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Effect of saliva contamination on induced sputum cell counts, IL-8 and eosinophil cationic protein levels

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Effect of saliva contamination on induced sputum cell counts, IL-8 and eosinophil cationic protein levels. J.L. Simpson, N.L. Timmins, K. Fakes, P.I. Talbot, P.G. Gibson. ©ERS Journals Ltd 2004.

ABSTRACT: Excessive salivary contamination of induced sputum samples prevents the satisfactory examination of lower airway inflammation. The effects of salivary contamination on different sputum fluid phase measures and the levels of salivary contamination preventing analysis are not defined. The present study sought to examine this by investigating the effect of increasing salivary contamination on induced sputum samples.

Sputum and saliva samples from subjects with asthma and healthy controls were collected, and treated with dithiothreitol (DTT). Saliva was then added to aliquots of dispersed sputum in increasing proportions (0% to 100%). The effect of increasing saliva contamination was assessed on sputum total cell count, viability, differential cell count and fluid phase levels of interleukin (IL)-8, eosinophil cationic protein (ECP) and total protein.

The addition of saliva to induced sputum reduced total cell counts and absolute cell counts but did not change the differential cell count. Levels of fluid phase ECP and IL-8 were significantly reduced with increased salivary contamination. There was a progressive reduction in ECP and IL-8, which reached significance at 70% and 80% saliva contamination, respectively. IL-8 levels corrected for total protein showed no change with increasing saliva concentrations.

Induced sputum differential cell counts expressed as the proportion of nonsquamous cells are robust measures that are not influenced by salivary contamination. Studies reporting total and absolute cell counts and fluid phase mediator levels require control for squamous contamination.

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Induced sputum has emerged as an important technique for the assessment of airway inflammation in diseases such as asthma, cystic fibrosis and chronic obstructive pulmonary disease [1]. Although sputum inflammatory cells have been analysed for many years [2], it has only been in the last decade that problems with sample collection and processing have been addressed, enabling reproducible cell counts to be obtained on multiple occasions [3–5].

Despite this progress, some aspects of sputum induction and analysis are incompletely standardised, particularly with regard to the effect of saliva contamination. Consequently, selection of lower respiratory portions from the expectorate has been advocated to overcome these problems [2]. The level of squamous contamination can vary widely between samples from different subjects. The effect of squamous contamination on fluid phase mediator assessment is unknown and may have important consequences when interpreting results.

The aim of this study was to evaluate the effect of salivary contamination on cellular and fluid phase markers of inflammation. The authors tested the hypotheses that excessive squamous contamination would have little impact on the differential cell counts but would lead to reductions in the concentrations of fluid phase markers. The study sought to identify the level of squamous contamination whereby inflammatory markers become erroneous to help determine the degree of squamous contamination that should lead to rejection of samples. In order to investigate these effects both induced sputum and saliva samples were collected and then increasing volumes of saliva were added to dispersed sputum samples.

Materials and methods

Study subjects

Subjects with asthma (n=7) and healthy controls (n=8) were recruited from the outpatient clinic at John Hunter Hospital and underwent a clinical assessment and sputum induction. The subjects had a mean (range) age of 45 (20–67) yrs and 11 (73%) were female. Subjects with asthma had a mean (range) forced expiratory volume in one second % predicted of 88.4% (63.5–122.7%). Written informed consent was obtained and the Hunter Area Research Ethics Committee approved the study.

Methods

Induced sputum and saliva collection. Subjects were asked to rinse their mouth out and gargle prior to the induction to reduce the risk of salivary contamination in the sputum sample.

Sputum induction was performed as described previously [6]. A saliva sample was collected after the sputum induction had been completed.

Induced sputum and saliva processing. Sputum mucocellular portions (400 μL) were selected and dispersed using dithiothreitol (DTT) (9.05 μg·mL^{-1}; Calbiochem, La Jolla, CA, USA). DTT was also added to 400 μL of saliva in a separate container. Both sputum and saliva samples were processed concurrently in separate tubes using the sputum processing method described previously [6]. Briefly, the sputum/DTT and the saliva/DTT solutions were rocked in a shaking waterbath at 37°C for 30 min before the addition of phosphate-buffered saline (PBS). The samples were then filtered. DTT-treated saliva was added to DTT-treated sputum (cells and supernatant) in increasing concentrations from 0 to 100% volume. The combined samples were then centrifuged to prepare a cell-free supernatant and a cell pellet. The cell pellet was then resuspended in PBS and cytospin slides were prepared.

