Exhaled breath condensate biomarkers in asbestos-related lung disorders

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KEYWORDS
Exhaled breath condensate; Asbestos; Exhaled nitric oxide; Oxidative stress

Summary
Objectives: Asbestos induces generation of reactive oxygen and nitrogen species in laboratory studies. Several such species can be measured non-invasively in humans in exhaled breath condensate (EBC) but few have been evaluated. This study aimed to assess oxidative stress and lung inflammation \textit{in vivo}.

Methods: Eighty six men were studied: sixty subjects with asbestos-related disorders (asbestosis: 18, diffuse pleural thickening (DPT): 16, pleural plaques (PPs): 26) and twenty six age- and gender-matched normal individuals.

Results: Subjects with asbestosis had raised EBC markers of oxidative stress compared with normal controls [8-isoprostane (geometric mean (95% CI) 0.51 (0.17 to 1.51) vs 0.07 (0.04 to 0.13) ng/ml, p < 0.01); hydrogen peroxide (13.68 (8.63 to 21.68) vs 5.89 (3.99 to 8.69) \textmu M, p < 0.05), as well as increased EBC total protein (17.27 (10.57 to 28.23) vs 7.62 (5.13 to 11.34) mg/ml, p < 0.05), and fractional exhaled nitric oxide (mean ± SD) (9.67 ± 3.26 vs 7.57 ± 1.89 ppb; p < 0.05). EBC pH was lower in subjects with asbestosis compared with subjects with DPT (7.26 ± 0.31 vs 7.53 ± 0.24; p < 0.05). There were no significant differences in exhaled carbon monoxide, EBC total nitrogen oxides and 3-nitrotyrosine between any of the asbestos-related disorders, or between these and controls.

Conclusion: In asbestos-related disorders, markers of inflammation and oxidative stress are significantly elevated in subjects with asbestosis compared with healthy individuals but not in pleural diseases.

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Introduction

Lung disease due to inhalation of asbestos fibres continues to be a significant problem worldwide. Several different diseases occur, including asbestosis (diffuse interstitial pulmonary fibrosis due to asbestos inhalation) and pleural plaques (PPs). PPs are the commonest asbestos-related abnormality and generally regarded as benign markers of asbestos exposure. In contrast, diffuse pleural thickening (DPT) is rarer and results in a restrictive ventilatory defect and significant symptoms even in the absence of asbestosis. Distinguishing between asbestosis and asbestos-related pleural disorders is important not only for overall disease prognosis but also because of the acknowledged link between asbestosis and DPT and the development of lung cancer. The risk of lung cancer with asbestos exposure in the absence of asbestosis is significantly lower, although still under active debate.

Asbestos induces inflammation and cell death in animal models, modulating cell proliferation and causing DNA damage. It activates lung inflammatory cells such as neutrophils and macrophages, generating reactive metabolites such as hydrogen peroxide, superoxide anions, hydroxyl radicals and nitric oxide. Traditional methods of assessing lung inflammation (such as bronchoalveolar lavage (BAL), biopsy) in asbestos-related disorders are hampered by the fact that these are invasive, and patients affected are usually elderly, with poor lung function and frequent concurrent disease. Simple, non-invasive tests which accurately reflect lung pathology would therefore be of considerable use in diagnosis and possibly also in monitoring progress of these disorders.

Recently, several novel non-invasive techniques to assess lung disease have been developed. Exhaled breath consists of a gaseous phase containing volatile mediators (e.g. NO, CO) and a liquid phase which can be collected as exhaled breath condensate (EBC). Fractional exhaled nitric oxide (FeNO) has been measured in many lung diseases and is raised in asbestosis as well as in asthma and pulmonary fibrosis. However, it is non-specific. A more specific pattern of biomarkers might emerge using additional non-invasive techniques, in addition to elucidating underlying disease mechanisms. EBC could prove helpful in this regard. Inflammatory markers as well as those of oxidative imbalance can be measured in EBC and an increasing number of EBC biomarkers are being described. American Thoracic Society (ATS) and the European Respiratory Society (ERS) have recently published recommendations which summarise knowledge in this area. EBC is minimally invasive, easy for the patient, and can be repeated frequently. However, much still remains to be learnt about this new area.

