

Sputum induction: effect of nebulizer output and inhalation time on cell counts and fluid-phase measures

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Summary

Background A knowledge of the factors that can affect induced sputum results is essential in order to standardize the procedure.

Objective We investigated the influence of nebulizer output on sputum cell counts and fluid phase measurements at increasing times of sputum induction.

Methods Eighteen adults with stable asthma inhaled an aerosol of 3% hypertonic saline to induce sputum after 7, 14 and 21 min on 2 days separated by 48 h. On one day, in random order, the ultrasonic nebulizer used had a relatively low output of 0.87 mL/min (particle size 5.58 μ m mass median aerodynamic diameter, MMAD) and, on the other, a higher output of 1.90 mL/min (particle size 4.14 μ m MMAD). The sputum was selected from each expectorate and examined blind to the induction procedures.

Results With both nebulizers, the 14- and 21-min samples were lower in weight, neutrophils, eosinophils, eosinophil cationic protein (ECP) and interleukin (IL)-8 and higher in macrophages. The higher output nebulizer induced sputum with higher cell viability and lower ECP and IL-8.

Conclusion The results identify that the volume of hypertonic saline inhaled in sputum induction influences the fluid-phase measurements. The duration of induction does alter the cell counts and suggests that the later expectorated sputum samples originate from more peripheral airways. The results draw attention to the need to standardize the volume and time of nebulization to accurately interpret and compare results.

Keywords: induced sputum, sputum cell counts, sputum chemistry

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Introduction

Airway diseases are considered to be caused and exacerbated by airway inflammation. The presence and intensity of the inflammation can be examined in sputum induced with an aerosol of hypertonic saline [1,2] Inflammatory cell counts and some fluid-phase measurements can be reproducible, valid and longitudinally responsive [3]. Sputum analysis has therefore become useful in research and there is growing evidence that it has use in clinical practice [4].

Understanding the factors which alter sputum results is necessary to avoid misinterpretation. Several of these factors have already been evaluated [5–9]. Two abstracts [10,11] and one full report [12] have recently suggested that the proportion of cells in induced sputum is altered by the duration of hypertonic saline inhalation. High output ultrasonic nebulizers were used for the sputum induction. The sputum samples harvested within a few minutes of induction contained more neutrophils and eosinophils, while later samples contained more macrophages and lymphocytes, similar to bronchoalveolar lavage (BAL), suggesting that they originated from more peripheral airways. Furthermore, the sputum fluid phase measurements

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in the later samples were lower, suggesting a possible dilution effect of the inhaled saline. These observations are important to confirm using the lower output ultrasonic nebulizer that we use, since they are relevant to standardize methods of sputum induction and accurately interpret results. We have therefore compared, in subjects with stable asthma, the effect of sputum induction performed with the low output Medix nebulizer with the higher output DeVilbiss nebulizer used by Gershman *et al.* [11,12] on sputum cell counts and fluid-phase measurements in induced sputum selected from the expectorate. We also examined the effect of the duration of sputum induction by taking samples after 7, 14 and 21 min of induction.

Materials and methods

Subjects

Eighteen non-smoking adults with asthma volunteered for the study (Table 1) but only 12, who were able to expectorate sputum after each induction, were analysed. The diagnosis of asthma was based on a history of symptoms of asthma and reversible airflow limitation indicated by an increase in forced expiratory volume in 1 s (FEV₁) of $\geq 15\%$ after salbutamol 200 mg, or by airway hyper-responsiveness with a provocation concentration of methacholine to cause a fall in FEV₁ of 20% (PC₂₀) of < 8 mg/mL. The subjects had either no or mild occasional daytime symptoms. They were stable and medications were kept the same during the study. None had evidence of infection and none were exposed to seasonal allergens to which they were allergic for 4 weeks before or during the study. The study was approved by the Research Ethics Board of St Joseph's Healthcare and all subjects gave written informed consent.

