhaled frusemide and / or heparin (Van Schoor J et al., 2000)
- In patients with asthma, airway responsiveness to an indirect airway challenge is more closely associated with airway inflammation than airway responsiveness to a direct stimulus (Van Den Berg G et al., 2001)
- Airway responsiveness to an indirect stimulus may also better reflect acute changes in airway inflammation induced by allergen avoidance (van Velzen E et al., 1996) or by treatment with inhaled steroids. (Taylor DA et al., 1999; Hofstra WB et al., 2000)

The major advantage in using indirect stimuli to test for active asthma is their capacity to act on many different cells so that a wide variety of substances contribute to the narrowing that follows. Late responses after these challenges however are rare. (Phillips GD et al., 1990; O'Sullivan S et al., 1998) Another important advantage of challenge with indirect stimuli is the lack of false-positive tests. Healthy subjects respond with mean falls in

FEV_1 of 2 to 6% (Riedler J et al., 1994 & 1998; Hurwitz KM et al., 1995; Rabone SJ et al., 1996; Godfrey S et al., 1999), or small change in conductance. (Cushley MJ 1983)

Indirect challenges act by causing the release of endogenous mediators that cause the airway smooth muscle to contract, with or without effect in inducing micro-vascular leakage. Because the responses to these challenges are modified or even completely inhibited by inhaled steroids, the airway response to these challenges may be a closer reflection of active airway inflammation. They are thus potentially useful in the evaluation of efficacy and to monitor the response to treatment. (Jonasson G et al., 2000). A negative response to an indirect challenge suggests that the person either does not have asthma or that the asthma is currently under control with treatment.

When the airway response to an indirect stimulus is inhibited after regular treatment with steroids, it is unlikely that the inflammatory cells and mediators are present in significant numbers or concentration to cause airway narrowing. This concept is important because the airways of asthmatics generally remain hyper-responsiveness when the agonist is given directly to the airways, as it is with a pharmacological challenge. (Vathenen AS et al., 1991; Sont JK et al., 1999; Reddel HK et al., 2000)

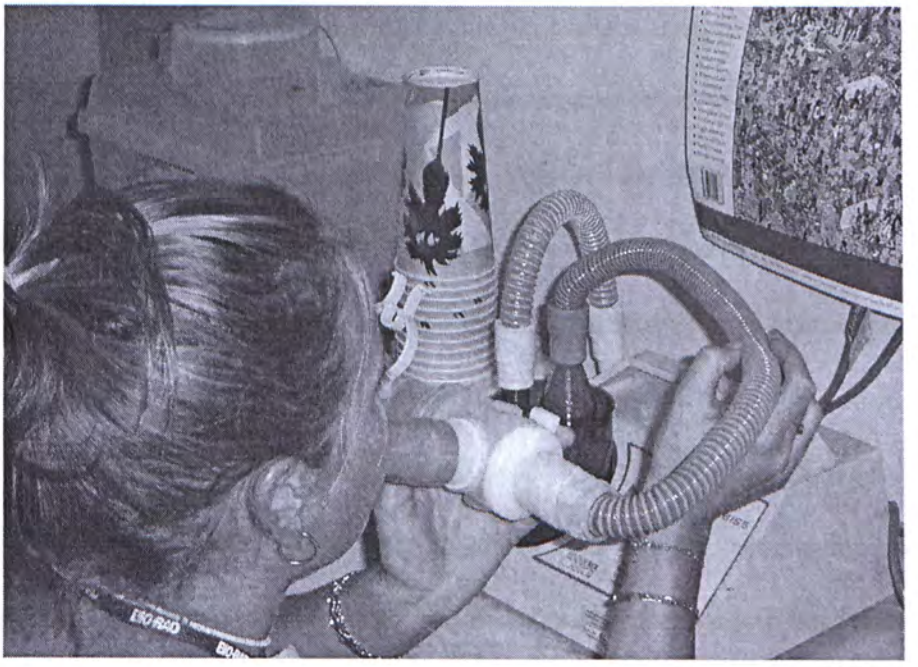


Fig. 2.5 Hypertonic saline challenge

2.3.6 Airway inflammation measurement

Airway inflammation is defined as the presence of activated inflammatory cells in the airways. Airway inflammation is a major characteristic of childhood asthma that usually has an eosinophilic component. Airway inflammation is important in the pathogenesis of asthma. Pathophysiologically, as well as accumulation of eosinophils and CD4 lymphocytes, there is activation of the epithelium and smooth muscle, mucus hypersecretion, thickening of the sub-epithelial collagen layer, mast cell degranulation, and smooth hypertrophy and hyperplasia. (Kay AB, 1996) Therefore, prevention and treatment of airway inflammation is the mainstay of asthma management. (Boulet LP, 1999)

The nature and extent of airway inflammation has been assessed using direct, invasive bronchoscopy with bronchial washings or biopsy or

bronchoalveolar lavage (BAL), or by indirect measurements of symptoms, spirometry, peak expiratory flow, tests of airway hyper-responsiveness or peripheral blood inflammatory markers (Pauwels R, 1996) Bronchoscopy with examination of cellularity provides the gold standard assessment of airway inflammation, which is limited by discomfort to the patient, expense and risks of the procedure, especially in school-age children. The indirect measurements relate variably with each other and reflect more than airway inflammation, for which they are nonspecific. As a result, not surprisingly, they may not correlate with direct measurements of airway inflammation. (Haley K & Drazen J, 1999)

Examination of induced sputum has evolved as a direct, relatively non-invasive, valid method of measuring airway inflammation over the last decade for children. (Pin I et al., 1992) It has emerged as an important and useful technique to study airway inflammation in childhood asthma because it is non-invasive and allows sample to be collected on repeated occasions. Sputum induction has been used to study asthma in children since the first description of the technique by Pin I et al in 1992 (Pin et al., 1992) Sputum is induced by inhalation of isotonic or hypertonic solutions administered by nebulisation. The aim of sputum induction is to collect an adequate sample of secretions from lower airways in subjects who do not produce sputum and expectorate it spontaneously.

In general, sputum induction in children of more than 6 years old is safe and has a successful rate varies from 68% – 100%. (Gibson PG et al., 2002) Sputum induction in younger children is limited by their poor spirometric technique and low tidal volume, which limits the dose of saline that can be delivered. (Riedler J and CF Robertson, 1994) In most studies, the procedure is well tolerated by children and possible side effects reported include cough, bronchospasm, vomiting and anxiety. (Pizzichini E et al., 2002; Covar RA et al., 2004; Rytila P et al., 2004) The procedure is equally well tolerated by children with severe asthma and those with an acute exacerbation. (Twaddell SH et al., 1996)

The proposed mechanism of sputum induction is that during the inhalation of isotonic or hypertonic solution, the increased osmolarity of the airway lining fluid increases vascular permeability in the bronchial mucosa and induces production of mucus by submucosal glands, which has been demonstrated in animal studies. (Umeno E et al., 1990) However, in vivo instillation of hypertonic solution into the airways of animals and humans induces an increase in the levels of several mediators but no rise in levels of albumin or other markers of increased vascular permeability. (Gravelyn TR et al., 1988; Freed AN et al., 1994) Therefore, the hypothesis has not been completely confirmed and only preliminary measurements of sputum osmolarity after induction have been reported with conflicting results. (Louis R et al., 1999)

When the induced sputum processed under standardized procedures, the results were reproducible. (Fahy JV et al., 1993 & Pizzichini E et al., 1996) The procedure is well tolerated by children with a success rate up to 80% (Gibson PG et al., 2000) The inflammatory findings correlate best with bronchial washings and more variably with bronchial biopsies and BAL because they reflects differences in different airway compartments. (Fahy JV et al., 1995; Maestrelli P et al., 1995; Grootendorst DC et al., 1997; Keatings VM et al., 1997; Pizzichini E et al., 1998) Induced sputum and bronchial washings reflect secretions from the more central airway lumen, BAL from the peripheral lumen and bronchial biopsies from the more central airway walls. In addition, sputum eosinophils are more sensitive and specific for the presence of asthma than blood eosinophils or eosinophilic cationic protein (ECP) (Pizzichini E et al., 1997)

The value of using sputum eosinophils as assessment tool in airway inflammation has been highlighted in both adult and children studies. (Pavord ID et al., 1997; Keatings VM et al., 1996) In selected published studies, up to 80% of corticosteroid-naïve children (Green RH et al., 2002; Pizzichini E et al., 1996; Silkoff PE et al., 2003) and more than 50% of corticosteroid treated children (Pavord ID et al., 1997) with concurrent symptoms have sputum eosinophils count outside the normal range. Thus the monitoring of sputum eosinophils may allow better asthma control and provide a useful guide in management. Recent evidence suggests that a treatment strategy directed at normalization of airway eosinophil reduces asthma exacerbations and hospital admissions in children. (Gibson PG et al., 2000)

In asthmatic patients, the number of eosinophils is elevated when compared with normal controls. There is a correlation between measurements of eosinophils counts obtained in induced sputum with BAL fluid in adult. (Keatings VM et al., 1997) In addition, there is a correlation between the change in airway responsiveness and the change in sputum eosinophils count after allergen challenge (Pin I et al., 1992) and sputum eosinophils are reduced by steroid treatment in a dose-dependent manner in adult. (Gibson PG et al., 2000) Sputum eosinophils are elevated in some patients with severe intractable asthma. (Jatakanon A et al., 1998; Wenzel SE et al., 1997)

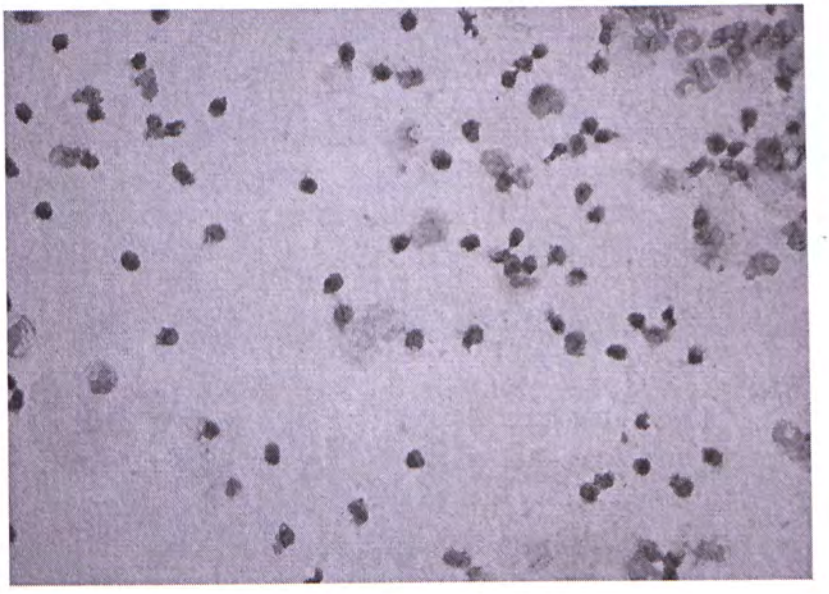


Fig. 2.6 Sputum eosinophilia

Although induced sputum is a direct, relatively non-invasive, valid and repeatable method of measuring airway inflammation; sputum examination has been traditionally limited by the difficulty in obtaining adequate samples, especially in children. The use of 4.5% hypertonic saline to facilitate sputum production and dithiothreitol to improve cell dispersion has allowed this procedure to be applied successfully. Combined hypertonic saline challenges and sputum induction is a non-invasive, safe, cost saving and reproducible method in assessing childhood asthma. HSCSI can assess airway inflammation and airway hyper-responsiveness at the same time providing important information in relation to the underlying airway abnormalities.

2.4 The use of HSCSI in clinical trial

2.4.1 Role of sputum eosinophil count in childhood asthma

The sputum eosinophil plays an important role in the evaluation of airway inflammation in childhood asthma. By assessing the degree of airway inflammation and targeting treatment in relation to response, we could prevent children from being exposed to unnecessarily high dose of inhaled corticosteroid and suffer from its potential side effects. In addition, asthma frequently begins in childhood and becomes chronic by adulthood; therefore, it is necessary to evaluate the role of airway inflammation in children in order to study the role of airway inflammation in the genesis of asthma. Thus, the greatest clinical application of sputum induction is to study the extent of airway inflammation non-invasively in children as the use of bronchoscopy or biopsies have been very limited due to ethical and safety reasons. (Gibson PG et al., 1998) Induced sputum may further provide important insight into the pathology and mechanism of asthma and its determinants of severity. Sputum induction allows the analysis of mediators such as proteins and cytokines that are present in the fluid phase of the sputum sample. (Pin I et al., 1992; Fahy JV et al., 1995; in't Veen JC et al., 1999; Gibson PG et al., 2000) These mediators in sputum can be assessed by ELISA (Enzyme-Linked ImmunoSorbent Assay) or bioassays techniques. However, some components in sputum such as the salivary proteins (mucins), degradation products such as DNA and actin released from dead cells may interfere with both the quantitative and qualitative measurement of cytokine function. (Louis R et al., 1999; Perks B et al., 2000) A standardized method is not established currently for the proper assessment of these fluid-phase mediators.

2.4.2 Role of sputum induction in predicting asthma exacerbation

In a study by Green RH et al., they found that higher level of sputum eosinophils correlated with greater airway obstruction and the increase in sputum eosinophils can be considered as a predictor for asthma exacerbations. (Green RH et al., 2002) Apparent stable asthmatics with high eosinophil counts are more likely to develop an exacerbation on reduction of their regular corticosteroid therapy. In their study, they found that the extent of control of eosinophilic airway inflammation and exacerbation was greater in the sputum management group, which targeted at lowering the airway inflammation, than in the BTS management group. They concluded that a treatment strategy targeted at normalization of sputum eosinophilia would reduce asthma exacerbations and hence a better asthma management. (Green RH et al., 2002) Similar study is lacking in the paediatric population but the same theoretical rationale should apply.

In another study by Zacharasiewicz A et al., Forty children with stable asthma eligible for inhaled steroid reduction were recruited and reviewed every 8 weeks, and their inhaled steroid dose halved if clinically indicated. eNO, combined HSCSI, and exhaled breath condensate collection were performed at each visit to predict success or failure of reduction of inhaled steroids. Thirty of 40 (75%) children tolerated at least one dose reduction, 12 of 40 (30%) were successfully weaned off, and in total, 15 of 40 (38%) children experienced loss of asthma control. Treatment reduction was successful in all children who had no eosinophils in induced sputum before the attempted reduction. By using multiple logistic regression, increased eNO (odds ratio, 6.3; confidence interval, 3.75–10.58) and percentage of

sputum eosinophils (odds ratio, 1.38; confidence interval, 1.06–1.81) were significant predictors of failed reduction. These findings suggest that monitoring airway inflammation may be useful in optimizing treatment in children with asthma. (Zacharasiewicz A et al., 2005)

2.4.3 Role of sputum induction in differentiate types and causes of airway inflammation and treatment strategies

Sputum induction can be used as an assessment tool in differentiating eosinophilic and non-eosinophilic inflammation. The differentiation is important with respect to treatment. An increase in sputum eosinophils is regarded as characteristic of asthma and can be induced by inhaled allergen (Gauvreau GM et al., 1996 & 1999) and by a reduction in steroid treatment in steroid dependent asthma. (Gibson PG et al., 1992; In't Veen JC et al., 1999; Pizzichini MM et al., 1999)

In healthy subjects, sputum differential cell counts show that macrophages and neutrophils predominate and eosinophils and lymphocytes are few. (Belda J et al., 2000) Abnormalities in differential cell counts re-emphasize the occurrence of different types of inflammation and their different causes.

Persistent sputum eosinophilia in patients with "difficult asthma" should raise the possibility of under treatment or noncompliance with corticosteroid medication. Noncompliance can be confirmed by comparing the level of sputum eosinophilia before and after direct supervision of administration of medication. (Parameswaran K et al., 1999) This issue is

particularly important in paediatric asthma as poor compliance has a direct link with frequent exacerbations.

In addition, sputum induction can help to identify appropriate add on therapy in childhood asthma. Long acting beta-agonists significantly improve symptoms and pulmonary function but they could mask underlying airway inflammation as they do not possess anti-inflammatory action (Claman DM et al., 1994) McIvor RA et al. has demonstrated that salmeterol controlled symptoms and lung function in asthmatic subjects undergoing corticosteroid withdrawal allowing greater sputum eosinophilia to develop before a clinically recognizable exacerbation. (McIvor RA et al., 1998) Gibson PG et al., has demonstrated that, on the other hand, long acting beta-agonists improve asthma control in symptomatic patients with normal cell counts on corticosteroid therapy (Gibson PG et al., 1999).

For sputum neutrophilia, the known causes include chronic airflow limitation associated with cigarette smoking, (Pizzichini E et al., 1996; Stanescu D et al., 1996; Keatings VM et al., 1997) pollutants such as ozone (Fahy JV et al., 1995; Holz O et al., 1999; Vagaggini B et al., 1999), endotoxin (Nightingale JA et al., 1998) and infection (Pizzichini MM et al., 1998). The intensity of the neutrophilia is indicated by the total cell count and is most pronounced in bronchial infection. The likely cause of non-eosinophilic but neutrophilic exacerbations of asthma is viral infection, (Fahy JV et al., 1995) although the cause and incidence has not been confirmed. (Pizzichini MM et al., 1998; In't Veen JC et al., 1999)

For normal sputum cell counts or neutrophilia with a normal total cell

count, other conditions such as gastroesophageal reflux (Parameswaran K et al., 2000), habit cough need to be considered.

2.4.4 Role of sputum induction in evaluating chronic cough in asthma

Childhood chronic cough is a common and troublesome problem. It is a non-specific symptom that is associated with several unrelated mechanisms and has various causes including asthma. Sputum induction can be used to recognize eosinophilic bronchitis without asthma. (Gibson PG et al., 1989 & 1995) This condition was observed in patients with a chronic cough who had normal spirometry and normal airway responsiveness to methacholine and adenosine monophosphate. The sputum contained an increase in eosinophils, and this plus the chronic cough were reversed by corticosteroid treatment though eosinophilic bronchitis is rarely seen in children.

Furthermore, it has been demonstrated that chronic cough usually is associated with predominant sputum neutrophilia, but up to 40% of subjects with cough have sputum eosinophil count of more than 3%. (Brightling CE et al., 1999; Gibson PG et al., 2001) In chronic cough with sputum eosinophilia, inhaled steroids are usually effective but prednisone may be required transiently (Wong AG et al., 1996) or regularly (Hargreave FE et al., 1999). In contrast, chronic cough without sputum eosinophilia is not going to respond to corticosteroids (Pizzichini MM et al., 1999). Sputum induction in this scenario is a useful and cost effective tool for diagnosing the group of patients with chronic cough that is going to be responsive to anti-asthma therapy.

2.5 Problems of sputum induction in children

The method of sputum induction has many advantages but it has a few major drawbacks that limit its use in daily clinical practice. The process of sputum induction needs dedicated, highly qualified laboratory technicians and the process is rather time consuming. From our experience, it can be estimated that even if a patient produces a suitable sputum sample within 10 min of starting induction, the total time required for preparation of equipment, induction process, processing of the sample, staining and performing a differential cell count will amount to ~180 min. The method has therefore been used mainly for research purposes and less often in clinical practice.

In addition, the need to process sputum within 2 h after induction limits the number of patients that can be examined in parallel and restricts its use in most out-patient settings Storage of sputum could cause a lower quality of cytospins and a change in sputum compositions. (Bettschart RW et al., 1998) Two methods were suggested in literature to overcome the problems. One method consists of simultaneous homogenization and fixation of freshly expectorated sputum with Saccomanno's fixative plus 0.2% dithiothreitol (DTT). (Nielsen L et al., 1998) Although it enables longer storage of sputum sample, cell counts are still difficult to perform due to cellular shrinkage after fixation. The second method utilizes dimethylsulphoxide (DMSO) to freeze the sputum cells to avoid water crystallization. (Popov T et al., 1998) Although there is an impairment of cellular morphology, the extent does not affect the differential cell count. Preliminary data show a good correlation between frozen samples and

corresponding freshly processed aliquots. (Holz O et al., 1999)

Gibson PG et al., has demonstrated a different approach to simplify sputum analysis. He and his colleagues lysed the cell pellet after homogenization and measured the amount of eosinophilic cationic protein (ECP) released as a marker, instead of preparing cytospins to quantify eosinophils. (Gibson PG et al., 1998) This automatic method requires ~3 hours for analysis of ECP and a good correlation between the number of eosinophils and the concentration of ECP in the cell lysate suggests that this could be an easier way to perform large scale studies. However, further evidence and validation are needed

In order to incorporate sputum induction as a cost effective tool in our routine clinical practice, the time and manpower needed for processing have to be shortened. Sputum induction in its current state is generally considered as a research tool only, but it is rapidly being recognized as a useful adjunct in the management of asthma.