In this study, we aimed to assess lung oxidative stress and inflammation in vivo in subjects with asbestos-related disorders and compare them with age matched controls. Although a limited number of EBC biomarkers have been examined in asbestosis in one report to date, there have been no studies examining subjects with other asbestos-related lung disorders. We hypothesized that, similar to animal and in vitro studies, markers of oxidative stress and those of lung inflammation would be elevated in asbestosis compared with controls but to a lesser extent in subjects with asbestos-related pleural disorders.

Methods

Study subjects

Subjects were recruited from the Dust Diseases Board (DDB) of New South Wales, and from St Vincent’s Hospital, Sydney. The study was approved by the Human Research Ethics Committee (HREC) and informed consent was obtained from all participants.

All subjects had a confirmed history of workplace asbestos exposure other than controls and were classified into three groups (asbestosis, diffuse pleural thickening (DPT) and pleural plaques (PPs)) according to current ATS recommendations. Smokers were excluded, but ex-smokers (who had stopped smoking ≥1 year) were included provided there was no evidence of other lung diseases. Age and sex-matched controls were recruited from St Vincent’s Hospital and the DDB. All control subjects were never or ex-smokers without any evidence of asbestos-related or other lung disease after screening. Subjects with asthma, COPD or any other factors which were likely to affect EBC or FeNO (e.g. inhaled glucocorticosteroids, nitrates) were excluded from the study.

Pulmonary function testing

All subjects had lung function performed according to ATS/ERS guidelines on the same day of the EBC collection. Forced expiratory volume in 1 s (FEV1 (L)), forced vital capacity (FVC (L)), vital capacity (VC (L)) and diffusing capacity for carbon monoxide (DLCO (ml/mmHg/min)) were measured via spirometry or body plethysmography (Vmax Spectra 22D, SensorMedics, CA, USA). Percentage of predicted values (% pred) was calculated using the European Coal and Steel regression equations.

Exhaled breath condensate biomarkers and exhaled breath markers

EBC was collected using a dedicated breath refrigeration circuit Ecocscreen (Erich Jaeger GmbH, Hochberg, Germany) according to ERS/ATS recommendations. Subjects first rinsed their mouths with distilled water and breathed tidally through a mouthpiece connected to a two-way non-rebreathing valve with nose-clip on, containing a saliva trap. One to two millilitres of condensate was collected after 10 min. In order to minimize thaw-freeze cycles and stabilize EBC biomarkers, the cooled condensate was immediately separated in aliquots and stored at −80 °C for further assays within 3 months. Enz-Chek Ultra Amylase kit (Molecular Probes, Invitrogen, Oregon, USA) was used to test the presence of α-amylase in samples (sensitivity: down to 2 mU/ml). Saliva contaminated EBC samples were excluded. FeNO and exhaled CO were measured online according to ERS and ATS specifications, using a rapid-response chemiluminescence NO and CO analyzer (LR 2500 (I); Logan Research, Rochester, UK). A constant expiratory flow rate of 250 ml/min was used, which was chosen to more closely reflect alveolar NO flux.
Laboratory analysis