Table 1. Subject characteristics

<i>n</i> (males)	12 (6)
Age (yr)	25 (5.2)
Atopic (<i>n</i>)	11
FEV ₁ (L)	2.72 (0.9)
VC (L)	3.8 (1.1)
FEV ₁ /VC (%)	70 (12)
PC ₂₀ methacholine (mg/mL)*	2.2 (1.6)
Inhaled corticosteroid (<i>n</i>)	11

Mean (SD), *geometric mean (geometric SD). Atopic means one or more positive allergy skin prick tests with common allergen extracts.

Study design

The study was cross-sectional, randomized and crossover, with blind assessment of outcome measurements. The subjects were seen on 3 days separated by 48 h (to exclude an effect of the previous sputum induction on baseline sputum outcomes) within 1 week at the same time of day. At the first visit, the suitability of the subject for the study was determined by a questionnaire and spirometry or methacholine challenge test. On the second and third visits, the stability of the subject was checked by a questionnaire of symptoms, need for β_2 -agonist and FEV₁ (change $< 10\%$). Sputum induction was performed with an aerosol of hypertonic saline generated for 21 min by one of two nebulizers, the order being determined by a table of random pairs. The subject was asked to cough sputum into a separate container at 7, 14 and 21 min of induction. The subject was unaware of the features of each nebulizer and each container was coded with no indication of the nebulizer or time of collection and was sent for processing within 2 hours.

Methods

The FEV₁ was measured according to the American Thoracic Society specifications [13] with a Koko-Trek spirometer (Trudell Medical International, London, Ontario, Canada). The methacholine inhalation test was carried out as described by Juniper and coworkers [14] and the results were expressed as the provocation concentration to cause a fall in FEV₁ of 20% (PC₂₀) in non-cumulative units.

The method of sputum induction was that described by Pizzichini *et al.* [15], with the exception of the nebulizers used and the concentration of saline, which was kept at 3%. The nebulizers were the Medix® ultrasonic nebulizer (Canadian Medical Products Ltd, Markham, Ontario, Canada), with an output of 0.87 mL/min and particle size of 5.58 mm mass median aerodynamic diameter (MMAD), and the DeVilbiss Ultraneb 99® nebulizer (DeVilbiss Co., Somerset, PA, USA), with an output of 1.9 mL/min and particle size of 4.14 mm MMAD. With the Medix, 9 mL of saline were placed in the reservoir for each 7-min period of induction. Because of overheating of nebulizer after running for 7 min, a separate nebulizer was used for each induction period. The output for the Medix was calculated by weighing the nebulizer before and after running for 7 min. The DeVilbiss nebulizer was operated with 25 mL of saline in the chamber for each period and the output wheel at two-thirds of maximum power. It was connected to a tube of 162 cm in length and 2.2 cm in diameter. The output was calculated from the loss of weight of the nebulizer chamber before and after operation for 7 min, minus the change in weight of the tube.

The sputum was processed and examined by trained technologists as described by Pizzichini *et al.* [15]. Briefly, the fresh expectorate was poured into a Petri dish and all portions that appeared to be macroscopically different to saliva (opaque, dense, more viscid) were placed in a 15-mL polystyrene tube. Then it was treated with four times the weight (in g) of selected sputum in mL of 0.1% dithiothreitol (DTT) (Sputolysin, Calbiochem Corp., San Diego, CA, USA) freshly diluted 1 : 10 with distilled water, vortexed for 15 s and rocked for 15 min. A further four volumes of phosphate buffered saline (PBS) was added to the suspension, which was then filtered through 48 µm nylon mesh (B & SH Thompson, Scarborough, Ontario, Canada). Total cell counts and trypan blue cell viability were determined in a haemocytometer and differential cell count was obtained from counting 400 non-squamous cells in stained cytopspins. The remaining cell suspension was centrifuged and the supernatant stored at -70°C for later fluid-phase measurements. The concentration of eosinophil cationic protein (ECP) ($\mu\text{g/L}$) in the thawed supernatant was determined using a radioimmunoassay (Kabi Pharmacia Diagnostics AB, Uppsala, Sweden). Interleukin (IL)-8

and α_2 -macroglobulin were measured using an enzyme-linked immunosorbent assay (Quantikine: R & D Systems Inc., Minneapolis, MN, USA). The fluid-phase results were corrected for the dilution of the weight of selected sputum.