2.6 Conclusion

Diagnosis and assessment of asthma has traditionally been relied on clinical history, which depends on the integrity of patients and the accuracy of the reporting, which is subjective in nature. Lung function tests such as spirometry and peak flow measurement indirectly reflect the degree airway inflammation. Examination of cellularity by means of BAL or bronchoscopy is the gold standard in assessing airway inflammation; however, they are invasive, expensive and potentially hazardous in children. Since controlling airway inflammation is the core of asthma management, non-invasive markers of airway inflammation are needed to guide clinical practice and for research purposes. Therefore, HSCSI has become an attractive assessment tool in assessing asthma severity as it can assess airway inflammation as well as airway hyper-responsiveness at the same time in a non-invasive manner.

Sputum induction in children is currently regarded as a useful technique in assessing airway inflammation because of its non-invasive nature. It has its place and value in clinical practice such as assessing the airway inflammation in persistence asthma, evaluating the efficacy of inhaled corticosteroid in reducing sputum eosinophilia, distinguishing the eosinophilic asthma from the non-eosinophilic asthma, and predicting asthma exacerbation. However, the long induction and processing time limits its regular use in the outpatient clinic.

Chapter 3 HSCSI – Methodology and Materials

3.1 Introduction

Hypertonic saline aerosols are potent stimuli for airflow limitation in asthmatics, whereas normal subjects only rarely react (Schoeffel RE et al., 1981; Anderson SD et al., 1983; Smith CM & Anderson SD, 1986; Araki H & Sly PD, 1989; Riedler J et al., 1994). The potential for aerosols of hypertonic solutions to be used for airway provocation testing was first recognized in 1981. (Schoeffel RE et al., 1981) From the studies of Schoeffel and Smith (Smith CM & Anderson SD, 1986), it was apparent that the rate of change of the airway osmolarity was an important determinant for a positive response in an asthmatic. Therefore, a more concentrated hypertonic saline (4.5%) is now used rather than the original concentration (3.6%) reported in 1983. (Anderson SD et al., 1983) The original protocol, which is used for adults, has been slightly modified for use in children by Riedler et al. (Riedler J et al., 1994 & 1998; Riedler J & Robertson CF, 1994).

The protocol for combined HSCSI described here has been successfully implemented in our paediatric pulmonary function laboratory at Prince of Wales Hospital. The protocol was chosen over others because it was the best described, and has been shown to be practical and safe for use both in adults and children.

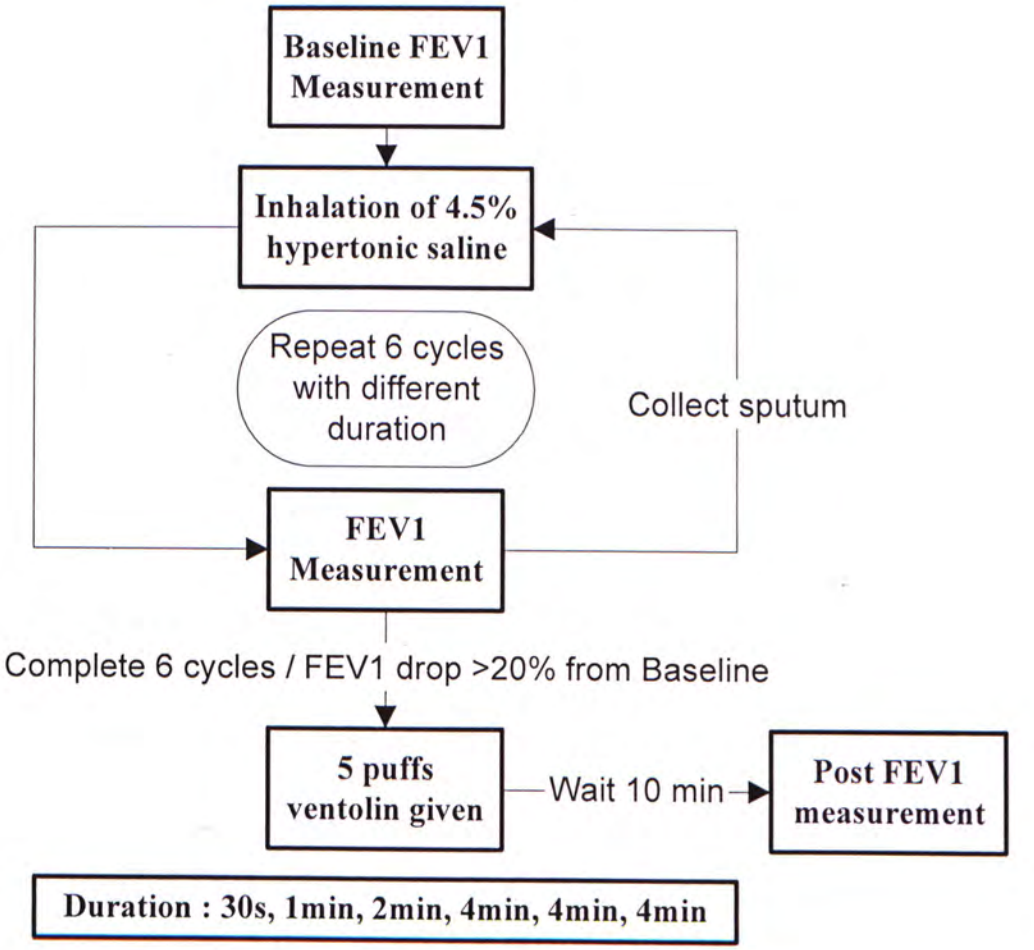


Fig. 3.1 Procedures for combined HSCSI

3.2 HSCSI – Methodology

3.2.1 Patient preparation

Medications used in treatment of asthma have been found to inhibit or even completely prevent responses to the hypertonic saline challenge. Therefore, patients should withhold their pulmonary medications for the appropriate times before hypertonic saline challenge – no short acting bronchodilators, sodium cromoglycate, nedocromil sodium, or ipratropium bromide for 8 hours; no long acting or sustained-release bronchodilators or antihistamines for 48 hours; or leukotriene antagonist for 4 days. (Kemp JP et al., 1998)

No caffeine should be taken on the morning of the study, although some laboratories request 24 hours withholding of beverages or food containing caffeine. No vigorous exercise should be undertaken for at least 4 hours before attendance at the laboratory, and it would be preferable to have no vigorous exercise on the day of study (Dessanges JF et al., 1999) because of the possibility of cross-refractoriness. Steroids can be withheld on the day of the study, but this is flexible depending on the question being investigated. In addition, recent exposure to allergens, respiratory infection, and cigarette smoke may affect responses to the hypertonic saline challenge test.

HSCSI is performed in a dedicated area with negative pressure facilities. Before the start of the procedure, the patient is examined by a physician and his temperature is checked. The procedure will be thoroughly explained to the patient and the technique of proper coughing and

expectoration are demonstrated. Relatives and parents are asked to wait outside the laboratory to minimize interference. During the procedure, a medical personnel is usually present during the process and in addition bronchodilators together with other essential resuscitation equipments are readily available.

Fig. 3.2 Laboratory for combined HSCSI (7/F Ward AB Treatment Room)

3.2.2 Hypertonic saline preparation

Preservative-free sterile solutions of 4.5% saline are prepared; about 200 – 250ml of the solution is required for each challenge. The solution is disregarded after a single use. The solution should be refrigerated at 4°C and warmed to room temperature (21 – 23°C) for 30 min before use, as low temperatures may reduce the output of aerosol from the ultrasonic nebulizer.

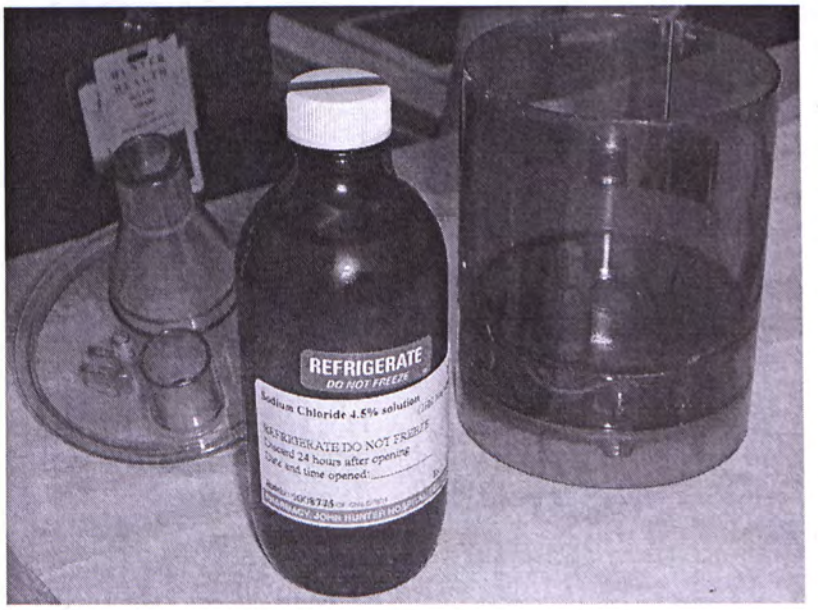
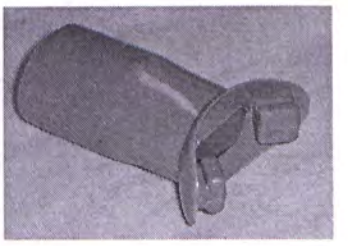


Fig. 3.3 4.5% Hypertonic saline and nebulizer cup

3.2.3 Equipments and materials

- Refrigerator for the storage of 4.5% hypertonic saline and specimen
- ULTRA-NEB™ ultrasonic nebuliser, model 2000, on the maximum output setting (DeVilbus)
- Salbutamol with a spacer device
- Stop watch

- Hans Rudolph valve box, large 2 way no. 2700 (Fig 3.4)



- VacuMed 1001 mouthpiece (Fig. 3.5)

- Spirometer (Spirolab II, MIR, Italy) and mouthpiece (Fig. 3.6)
- Sputum collection jar
- Calculator and record sheet (Appendix I)
- Nose clip
- Absorbent sheet on which to place valve box
- Benchtop Digital Scales

3.2.4 Aerosol generation and delivery

Ultrasonic nebulizers are used to generate high density hypertonic saline aerosols. It is important to ensure that the particle size generated is suitable and that the actual volume of output of the nebulizer is sufficient for airway provocation testing. Nebulizer with large volume canisters (400ml capacity) which can be easily detached for weighing will be suitable for the use in the procedure.

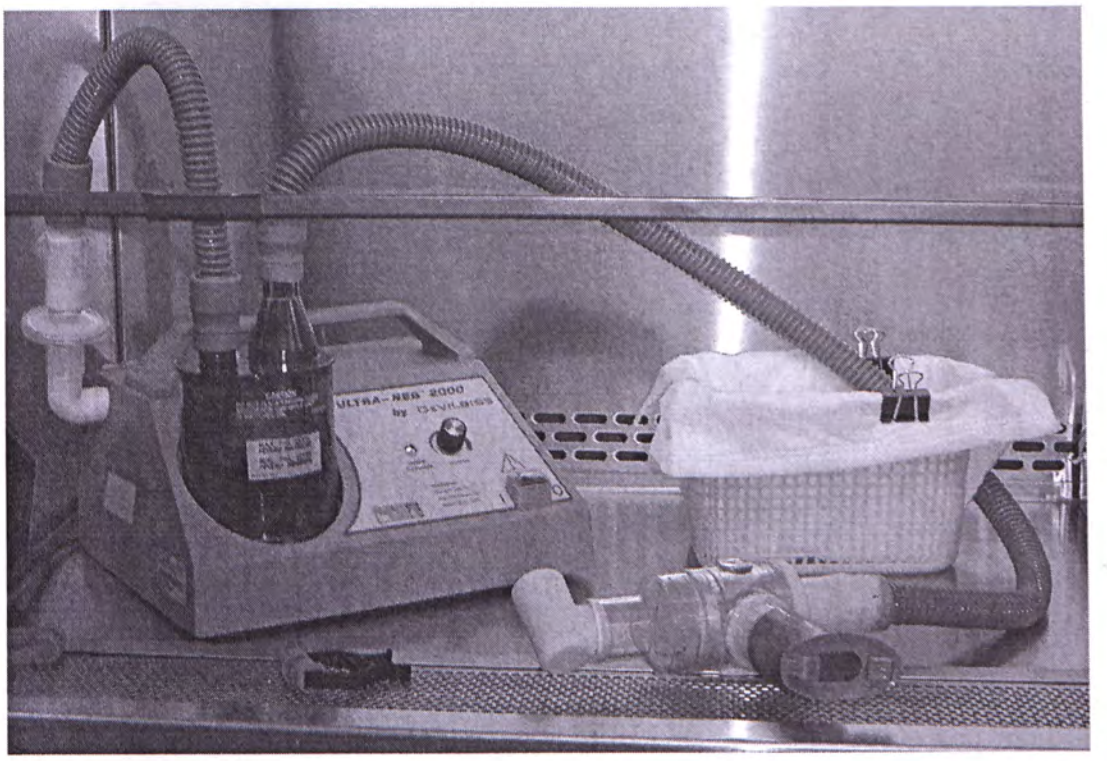


Fig. 3.6 Assembled DeVilbis 2000 Nebulizer filled with 4.5% hypertonic saline, Hans Rudolph valve box and mouthpieces in Nuaire Biological Safety Cabinets Class II Type B2.

DeVilbis 2000 produces aerosol particles of 2 – 6 μg when water is used. After passing through the tubing and valve that filter and trap large particles, the dispersion profile of the aerosol particles inhaled by the subject may be very different from the profile of those leaving the canister. When the tubing and valve is attached, a minimum output of 1.5mL/min is recommended, although a minimum output of 1.2mL/min is sufficient for adults.

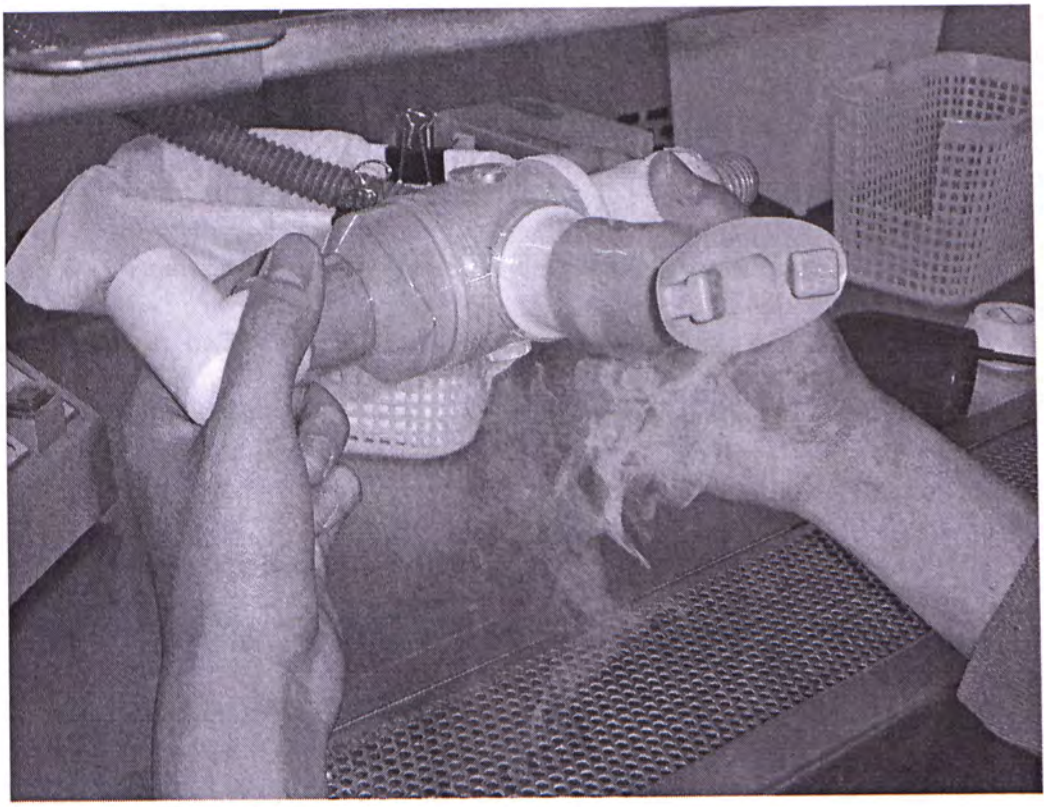


Fig. 3.7 4.5% hypertonic saline aerosol

The following factors will affect the output of aerosol from the nebulizer and the dose delivered to the child.

- The room temperature for the nebulizer to operate should be 21°C or above an initial exposure to lower temperatures results in lower output by the nebulizer, especially DeVilbis.
- The output can vary with the volume of fluid in the nebulizer (Anderson SD & Schoeffel RE, 1985), so a constant starting volume between 200 – 250mL is recommended. (Anderson SD et al., 1995)
- The nebulizer output is decreased with use of two-way valves, as aerosol particles impact on neoprene diaphragms. Multiple small valves such as Hans Rudolph Series 7200 should not be used. Hans Rudolph Series 2700 is recommended for both adults and

children. The advantage in using valve system is that the aerosol is not dissipated when the subject exhales; instead, it accumulates in the tubing and canister and is inhaled with the next breath.

- Face masks are not recommended as excessive amounts of aerosol can be accidentally delivered by this techniques. The nebulizer output may be reduced to an unacceptable value (<1.2mL/min) by the use of a disposable medication cup, thus leading to false-negative tests.
- The main canister is used to hold the solution rather than a cup unless there is a question of hygiene, in which case the cup device should be used.
- The condensate on the tubing that falls back into the canister should not be in contact with the patient because there is a valve on the inspiratory port.
- Tubing with a smooth interior surface, 60 – 70cm long with an internal diameter of 22mm is recommended. The longer the tubing between the canister and valve, the fewer aerosols will be delivered to the inspiratory port of the two way valve.
- The dose delivered can vary according to the tidal volume of the subject. Riedler et al observed very large variations in children (Riedler et al., 1994)
- The nebulizer output can diminish over many months of regular use, and the piezo electric crystal may need to be changed. Loss of output should be detected readily, as the volume delivered to each patient is recorded by weighting.

- The canister and tubing, but not the valve are weighted before and after the challenge and can be weighted during the challenge.
- Care must be taken to allow the solution that has collected in the tubing to run back into the nebulizer canister. The valve is not weighted because it frequently contains saliva.
- It is recommended that once the nebulizer is characterized, the tubing and valve remain the same and the same nebulizer is used for the same person if multiple tests are performed.

3.3 Protocol for combined HSCSI

1 Place 250ml of 4.5% hypertonic saline into the nebuliser cup and weight along the cover and the delivery tube but does not including the two way valve

2 Ascertain that medication has been withheld for the specified time and document the type, amount and time of last medication.

3 Explain the purpose of the test to the subject – how it is conducted and possible side effects e.g. cough, dry mouth, gagging, chest tightness, wheezing, dyspnoea, nausea and excess salivation.

4 Instruct and demonstrate how to obtain sputum from the lungs by coughing and clearing throat.

5 Perform baseline spirometry (FEV_1 and FVC)

6 Calculate a 20% fall of the highest FEV_1 and record (FEV_1 x 0.8 =20%).

7 Ask the subject to rinse his/her mouth in order to eliminate squamous cell contamination of the specimen.

8 Ask the subject to apply the nose clip and insert the mouthpiece into their mouth, in a similar manner to a snorkel.

9 When the subject is comfortable, turn on the nebulizer and start the stopwatch. Instruct the subject to breath "normally" (tidal breathing) through the mouthpiece. Turn the machine off after thirty seconds have elapsed.

10 The subject is allowed to drink water during the process if he / she feels thirsty.

11 Place the mouthpiece, opening down, on the absorbent sheet so any excess residue can be drained. This prevents a potential backwash of pooled secretions through the one way valve.