Outcomes assessed. A total cell count (TCC), cell viability (using trypan blue exclusion), and differential cell counts (using May Grunwald Geimsa staining) were performed on cytospins. Supernatant samples were assessed for interleukin (IL)-8 (enzyme-linked immunosorbent assay; R&D Systems, Minneapolis MN, USA), eosinophil cationic protein (ECP) (radioimmunoassay; Pharmacia Diagnostics, Uppsala, Sweden) and total protein (Coomassie Plus; Pierce Rockford, IL, USA).

Statistical analysis

Results are reported as median (interquartile range (IQR)). Nonparametric tests were used to compare data. Friedman's test was used to examine the changes in parameters following the addition of saliva, with significance accepted at the $p<0.05$ level. Posthoc testing using the signed-rank test with bonferroni corrected p-values was performed. For posthoc tests, significance was accepted at $p<0.0125$.

Results

Characteristics of induced sputum and saliva

DTT-treated sputum contained a median of 2.5 (1–8.1)% squamous cells. The TCC was 36 (20–52) $\times 10^4$ cells·mL^{-1}. Sputum differential cell count results are shown in table 1. Sputum contained a median (IQR) of 346 (236–610) μg·mL^{-1} protein, 47 (13–318) ng·mL^{-1} ECP, and 0.7 (0.3–1.7) ng·mL^{-1} IL-8.

The percentage of sputum eosinophils was higher in subjects with asthma compared to healthy controls (1.6%

Table 1. – Effect of salivary contamination on sputum viability, differential, total and absolute cell counts

	Saliva concentration vol %						p-value
	0	11	20	50	70	80	
Viability %	82 (68–87)	80 (61–88)	82 (55–86)	83 (63–87)	71 (58–88)	74 (58–88)	NS
Differential cell count %							
Neutrophils	28.9 (19.5–46.8)	36.8 (17.7–50.9)	24.4 (16.7–38.2)	30.3 (20.8–51.4)	24.8 (12.4–51.1)	23.8 (13.4–50.4)	NS
Eosinophils	0.3 (0–1.8)	0.6 (0–3.1)	0.1 (0–3.6)	0.8 (0–4.2)	0 (0–2.9)	0.7 (0–2.4)	NS
Macrophages	63.5 (51.3–75.3)	58.1 (45.3–78.2)	69.6 (54.4–79.8)	61.1 (43.9–75.1)	70.9 (45.5–83.2)	73.0 (44.4–80.1)	NS
Lymphocytes	0.8 (0.2–0.8)	1.6 (0.4–2.3)	0.8 (0.4–1.2)	0.8 (0.3–1.5)	0.4 (0–0.7)	0.2 (0–0.7)	NS
Columnar epithelial cells	1.4 (0.7–3.5)	1.2 (0.3–2.3)	0.8 (0.5–2.5)	0.6 (0.2–4.1)	0.9 (0–2.9)	0.4 (0–1.5)	NS
Squamous epithelial cells	2.5 (1–8.1)	4.1 (2.9–11.4)	7.8 (4.3–15.7)	13.8 (8.2–36.8)	21.1 (13.3–48)	42.4 (18.9–63.3)	<0.0001
Absolute cell counts $\times 10^4$·mL^{-1}							
Total cells	36 (20–52)	29.5 (19–49)	28.5 (13–68)	20.5 (11–31)	13.5 (6–27)	7 (4–10)	<0.0001
Neutrophils	8.7 (4.7–20.4)	9.1 (4.6–14)	4.9 (2.8–15.1)	4.6 (2–8.9)	1.4 (0.7–4.5)	0.7 (0.3–1.8)	<0.0001
Eosinophils	0.13 (0–0.43)	0.17 (0–0.55)	0.05 (0–0.42)	0.16 (0–0.38)	0 (0–0.17)	0.05 (0–0.1)	0.004
Macrophages	25.3 (10.6–33.5)	23.3 (8.6–25.9)	20.4 (8.0–43.4)	13 (6.7–21.8)	10.4 (3.4–16.2)	4.4 (2.4–5.4)	<0.0001
Lymphocytes	0.30 (0.05–0.39)	0.34 (0.13–0.82)	0.28 (0.08–0.41)	0.17 (0.07–0.36)	0.04 (0–0.22)	0.01 (0–0.03)	<0.0001
Columnar epithelial cells	0.57 (0.36–0.72)	0.27 (0.16–1.02)	0.34 (0.12–0.61)	0.19 (0.10–0.42)	0.11 (0–0.19)	0.02 (0–0.14)	<0.0001