Total nitrogen oxides (NOx) were measured after enzymatic reduction of nitrate using a fluorimetric modification of the Greiss reaction.\(^{14,20}\) Lower limit of detection of this assay was 2 µM. Total protein concentration was measured using a QuantiPro BCA assay kit (Sigma–Aldrich, Sydney, Australia), with lower limit of detection at 4 µg/ml. 8-Isoprostane was measured using a specific enzyme-immunoassay (ELISA) kit (Cayman Chemical, Ann Arbor, MI, USA), validated to obtain a high correlation (0.95) with known amounts of 8-isoprostane and with lower detection limit 5 pg/ml.\(^{21}\) Hydrogen peroxide (H\(_2\)O\(_2\)) was measured spectrophotometrically by horseradish peroxidase-catalysed oxidation of tetramethylbenzidine.\(^{13,14}\) The nitration product of tyrosine, 3-nitrotyrosine, was measured via enzyme immunoassay (EIA) (Cayman Chemical, Ann Arbor, MI, USA). Lower limit of detection was 4 ng/ml. EBC acidity was measured immediately after defrosting frozen EBC samples with a pH sensor (IQ125 Minilab Professional pH meter, Merck, CA, USA). pH measurement range was 2–12 (±0.1 pH).

Statistical analysis

Where data did not conform to Normal distribution, these were log transformed. Data were expressed as means ± standard deviation (SD) and as geometric means (95% confidence intervals (CI)) when log transformed unless otherwise stated. Sample size was calculated based on pH confidence intervals (CI) when log transformed unless otherwise stated. Sample size was calculated based on pH confidence intervals (CI) when log transformed unless otherwise stated. Results were considered significant if \(p < 0.05\). Correlation between biomarkers and lung function parameters was performed using Pearson’s correlation coefficient.

Results

Subjects

Eighty-six male subjects were studied (48 ex-smokers and 38 never smokers). None of the subjects was using inhaled corticosteroids. Demographic data are shown in Table 1.

EBC nitrogen oxides (NOx) and 3-nitrotyrosine

EBC NOx was measurable in all groups. A trend towards increasing EBC NOx concentrations in patients with DPT and asbestosis compared with normal controls was observed but did not reach statistical significance. EBC levels of NOx did not correlate with 3-nitrotyrosine or hydrogen ion (H\(^+\)) concentration in EBC. However, NOx correlated directly

<table>
<thead>
<tr>
<th>Subject characteristics</th>
<th>Normal</th>
<th>Pleural plaques</th>
<th>DPT</th>
<th>Asbestosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of patients</td>
<td>26</td>
<td>26</td>
<td>16</td>
<td>18</td>
</tr>
<tr>
<td>Age (years)</td>
<td>71 ± 9.19</td>
<td>70 ± 5.23</td>
<td>72 ± 7.04</td>
<td>75 ± 5.58</td>
</tr>
<tr>
<td>Ex-smokers</td>
<td>12 (46%)</td>
<td>11 (42%)</td>
<td>11 (69%)</td>
<td>14 (78%)</td>
</tr>
<tr>
<td>Lung function results</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FEV(_1) (%pred)</td>
<td>91.65 ± 15.41</td>
<td>89.12 ± 16.41</td>
<td>78.31 ± 19.68*</td>
<td>78.47 ± 26.3*</td>
</tr>
<tr>
<td>FVC (%pred)</td>
<td>91.88 ± 16.46</td>
<td>91.73 ± 16.04</td>
<td>82.88 ± 18.47</td>
<td>77.71 ± 20.11*</td>
</tr>
<tr>
<td>VC (%pred)</td>
<td>98.18 ± 15.80</td>
<td>100.0 ± 10.98</td>
<td>87.00 ± 17.91</td>
<td>3.23 ± 24.24*</td>
</tr>
<tr>
<td>DLCO (%pred)</td>
<td>89.43 ± 15.26</td>
<td>86.69 ± 16.06</td>
<td>75.31 ± 22.13*</td>
<td>56.94 ± 20.87***</td>
</tr>
</tbody>
</table>

Data presented as means ± standard deviations unless otherwise specified. Significant difference between subjects with asbestosis and DPT and normal controls (***p < 0.001, *p < 0.05) and also between subjects with asbestosis and those with pleural plaques (**p < 0.001).
with protein ($r = 0.64, p < 0.0001$), 8-isoprostane ($r = 0.59, p < 0.0001$) and H$_2$O$_2$ ($r = 0.61, p < 0.0001$) in EBC in all groups.