12 Wait for 60 seconds after each nebulisation episode in order to record maximal bronchoconstriction.

13 FEV_1 is recorded at the end of one minute. The nebulisation resumes immediately after the percentage of FEV_1 fall has been calculated.

14 The dose is cumulative over the time of challenge. Keep time for spirometry at each dose level to a minimum to ensure cumulative effects.

15 Percentage fall of $FEV_1 = \frac{(\text{Baseline FEV1} - \text{FEV1}) \times 100}{\text{Baseline FEV1}}$

16 Repeat steps 11-14, increasing the nebulisation time by doubling the time period: one minute, two minutes, and three periods of 4 minutes.

17 Between each period, there is a one minute break before the FEV_1 is recorded. Use this time to encourage the subject to produce sputum. Ask them to cough, clear their throat and deposit all or any oral contents into the specimen container.

18 It usually takes a cumulative time of about 11 minutes to produce an adequate specimen. Listen for a moist cough as a sign the subject is ready to produce sputum.

19 If the FEV_1 falls below 20% and there is not enough sputum produced, instruct the subject to inhale a beta-agonist and wait for 10 minutes, or until the FEV_1 has returned to within 10% of the baseline measurement.

20 Restart the nebulisation of 4.5% hypertonic saline, delivered in 4 minute doses, until sputum is obtained or a cumulative nebulisation

time of 20 minutes is reached. The nebulisation time is continued at 2 minute intervals if the subject demonstrates severe airway hyper-responsiveness to 4.5% saline (PD_{20} is < 2ml).

21 Once an adequate specimen has been obtained, refrigerate. Processing must take place within a maximum of 4 hours.

22 Stop the nebulisation if:

a) An adequate specimen has been produced and the FEV_1 has dropped 20%

b) An adequate specimen has been produced and the cumulative nebulisation time is 20 minutes

c) The subject requests the nebulisation to stop.

23 Ensure the subject's FEV_1 has returned to within 10% of the baseline measurement and that they are experiencing minimal discomfort before allowing them to leave a supervised area.

24 Following the end of the procedure once again weigh to 1 decimal place, the remaining saline, container and tubing (without the mouthpiece).

Fig. 3.8 Lung function measurement during combined HSCSI

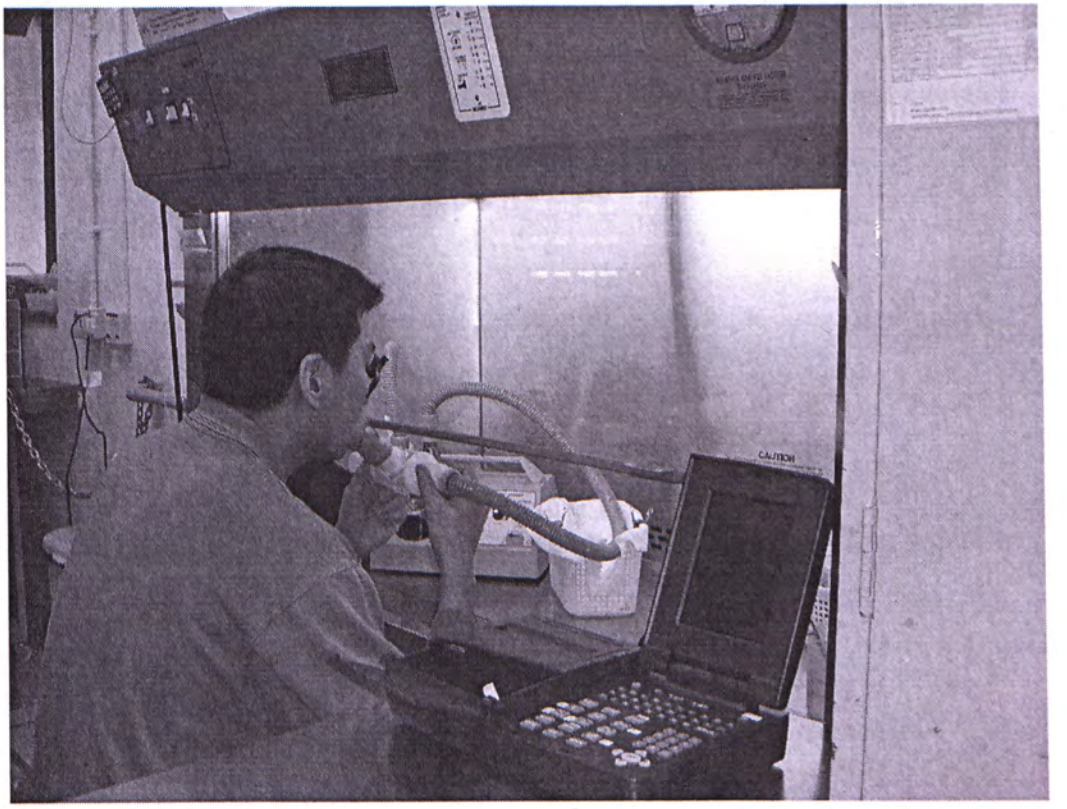


Fig. 3.9 Inhalation of 4.5% hypertonic saline by in combined HSCSI

3.4 Practical considerations in HSCSI

- If the subject is clinically unstable, or becomes symptomatic during the procedure, caution should be exercised when determining the length of each nebulisation.

- Monitor the FEV_1 at 1 to 2 minute intervals during each nebulisation if there is reason for concern.

- If the subject wishes to cough during the procedure, they should be encouraged to cough into the nebuliser and not remove the mouthpiece

- Between nebulisations encourage coughing in order to obtain a specimen. Many subjects will cough immediately the nebulisation finishes. This opportunity can be used to obtain sputum but exercise moderation so as to not tire the subject or cause a sore throat.

- Listen to the subject to ensure the sample is from the lungs and not post nasal secretions.

- All sputum induction procedures should be conducted in areas where in case of emergency resuscitation equipment is available.

3.5 Expression of airway response

The airway responsiveness to hypertonic aerosols is expressed as the dose of 4.5% saline required to provoke 20% fall in FEV_1. To calculate this, the response-dose curve can be constructed – expresses as a percentage of the baseline value for FEV_1 (fall in FEV_1) against the cumulative dose of aerosol.

The cumulative dose of aerosol is determined for each time interval by dividing the total dose delivered to the inspiratory port of the two-way valve divided by the total time for delivery.

The percentage fall in FEV_1 is plotted against the cumulative dose. A PD_{20} value can be obtained by linear interpolation, and is expressed in ml. One ml of 4.5% saline is assumed to be 1 g, but the factor for relative density (1.003) can be used to correct the volume.

PD_{20}	< 2mls	Severe hyperresponsiveness
	2.1 - 3.0	Moderate - severe hyperresponsiveness
	3.1 - 6.0	Moderate hyperresponsiveness
	> 6mls	Mild hyperresponsiveness

Table 3.1 Interpretation of PD_{20} in HSCSI

It is acknowledged that the dose that enters and deposits in the airways, but using the technique and equipment described, it is estimated that 20% of the hypertonic saline will reach the lower airways. (Anderson SD et al., 1995; Gonda I, 1986)

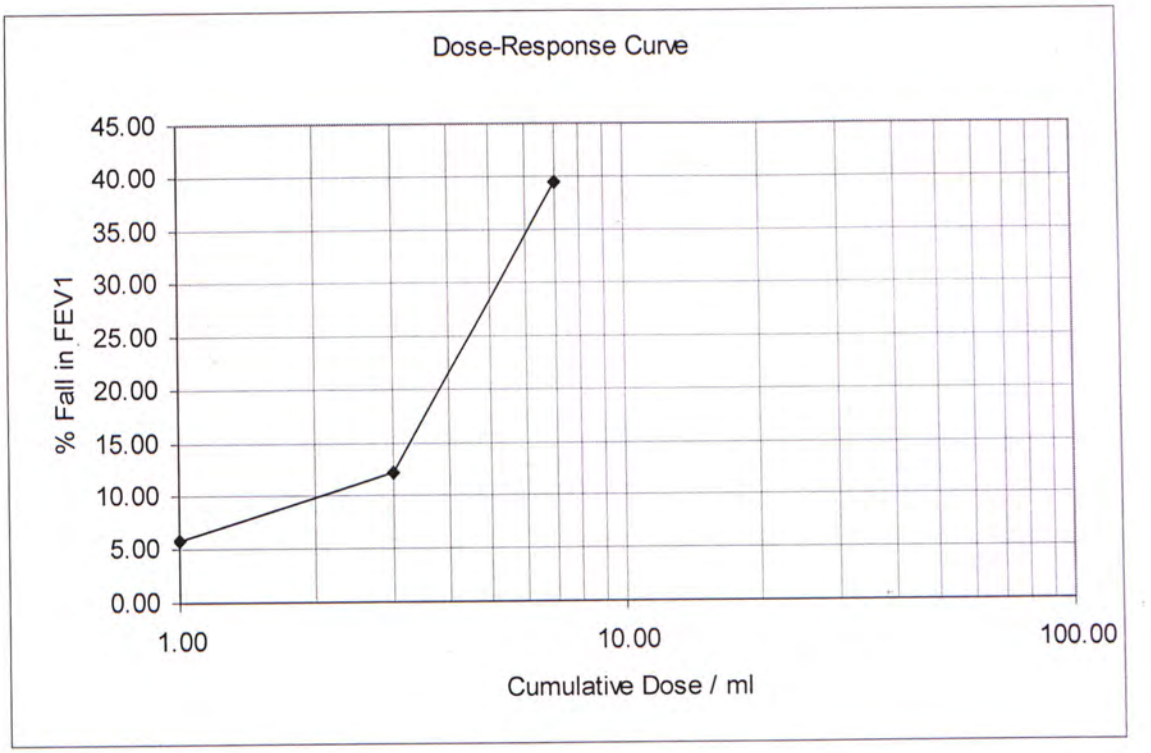


Fig. 3.10 Dose response curve in HSCSI

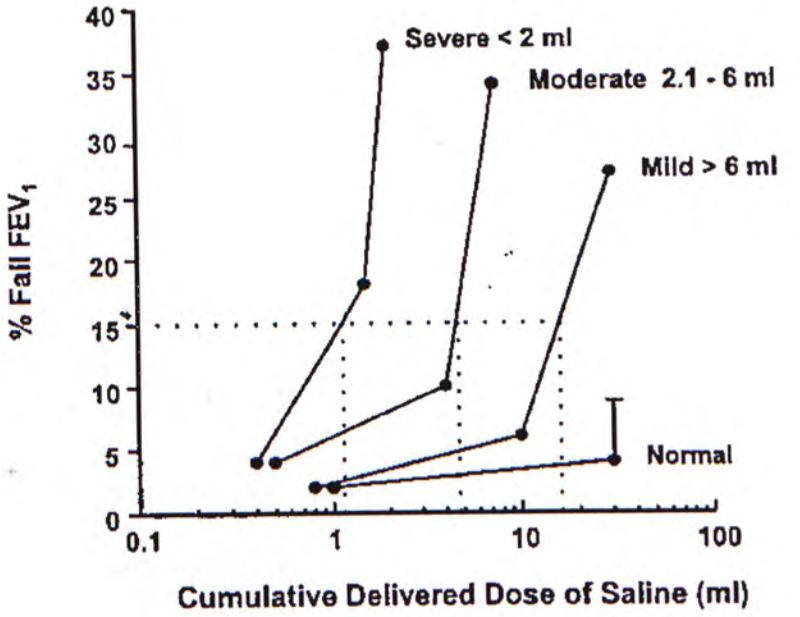


Fig. 3.11 Dose response curve in differentiating different severity of airway hyperresponsiveness

3.6 Safety issue in HSCSI

The hypertonic saline solution is best prepared by a trained person such as pharmacist. It is recommended that two people familiar with the standardized protocol, symptoms, signs of respiratory distress, and safety procedures, be in attendance or nearby.

If a subject appears to be distressed during the challenge, the ventilation is reduced, or the oxygen saturation is falling, the challenge should cease. Under these circumstances, an FEV_1 measurement is made immediately and a bronchodilator administered if required.

It is usual to administer a bronchodilator at the completion of study to anyone who has 10% or greater fall in FEV_1. A person should always stay with a subject after challenge in order to make FEV_1 measurements and to ensure recovery to baseline of lung function.

The protocol described here was suitable for mild or well-controlled asthmatics; those with severe disease often have reductions in FEV_1 of 40% or more, even when the pre-challenge lung function is normal. The situation can rapidly reverse after inhaling a bronchodilator.

If the subject is taking beta agonists either short acting or long acting on a daily basis; the dose required to reverse the induced changes in FEV_1 may be 2 to 3 times greater than the clinically recommended dose. (Hancox RJ et al., 2002)

The bronchodilator can be delivered using a large or small volume spacer device. In the cases of severe bronchoconstriction, oxygen should be administered together with bronchodilator. The equipment to make measurements and to administer a bronchodilator must be portable. Some form transportation should be available to allow rapid access to the nearest emergency department.

Fig. 3.12 Bronchodilator with volumatic spacer

3.7 Approach in our studies

HSCSI is an important tool in assessing airway hyperresponsiveness, especially in distinguishing mild, moderate and severe asthma. In our following studies, all of our subjects were clinically classified as stable and mild asthma and no severe asthma subjects recruited. Therefore, we concentrated on the interpretation of sputum differential cell counts instead of airway response as a technique in the management of childhood asthma and in our clinical studies only results from sputum induction are reported.

3.8 Sputum processing

Sputum obtained in hypertonic saline challenges and sputum induction is a variable mixture of tracheobronchial secretions, saliva and hypertonic saline. The contamination of saliva is considered as a problem as it can confound the interpretation of the results. In order to cope with this problem, the sputum plugs are generally selected to reduce contamination with upper respiratory tract material (although not all groups do this), and the cells dissociated from mucus using a reducing agent such as dithiothreitol (DTT). Good quality cytoslides can be routinely obtained and a reliable cell differential generated. (Fahy JV et al., 1993; Gibson PG et al., 1989; Pavord ID et al., 1997)

3.8.1 Sputum sample for processing

Some asthmatics, in particular those with acute exacerbations or severe current symptoms produce sputum spontaneously. (Schoonbrood DF et al., 1994; Hill AT et al., 1999) Spontaneously sputum contains similar percentages of inflammatory cells and mediators to induced sputum. However, cell viability in spontaneous sputum is considerably lower than that in induced sputum (Pizzichini MM et al., 1996; Bhowmik A et al., 1998) and the quality of samples is poorer, particularly in patients with more severe asthma (Bartoli ML et al., 2002). The reason is possible that a longer residence time of mucus secreted into the airways leads to reduced cell viability and a poorer distinction between different types of inflammatory cell.

Induced sputum obtained from hypertonic saline challenge is actually a variable mixture of tracheobronchial secretions, saliva and hypertonic saline. Saliva is a contaminant that can confound the interpretation of sputum results. There are two methods in dealing with the salivary contamination. They are the selected sputum method and the whole sputum method. Selected sputum method minimized salivary contamination by microscopic selection of viscid mucocellular portions of sputum for analysis. (Gibson PG et al., 1989; Pin I et al., 1992) In whole sputum method, the sample collected is analysed as a whole, ie sputum and saliva together; the salivary results are subtracted from the sputum results to give a "corrected cell-count". (Fahy JV et al., 1993) At present, there are no recommendations or guidelines to indicate which method should be used under what circumstances because insufficient comparative studies have been conducted. Both methods have been shown to be useful in assessing airway inflammation in adults; however, studies have shown that the selected sputum method may provide more viable cells, more eosinophils and a higher concentration of eosinophil cationic protein (ECP) (Spanevello A et al., 1998). Although there are no formal comparisons between two methods, the majority of studies in children have used the selected sputum method and only two studies have used the whole sputum method. (Grootendorst DC et al., 1999; JW et al., 1999) Therefore, we manually select viscid mucocellular portions of sputum for analysis in our protocol.

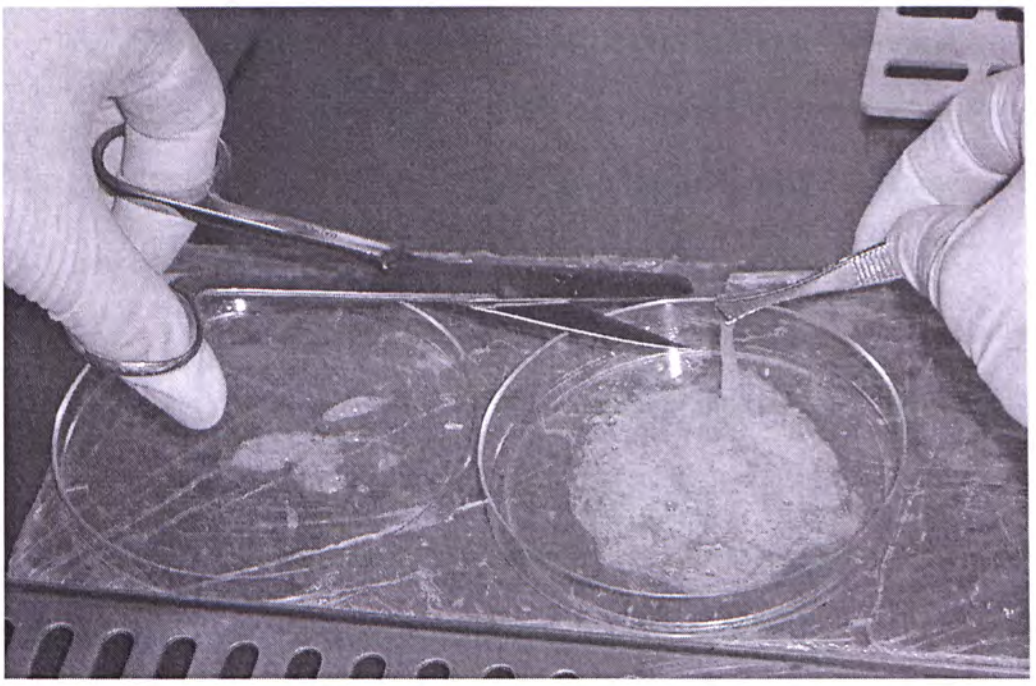


Fig. 3.13 Selected sputum method

3.8.2 Method for sputum processing

The sputum sample must be placed in ice after collected and dispersed within 2 hours at 4°C prior to cytocentrifugation to avoid cell and mediator degradation. (Pizzichini MM et al., 1996)

The examination of unprocessed smears has the disadvantage of sampling only a circumscribed part of the sputum. In addition, leucocytes may be entrapped in mucus and aggregated, making them difficult to identify. Dithiothreitol (DTT) is used to disperse sputum sample by breaking up disulfide bonds in the large mucin molecules which enable the release of cells and mediators from the mucus. Thus a monolayer of sputum cells is generated (Fleury-Feith J et al., 1987) and allowing the qualification and characterization of cells to be more reliable. (Pizzichini E et al., 1996)

DTT has been shown to be far superior for making high-quality cytospins, but it can interfere with the detection of soluble mediators by way of their effects on disulfide bonds, as is the case with chemokines. This makes soluble mediators susceptible to denaturation by DTT thus potentially altering epitopes and the immunological detection of these proteins. DTT appears not to affect detection of interleukin-5 and fibrinogen, but clearly increases the detectable levels of ECP and IgA, (Louis R et al., 1999) not because of cell activation but because of increased release from mucins. After the dispersion of sputum sample and cytocentrifugation, the cytoslides is stained and 400 non-squamous cells are counted.