Statistical analysis

Descriptive statistics were used to summarize the clinical and demographic characteristics of the sample. Measurement results were decoded and sorted by nebulizer and time. Data are reported as arithmetic mean (standard deviation). The repeated measures analysis of variance model was used to analyse the effects of the two within-subjects factors (nebulizer output and duration of sputum induction) on cell counts and fluid phase measurements. From this analysis, estimated mean effects were calculated. A dummy variable representing the order of the nebulizers was also included in the model. As an example for clarity, Fig. 1 represents different combinations of the effects of the output of the nebulizer and time of nebulization. Significance was accepted at the 95% level. The statistical

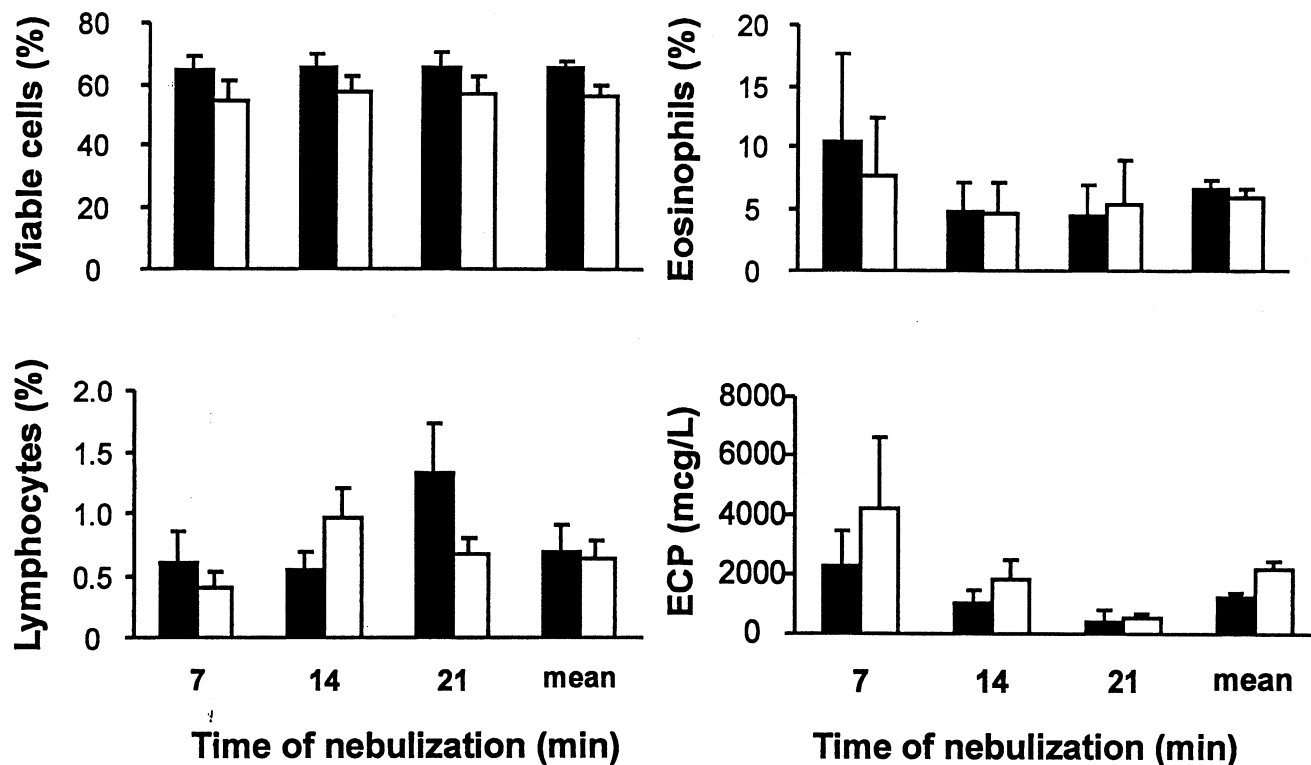


Fig. 1. The combination of the effects of nebulizer output and the time of nebulization on selected sputum outcomes. Closed bars represent the high output nebulizer and open bars the low output nebulizer. Dispersion bars are standard errors. An effect of time of nebulization is represented by a trend to progressively decrease or increase in both nebulizer bars (eosinophils, top right), an effect of nebulizer output is represented by a clear separation between both nebulizer bars (viability, top left), both effects together (ECP, bottom right) and no effect at all (lymphocytes, bottom left).