3-Nitrotyrosine was difficult to detect in EBC of normal subjects. Most of the results were close to the limit of detection of the assay and below this in 20 patients. However, there was no significant difference in EBC 3-nitrotyrosine between normal subjects and patients with asbestos-related diseases, and no significant correlation between 3-nitrotyrosine and either FeNO levels, other EBC biomarkers or lung function.

EBC pH ($H^+$ concentration)

Subjects with asbestosis had significantly lower EBC pH than individuals with DPT (7.26 ± 0.31 versus 7.53 ± 0.24, $p < 0.05$; Fig. 2B). EBC pH was lower in asbestosis than in controls but this did not reach statistical significance (7.26 ± 0.31 versus 7.44 ± 0.32, $p = 0.07$). There were no significant EBC pH differences between other groups. No significant correlation between $H^+$ and other biomarkers or lung function was observed.

Exhaled breath markers: fractional exhaled nitric oxide (FeNO) and exhaled carbon monoxide (eCO)

FeNO was significantly increased in patients with asbestosis compared with controls (9.67 ± 3.26 versus 7.57 ± 1.89 ppb; $p < 0.05$) (Table 2, Fig. 3). There was no significant correlation between FeNO and lung function, or with any EBC biomarker. There was no significant difference in eCO between the different asbestos-related diseases nor compared with controls, neither any significant correlation between eCO and lung function or EBC biomarkers.

Discussion

Non-invasive techniques have not yet been widely investigated in occupational lung disease. These methods are totally non-invasive, inexpensive and easy to repeat, providing a rapid method of assessing biomarker levels. FeNO has evolved over the last decade from a research tool into a clinical measurement useful for diagnosing and
monitoring asthma, and is also likely to prove useful in occupational asthma.23

Much less information is available about EBC biomarker analysis, particularly with regard to asbestos-related disorders, with only two reports to date published on this topic.11,24 Our results confirm our own previous findings that FeNO is raised in asbestosis10 and are compatible with that of others11,25,26 as well as revealing new information regarding EBC biomarkers in the range of asbestos-related disorders. We have confirmed laboratory evidence suggesting that reactive oxygen species (ROS) and reactive nitrogen species (RNS) play an important role in inducing lung toxicity from asbestos7,27 in vivo, and that levels of oxidative stress differ between the different asbestos-related disorders. Although our study was not designed specifically to compare conventional methods with newer techniques, it seems likely that non-invasive methods may prove a useful addition to these in the future, particularly in elderly or infirm patients.

Asbestos fibres are cytotoxic both in vitro and in vivo.7,17 Asbestos produces ROS (superoxide, H₂O₂ and hydroxyl radicals) and RNS (nitric oxide, nitrogen oxides and peroxynitrite) by at least two mechanisms.28 ROS contribute towards asbestos-induced alveolar cell injury through lipid peroxidation and release of inflammatory cytokines, leading to apoptosis, persistent lung inflammation and potentially also to malignant transformation. A variety of different cytokines are probably involved, including TNF isoforms,36 macrophage inflammatory protein 2 (MIP-2), PDGF isoforms40 and also interleukins such as IL-1 and IL-8.28 Although the current study did not examine the full range of such cytokines, several of these have been measured in EBC in our laboratory.10,14 and this study has confirmed the feasibility of their measurement in future research into asbestos-related disorders.