Combining hypertonic saline challenge with sputum induction allows the assessment of bronchial hyper-responsiveness and degree of airway inflammation during the same procedure. In adult patients, hypertonic saline challenge has been shown to be sensitive, reliable and it correlates better with serum markers of inflammation than methacholine challenge. (Sont JK et al., 1993) Whether the same holds true for childhood asthma remains to be seen. Although combined hypertonic saline challenge with sputum induction provides a useful means to compare airway hyper-responsiveness and airway inflammation at the same time, it was reported that its success rate in obtaining adequate sputum allowing for the preparation of good quality cytoslides for differential cell count was lower than sputum induction alone. (Jones PD et al., 2001)

Sputum induction is performed using an inhalation of 4.5% hypertonic saline (HS) through a mouthpiece and large two-way non-rebreathing valve (Hans Rudolph 2700, Hans Rudolph Inc, Kansas City USA) connected to a DeVilbiss ultrasonic nebulizer set at the maximum output (6 litres/minute). The child is asked to rinse his mouth with water to clear debris and squamous epithelial cells. A nose-clip is worn and baseline forced expiratory volume in 1 second (FEV_1) is measured. Sputum induction is only carried out if the subject's FEV_1 is at least 65% predicted using local reference values. The child then inhales HS for a period of 30 seconds. Lung function is repeated 1 minute after the inhalation. If no sputum is obtained and lung function remains greater than 80% of the baseline value, the test continues. The child then continues inhalation of HS for periods of 1 min, 2 min, and then three periods of 4 min each. He is encouraged to cough up any sputum after each dose of HS and the sputum sample is collected in a specimen bottle, kept at 4°C, and processed within 2 hours. A record of any side effects experienced by the child undertaking the test and repeat measurements of FEV_1 are made at the end of each elapsed inhalation time period. The study concludes either when the child develops troublesome symptoms, when lung function drops below 80% of the baseline value, or if the child cannot be persuaded to complete the whole inhalation procedure. If the child has a greater than 20% drop in FEV_1, 500 mcg of salbutamol is administered using a metered-dose inhaler with a spacer, and recovery monitored. The subject is allowed to leave only when the FEV_1 returns to its baseline value. We will also stop the procedure if no sputum is obtained after 20 minutes of inhalation despite stable FEV_1. For sputum processing, our laboratory uses the selected sputum plug method as

described above. The volume of the selected sputum is measured and 0.1% dithiothreitol (DTT) (Sigma Chemicals, Poole, UK) is added to the sputum in a 4:1 ratio in order to break up the disulfide bonds and disperse the cells. The cell suspension is aspirated until homogenized and filtered to remove any remaining debris. Phosphate buffered saline is then added to the cell suspension. The non-squamous cell count and cell viability (with trypan blue) are determined in a haemocytometer. The cell suspension is centrifuged at 400G for 10min and cytospins are made and stained by May-Griunwald Giemsa stain. 400 non-squamous cells are counted and adequate sample is defined as having less than 50% squamous cell. The eosinophil count is then expressed as a percentage of total cell count.

3.8.3 Equipment and material for sputum processing

Equipment:

- Observation base plate (black)
- Inverted microscope
- 100µl Positive displacement Pipettes with tips
- Thermoline Shaking water bath 22°C
- Centrifuge
- Millipore nylon filter 60µl and filter holders
- Haemocytometer and cover slip
- Cytocentrifuge and its accessories

Material:

- Sputolysin (0.01g DTT + 10ml PBS)
- Trypan Blue
- Distilled water
- PBS
- Petri dish
- Glass slides
- Disposable Pipettes
- 15ml falcon tubes
- 2.5ml and 10ml syringes
- 1.5ml eppendorf tubes

3.8.4 Protocol for sputum processing using selected sputum method

1. Fill out the sputum worksheet details for visit number and collection details (include worksheet – Appendix I)
2. Invert sputum sample jar into a clean open petri dish in the hood
3. Use clean forceps to separate mucus clumps from saliva bubbles
4. Evaluate quality of sputum (volume, color and number of mucus clumps)

   Remarks:

   - Small white space 1.5mm x 3mm on observation base plate is the size of 1 mucus clumps
   - If it is uncertain whether the sample contains lower respiratory clumps, inspect sample in petri dish using inverted microscope and find the non-squamous cell areas
5. Use forceps to pull up all mucus clumps

6. Make Sputolysin 1:10 (1ml stock solution to 9ml distilled water in 15ml falcon tube) Label tube with date made and initials.

   Remark: The stock solution is stable for 48hours at 4 °C

7. Label 15ml falcon tube with patient Lab number
8. Pipette all suitable mucus clumps (minimum 100µl but <1 ml) into labeled 15ml tube by a positive displacement pipette. Record the actual volume of the mucus used on the worksheets
9. Add 4 times the volume of diluted Sputolysin to sample in tube, mix up and down with disposable pipette (e.g. 300µl sputum + 1.2ml diluted sputolysin)
10. Cap tube and place in shaking water bath at 22°C for 30 minutes to dissolve mucus
11. Add the same volume of PBS as Sputolysin (e.g. 300µl sputum + 1.2ml PBS) and mix with a disposable pipette and shake for further 5 minutes in water bath
12. Filter sputum and sputolysin mixture through nylon filter apparatus

    Procedure:

    - Wet filter with PBS prior to filtering sample to ensure no loss of sample during filtering.
    - Insert 2.5 / 5 / 10ml syringe (depending on volume to be filtered) into mixing cannula.
    - Use syringe to draw up sample then slowly filter sputum and sputolysin mixture through nylon mesh filter and down the side of the tube (1 drop per 1s)

13. Record the post-filtered volume on data sheet (used for total cell count, TCC)

14. Mix 20µl sample with 20µl Trypan Blue into a eppendorf tube
15. Pipette 20µl mixture onto the Haemocytometer
16. Use a light microscope to perform a Total Cell Count
17. Centrifuge the remaining cell suspension at 400G / 1600rpm for 10 minutes
18. Evenly aspirate the supernatant into eppendorf tubes labeled with a lab number and freeze immediately at –20 °C / –70 °C
19. Resuspend the pellet in a volume of PBS determined by the following equation:

    V2 = Total cell count/ml x Post Filtered Volume (V1)
20. Assemble Cytospin apparatus (slide, filter paper, sample bucket, and metal clip), making sure that the filter card circle lines up with the filter cup circle by observing the reverse side of the metal clip when assembled.
21. Using disposable pipette add required volume (V2) of PBS to dry cell pellet, mix up and down a few times to ensure sample is completely dispersed (sample now at 1 x 10^6 cells per ml)
22. Add 70µL of the sample to each cytospin sample bucket, and centrifuge sample (500 rpm for 5 minutes)
23. Carefully and quickly remove cytospins from apparatus, making sure to flip off the filter paper, air dry slides. Label with a lab number and the appropriate fixative, leaving spare slides labelled but unfixed.
24. If DTT is still in date, put in the fridge. (DTT lasts for two days after it has been made)

Remarks: Calculation:

Formula to calculate TCC and viability:

$$TCC = \frac{\text{inflammatory cell count}}{\text{number of quadrants}} \times 0.02$$

$$= x.xx \times 10^6/ml$$

$$\% \text{ Viability} = \frac{\text{number of live cells}}{\text{total number of cells}} \times 100$$

3.8.5 Equipment and Material for May Gruneald – Giemsa (MGG) staining

Equipment:

- Coplin Jars
- 100mm Glass Funnel
- Tripod
- 50 – 100mL Glass Measuring Cylinder
- 200mL Glass Beaker
- Filter Paper - Whatmans Grade 1 (DMB Cat no: F110)
- Tweezers
- Microscope
- Glass pipette
- Macropipette controller
- Aluminium or stainless steel tray
- Glass coverslips - 22 x 22mm (DMB Cat no: MHO)
- Glass screw top jar

Materials:

- May and Grunwald stain (Australian Scientific Cat no: 35025 4R)
- Giemsa (Australian Scientific Cat no: 35014 4M)
- Ethanol

- pH 6.8 Buffer Tablets (Australian Scientific Cat no: 33199)
- Distilled water
- Incohelp Pads (Oracle Cat no: M 12796)
- DPX (Australian Scientific Cat no: 36029 4H)
- Tissues
- Pasteur pipette (Uni store: Cat no: P250)
- 50mL Falcon Tubes (Uni store Cat no: T280)

3.8.6 Protocol for May Gruneald – Giemsa (MGG) staining

Before experiment:

1. Prepare the pH 6.8 buffer by dissolving 1 buffer tablet in 1000mL of distilled water
2. The Giemsa solution is made up by adding 5mL of Giemsa to 45mL of pH 6.8 buffer solution
3. The filter paper is folded in half four times
4. Place the funnel in the tripod, and the filter paper in the funnel, place this over a coplin jar
5. Pour the Giemsa solution into the funnel, which is positioned, over the glass coplin jar
6. Pour approximately 50mL of May and Grunwald into a glass coplin jar. This is stable for 3 months (Label with Expiry date)
7. Place tap water into water jars
8. Place 100% ethanol into fixative jars

Staining Procedure:

1. Place slides in 100% ethanol for 10 minutes
2. Move the slides from the ethanol to the coplin jar containing the May and Grunwald
3. Stain in May and Grunwald for 10 minutes
4. Move the slides to a coplin jar containing water and give slides a rinse to remove excess stain
5. Remove from the water and place in the Giemsa solution for 5 minutes
6. Wash in water twice, by placing the coplin jar under running tap water, then emptying the jar and repeating this step.
7. Wipe back of slides with Kimwipes (be careful not to wipe the wrong side, as this will remove the cells)
8. Allow slides to airdry
9. Add Coverslip to slides
    - Lay slides on bench, cell side up
    - Get out coverslips as required, and line them up with the slides
    - Put a drop of DPX on the coverslip using a pasteur pipette that has had about a centimeter cut off the end
    - Slowly lower the slide onto the coverslip ensuring that the mountant covers the cells with no air bubbles
10. Place back up the other way and let the mountant dry
11. The next day a differential count will be done

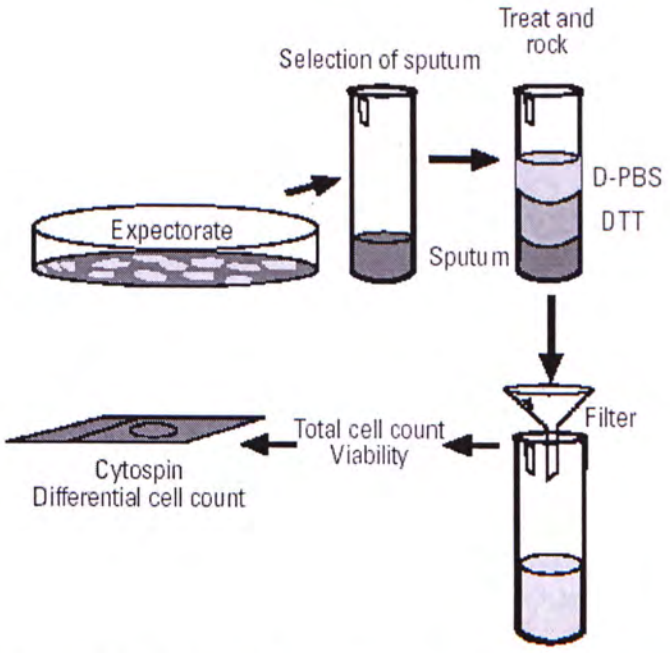


Fig. 3.14 Illustrated diagram for sputum processing. (Gibson PG et al, 2000)

D-PBS: Dulbecco's phosphate buffered saline;

DTT: dithiothreitol.

3.9 Interpretation of sputum differential cell counts

Differential cell counts on sputum cytopsins have good inter-observer consistency. (Boulet LP et al., 1987; Pin I, 1992; Popov T et al., 1994; Gelder CM et al., 1995; Pizzichini E et al., 1996) The repeatability of differential cell counts obtained on different days from clinically stable patients has been reported to be good. (Boulet LP et al., 1987; in 't Veen JC et al., 1996; Pizzichini E et al., 1996)

The normal ranges for sputum cell counts have been established by Cai Y et al. (Cai Y et al., 1998). The dominant cell in sputum from normal children is the macrophage, and the upper normal limit for sputum eosinophils in children is 2.5%.

Table 3.1 Sputum cell count for normal, atopic normal and nonatopic normal children (Cai Y et al., 1998)

	Normal	Atopic normal	Nonatopic normal
TCC 10^6 cells / ml	5.14 (1.2 – 9.08); 1.5 (0.8 – 3.9)	1.75 (0.89 – 2.6); 1.0 (0.55 – 2.15)	8.04 (0.63 – 15.5); 1.8 (1.05 – 6)
Eosinophils %	1.57 (0.62 – 2.52); 0.3(0 – 1.05)	2.16 (0.83 – 3.48); 0.5 (0 – 2.8)	1.13 (0 – 2.54); 0 (0 – 0.6)
Mast Cells %	0.024 (0 – 0.05); 0 (0 – 0)	0.03 (0 – 0.07); 0 (0 – 0)	0.02 (0 – 0.06); 0 (0 – 0)

Data are presented as mean (95% confidence interval); median (interquartile range). TCC: total cell count

Table 3.2 Sputum cell counts in normal subjects (NC), controlled asthma (CA) on inhaled corticosteroid, symptomatic asthma (SA) and those with exacerbation of asthma (EA) (Cai Y et al., 1998)

	NC N = 72	CA N = 15	SA N = 16	EA N = 11
TCC 10^6 cells / ml	1.5 (0.8, 3.9)	1.9 (1.0, 7.5)	1.7 (0.9, 4.1)	2.5 (1.6, 5.4)
Eosinophils %	0.3 (0, 1.05)$^+$	2.5 (1.5, 0.75)	3.8 (2.4, 15.1)	8.5 (1.5, 20.0)
Neutrophils %	35 (12.0, 88.0)	46.5 (29.5, 58.5)	47.0 (24.8, 57.8)	27.0 (22.5, 42.0)
Epithelial cell %	1.5 (0.8, 3.0)	10.5 (5.0, 17.5)	11.5 (5.5, 21.3)	18.0 (6.0, 28.0)

Values are presented as median and interquartile range, or as absolute number or percentage.$^+$:p=0.0005, using Kruskal Wallis test. N = 47 for total cell count (TCC) from normal subjects.

Mast cells are seldom seen in the sputum of healthy children (Pin I et al., 1992; Cai Y et al., 1998; Gibson PG et al., 1998). They may be infrequently seen in sputum from children with asthma, where their levels are correlated with airway responsiveness to 4.5% saline. (Gibson PG et al., 1998)

Shed epithelial cells can be detected in sputum in increased numbers in unstable asthma. (Cai Y et al., 1998) When asthma control improves with allergen avoidance, epithelial desquamation is reduced. (Piacentini GL et al., 1998)

Sputum eosinophil counts may reflect different severity of childhood asthma. Elevated level of sputum eosinophil counts are seen in exacerbations and acute asthma (Twaddell SH et al., 1996; Cai Y et al., 1998; Oh JW et al., 1999; Gibson PG et al., 1999; Norzila M et al., 2000) Sputum eosinophil cell counts decreases after corticosteroid therapy. (Oh JW et al., 1999) Sputum eosinophils correlate well with other objective markers of airway inflammation. Studies comparing bronchial wash and bronchoalveolar lavage (BAL) to induced sputum in adults show that there is good agreement between the type of cells recovered by sputum and bronchoscopic samples. (Grootendorst DC et al., 1997; Pizzichini E et al., 1998) Selected sputum is more concentrated than BAL fluid, having a higher density of recovered cells and higher levels of fluid-phase markers (Pizzichini E et al., 1998).

Sputum eosinophilia has a sensitivity of 70% and specificity of 90% in differentiation of asthma subjects from normal control when compared to serum ECP level, which has a sensitivity of 50% and specificity of 50% (Pizzichini E et al., 1997). In children, sputum ECP level was not correlated with serum ECP level (Piacentini GL et al., 1999) and the improvement in clinical asthma with inhaled corticosteroids was associated with a fall in sputum ECP level but no change in serum ECP level. (Sorva R et al., 1997) Higher levels of induced sputum eosinophils are associated with greater airflow obstruction as reflected by a reduced forced expiratory volume in one second (FEV_1) (Twaddell SH et al., 1996; Sorva R et al., 1997; Grootendorst DC, et al., 1999; Timmins N et al., 2000). The same association is reported in severe asthma, in which sputum eosinophil counts

correlated with the degree of airflow obstruction in children attending the emergency department with severe asthma (Piacentini GL et al., 1998).

Degree of sputum eosinophilia is also associated with the degree of airway responsiveness, (Gibson PG et al., 1998; Piacentini GL et al., 1998; Pin I et al., 1993) and eosinophil numbers are reduced with allergen avoidance. (Piacentini GL et al., 1996; Lonnkvist K et al., 1999) These data suggest that direct measures of airway inflammation using selected sputum may be more useful than indirect measures in childhood asthma.

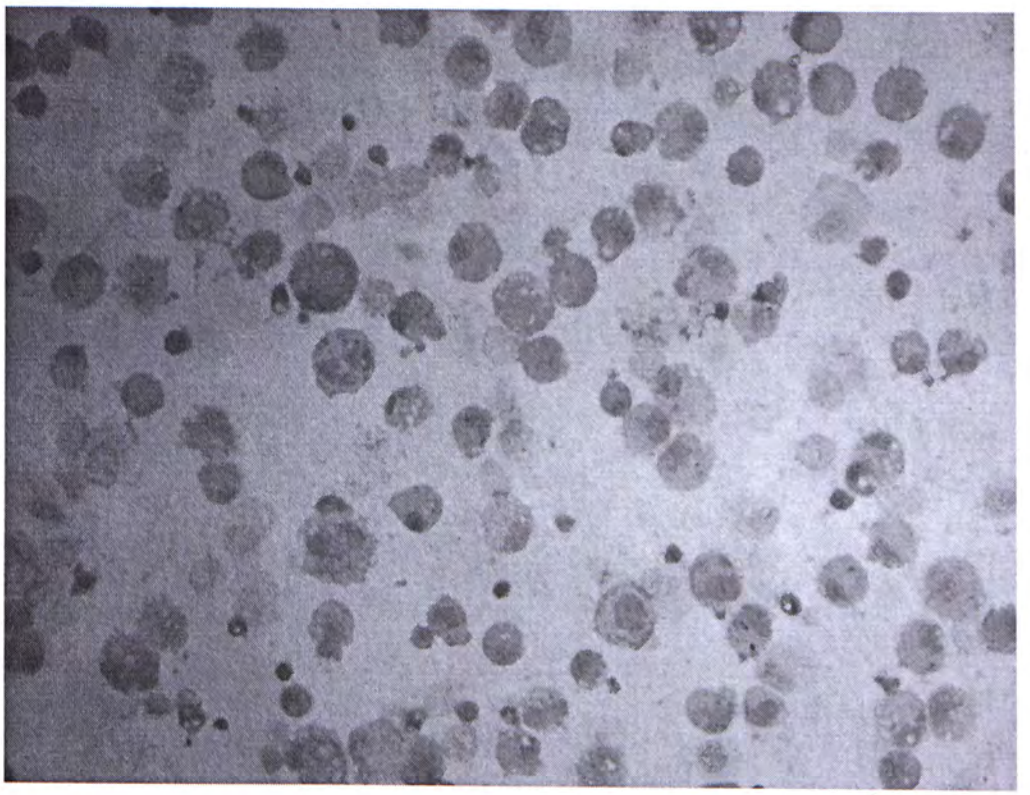


Fig. 3.15 High quality cytospin slide for differential cell counts. 400 non-squamous cells are counted and the result is expressed as percentage eosinophilia.

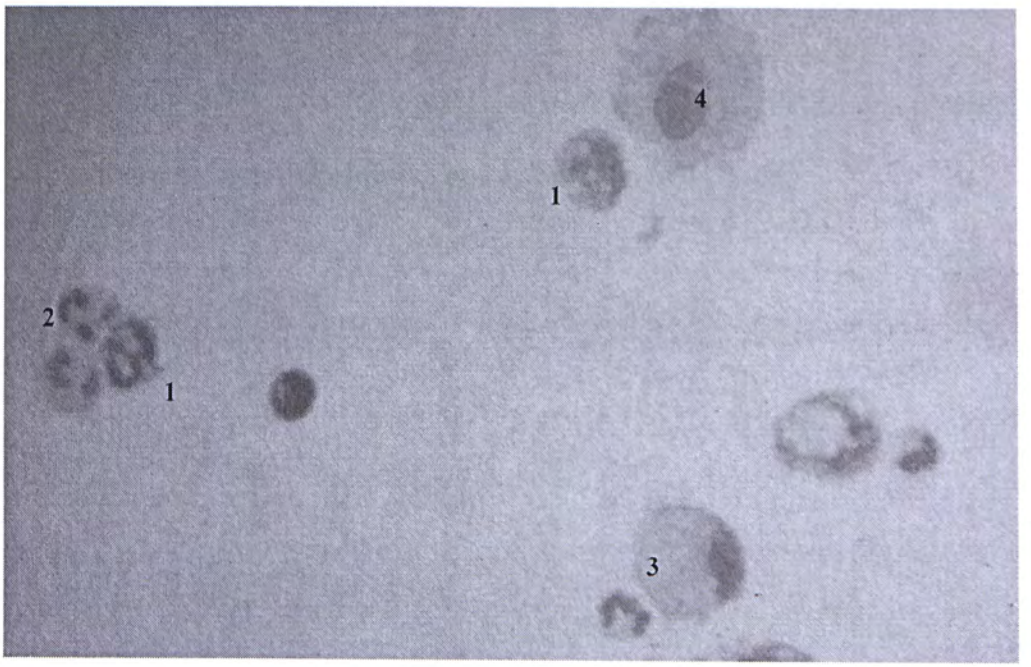


Fig. 3.16 Differential cell counts in cytoslide for eosinophil (1); neutrophil (2); macrophage(3); epithelial cell (4)

3.10 Conclusion

In this chapter, we have reviewed methodology of HSCSI and protocol for sputum processing, staining and differential cell counts.