Data are presented as median (interquartile range). NS: nonsignificant.

Table 2. – Sputum cell count and fluid phase mediators for subjects with asthma and healthy controls

	Asthma	Healthy controls	p-value
Subjects n	6	4	
Total cells $\times 10^4$·mL^{-1}	35 (20–52)	38 (22–80)	NS
Viability %	78 (70–87)	82 (72–89)	NS
Neutrophils %	22.6 (16.4–33.1)	44 (29.7–58.3)	NS
Macrophages %	70.8 (64.4–78.8)	53.1 (38.9–62.4)	NS
Eosinophils %	1.6 (0.3–1.8)	0 (0–0.13)	0.02
Columnar epithelial cells %	1.3 (0.8–3.0)	2.1 (1.0–8.2)	NS
Lymphocytes %	0.5 (0–1.0)	0.77 (0.54–0.77)	NS
Squamous Cells %	2.8 (1–5.3)	2.5 (1.6–11.6)	NS
IL-8 ng·mL^{-1}	1.7 (0.9–2.5)	0.8 (0.3–2.7)	NS
ECP ng·mL^{-1}	547 (182.1–930.4)	38.3 (72–193.6)	NS
Total protein μg·mL^{-1}	606.4 (402.5–643.3)	385 (248.6–542.5)	NS

Data are presented as median (interquartile range). IL-8: interleukin-8; ECP: eosinophil cationic protein. NS: nonsignificant.

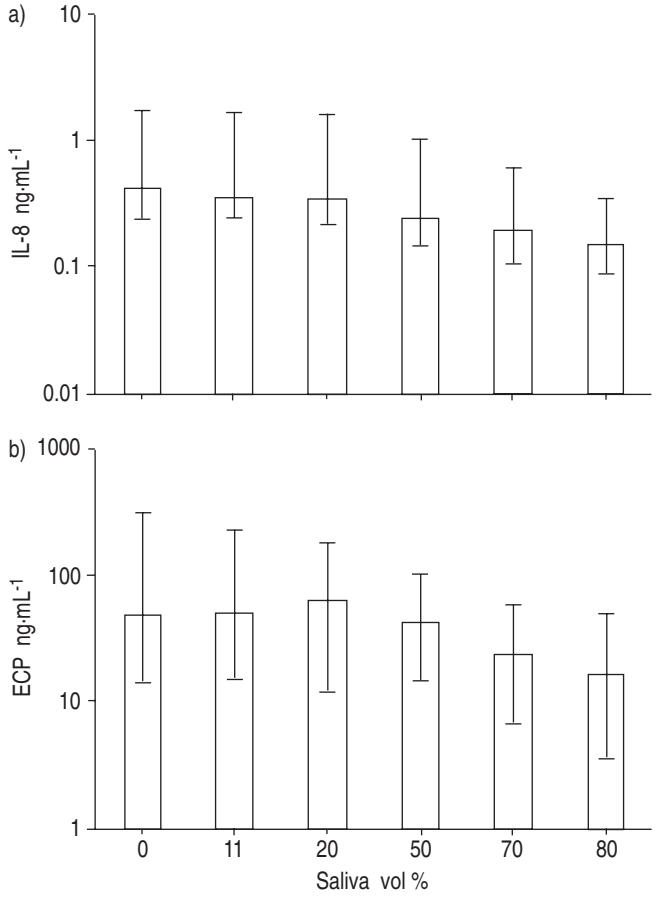

Fig. 1. – Effect of increasing salivary contamination on the concentration of a) interleukin (IL)-8 and b) eosinophil cationic protein (ECP) in sputum supernatant. Data are presented as median levels with interquartile range indicated as error bars.