Information is still evolving about technical aspects of EBC, its clinical application, and its potential confounding factors.13,15 A wide variety of different biomarkers can be measured,14,31 and lung acidification assessed,32 but the exact origin of EBC and all the factors affecting biomarker concentrations have yet to be fully elucidated. While acknowledging this, we carefully designed our study to exclude known factors such as smoking33,34 and inhaled corticosteroids15,36 that affect EBC biomarker levels, utilized age and sex-matched controls, and collected and analyzed our samples according to published recommendations. One potential confounding factor is the fact that our subjects (although current non-smokers and age- and sex-matched) were not matched for past smoking habit. However, we believe this to be unlikely as an explanation of our results, as we excluded all subjects with a past medical history of asthma or emphysema, and none had airflow obstruction on lung function testing. Also, the proportion of ex-smokers in our group with DPT was similar to that in our group with asbestosis; yet significant differences in biomarkers were found only in the latter. Several studies37,38 have shown poor reproducibility of biomarkers in EBC, although pH is probably more reproducible,33,39,40 and there is still on-going debate regarding potential salivary contamination for NOx in particular. However, this is less relevant to our study because we used identical techniques for subjects with disease and controls, so any error should apply equally to both techniques, and we included an adequate number of subjects. We used the EcoScreen® commercial device for EBC sampling which collects EBC as ice, which is thought to be beneficial for unstable markers, and the methodology recommended by the ATS/ERS task force.13 We did not de-aerate EBC with

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**Table 2** Exhaled breath condensate (EBC) biomarker levels.

<table>
<thead>
<tr>
<th>EBC biomarkers</th>
<th>Normal</th>
<th>Pleural plaques</th>
<th>DPT</th>
<th>Asbestosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>eCO (ppm)</td>
<td>2.55 ± 0.96</td>
<td>2.60 ± 0.85</td>
<td>2.80 ± 0.81</td>
<td>1.82 ± 1.06</td>
</tr>
<tr>
<td>NOx (μm)</td>
<td>28.72 ± 17.11</td>
<td>28.54 ± 17.97</td>
<td>36.68 ± 24.51</td>
<td>35.94 ± 22.74</td>
</tr>
<tr>
<td>3-Nitrotyrosine (ng/ml)</td>
<td>0.39 (0.13–1.18)</td>
<td>1.47 (0.60–3.61)</td>
<td>2.23 (0.45–11.18)</td>
<td>0.20 (0.05–0.86)</td>
</tr>
<tr>
<td>FeNO (ppb)</td>
<td>7.57 ± 1.89</td>
<td>8.38 ± 3.43</td>
<td>8.92 ± 4.08</td>
<td>9.67 ± 3.26*</td>
</tr>
<tr>
<td>pH</td>
<td>7.44 ± 0.32</td>
<td>7.40 ± 0.37</td>
<td>7.53 ± 0.24</td>
<td>7.26 ± 0.30†</td>
</tr>
<tr>
<td>H⁺ ions (μm)</td>
<td>0.05 ± 0.04</td>
<td>0.08 ± 0.19</td>
<td>0.03 ± 0.02</td>
<td>0.07 ± 0.06†</td>
</tr>
<tr>
<td>Protein (μg/ml)</td>
<td>7.62 (5.13–11.34)</td>
<td>8.05 (5.27–12.29)</td>
<td>12.94 (9.00–18.59)</td>
<td>17.27 (10.57–28.23)*</td>
</tr>
<tr>
<td>8-isoprostane (ng/ml)</td>
<td>0.07 (0.04–0.13)</td>
<td>0.12 (0.06–0.23)</td>
<td>0.22 (0.07–0.71)</td>
<td>0.51 (0.17–1.151)**</td>
</tr>
<tr>
<td>H₂O₂ (μm)</td>
<td>5.89 (3.99–8.69)</td>
<td>6.96 (5.03–9.63)</td>
<td>9.16 (6.24–13.44)</td>
<td>13.68 (8.63–21.68)*</td>
</tr>
</tbody>
</table>

Data presented as means ± standard deviation and those log transformed expressed as geometric means (95% confidence interval); significant difference between subjects with asbestosis and normal controls (**p < 0.01; *p < 0.05) and also between those with asbestosis and DPT (p < 0.05).