Chapter 4 Factors predicting successful sputum induction

4.1 Introduction

Asthma is characterized by variable airway obstruction, airway hyper-responsiveness and influx of inflammatory cells, especially eosinophils, into the bronchial mucosa. (Wardlaw AJ wt al., 2000) It is a common disease that can cause much morbidity and mortality. (Campbell MJ et al., 1997; Janson C et al., 1997; Dente FL et al., 2004) Management decisions in childhood asthma have traditionally been based on assessment of symptoms, results of peak expiratory flow rate (PEFR) or simple spirometry and the frequency of use of rescue medication. However, particular in mild childhood asthma, PEFR and spirometry have their limitations; as abnormal airway physiology is often absent. (Clancy K, 2004; Enright PL et al., 1994; Hunter CJ et al., 2002) In addition, these measurements do not correlate closely to the underlying eosinophilic airway inflammation, which is a predictor of asthmatic exacerbation and precursor of airway remodeling. (Crimi E et al., 1998) Up to 80% of corticosteroid-naïve subjects (Green RH et al., 2002; Pizzichini E et al., 1996; Silkoff PE et al., 2004) and more than 50% of corticosteroid treated subjects (Pavord ID et al., 1997) with concurrent symptoms have sputum eosinophil counts outside the normal range yet their airway physiology may remain normal. Thus the monitoring of sputum eosinophil count may allow better asthma control and provide a useful guide in management. Recent evidence suggests that a treatment strategy directed at normalization of airway eosinophil count reduces asthma exacerbations and hospitalization. (Gibson PG et al., 2000) Current research has also highlighted the potential use of sputum

induction as a non-invasive method in assessing airway inflammation. (Louis R et al., 2000)

The successful rate of sputum induction in children with asthma has been reported to vary from 68 – 100%. (Gibson PG et al., 2000) In young children the procedure is limited by the poor spirometric technique and low tidal volume that can be generated by the subject, and that limits the dose of saline delivered. (Riedler J., et al., 1994) In most study series utilizing sputum induction, the procedure is well tolerated but side effects including cough, bronchospasm, vomiting and anxiety have been reported. (Pizzichini E et al., 2002; Covar RA et al., 2004; Rytila P et al., 2004) It will therefore be clinically useful to have predictors that are associated with successful sputum induction so that children unlikely to produce an adequate sputum sample are not subjected to such procedure. In this study, we aimed to find out predictive factors for successful sputum induction in children with stable asthma.

4.2 Methods

4.2.1. Patient selection

All children with stable asthma attending the Paediatric Chest Clinic of the Prince of Wales Hospital between the period October 2003 and December 2004 were recruited. The ages of the children were between 6 and 18 years and all were able to cooperate with the tests. The diagnosis of asthma was made on standard grounds. (Warner JO, 1992) In addition, we defined stable asthmatics as those with (i) no asthmatic exacerbation for the preceding 4 weeks necessitating oral prednisolone or an increased use of inhaled corticosteroids, (ii) the use of rescue treatment no more than three times a week, and (iii) no clinical indication for change in treatment medication. We excluded from our study children who had other concomitant non-asthmatic chronic airway diseases such as bronchiectasis; those who used any prescription or over-the-counter medication that may affect the course of asthma or its treatment (such as Traditional Chinese Medicines); and children who were currently involved in any other asthma treatment trial. We obtained approval for the study from the Ethics Committee of The Chinese University of Hong Kong, and the children's parents or guardians gave written informed consent.

4.2.2 Study design

This was a prospective observational study. A detailed history was taken and thorough physical examination performed on each child. Weight and height were recorded using standardized equipments. The recruited subjects were required to undergo the following assessments as part of their asthma

management.

(1) Asthma severity by visual analogue score, a subjective score from 0 to 10 (0 = asymptomatic, 10 = severe disabling asthma) was obtained from the subjects. Similar visual analogue score to assess patients' daytime and night-time cough severity (0 = no cough, 10 = severe disabling cough) and how asthma and cough were affecting the subjects' well being and daily activity were also asked. (0=poor quality and 10=good quality).

(2) Skin prick test to five groups of aero-allergens (house dust mite, cat and dog dander, cockroaches, grass and tree pollens and mixed moulds) was done according to standard procedures, using purified allergen extracts. A child was considered atopic if he had at least one skin test result that showed an induration with a diameter of at least 2 mm greater than the negative (normal saline) control.

(3) Measurement of exhaled nitric oxide (eNO) using a chemilumiscence analyzer (NOA280i, Sievers Instruments, Boulder, CO, USA), sensitive to NO from 1 ppb to 200 ppm and with a resolution of 1 ppb and accuracy of ±1 ppb, according to the American Thoracic Society guidelines. The subject were comfortably seated without nose clip, inhaled NO-free air from a reservoir and subsequently exhaled against a resistor. The flow rate was set at 50 ml/s. This on-line measurement was taken in triplicate and the average recorded.

(4) Spirometry (Spirolab II, MIR, Italy) using standard technique measuring forced expiratory volume in the first second (FEV_1) and forced vital capacity (FVC). The obtained best of three efforts was compared with local

age- and sex-matched reference values. (Ip MS et al., 2000)

(5) Sputum Induction. Hypertonic saline (4.5% HS) was used and sputum induction carried out using the standard technique. (Smith CM & Anderson SD, 1990; Fahy JV et al., 2001) The test was only performed if the child's FEV_1 was at least 65% predicted. The procedure was explained to the child, who would rinse his or her mouth with water to clear debris and squamous epithelial cells. The best of three FEV_1 obtained from spirometry was used as the baseline value. A nose-clip was worn and the child began inhalation of 4.5% HS for a period of 30 s. Spirometry was repeated 1 minute after the inhalation period. If no sputum was obtained and FEV_1 was greater than 80% of the baseline value, the test continued. The child would then continue inhalation of HS for periods of 1 min, 2 min, and then three periods of 4 min each. FEV_1 was repeated after each inhalation period. The child was encouraged to cough up any sputum after each dose of HS and the sputum was collected into a polypropylene tube. A record of any side effects experienced by the child undertaking the test was made at the end of each elapsed time period. The study concluded either when the child developed troublesome symptoms, when lung function had dropped below 80% of the baseline value, or if the child could not be persuaded to complete the whole inhalation procedure. If the child had a greater than 20% fall in FEV_1, 500 mcg salbutamol were administered using a metered-dose inhaler and spacer, and recovery monitored. The sputum sample was processed within 4 hours. The sputum was poured into a Petri dish and the viscid mucocellular portions selected. After treatment with

0.1% Dithiothreitol (DTT) (Sigma Chemicals, Poole, UK) and Phosphate buffered saline (PBS), the cell suspension was aspirated until homogenized and subsequently filtered with a 48 mm nylon gauge. The cell suspension obtained was then centrifuged at 400G for 10 minutes. Cytospin samples were prepared and stained by May-Griunwald Giemsa stain prior to performing a differential cell count by an individual who was unaware of the clinical data of the subjects. Specimens containing squamous epithelial cells of less than 50% of the total inflammatory cell number were considered adequate, ie successful induction. At least 400 inflammatory cells were counted for each specimen. Eosinophil count was expressed as percentage of total cell count.

4.2.3 Statistical analysis

Descriptive statistics were used to summarize the baseline characteristics of the patients and the results were expressed as median with interquartile ranges. Potential predictors were first evaluated individually by chi-square test and Mann-Whitney test for association with successful sputum induction. Predictors with $p < 0.25$ were then analyzed by multivariate logistic regression analysis, using a forward stepwise selection strategy. When two or more potential predictors were highly correlated, the predictor that was clinically important was selected for entry. P value of < 0.05 was considered significant. SPSS version 11.5 for Windows was utilized for all statistical analysis.

4.3 Results

One hundred and thirty subjects with stable asthma were recruited. The median age was 11 years [IQR: 5]. There were 93 boys and 37 girls. All subjects by definition were atopic and 120 of them (92.3%) had positive skin prick test to more than one aeroallergen. 67 patients (51.5%) were using inhaled corticosteroids and the median beclomethasone equivalent dosage was 125 mcg (range 50mcg – 500mcg, assuming budesonide has equal potency and fluticasone is twice as potent as beclomethasone). 55 patients (42.3%) were using long-acting beta agonists and 8 patients (6%) were taking a regular leukotriene receptor antagonist. Asthma was well controlled in all patients and the baseline asthma severity score was low with a median of 0.7. All except two of the subjects had normal percent predicted FEV_1 (> 80%). The two cases had moderate obstructive deficit demonstrated on spirometry, and their predicted FEV_1 were 63% and 57%. The median eNO was 48.95 ppb (range from 4.8ppb – 190ppb). The demographic characteristics of the subjects are shown in Table 4.1.

Sputum induction was well tolerated by all subjects. Sore throat was the most common complaint after the procedure, occurring in 20 subjects (15%), followed by chest discomfort, affecting 8 subjects (6%). The procedure was prematurely terminated in three cases, in two because of vomiting and in the remaining one because of the unpleasant taste of 4.5% HS. None of the subjects developed significant clinical bronchospasm during the procedure. 93 subjects (74.5%) were able to produce an adequate sputum sample.

The severity of asthma assessed by spirometry, use of second line asthma medications (long-acting beta agonists and leukotriene receptor antagonist) and age were not associated with successful sputum induction. Using multivariate analysis, only eNO was found to be a significant predictor ($p = 0.022$ Mann-Whitney U test) (Table 4.2). Area under receiver operator characteristics (ROC) curves showing sensitivity and 1-specificity was 0.634 (Table 4.3).

	Male	Female	Total
Male : female	93	37	93:37
Height	144.0 (30.5)	144.7 (22.5)	144.35 (26.5)
Age, year	11 (5.0)	10 (10.0)	11 (5)
FEV1	1.97 (1.3)	1.83 (0.8)	1.94 (1.2)
% FEV1	92.27% (17%)	86.36% (16%)	90.28% (17%)
eNO, ppb	50.1 (63.7)	47.8 (58.05)	48.95 (61.37)
Child score (Asthma)	9.5 (1.5)	9.6 (1.3)	9.5 (1.4)
Severity of asthma score	0.7 (1.9)	0.6 (3.0)	0.7 (2.0)
Child score (Cough)	9.5 (2.0)	9.5 (2.0)	9.5 (2.0)
Day time cough score	0.3 (1.25)	0.3 (0.9)	0.3 (1.2)
Night time cough score	0.3 (1.45)	0.2 (0.8)	0.3 (1.2)

Table 4.1 Demographic characteristics of subjects – Median (IQR)

4.4 Discussion

In this study involving children with stable asthma, we found that the procedure of sputum induction was well tolerated by all participants. Exhaled nitric oxide but not age, was a significant predictive factor for successful induction.

It is important to objectively measure airway inflammation as this can play a crucial part in the diagnosis and management of asthma in children and adults alike. Inhaled corticosteroids are the treatment of choice for eosinophilic airway inflammation. By assessing the degree of airway inflammation and targeting treatment in relation to response, we could prevent children from being exposed to unnecessarily high doses of inhaled corticosteroids and as a result suffer from their potential side effects. (Drake AJ et al., 2002) In this context, sputum induction provides a well-tolerated, safe and non-invasive method for the examination of the extent of airway inflammation in asthmatic patients. (Gibson PG et al., 2000 & Covar RA et al., 2004) Among various cellular and fluid phase markers found in sputum, sputum eosinophilia is well validated as a marker of airway inflammation. (Gibson PG et al., 2000) Cai *et al.* have established the reference range of sputum eosinophil count for both normal and asthmatic children: the upper limit normal was found to be 2.5%. (Cai Y et al., 1998) Sputum eosinophil counts have also been found to have good correlation with asthma severity. (Gibson PG et al., 2000 & Louis R et al., 2000) Green *et al.* found that higher levels of sputum eosinophils correlated with greater airway obstruction and that the increase in sputum eosinophils can

be considered as a predictor for asthma exacerbations. (Green RH et al., 2002) In the same study, the authors found that the extent of control of airway inflammation and asthma exacerbation was better in the sputum management group, which targeted at normalizing airway eosinophilia, than in the traditional symptom based management group. There is accumulating evidence to suggest that sputum examination is a reliable and clinically useful method in the management of asthma, and this data is also extending to the paediatric population. (Gibson PG et al., 2000; Louis R et al., 2000; Covar RA et al., 2004; Lex C et al., 2005)

Our success rate in obtaining an adequate sputum sample is in the range of 70 to 80% which is similar to that reported in the literature and reiterates the feasibility of this procedure in children with asthma. (Gibson PG et al., 2000) Tolerability of the procedure has also been demonstrated in children with severe asthma. (Lex C et al., 2005) Although significant adverse effects associated with sputum induction were rare, the procedure can be unpleasant. Furthermore complications such as bronchospasm, vomiting, sore throat and chest discomfort have all been reported. (Pizzichini E et al., 2002; Covar RA et al., 2004; Lex C et al., 2005) In our current study, the procedure was terminated prematurely on three occasions (2.3%). Fifteen percent of our subjects complained of sore throat and up to six percent had chest discomfort after the procedure. In the study by Lex *et al*, seven of 38 children complained of dyspnoea and/or wheeze during the induction and five (13%) had to terminate the procedure prematurely. (Lex C et al., 2005) It will therefore be useful to

have clinical parameters associated with successful induction so that children who do not satisfy such criteria are not subjected to the procedure. In the study by Lex *et al.*, sputum induction was successful in 28 out of 38 children (74%) with difficult asthma. 50% of those less than 12 years of age were able to produce an adequate sample, compared to 88% of those who were older than 12 years (Fisher's exact test, P=0.02). (Lex C et al., 2005) The authors suggested that age was an important predictor for successful sputum induction. By comparison, in our experience, the success of sputum induction instead appears to depend on the enthusiasm of the technician, degree of participation and cooperation and understanding of the procedure by the children involved. Although sputum induction in younger children often requires more demonstrations and explanations than older children, we did not find age to be an important predictive factor for success. Recently, a group reported success rate of 67% in obtaining adequate sputum on induction in children aged 5 years. (Rytila P et al., 2004)

Exhaled nitric oxide was found to be a significant predictor for successful sputum induction in the current study. It has been reported that the magnitude of eNO was increased in proportion to bronchial wall inflammation or induced-sputum eosinophilia as well as to airway hyperresponsiveness. (Gibson PG et al., 2000; Fahy JV et al., 2001; Covar RA et al., 2004; Lex C et al., 2005,) The raised eNO may simply reflect an actively inflamed airway that is edematous and packed with numerous inflammatory cells and cytokines. With such inflammation, there are theoretically larger volumes of secretions, making

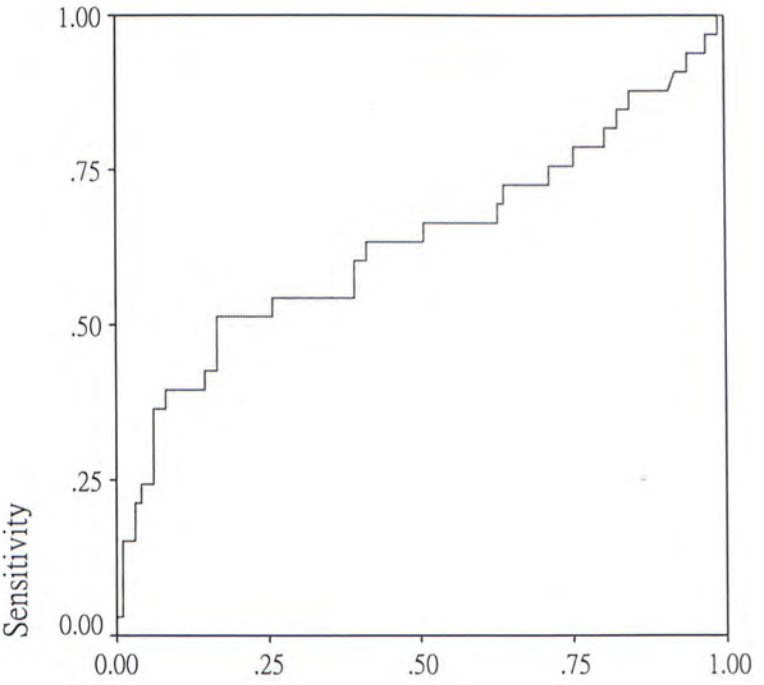
it easier for us to obtain a sputum sample. It would be interesting to investigate whether sputum is as likely to be induced in the same subject once the underlying airway inflammation has been better controlled, i.e. reduced eNO levels. In a study involving children with mild asthma, the success rate for sputum induction fell from 82% to 68% after the initiation of inhaled corticosteroids. (Rytila P et al., 2004) It was observed in the study by Lex *et al.* that 33 out of 38 children had elevated eNO (> 23ppb) and of the 33 children, 28 were successful in sputum induction to produce an adequate sample.[16] If we had used a cut-off of 23 ppb, 73 out of 98 children would have had a successful induction. The corresponding sensitivity, specificity, positive predictive and negative predictive values were 75.3%, 24.2%, 74.5% and 25.0% respectively. Despite acceptable sensitivity, the low specificity and negative predictive value would limit the clinical usefulness of this eNO cutoff value.

There are certain limitations to our study. Firstly, the present study did not involve subjects with severe asthma. All except two of our study subjects had normal percent predicted FEV_1 at baseline. We therefore are not in a position to comment on the applicability of our results to patients with severe asthma. However, in our clinical practice, severe asthma constitutes only a small proportion of our asthma workload and most patients belong to the category of asthma subgroup similar to our study population. Therefore, the result generated from this study is still applicable to our daily practice. Secondly, as the area under ROC curve was too small, we were unable to establish a reliable cutoff with acceptable sensitivity and specificity. Larger sample size would be

needed to derive more accurate results. Lastly, we did not include patients with other respiratory diseases such as chronic cough, bronchiectasis, or pulmonary tuberculosis. The predictive factors for sputum induction in different conditions might be different.

	Mann-Whitney U	Wilcoxon W	Z	Asymp. Sig. (2-tailed)
AGE	1421.000	1982.000	-.966	0.334
HEIGHT	1398.500	1959.500	-1.081	0.280
FEV1	1246.500	1807.500	-1.894	0.058
FVC	1252.000	1813.000	-1.864	0.062
FEV1 - Predicted	1400.500	1961.500	-1.070	0.285
FVC - Predicted	1386.500	1947.500	-1.145	0.252
% FEV1	1287.000	1848.000	-1.677	0.094
% FVC	1232.000	1793.000	-1.971	0.049
FEV1/FVC	1571.000	6324.000	-.158	0.875
eNO	1172.500	5925.500	-2.290	0.022*
Child Score (Asthma)	1552.000	6305.000	-.262	0.793
Asthma Severity Score	1586.000	6339.000	-.078	0.938
Child Score (Cough)	1433.000	6186.000	-.907	0.364

Table 4.2 Mann-Whitney Test Result



ENO (ppb)	Sensitivity	Specificity	Positive predictive value	Negative predictive value
10	93.8%	9.1%	75.2%	33.3%
15	86.6%	12.1%	74.1%	22.2%
20	77.3%	21.2%	74.3%	24.1%
23	75.3%	24.2%	74.5%	25.0%
40	55.7%	33.3%	71.1%	20.4%
60	36.1%	45.5%	66.0%	19.5%

Table 4.3 Area under Receiver operator characteristics (ROC) Curves for eNO in predicting successful sputum induction

4.5 Conclusion

In this study, we have demonstrated that sputum induction was feasible in children with asthma and a success rate of around 80% was reported. The majority of our patients tolerated the induction process well and no significant adverse effects were documented during the procedure. We have also identified exhaled nitric oxide, not age as a predictive factor for successful sputum induction. This could be clinically relevant to practicing clinicians who use this technique in their management of asthma, so that children unlikely to produce an adequate sample are not subjected to this procedure. However, more studies are needed to verify our results and derive reliable cutoff predictive values.