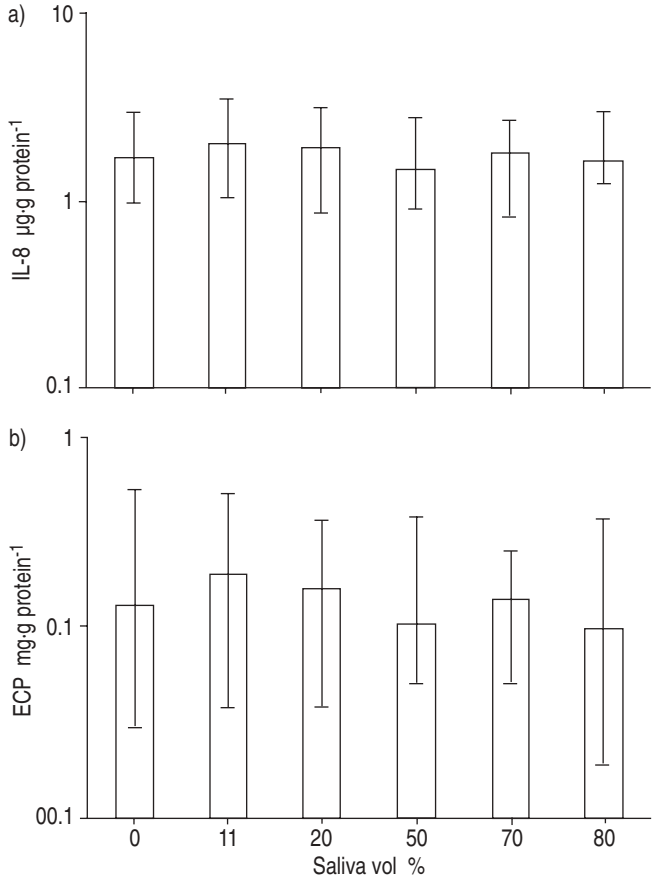

Fig. 2. – Effect of salivary contamination on a) interleukin (IL)-8 and b) eosinophil cationic protein (ECP) concentration corrected for protein content of sputum supernatant. Data are presented as median levels with interquartile range indicated as error bars.

versus 0%, p=0.02, table 2). There were no differences in the proportion of other cells including squamous epithelial cells between subjects with asthma and healthy controls (table 2). Levels of IL-8, ECP and total protein were similar between subjects with asthma and healthy controls (p>0.05, table 2).

Saliva contained a median of 0 (0–3)×10^4 cells per mL (data not shown). Three samples (20%) had enough cells to prepare a cytospin slide, which contained a median of 100% squamous cells. Neutrophils and macrophages were observed on only one of the cytospins for 100% saliva concentration. The concentration of IL-8 in saliva samples was 0.09 (0.05–0.123) ng·mL^{-1} and was measurable in 10 (67%) of samples. ECP levels were 4.6 (2.5–25.7) ng·mL^{-1} and was measurable in only 7 (47%) of samples (data not shown).

Effect of saliva contamination on induced sputum

Ten (67%) sputum samples (6 asthmatic) were of sufficient volume to obtain cell count data, collected in the saliva range of 0% to 80% (table 1). This data set was used for the analysis reported below.

Increasing saliva contamination resulted in a reduced TCC from 36×10^4 to 7×10^4 cells·mL^{-1} (p<0.0001), but had no effect on cell viability (table 1, p>0.05). There was no significant effect of salivary contamination on sputum differential cell counts (table 1, p>0.05). In contrast, the addition of saliva significantly reduced the absolute cell counts for all cell types (table 1).

The addition of saliva to induced sputum samples resulted in lower levels of protein in the supernatant (data not shown, p<0.0001). There was also a significant reduction in the levels of ECP and IL-8 in samples containing higher proportions of saliva than in neat samples of induced sputum (figs 1a, 1b, p<0.0001). Corrected IL-8 results, expressed as per µg of total protein, were not influenced by increasing dilution with saliva (p=0.228, fig. 2a). Statistically significant differences in ECP levels, after saliva dilution, remained after correction of ECP levels for protein (p=0.02, fig. 2b).