software used was SPSS for Windows® Version 6.1 (SPSS Inc., Chicago, IL, USA).

Results

Twelve subjects were able to expectorate all three samples of induced sputum (from each of the two nebulizers) and were analysed. The other six subjects were removed because they failed to produce sufficient sputum to be examined; three subjects failed with the higher output nebulizer at 14, 14 and 21 min and three with the lower output nebulizer at 7, 7 and 14 min). There was no significant effect of nebulizer or time on FEV₁, which fell by less than 10%.

We examined the effect of nebulizer output on the cell and fluid-phase measurements (Table 2). The higher output nebulizer resulted in significantly higher cell viability but lower ECP (Fig. 1) and IL-8 levels than the lower output nebulizer, independent of duration of nebulization. The estimated mean differences (confidence interval 95%, CI) between the nebulizers were 9.1 (0.3–18.0)%, –945.1 (–410.7 to –1479.5) µ/L and –727.4 (–1 to –3484.5) pg/mL, respectively.

As the duration of nebulization increased, the samples had a lower weight and percentage of neutrophils and eosinophils (Fig. 1), and a higher percentage of macrophages. This effect was significant and independent of the nebulizer used. An estimate of the magnitude of this effect was given by the mean differences (from the average of both nebulizers) between 21 and 7 min (CI) and was –11.6 (–0.1 to –35) mg, –15.5 (–4.7 to –26.3)%, –4.2 (–3.3 to –5.2)% and 17.4 (7.8–26.9)%, respectively. This effect was also found using the absolute cell counts for

the same measurements. ECP and IL-8 were also affected by the duration of nebulization, the 21-min sample being –2771.8 (–2554.9 to –2998.7) µ/L and –5682.2 (–3331.1 to –8033.4) pg/mL lower than the 7-min sample. The α₂-macroglobulin variability in measurements was too great to identify any trend. The magnitude of the changes in sputum outcomes did not relate to the baseline FEV₁, the $r = 0.46$ ($P = 0.22$) for eosinophils, 0.29 (0.45) for neutrophils, 0.52 (0.17) for ECP, and 0.01 (0.97) for IL-8.

Given these differences due to nebulizer and duration, we questioned what the results would have been had we used our usual procedure of combining all three samples to express the results. We therefore estimated the cell counts and fluid measurements for the whole sample for each nebulizer by adding the three absolute cell counts and dividing by the total number of cells in all three samples, or by adding the three values and dividing by three in the case of fluid measurements [Table 2]. This estimation of the whole sample generally fell between the results for the 7- and 14-min samples; it was significantly different from 21 min but not from the 7-min or 14-min samples.

The order of the nebulizer used produced no significant effect on any outcome variable, indicating that the results of the second sputum induction were not influenced by the first induction.

Discussion

The results of this study indicate that the induction of sputum with a high output DeVilbiss ultrasonic nebulizer (1.9 mL/min) gives specimens of selected sputum with significantly lower ECP and IL-8 levels than the lower output Medix ultrasonic nebulizer (0.87 mL/min). They

Table 2. Effect of output of nebulizers and duration of nebulization on some cell counts and fluid phase measurements