Chapter 5 Use of once-daily fluticasone propionate in children with stable asthma – study on airway inflammatory markers

5.1 Introduction

Asthma is primarily an inflammatory disorder of the airways and the most appropriate clinical management is to focus on preventing or alleviating the inflammation. Inhaled corticosteroids are effective treatment since they reduce airway inflammation, decrease bronchial hyper-reactivity and improve the long-term prognosis of asthma. (Haahtela T et al., 1991; Geddes DM, 1992) But ineffective asthma management resulting from poor adherence to prescribed treatment is common, and non-compliance rates have been reported to range from 30 to 70%. (Mawhinney H et al., 1991; Bender B et al., 1997; Apter AJ et al., 1998) A low rate of adherence to inhaled corticosteroid use is associated with more frequent asthma exacerbations in children. (Milgrom H et al., 1996) A simplified treatment plan that includes fewer medication administrations per day may improve adherence (Tashkin DP, 1995) and an attractive regimen is the use of once-daily medication.

Fluticasone propionate (FP) is a potent inhaled corticosteroid with negligible oral systemic bioavailability due to incomplete absorption and rapid first-pass metabolism. (Harding SM, 1990) It has also been shown to have high lipophilicity and affinity for the glucocorticoid receptor, a prolonged absorption phase, and an increased uptake and retention in the lungs. (Hogger P & Rohdewald P, 1994; Derendorf H et al., 1998) A human in vivo study demonstrated that FP had longer retention and a 100-fold greater concentration

in peripheral lung tissues compared with serum. (Esmailpour N et al., 1997) These properties, which help in maintaining asthma control at low doses while minimizing systemic effects, are ideal for reducing the dose frequency. But the results of trials assessing the efficacy of once-daily FP have been mixed. (Ayres JG et al., 1995; Boulet LP et al., 2000; LaForce CF et al., 2000; Nathan RA et al., 2000; Wolfe J et al., 2000; ZuWallack R et al., 2000; Berger WE et al., 2002)

Results from an 8-week, double-blind trial involving 328 children with stable, mild-to-moderate asthma demonstrated that once-daily dose of 100mcg FP powder administered via accuhaler in the evening was as effective as a twice-daily dose of 50mcg. (Hodges I & Netherway T, 2005) The main outcome measures in this study were asthma exacerbation and lung function. Whether improvements in these parameters translate to reduction in airway inflammation is unknown. In two studies involving children (LaForce CF et al., 2000; ZuWallack R et al., 2000), twice-daily dosing of FP was found to provide significantly greater improvement in forced expiratory volume in the first second (FEV_1), reduction in the frequency of bronchodilator usage, and fewer subjects withdrew due to lack of efficacy when compared to the once-daily dosing. A recent review article concluded that once-daily administration of FP is not currently recommended. (Purucker ME et al., 2003)

In this study, we assessed whether in patients with stable asthma, once-daily FP was as effective as twice-daily in the control of airway inflammation as assessed by exhaled nitric oxide (eNO) and eosinophil count in induced sputum. We hypothesized that the once-daily regimen had similar efficacy as the twice-daily regimen in maintaining or reducing airway inflammation.

5.2 Methods

5.2.1 Patient selection

All children with stable asthma requiring twice-daily use of inhaled FP for 3 months immediately prior to the study were recruited from the Paediatric Chest Clinic of the Prince of Wales Hospital. The age of the children was between 7 and 18 years and all were able to cooperate with the tests. The diagnosis of asthma was made on standard grounds. (American Thoracic Society, 1987) We defined stable asthma as no asthmatic exacerbation for the preceding 4 weeks necessitating oral prednisolone or an increased use of inhaled corticosteroids, the use of rescue treatment no more than three times a week, and with no clinical indication for change in treatment medication. Children who had other concomitant non-asthmatic chronic airway diseases such as bronchiectasis; those who used any prescription or over-the-counter medication which might have affected the course of asthma or its treatment; and children who were currently involved in any other asthma treatment trial were excluded. We had obtained approval for the study from the Ethics Committee of the Chinese University of Hong Kong, and written informed consent were given to the children's parents or guardians

5.2.2 Study design

This was a prospective observational study. The recruited subjects were required to attend an asthma education session organized by a specialist asthma nurse before the start of the study. During the session, correct use of inhalers was checked and recording in the asthma diary was taught. The subjects were then entered into a run-in period that lasted for two weeks with continuous diary card recordings in the last 7 days. Criteria for acceptable asthma stability during the last 7 days of the run-in period included no more than 3 days of more than 3 times use of bronchodilator medication per day and no more than one night with awakenings resulting from asthma that required treatment with bronchodilator.

The two-week run-in period was to confirm adequate asthma control on the current dosage of FP and it was followed by 8 weeks when the subjects were given the same dosage of FP to be taken once-daily in the evening (for example, FP 125 micrograms twice daily was changed to FP 250 micrograms daily). The same inhaler device was used. The subjects were given rescue medication for use as required, in the form of metered dose inhaler ventolin with or without a spacer. No other bronchodilator or anti-inflammatory medications were permitted during the study. Nasal corticosteroids and antihistamines were allowed if required. The subjects were then required to return for repeat assessment after 8 weeks of once-daily FP. The following parameters were assessed at baseline after the run-in and after 8 weeks of once-daily FP:

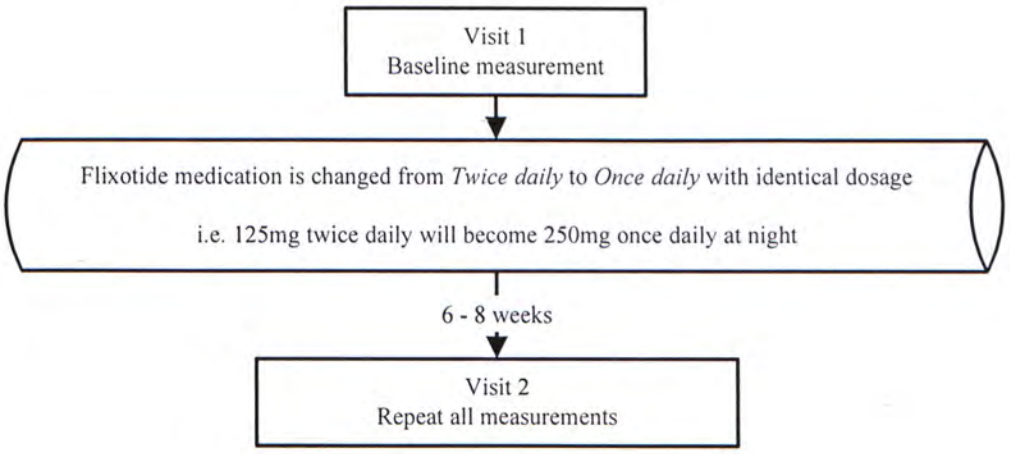


Fig. 5.1 Study Plan

(1) Patients had to keep daily diary cards and to record use of FP, use of bronchodilator, daytime and night-time asthma symptoms (cough, wheeze) and sleep disturbance. Asthma severity by visual analogue score, a subjective score from 0 to 10 (0 = asymptomatic, 10 = severe disabling asthma) was obtained at the start and end of the study (start = after the run-in period, end = 8 weeks after the subjects had been on once- daily FP) from the patients. Similar visual analogue scores to assess (a) patients' preference of dosing regimen (0 = least preferred, 10 = most preferred), and (b) patients' daytime and nighttime cough severity (0 = no cough, 10 = severe disabling cough) were also obtained from the subjects.

(2) eNO was measured by a rapid-response chemiluminescent method using the Sievers 280i NOA analyzer (Sievers, Boulder, CO, USA), with sensitivity from 1 ppb to 200 ppm, and a resolution of 1 ppb and accuracy of ±1 ppb, according to the American Thoracic Society guidelines.

(American Thoracic Society, 1999) The subject were comfortably seated without nose clip, inhaled NO-free air from a reservoir and subsequently exhaled against a resistor. The flow rate was set at 50 ml/s. This on-line measurement was taken in triplicate and the average recorded.

(3) Spirometry (Spirolab II, MIR, Italy) using standard technique measuring FEV_1 and forced vital capacity (FVC). The obtained best of three efforts was compared with local age- and sex-matched reference values. (Ip MS et al., 2000)

(4) Sputum induction. 4.5% hypertonic saline (HS) was used and sputum induction carried out using the standard technique. (Smith CM & Anderson SD, 1999 & Fahy JV et al., 2001) The test was only performed if the child's FEV_1 was at least 65% predicted. The procedure was explained to the child, who would rinse his or her mouth with water to clear debris and squamous epithelial cells. The best of three FEV_1 obtained from spirometry was used as the baseline value. A nose-clip was worn and the child began inhalation of HS for a period of 30 s. Spirometry was repeated 1 minute after the inhalation period. If no sputum was obtained and FEV_1 was greater than 80% of the baseline value, the test continued. The child would then continue inhalation of HS for periods of 1 min, 2 min, and then three periods of 4 min each. FEV_1 was repeated after each inhalation period. The child was encouraged to cough up any sputum after each dose of HS and the sputum was collected into a polypropylene tube. A record of any side effects experienced by the child undertaking the test was made at the end of each elapsed time period. The study concluded either when the child

developed troublesome symptoms, when lung function had dropped below 80% of the baseline value, or if the child could not be persuaded to complete the whole inhalation procedure. If the child had a greater than 20% fall in FEV_1, 500 mcg salbutamol were administered using a metered-dose inhaler and spacer, and recovery monitored. The sputum sample was processed within 4 hours. The sputum was poured into a Petri dish and the viscid mucocellular portions selected. After treatment with 0.1% Dithiothreitol (DTT) (Sigma Chemicals, Poole, UK) and Phosphate buffered saline (PBS), the cell suspension was aspirated until homogenized and subsequently filtered with a 48 μm nylon gauge. The cell suspension obtained was then centrifuged at 400G for 10 minutes. Cytospin samples were prepared and stained by May-Griunwald Giemsa stain prior to performing a differential cell count by an individual who was unaware of the clinical data of the subjects. Specimens containing squamous epithelial cells of less than 50% of the total inflammatory cell number were considered adequate. At least 400 inflammatory cells were counted for each specimen. Eosinophil count was expressed as percentage of total cell count.

5.2.3 Statistical analysis

Descriptive statistics were used to summarize the baseline characteristics of the patients and the results were expressed as median (IQR). Wilcoxon test was used for comparing paired data and $p < 0.05$ was considered as statistically significant. SPSS version 11.0 for Windows was utilized for all statistical analysis.

5.3 Results

Thirty subjects with stable asthma were recruited but one failed to return for his second assessment and he was excluded from the final analysis. There were 18 boys and 11 girls. The median age was 10 years (IQR: 3.5). The dosage of FP ranged from 100 micrograms to 500 micrograms per day and none of the subjects were taking any other anti-inflammatory drugs for their asthma. The demographic characteristics of the subjects, results of their spirometry, eNO, percentage sputum eosinophil count and visual analogue scores at baseline and after 8 weeks of once-daily FP are shown in Table 1. Sputum induction was well tolerated by all subjects and no adverse effects were reported. Twenty-three subjects (79%) were able to produce an adequate sputum sample on the first visit and eighteen of them on both visits. Asthma was well controlled in all patients and the baseline asthma severity score was low (median 1.0). All except two of the subjects had normal percent predicted FEV_1 (> 80%), the two cases had moderate obstructive deficit demonstrated on spirometry, and their percent predicted FEV_1 were 63% and 57% respectively.

None of the subjects reported worsening of their asthma during the 8-weeks study period. There was a statistically significant improvement in eNO [40.5 ppb (58.5) vs. 33.9 ppb (34.8), $p = 0.023$], and sputum eosinophil count [4.25% (10.25%) vs. 1.00% (2.6%), $p = 0.023$] after the medication was changed to once-daily use. There was also a trend of improved asthma severity assessed by the visual analogue score after 8 weeks of once-daily FP [1.0 (3.5) vs. 0 (1.5), $p = 0.80$]. There was however, no significant difference in FEV_1, p

= 0.347, night-time cough scores, p = 0.351 and daytime cough scores, p = 0.843 after treatment was changed to once-daily FP.

All subjects preferred the once-daily dosing regimen and the median preference score was 10 (p = 0.033). All visual analogues scores before and after treatment change were summarized in Table 5.1 and fig. 5.2 – 5.5.

	At baseline after run-in	After 8 weeks of once-daily FP
Age (years)	10 (3.5)	
Height (cm)	141 (21.5)	
FEV_1 (% predicted)	88.49% (16.1%)	90.50% (21.2%)
FVC (% predicted)	90.02% (18.1%)	89.84% (16.9%)
FEV_1 / FVC	91.85% (9.9%)	97.11% (16.6%)
eNO (ppb)	40.5 (58.5)	33.9 (34.8)
Sputum Eos (%)	4.25% (10.25%)	1% (2.6%)
Asthma severity	1.0 (3.5)	0 (1.5)
Night time cough	0 (0)	0 (0)
Day time cough	0 (1.0)	0 (1.0)
Preference score	10 (4.5)	10 (0.5)

Table 5.1 Demographic characteristics, spirometry, inflammatory markers and visual analogue scores of the subjects at baseline and end point – Median (IQR)

Fig. 5.2 Visual analogue score – Severity of Asthma

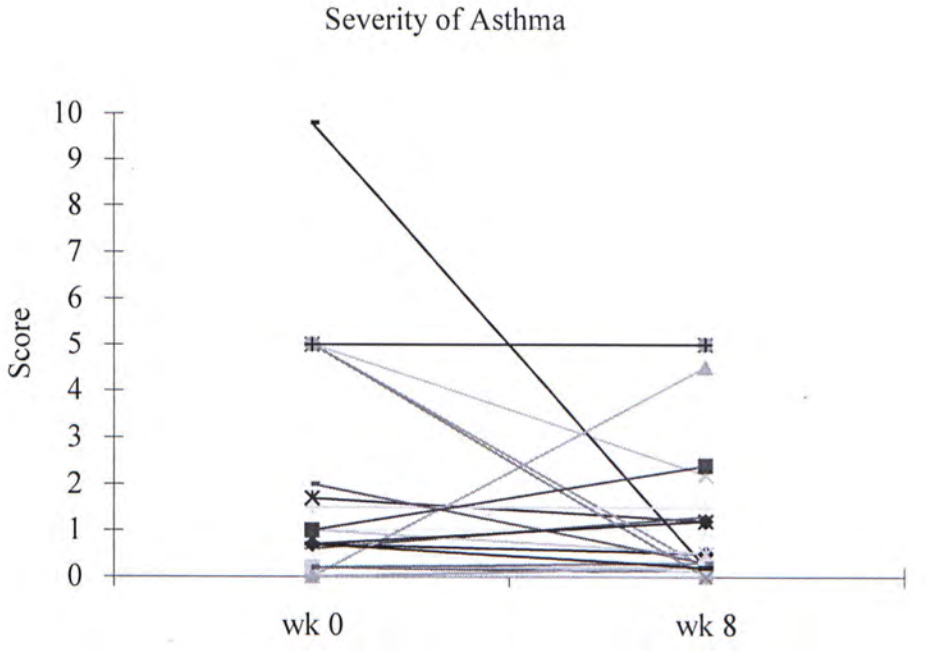


Fig. 5.3 Visual analogue score – Preference Score

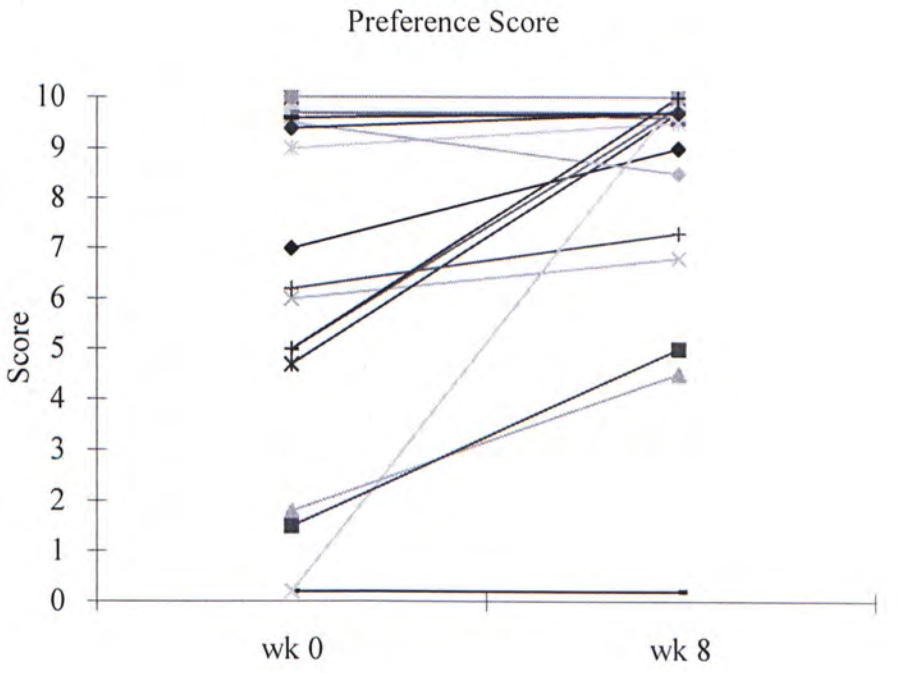


Fig. 5.4 Visual analogue score – Day Time Cough

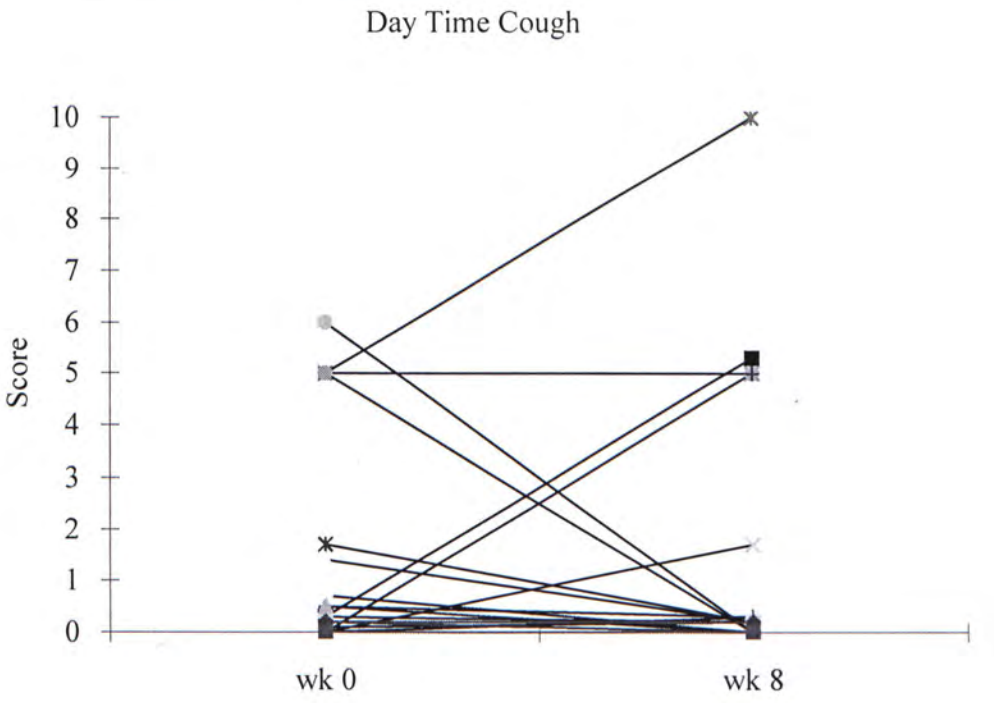
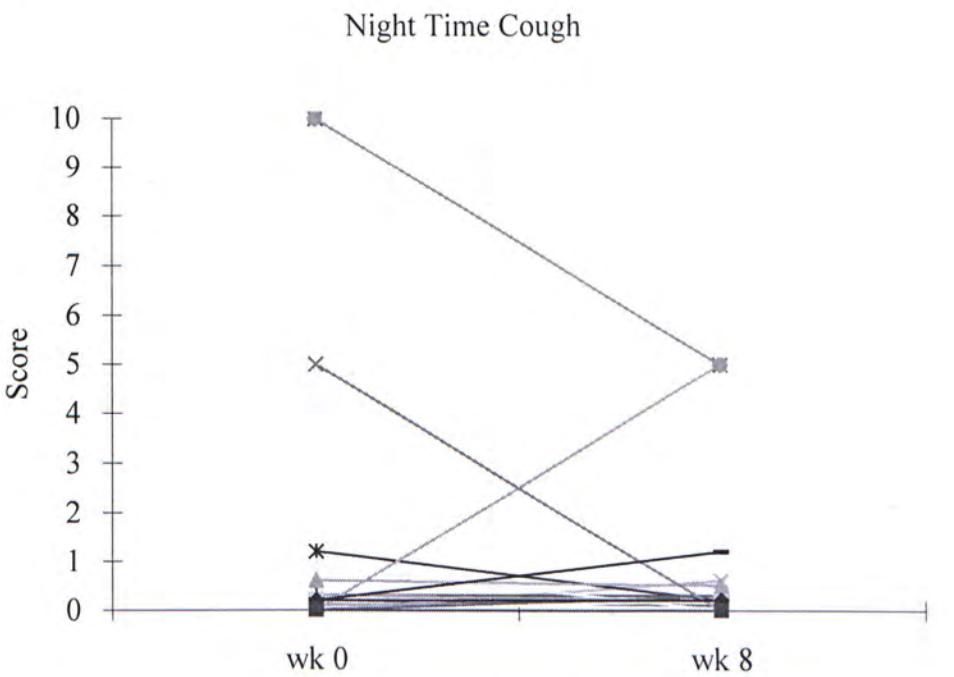


Fig. 5.5 Visual analogue score – Night Time Cough



5.4 Discussion

In this study we were able to demonstrate that the use of once-daily FP was at least as effective as twice-daily dosing in the control of airway inflammation in a cohort of children with stable asthma. There was significant improvement in the airway inflammatory markers after the medication was changed to once-daily use. This dosage frequency was also the preferred regimen by the subjects.