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**Figure 3** Fractional exhaled nitric oxide (FeNO) measured in parts per billion (ppb) in normal subjects (n = 19), PPs (n = 17), DPT (n = 12) and asbestosis (n = 16). (Bars = means.)
argon gas prior to pH measurement because our study was initiated before this was recommended, when opinion regarding the advisability of this procedure was divided. However, our results are consistent with our previous data which showed a close correlation between aerated and de-aerated samples, and were not the primary outcome variable of the study.

The elevated FeNO we once again found in asbestosis is likely to reflect on-going lower respiratory tract inflammation and is probably alveolar in origin. We did not have the facility for extended NO measurement, but Lehtonen and colleagues, have studied this and found that alveolar NO concentrations were raised in asbestosis compared with healthy controls whereas bronchial NO flux was similar in both groups. Exhaled CO2, which has been variably shown to be increased in asthma did not differ among the groups in our study. We also found markers of oxidative stress (8-isoprostanate and hydrogen peroxide) to be elevated in subjects with asbestosis but not other asbestos-related lung disorders. 8-Isoprostanate is a stable end product of lipid peroxidation which is released from inflammatory cells activated by asbestos fibres. H2O2 is produced by several inflammatory cells including pulmonary macrophages and by parenchymal epithelial cells. 8-Isoprostanate has been reported to be increased in EBC of patients with different respiratory diseases, including in asbestosis. Our findings confirm these findings and extend them to include H2O2, where elevated H2O2 levels were similar to subjects with idiopathic pulmonary fibrosis.

We assessed EBC total nitrogen oxides (NOx) and 3-nitrotyrosine. 3-Nitrotyrosine is an end product formed upon reaction of free or protein-bound tyrosine with NOx. Unlike FeNO, EBC 3-nitrotyrosine and NOx were not significantly increased in the asbestos-exposed group compared to controls, nor did these correlate with FeNO. However, EBC NOx did show some non-significant trend towards a relationship with increased lung inflammation, with a gradation in levels from the PPs to the asbestosis groups. EBC NOx are in general unstable and have been well documented to be affected by several other factors e.g. oral bacteria, diet, NOx contamination from the collection device. 3-Nitrotyrosine proved difficult to measure and did not add to our findings. Overall, our results indicate that NOx and 3-nitrotyrosine are currently not useful for assessing asbestos-related disease.

The stability and bioactivities of many RNS and ROS are pH dependent, and thus airflow pH is likely to be relevant to the effects of asbestos on the lung. Airway pH changes in a variety of different lung diseases including asthma, COPD and also with gastro-oesophageal reflux, but has not been previously assessed in asbestos related lung diseases, including in asbestosis. The facility for extended NO measurement, but Lehtonen and colleagues, have studied this and found that alveolar NO concentrations were raised in asbestosis compared with healthy controls whereas bronchial NO flux was similar in both groups. Exhaled CO2, which has been variably shown to be increased in asthma did not differ among the groups in our study. We also found markers of oxidative stress (8-isoprostanate and hydrogen peroxide) to be elevated in subjects with asbestosis but not other asbestos-related lung disorders. 8-Isoprostanate is a stable end product of lipid peroxidation which is released from inflammatory cells activated by asbestos fibres. H2O2 is produced by several inflammatory cells including pulmonary macrophages and by parenchymal epithelial cells. 8-Isoprostanate has been reported to be increased in EBC of patients with different respiratory diseases, including in asbestosis. Our findings confirm these findings and extend them to include H2O2, where elevated H2O2 levels were similar to subjects with idiopathic pulmonary fibrosis.

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In conclusion, our study has confirming the relevance of oxidative stress and nitrogen species in asbestosis, and suggests that EBC biomarkers may prove useful non-invasive tools in diagnosing and distinguishing between the different asbestos-related disorders in the future.

Conflict of interest statements
SC, CC, AS and ARJ have none declared.
PST has received fees for educational presentations from Boehringer Ingelheim and Altana.
DHY has received a speaker’s fee from AstraZeneca and is a co-investigator peer reviewed grant funded by Actelion, but has received no other funding from any pharmaceutical company.

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