Time (Neb)	Weight		TCC		Vab		N		Eo		M		L		ECP		α ₂ M		IL-8	
	Hi	Lo	Hi	Lo	Hi	Lo	Hi	Lo	Hi	Lo	Hi	Lo	Hi	Lo	Hi	Lo	Hi	Lo	Hi	Lo
7 min	214	207	5.5	4.2	64.7	54.4	54.9	53.8	10.4	7.6	33.7	37.5	0.6	0.4	2270	4202	5.6	5.8	10.1	10.3
14 min	207	186	3.5	2.8	65.2	57.2	47.7	44.0	4.8	4.6	48.2	48.7	0.5	1.0	1034	1813	11.3	5.4	7.0	9.0
21 min	186	190	2.8	3.0	65.6	56.5	38.9	38.8	4.4	5.2	54.7	51.3	1.3	0.7	402	526	5.3	5.7	4.5	4.5
Mean	201	194	3.9	3.3	64.9	57.0	50.6	47.5	7.1	6.4	41.0	44.0	0.7	0.6	1235	1865	10.8	5.6	7.2	7.9
(SD)	(63)	(71)	(3.6)	(2.5)	(11.0)	(18.8)	(21.3)	(24.4)	(15.7)	(14.2)	(17.7)	(22.0)	(0.7)	(0.5)	(1021)	(1521)	(8.1)	(4.2)	(5.9)	(7.1)
P Neb	0.720		0.131		0.034		0.705		0.547		0.600		0.920		0.001		0.469		0.009	
P Time	0.031		0.053		0.075		0.007		0.001		0.003		0.195		0.001		0.430		0.001	

Time = time of nebulization in minutes; Neb = nebulizer; Hi = high output nebulizer; Lo = low output nebulizer; Weight = weight of selected sputum (mg); TCC = total cell counts (10⁶ cells/g); Viab = viable cells (%); N = neutrophils (%); Eo = eosinophils (%); M = macrophages (%); L = lymphocytes (%); ECP = eosinophil cationic protein (µg/L); α₂M = α₂-macroglobulin (g/L); IL-8 = interleukin 8 (ng/mL). Values are arithmetic means (SD). Mean = estimated mean of the three samples. The *P*-values were obtained from analysis of variance of repeated measures.

also show that the duration of induction with expectoration at 7, 14 and 21 min resulted in a progressive lowering of sputum weight, total cell count, neutrophils and eosinophils, as well as ECP and IL-8, and a progressive heightening of macrophages. The results are relevant to the standardization of methods of sputum induction and to the accurate comparison of results within and between subjects and centres. The results add to those of previous studies. They are original in that they illustrate that the volume of aerosol inhaled influences the fluid-phase measurements in selected sputum. When the nebulizer output was higher, levels of fluid-phase ECP and IL-8 were lower. They also confirm the results of Holz and coworkers [10] and Gershman and coworkers [11], who observed a progressive effect of the duration of induction on cell counts and fluid-phase measurements. In the present study, the latter results, with the relatively small number of subjects studied, were only significant when comparing selected sputum after 7 min vs. 21 min. The 21-min sample, containing relatively more macrophages and fewer neutrophils and eosinophils, is closer to that seen in bronchoalveolar lavage (BAL) [16], and suggests that it comes from more peripheral airways. The lower fluid-phase measurements in this later sample are partly explained by this and partly by the dilution effect of the volume of inhaled saline aerosol, which would be greater with the higher output nebulizer or the longer times of induction. The dilution effect might be minimized by processing only selected portions of the expectorate. The results also indicate that the repeated inhalation of saline did not acutely influence parameters of inflammation. The relevance of these observations concerns the standardization of methods of sputum induction and the comparison of results within and between subjects and centres. Nebulizer output is particularly relevant to the fluid-phase measurements. The duration of induction is relevant to these plus the cell counts. Because the latter was only significant between the 7- and 21-min samples, it might be suggested that induction up to 14 min might minimize this effect providing sputum can be induced with this duration of induction. A similar induction period of 12 min was suggested by Gershman and coworkers [12]. However, it has not been established how often induction is unsuccessful at 14 min while successful at 21 min; until this has been established we would suggest that the 21 min is maintained, especially in research.

We conclude that for precise comparison of results within or between studies, sputum induction should be carried out using similar volumes of saline aerosol and duration of induction.

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