Fluticasone propionate (FP) is a potent inhaled corticosteroid with negligible oral systemic bioavailability. (Harding SM, 1990) It has high lipophilicity and affinity for the glucocorticoid receptors allowing prolonged retention in lung tissues. (Derendorf H et al., 1998; Hogger P & Rohdewald P, 1994) These properties make the drug an ideal candidate for reducing the dose frequency. However, in a recent review which included two paediatric studies, it was concluded that once-daily administration of FP is not currently recommended. (Purucker ME et al., 2003) The authors of the review article had found that twice-daily dosing was superior to once-daily dosing at the same nominal dose in change from baseline in the pre-dose FEV_1. There are major differences in study design between published trials and our current study. In the former, the subjects with persistent asthma were randomized to receive either once-daily or twice-daily dosing of FP and in many cases, the subjects had either been on inhaled corticosteroids of another class or they were steroid-naive. In our study, subjects had been on twice-daily FP for at least 3 months and they all underwent a dose frequency reduction to once-daily while

maintaining the same nominal dose. We looked at markers of airway inflammation as our primary outcome measure, unlike published studies where pre-dose FEV_1 or peak expiratory flow rate (PEFR) were their main outcome parameters. We decided that airway inflammation would provide more important information in relation to disease control, clinical management and prognosis. It has been shown that eosinophilic airway inflammation is a reliable predictor of asthmatic exacerbation and a precursor of airway remodeling. (Crimi E et al., 1998) Recent evidence also suggests that a treatment strategy directed at normalization of airway eosinophil count reduces asthma exacerbations and hospital admissions. (Gibson PG et al., 2000) We were able to demonstrate significant reduction in sputum eosinophil count and exhaled nitric oxide after the treatment was changed to once-daily use. This will have important long-term implications in relation to disease control and prognosis.

The use of once-daily inhaled corticosteroids offers convenience and improved acceptability to the patient. This was also the view expressed by our study subjects and all of whom preferred the once-daily regimen. It is possible that once daily treatment resulted in better medication compliance and hence improvement in disease control. This is especially important in the management of childhood asthma as compliance rate has consistently been demonstrated to be sub-optimal and a strong relationship has been established between poor compliance and risk of disease exacerbation. (Milgrom H et al., 1996) The optimal time for once-daily administration of medication has not been completely resolved as yet. The usual practice recommends taking the

medication in the evening as parents are more likely to be at home in the evening than in the morning, another measure to ensure parental supervision and medication compliance. Nevertheless the final decision will depend very much on the patient's preference and home circumstances. The issue of safety may be another concern as higher peak serum concentrations may conceivably surpass some critical "adverse event" threshold while maintaining an area under the curve (AUC) similar to that achieved by twice-daily dosing. Unpublished pharmacokinetic data from the US Food and Drug Administration however, suggest comparable systemic exposure (as measured by FP AUC concentrations) whether the same nominal dose of FP is given once-daily or divided into a twice-daily regimen. (Purucker ME et al., 2003)

There are certain limitations to our study. Firstly, the present study did not involve subjects with severe asthma. All except two of our study subjects had normal percent predicted FEV_1 at baseline. We therefore are not in a position to comment on the feasibility of using once-daily corticosteroids in patients with severe asthma. In our clinical practice, severe asthma constitutes only a small proportion of our asthma workload and most patients belong to the category of asthma subgroup similar to our study population. Therefore, the result generated from this study is still applicable to our daily practice. Besides, if once-daily use is associated with better medication compliance, that should be to the benefit of the patients regardless of their asthma severity. Secondly, the sample size was small as we only managed to recruit 29 subjects. We did not divide our subjects randomly into two groups comparing once-daily and

twice-daily use of medication concurrently. Our group of patients had mild asthma and they were well controlled on their initial dose of inhaled corticosteroids. We did not make an attempt to determine whether this was the lowest dose required. It is also possible that the improvement seen in the various parameters was a result of improved adherence with medications. Lastly, whether the use of once-daily regimen is also applicable to other classes of inhaled corticosteroids in relation to the control of airway inflammation is unknown.

5.5 Conclusion

The results of this study indicate that once-daily use of FP is equally effective as twice-daily treatment in maintaining asthma stability and reducing airway inflammation in children. These findings suggest that in children with stable asthma and well-controlled on inhaled fluticasone propionate, their treatment regimen can be changed from twice-daily to once-daily at the same nominal dose. At the end of the day, the best treatment for asthma is the one the patient would comply to once-daily treatment, being the preferred option, could result in better drug compliance, which could have favourable long-term effects on disease control and prognosis.

Chapter 6 Assessment of cough frequency in children with stable asthma: study on airway inflammatory markers

6.1 Introduction

Cough is a very common symptom of respiratory diseases such as viral infection and asthma in children. It is audible and can interfere with sleep for both patients and their parents. Cough can also be the first warning symptom of serious underlying problems such as foreign body inhalation or acute exacerbation of asthma. Parents are therefore understandably anxious and often seek medical advice for their children's cough. (Chang AB, 1999) In one study, parents reported that lack of sleep and choking were their main concerns for their children's cough. (Fuller P et al., 1998)

In childhood asthma, cough can be the only dominant symptom. (Chang AB et al., 1997; Rietveld S & Rijssenbeek-Nouwens LH, 1998). In a South African study, only 2.2% of children with classical asthma wheeze but do not cough. (Green R & Luyt D, 1997) Cough is also a dominant feature in childhood asthma exacerbation. In an unselected group of children hospitalized for an asthma exacerbation, almost 50% of parents reported that cough was usually or always present with an asthma exacerbation. (Chang AB et al., 1997) In a study involving children with moderate to severe asthma, Li AM et al, were able to find increased cough frequency in their subjects compared to normal controls. They also demonstrated a positive correlation between cough frequency and exhaled nitric oxide, a marker of underlying eosinophilic airway inflammation. Whether cough frequency in children with milder form of

asthma is also increased and whether a correlation still exists between cough and airway inflammation is unknown.

Problems and difficulties have been encountered by researchers in quantifying clinically relevant cough. Subjective recording of cough by means of diary cards and patient's report of cough frequency can be very variable and its reliability has been questioned (Archer LN & Simpson H, 1985; Thompson AH et al., 1987; Hoskyns EW et AL., 1991; Brunekreef B et al., 1992; Falconer A et al., 1993; Juniper EF et al., 1994; Pirila P & Sovijarvi ARA, 1995; Chang AB et al., 1998; Hamutcu R et al., 2002) Previous investigators have used conventional tape recorders to quantify cough objectively (Pirila P & Sovijarvi ARA, 1995; Falconer A et al., 1993). However, tape recorders are bulky and the lack of portability precludes their use in daytime cough assessments during normal activity. In addition, they rely only on a single audio signal. An audiovisual method for recording nocturnal cough has been successfully used in environments not suitable for sound only recording. (Picciotto A et al., 1998) Audiovisual recording allows accurate identification of the timing and number of cough episodes of the patient at night; however, this method is not practical during the day because it limits normal activity. Recently the use of an ambulatory cough monitoring device (LR 100) has been validated in both adults and children. (Munyard P et al., 1994; Hamutcu R et al., 2002) This is a portable, multi-parametric recording device, worn in a waist bag, and connected to the chest with three electromyographic (EMG) leads and a microphone. The device was highly acceptable to children and no adverse

effects during recording were reported. (Munyard P et al., 1996; Corrigan DL & Paton JY, 2003; Li AM et al., 2003; Zihlif N et al., 2005)

In this study, we aimed to (1) measure the cough frequency in children with stable asthma using a validated objective cough monitoring device, and (2) assess the correlation between the measured cough frequency with the degree and type of airway inflammation.

6.2 Cough Monitoring Machine LR 102

6.2.1 General Description

The LR102 cough recorder is an optimised ambulatory cough recording system. A simplified block diagram of the LR102 ambulatory cough recording system is shown in the following figure.

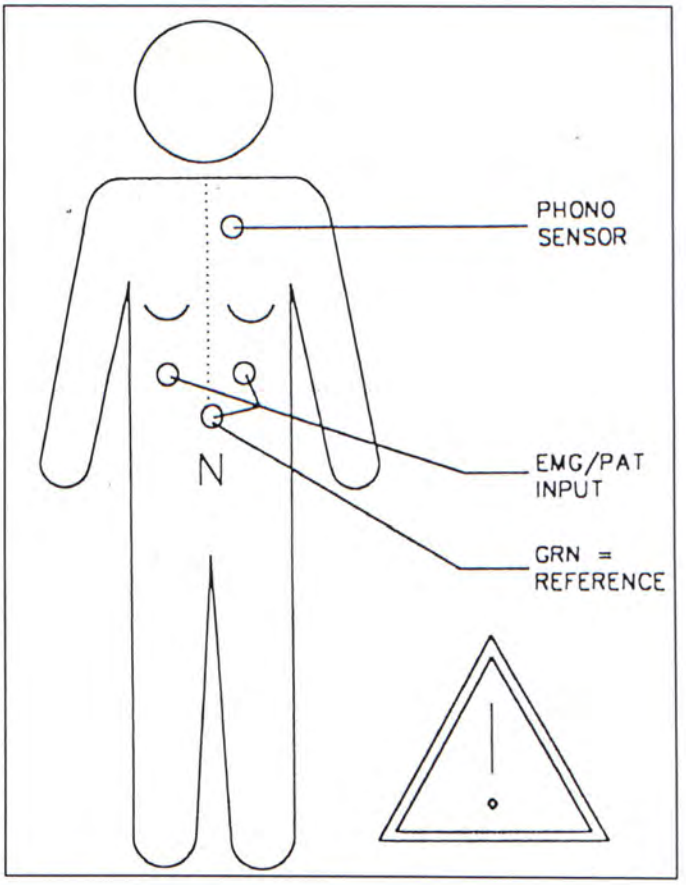


Fig. 6.1 LR102 ambulatory cough recording system

The LR102 cough recording system pre-processes the raw phono and EMG signals to provide a representative signal for cough. Both phono and EMG signals must be coincident for a cough to be tagged within the analysis program.

A combination of analogue and digital processing are used under the control of a dedicated set of microprocessors, which control acquisition and memory card recording.

The LR102 cough recorder has a built-in accelerometer, which allows the mean activity of the wearer, i.e. the patient, to be recorded at the same time. A pH channel is provided so that oesophageal reflux episodes could also be determined and correlated with the cough attacks. However, we did not use this function of the monitor in our study.

6.2.2 Real time communication with cough recorder

LR 102 cough recorder allows real time communication between the patient and the software so that the recording of the cough signals can be seen by the patient and the technician simultaneously. Therefore a baseline measurement and the position of the ECG leads and the Phono can be adjusted during installation. This will ensure correct position of the body leads and adequate signal pick-up before actual recording starts.

6.2.3 Placement of ECG electrode

The skin of the patient should be thoroughly cleaned to remove any grease. A distance between EMG electrodes A and B governs the sensitivity and, therefore, size of the tracing obtained. The patient should be asked to cough to establish a base line EMG response. The LED level indicator for EMG should light up when the cough machine is recording. If the LED is on all the time it

usually indicates that one of the electrodes has become detached.

6.2.4 Phono Sensor Placement

The phono sensor should be placed just off the midline of the body. The skin of the patient should be thoroughly cleaned to remove any grease and moisture. Double-sided adhesive rings should be used for attaching the Phono sensor to the skin.

6.2.5 Analysing Cough Data

LR 102 can automatically filter the "noise" in the data. Typical thresholds are set at trigger level = 50% and blanking period = 2 seconds or greater. On completion, the data will appear marked with green vertical tag bars where a cough has appeared within 1 second of the tag bar placement. Cough is identified by two signals: the surface EMG from the muscles of active expiration, and a filtered audio signal. A valid cough maneuver is defined as the presence of both signals. The EMG signal processing is such that voluntary muscle movement is filtered out, and the rapidly recruited abdominal wall muscles involved in coughing are recorded. Simultaneously the audio signal of the cough is recorded, thus giving two signals from which to verify that the child has coughed. The recording obtained will be played back, and displayed simultaneously visually on a personal computer. The recorded data were analyzed automatically and reviewed visually. Visual inspection confirmed that all cough epochs identified automatically were genuine. Coughing events were counted both as individual spikes and as clusters. We arbitrarily defined each

cluster (cough epoch) as a close succession of cough spikes (<2 sec between individual coughs) recorded by each trigger of the recorder. The cough data were expressed as total numbers of cough episodes (individual spike cough cluster) per recording time.

The installation of the whole set-up procedure takes about 15 minutes and the subject is then allowed to go home with the monitor and to return the next day for removal of the monitor.

6.2.6 Protocol for installation of cough machine

1. Clean the skin with Alcohol Prep Pad to remove any grease.
2. Stick the EMG electrodes according to the above figure (make sure the electrode is still moist)
3. Connect the EMG cable to the electrodes (Green to the bottom, red to the left and black to the right)
4. Place the Phono sensor just off the midline as indicated in figure and stick with double-sided adhesive ring.
5. Secure the attachment and position of the electrode with micropore.
6. Connect the Phono Sensor and EMG cable to the cough machine.
7. Insert the batteries and fill in the record sheet.
8. Real-time communication with the Cough Recorder and the PC
9. Choose Com 2 as the communication port
10. Plug in the serial communication cable, which has been connected to the Com port, to the serial port on the cough machine.

11. Insert the MMC card to switch on the cough machine.
12. Press "Zero blanking memory card" to blank the card.
13. Press "Monitor", then the recorder start to monitor real-time signal.
14. The patient is asked to perform baseline measurement.
15. Press "Stop Monitor" after the baseline measurement.
16. Detach the cable, secure the door and the patient are free to go.
17. When the patient returns, detach the cough machine, ECG leads and MMC card. The MMC card has recorded the data and will be used for analysis.

6.2.7 Cough Data Analysis

1. Insert the MMC card into the 4 in 1 adaptor and insert the adaptor in Omni Driver
2. Press "Import" → Enter the code → Press "Import"
3. Real time data will appear on screen on completion.
4. Press "Auto Tag" → "Set Tags"
5. On completion, all signals are screened and those unlikely cough signals are removed in accordance to the baseline.
6. The numbers of cough per hours are recorded and a histogram is rebuilt in Excel.

6.3 Methods

6.3.1 Subjects

Children with stable asthma between 7 – 18 years of age were invited to participate in the study. All children were first examined by a physician and patients suffering from upper respiratory tract infection were excluded. We defined stable asthma as no asthmatic exacerbation for the preceding 4 weeks necessitating oral prednisolone or an increased use of inhaled corticosteroids, the use of rescue treatment no more than three times a week, and with no clinical indication for change in treatment medication. All children were recruited from attendants to the paediatric chest clinic held at the Prince of Wales Hospital. Informed consent was obtained from all subjects and their parents.

6.3.2 Study design

A detailed history was taken and thorough physical examination performed on each child. Weight and height were recorded using standardized equipments. The participants underwent the following assessments:

(1) Asthma severity by visual analogue score, a subjective score from 0 to 10 (0 = asymptomatic, 10 = severe disabling asthma) was obtained from the subjects. Similar visual analogue score to assess patients' daytime and night-time cough severity (0 = no cough, 10 = severe disabling cough), and (b) surrogate quality of life score – asthma and cough (0=poor quality and 10=good quality) was also obtained from the subjects.

(2) Skin prick test

Five groups of aero-allergens (house dust mite, cat and dog dander, cockroaches, grass and tree pollens and mixed moulds) were done according to standard procedures, using purified allergen extracts. A child is considered atopic if he has at least one skin test result that shows indurations with a diameter of at least 3mm greater than the negative (normal saline) control.

(3) Exhaled nitric oxide (eNO) measurement

Exhaled nitric oxide (eNO) was measured by a rapid-response chemiluminescent method using the Sievers 280i NOA analyzer (Sievers, Boulder, CO, USA), with sensitivity from 1 ppb to 200 ppm, and a resolution of 1 ppb and accuracy of ±1 ppb, according to the American Thoracic Society guidelines. (American Thoracic Society, 1987). The subject were comfortably seated without nose clip, inhaled NO-free air from a reservoir and subsequently exhaled against a resistor. The flow rate was set at 50 ml/s. This on-line measurement was taken in triplicate and the average recorded.

(4) Spirometry

Spirometry (Spirolab II, MIR, Italy) using standard technique measuring FEV_1 and forced vital capacity (FVC). The obtained best of three efforts was compared with local age- and sex-matched reference values. (Ip MS et al., 2000)

(5) Sputum induction

Sputum induction. 4.5% hypertonic saline (HS) was used. The test was only performed if the child's FEV_1 was at least 65% predicted. The procedure was explained to the child, who would rinse his or her mouth with water to clear debris and squamous epithelial cells. The best of three FEV_1 obtained from spirometry was used as the baseline value. A nose-clip was worn and the child began inhalation of HS for a period of 30 s. Spirometry was repeated 1 minute after the inhalation period. If no sputum was obtained and FEV_1 was greater than 80% of the baseline value, the test continued. The child would then continue inhalation of HS for periods of 1 min, 2 min, and then three periods of 4 min each. FEV_1 was repeated after each inhalation period. The child was encouraged to cough up any sputum after each dose of HS and the sputum was collected into a polypropylene tube. A record of any side effects experienced by the child undertaking the test was made at the end of each elapsed time period. The study concluded either when the child developed troublesome symptoms, when lung function had dropped below 80% of the baseline value, or if the child could not be persuaded to complete the whole inhalation procedure. If the child had a greater than 20% fall in FEV_1, 500 mcg salbutamol were administered using a metered-dose inhaler and spacer, and recovery monitored. The sputum sample was processed within 4 hours. The sputum was poured into a Petri dish and the viscid mucocellular portions selected. After treatment with 0.1% Dithiothreitol (DTT) (Sigma Chemicals, Poole, UK) and Phosphate buffered saline (PBS), the cell suspension was

aspirated until homogenized and subsequently filtered with a 48 mm nylon gauge. The cell suspension obtained was then centrifuged at 400G for 10 minutes. Cytospin samples were prepared and stained by May-Griunwald Giemsa stain prior to performing a differential cell count and counted by a technician who was blinded to the clinical data of the subjects. Specimens containing squamous epithelial cells of less than 50% of the total inflammatory cell number were considered adequate. At least 400 inflammatory cells were counted for each specimen. Eosinophil count was expressed as percentage of total cell count.

(6) Cough monitoring

The LR 102 cough recorder was used in this study. The procedure of the operation has been described. The cough recorder allowed 24 hours recording. Once the cough recorder has been installed, the subjects and their parents were asked to complete a time activity recording sheet which included time the subject went to bed and woke up. All subjects returned the next day and underwent sputum induction.