Levels of supernatant IL-8 and ECP were correlated in sputum (saliva concentration 0%: r=0.74, p=0.036). A similar level of association was observed at 10 and 20% saliva concentration. However, at saliva concentrations above 50% this association was not observed.

IL-8 levels were significantly reduced compared to induced sputum (saliva concentration 0%) samples when the salivary concentration reached 80% (p=0.0117), while for ECP the levels became significantly reduced when the salivary concentration reached 70% (p=0.0117).

Discussion

This study investigated the effects of salivary contamination on induced sputum total and differential cell counts, and fluid phase ECP, IL-8 and protein levels. The induced sputum differential cell count seems particularly robust, since there was little change as a result of salivary contamination up to a

level of 42% squamous epithelial cells, which corresponded to a saliva concentration of 80% volume. However, salivary contamination led to a progressive and significant reduction in both total and absolute cell counts, as well as fluid phase ECP, IL-8 and protein levels. Correcting mediator levels for protein content could adjust for the effects of salivary contamination on IL-8 levels, but not ECP levels.

Squamous contamination is a confounding variable in the interpretation of sputum markers. There are differences in the extent of squamous contamination between different subjects and between the selected and whole expectorate methods of processing [4, 5, 7]. The data from this study indicate that while there is some effect of salivary contamination on the cellular differential, the effect is minimal even when salivary concentration is >70%. Although squamous contamination is greater with the whole expectorate method, the current data indicate that this is unlikely to have an impact on the differential cell counts reported as a percentage of nonsquamous cells, and comparative studies confirm this [7, 8]. Therefore, both the selected and whole expectorate methods of sputum processing give comparable results for the sputum differential cell counts.

The effect of squamous contamination on fluid phase markers such as ECP and IL-8 is more problematic. There was a progressive reduction in ECP concentration with increasing salivary contamination. Samples that were 70% saliva, which corresponds to a squamous cell count of >20%, would be unsatisfactory for ECP measurement. Furthermore, a correction for total protein did not resolve this. The current study data indicate that the value of ECP is critically dependent on the degree of salivary contamination. This is consistent with other published data reporting ECP levels in samples processed to reduce salivary contamination and is the likely explanation for greatly varying ECP concentrations between the selected [4] and whole expectorate methods of processing [5, 7–8].

There may be an assumption that as long as the salivary contamination between samples is fairly constant, there would be little effect on relative levels of ECP. The current study′s data suggest this is true in samples containing up to 70% saliva, but at concentrations above this no assumptions should be made about relative levels of ECP. In the current study 70% saliva concentration corresponds to a salivary proportion of 21% of cells.

In an earlier study FAHY *et al.* [5] have shown that ECP, histamine, tryptase and albumin levels are lower in the saliva of subjects with asthma compared to induced sputum samples. However, the level of squamous contamination in the sputum of subjects in this study were much higher (46%) compared to the current study (2.8%), and from the current results the present authors would speculate that these samples would have further increased mediator levels if they had been processed to decrease squamous contamination.

Little is known about the effects of the saliva contamination on IL-8 assessment. IL-8, like ECP, showed a reduction in concentration with increasing saliva contamination. However, this appeared to be successfully addressed by correcting for the protein content of each sample, which resulted in the IL-8 concentration per μg of protein being similar at all levels of saliva contamination. The generalisability of this result requires further study. Sputum protein content would increase in conditions of marked inflammation. It is possible that normalisation of IL-8 levels may not be adequate to account for the dilutional effect of saliva under these circumstances. However, as these conditions are not likely to alter saliva protein levels, but may influence both sputum IL-8 and protein content to some degree, it is also possible that sputum IL-8 levels corrected for protein may adequately address salivary contamination. The reason for the observed correction in IL-8 and not ECP is unknown. However, these results indicate that the dilution kinetics for ECP are different to those for total protein.

In conclusion, these experiments confirm that sputum differential cell counts, expressed as a proportion of nonsquamous cells are robust measures. Total cell counts, absolute cell counts and fluid phase markers are less robust and are sensitive to the effects of salivary contamination. For interleukin-8, the effects of salivary contamination may be overcome by protein correction. However, this requires further study in subjects with more marked inflammation. The results of this study reinforce the European Respiratory Society Taskforce recommendation that sputum results should be reported as the differential cell counts together with the extent of squamous contamination [9].

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