6.3.3 Statistical analysis

The data will be presented as median with interquartile range (IQR). Spearman's rank correlation coefficient was used to assess the association between the various parameters. SPSS for Windows statistical software (Release 11.0, SPSS Inc., Chicago, Illinois) was used in the analyses. The level of significance was set at 5% in all comparisons, and all statistical testings were two-sided.

6.4 Results

Thirty Six children (aged 7 – 17 years) with mean ages 11.68 years (IQR: 9 – 14) were recruited in this study. There were 24 boys and 12 girls. All subjects by definition were atopic and the commonest allergen was house dust mite. Eleven of them (31%) had more than one allergen sensitivity and all of them (100%) had sensitivity to house dust mite. Five patients were using inhaled corticosteroids and the range of beclomethasone equivalent dosage was between 50mcg and 250mcg, (assuming budesonide has equal potency and fluticasone is twice as potent as beclomethasone). The remaining 31 patients were using short-acting beta agonist on an as required basis. Therefore, by definition, all subjects had mild intermittent or persistent asthma. (National Asthma Education and Prevention Program Expert Panel Report 2, 1997)

Cough recording was successful in all children and 25 out of 36 children (69.4%) produced adequate sputum sample at induction. No adverse events or sleep disturbance were reported. The median number of cough episodes over a 24 hour period was found to be 25.5 (IQR 16 – 42.8) and this was significantly greater than that reported for normal children; 11 cough episodes per day ($p<0.001$) (Munyard P & Bush A., 1996) (Table 6.1)

	Mean	Median	IQR: 25	IQR: 75
Age / yrs	11.68	11.50	9.00	14.00
Height / cm	148.86	149.10	136.75	161.25
Weight / kg	40.70	36.70	30.80	47.50
Number of cough episodes recorded	32.36	25.50	16.00	42.75
eNO / ppb	65.74	56.05	37.38	105.00
% predicted FEV1	89.3%	83.3%	81.1%	97.6%
% predicted FVC	93.3%	84.0%	91.1%	100.0%
% Eosinophil	6.34	1.75	0.50	5.13
% Neutrophil	19.07	8.25	6.13	18.25
Quality of Life score – Asthma	8.75	9.70	8.00	10.00
Severity of asthma score	1.27	0.30	0.00	2.00
Quality of Life score – Cough	8.21	9.35	7.00	10.00
Day Time Cough	1.11	0.20	0.00	1.18
Night Time Cough	1.20	0.20	0.00	1.78

Table 6.1 Mean, median and IQR for various measuring parameters

It was found that the number of cough episodes correlated positively with the percentage of neutrophils ($p<0.005$) in sputum. eNO was found to correlate with the percentage of sputum eosinophils ($p=0.01$). We were not able to demonstrate any significant correlation between symptom scores, lung function parameters with the number of cough episodes detected. (Table 6.2)

	Correlation Coefficient	P value
eNO	- 0.185	0.279
FEV1	- 0.215	0.208
FVC	- 0.119	0.491
% Eosinophil	0.02	0.924
% Neutrophil	0.833	0.000*
Quality of Life score – Asthma	0.096	0.606
Severity of asthma score	- 0.077	0.679
Quality of Life score – Cough	- 0.100	0.592
Day Time Cough	- 0.090	0.630
Night Time Cough	- 0.068	0.717

Table 6.2 Correlation coefficient and p value of cough episodes to various measured parameter.

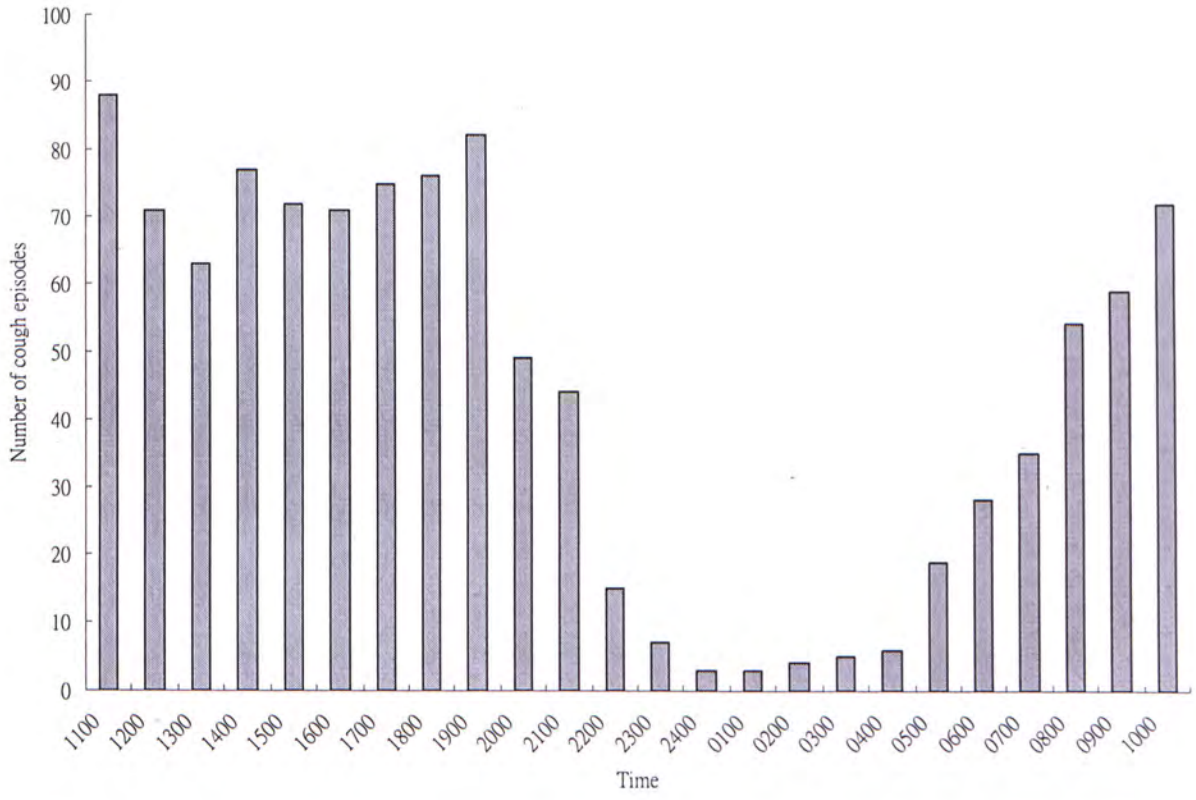


Fig. 6.2 Cough frequency distribution

The nighttime cough and daytime cough frequency were compared. We were not able to measure sleep stage, so we asked all patients and their parents to report the time when the patients slept and woke up in the morning. We found that all subjects slept at between 2200 – 2400 and woke up between 0700 – 0900. So we arbitrarily defined night cough as cough occurring between 2300 and 0700 hours and daytime cough as cough occurring at any other time. We calculated the hourly cough frequency for these two periods. Median daytime coughs / hours were significantly greater than nighttime coughs (71.5 / hour (IQR 56.50 – 76.25) vs 6.5 / hour (IQR 3.75 – 29.75), $p<0.001$).

6.5 Discussions

In this study, our aims were to assess the cough frequency in a group of children with stable mild asthma and to examine the relationship between the measured cough frequencies with airway inflammation. We were able to show, despite recruited at a time of apparent stability, that our cohort of mild asthmatics had increased cough frequency compared to normal children. (Munyard P & Bush A., 1996) The cough frequency was also found to correlate with percentage sputum neutrophil count.

We did not find any correlation between the subjective; self reported quality of life cough score ($p = 0.592$), daytime cough score ($p = 0.630$) and nighttime cough score ($p = 0.717$) with the actual cough frequency recorded. This finding is similar to what has been reported in the literature, questioning the reliability of subjective reported cough symptom. (Archer LN & Simpson H, 1985; Hoskyns EW et AL., 1991; Falconer A et al., 1993; Chang AB et al., 1998; Hamutcu R et al., 2002).

We have always believed that children with asthma would cough significantly more often than control subjects during exacerbations, but not during remission. (Rietveld S & Rijssenbeek-Nouwens L H., 1998) This is in contrast to the findings of our study where we found that children with stable asthma had an increased cough frequency compared with data previously obtained from normal school children. (Munyard P & Bush A., 1996) Similar to previously published work on stable moderate to severe asthmatics, in which

the authors also reported greater cough frequency compared to normal controls. In addition, we have found that cough frequency is greater during the day than at night, despite conventional teaching on the importance of night cough in asthma.

Persistent airway inflammation in stable asthmatics may explain our finding of increased total cough. A recent study showed that persistent airway inflammation was still evident in asthmatics that had been asymptomatic with normal lung function. (van den Toorn LM et al., 2001) On-going airway inflammation in our group of stable asthmatics may be supported by the finding that the median sputum eosinophil count was 1.75% (IR 0.5 – 5.13), compared to the values reported for normal controls which were 0.3% (IR 0.1 – 1.05) (Gibson PG et al., 2001). Although a direct relationship between cough frequency and sputum eosinophilia could not be demonstrated, we found a significant correlation between increased cough with the percentage sputum neutrophil count. Our findings are inconsistent with previous studies in cough and airway inflammation. Chang et al (Chang AB et al., 2002) found that increase in cough and eosinophilic inflammation were the characteristic of mild asthma exacerbation with insignificant change in lung function. Li et al. in their study of thirty-two (Li AM et al., 2003) children with stable moderate to severe asthma showed that the cough frequency was related to eNO. We were unable to explain the positive correlation between cough frequency and sputum neutrophilia. Previous investigators have reported the known causes of cough in relation to elevated sputum neutrophilia and that included chronic airflow

limitation associated with cigarette smoking, (Pizzichini E et al., 1996; Keatings VM et al., 1997; Stanescu D et al., 1996) pollutants such as ozone (Fahy JV et al., 1995; Vagaggini B et al., 1999; Holz O et al., 1999), endotoxin (Nightingale JA et al., 1998) and viral infection (Pizzichini MM et al., 1998). We did not obtain detailed information from our patients in relation to possible exposure to the above but none of subjects smoke. In fact, the median sputum neutrophil count from our cohort was 8.25% (IR 6.13 – 18.25) which was not elevated compared to what has been reported for normal controls; 35% (IR 12 – 88) (Gibson Am J Respir Crit Care Med 2001) Therefore this correlation may be a chance finding rather than a genuine relationship.

There are several limitations in our studies. Firstly, we recruited only 36 patients, whether an increased sample size would have given a significant correlation between cough frequency and other parameters especially sputum eosinophil count, is not known. We did not have normal Chinese children to serve as comparison to our cohort of patients for cough frequency. But we could not envisage race as an important determining factor in cough frequency. Secondly, we could not assess sleep stage or even whether the child was asleep. We relied mainly on the information provided by the parents. Thirdly, this study was a cross sectional study at a specific time point. We did not attempt to assess whether the current medications taken by the patients were optimal or not. Longitudinal studies to determine whether increasing asthma treatment or the use of anti-neutrophilic measures are useful strategy to treat increased cough should be carried out. The main strength of this study was the use of objective

cough monitor relying on two signals to confirm genuine coughing by the child. Sound quality may be affected by the sleep position of the patient and the actual recordings may be contaminated by sounds made by people nearby. By utilizing two signals to confirm an episode of cough greatly improved reliability and accuracy of the recording.

6.6 Conclusions

In summary, we were able to demonstrate increased cough frequency in children with mild asthma despite their disease being in remission. The increased cough might be driven by a neutrophilic inflammatory pathway, the significance of which will require further studies to elucidate.

Chapter 7 Summary and conclusion

Traditionally, assessment of asthma in children relies heavily on clinical history. Objective measurement by spirometry, PEFR, eNO, test of atopy by SKT have been used in assessing severity of asthma and predicting asthma exacerbations but they lack sensitivity and good correlation to underlying airway inflammation. BAL and bronchial biopsy are direct method in assessing airway inflammation. However, their use in clinical practice and especially in children is limited because of their invasive nature. Hypertonic saline challenge and sputum induction (HSCSI) is an important assessment method, especially in children. The combined procedure allows assessment of airway hyperresponsiveness and airway inflammation at the same time and it is non-invasive, safe and relatively well tolerated by all. In chapter 2, methods in assessing paediatric asthma, airway hyperresponsiveness and airway inflammation as well as various research studies conducted in relation to assessing the airway inflammation in persistence asthma, evaluating the efficacy of inhaled corticosteroids in reducing sputum eosinophilia, distinguishing eosinophilic asthma from non-eosinophilic asthma were reviewed.

In chapter 3, the protocol and the laboratory setup of HSCSI, at the Prince of Wales Hospital and other technical considerations such as data interpretation and safety aspects have been reviewed and evaluated. Besides, the protocol of sputum induction, processing and staining were discussed.

In Chapter 4, eNO was identified as a predictor associated with successful sputum induction; however, a cutoff point with satisfactory sensitivity and specificity was unable to be defined likely related to our small sample size and the heterogenous nature of the study population. Further research into this particular aspect of sputum induction would be clinically relevant to practicing clinicians who use this technique in their management of asthmatic patients, so that children unlikely to produce an adequate sample are not subjected to the procedure.

In chapter 5, sputum induction was used to evaluate the efficacy of once-daily inhaled corticosteroids (fluticasone proprionate) in maintaining asthma stability. It was found that once-daily dosage was equally effective in reducing airway inflammation in children. The findings suggest that in children with stable asthma and well-controlled on inhaled fluticasone propionate, their treatment regimen can be changed from twice-daily to once-daily at the same nominal dose. This study has demonstrated the use of sputum induction as a non-invasive maker in assessing severity of asthma and highlights the concept of targeting airway inflammation instead of symptoms in asthma management. At the end of the day, the best treatment for asthma is the one the patient would comply to. Once-daily treatment, being the preferred option by most children in our study, could result in better drug compliance, which could have favorable long-term effects on disease control and prognosis.

In chapter 6, a study with the use of the recently available cough monitoring machine LR102 and sputum induction in children was carried out. We were able to demonstrate that stable asthmatics had increased cough frequency compared to normal controls. The cough frequency was also found to be positively correlated with the degree of neutrophilic airway inflammation.

In conclusion, combined hypertonic saline challenges and sputum induction is safe and well tolerated among children. It has its clinical value as a non-invasive assessment tool of airway inflammation.

Future direction:

Combined HSCSI is now generally accepted as a research tool only as the procedure is time consuming and labour intensive; however, it may find its way into routine clinical practice in the future. Combined HSCSI may have its clinical value in three time scales. The first is the single timepoint application of the procedure in the diagnosis of airway disease. The second is the repeated use of the procedure in monitoring airway inflammation and response to treatment. The third refers to its potential in predicting outcome of the underlying airway disease.

A number of components in the fluid phase (supernatant) of sputum have been shown to be reproducible and sensitive treatment markers of asthma. However, difficulties in standardization, lack of normal control data and the problem associated with salivary contamination have led to their limited use in clinical studies. More research into the use of markers present within the sputum supernatant is definitely required to verify their clinical application.

In the future, more research work will be needed to provide a clinically friendly sputum induction procedure so that it can be performed routinely and cost-effectively in clinical practice. It is our hope that the procedure will one day be automated and the whole process can be completed within an acceptable time frame so that it can be made routinely available in hospitals and even in general practice.

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The Chinese University of Hong Kong
Department of Paediatrics - Pulmonary Function Assessment

A. Skin Prick Test, eNO, HSC, Simple Spirometry Assessment

I. Skin Prick Test (Visit 1 only)

1. Histamine	_____mm	7. Penicillium	_____mm
2. Saline	_____mm	8. Mould	_____mm
3. D. Pteronyssinus	_____mm	9. Pollen _____mm	
4. D. Farinae	_____mm	10. Grass _____mm	
5. Amer. Cockroach	_____mm	11. Dog	_____mm
6. Ger. Cockroach	_____mm	12. Cat	_____mm

PLACE LABEL HERE

Date :

Visit:

☐ Asthma (NCMG)
☐ Asthma (CMG)

Phone:

II. eNO Measurement (Sievers NOA)

Smoking: ACTIVE / PASSIVE / NIL

Data: 1.	2.	3.	4.
Procedure: Done / Failed	No of trial :	eNO:	% dev:

IV. Hypertonic Saline Challenge

Rinse mouth: Yes

Nebuliser cup : (pre-weight)	g
Nebuliser cup : (post-weight)	g
Difference in weight :	g
Cumulative time :	min
Output (g/min) :	
Baseline Measurement (FEV1/FVC) :	Best
Predicted : /	
1st trial : /	
2nd trial : /	
3rd trial : /	
4th trial : /	
5th trial : /	

Asthma medication and time last taken:

Time	FEV1	Change in %	Sputum	Remarks
Baseline		%	Y/N	
0.5 min		%	Y/N	
1 min		%	Y/N	
2 min		%	Y/N	
4 min		%	Y/N	
4 min		%	Y/N	
4 min		%	Y/N	
post		%	Y/N	

FEV1 20% fall:
(Best FEV1 x 0.8)

Result: Sputum Induced / Saliva only / No Sputum

V. Simple Spirometry

Pre	Predicted	Observed	% predicted
FEV1:			
FVC			
FEV1/FVC			

Post	Observed	% Predicted
FEV1:		
FVC		
FEV1/FVC		

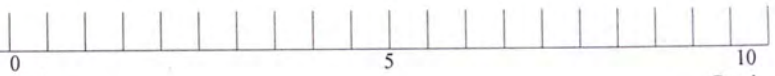
Remarks / Side Effects reported :

The Chinese University of Hong Kong
Department of Paediatrics - Questionnaire

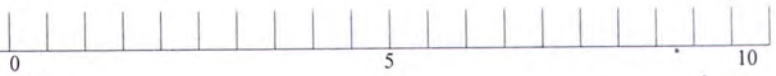
Symptom Score, Disease Severity Score, Convenience score, Cough score

Date: Visit no:

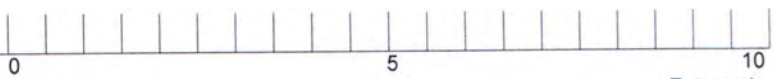
1. Asthma Score



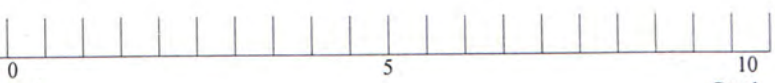
2. Severity of Asthma



3. Convenience score



4. Cough Score



5. Day Time Cough

6. Night Time Cough

Remarks / Chest Examinations:

Sputum Processing and Cytoslide Analysis

I. Macroscopic Examination and Cell counting (Choose the best one)

Est. Volume:	ml
Color:	opague / green / yellow / jelly
Sputum used:	
Comment:	Saliva only / Poor but acceptable / Good
Sample 1 : Alive:	Dead:
TCC = Total cell count in 5 squares /5 x 0.02 x 10^6 /ml	
= (	) /5 x 0.02 = x 10^6 /ml
Sample 2 : Alive:	Dead:
TCC = Total cell count in 5 squares /5 x 0.02 x 10^6 /ml	
= (	) /5 x 0.02 = x 10^6 /ml

Re-suspension volume (1ml ~~ 10^6 /ml cell)
TCC x Post filtration volume
= ml
Viability = %
TCC x 9 = x 10^6 /ml
III. Cytoslide (minimum 2 slides, maximum 4 slides)
Code:
IV. Supernatant
Code:

II. Cytoslide Quality	Slide no:			
1. Debris				
2. Cell Outline				
3. Nuclear Morphology				
4. Squamous				
5. Overall impression				
6. Slide Macrophage present				
7. No of cells on slide				
Total Score				

III. Differential Cell counting	Slide no:							
1. Neutrophils								
2. Eosinophils								
3. Macrophages								
4. Lymphocytes								
5. Col. Epithelial cell								
6. Squamous								

Cough Assessment

Date of Installation:	Time:	
Date of Return:	Time:	
1. Cough machine with door secure screw		
2. Waist bag and batteries (4AA)		
3. MMC card, Phono sensor and EMG leads		
Patient:	Checked by:	

Baseline measurement:	Phono	EMG
1. Deep cough		
2. Speaking		
3. Deep Breathe		
4. Laughing		
5.		
6.		

Cough data: (no of tags)

1	2	3	4	5	6	7	8	9	10	11	12
13	14	15	16	17	18	19	20	21	22	23	24

Start: (press 3 consecutive signals)

End: (press 3 consecutive signals)

Remarks / Side Effects / Damage :