Dose response of CPAP on nasal symptoms, obstruction and inflammation \textit{in vivo} and \textit{in vitro}

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Running Head: Effect of CPAP on the airway and inflammation.

ABSTRACT
Obstructive sleep apnoea (OSA) is a common condition associated with cardiovascular risk. Continuous positive airway pressure (CPAP) is an effective treatment but associated with nasal side effects, which hinder compliance and may result from inflammation.
We investigated whether CPAP was pro-inflammatory to human subjects \textit{in vivo}, and to cultured bronchial epithelial cells \textit{in vitro}. \textit{In vivo}, we further investigated whether induction of nasal inflammation was associated with the development of systemic inflammation, nasal symptoms, and changes in nasal mucociliary clearance.

\textit{In vitro}, CPAP resulted in cytokine release from cultured BEAS-2B cells in a time- and pressure (dose)-dependent manner. \textit{In vivo}, CPAP resulted in dose-dependent up-regulation of nasal inflammatory markers associated with the development of nasal symptoms, and reduced mucociliary clearance. CPAP also up-regulated selected markers of systemic inflammation.

CPAP results in dose-dependent release of inflammatory cytokines from human epithelial cells \textit{in vitro} and \textit{in vivo}. \textit{In vivo} responses were associated with systemic inflammation, reductions in nasal mucociliary function and the development of nasal symptoms. This emphasises the need for novel strategies to reduce nasal inflammation and therefore aid compliance.

\textbf{Keywords:} airway symptoms, bronchial epithelial cells, inflammatory markers, continuous positive airway pressure, mucociliary clearance
INTRODUCTION

Obstructive sleep apnoea (OSA) syndrome is the most common sleep disorder, affecting up to one-fourth of the Western adult population [1]. It occurs when the pharyngeal airway becomes narrow due to the natural relaxation of muscles during sleep. Nasal continuous positive airway pressure (CPAP) has become the gold-standard management of clinically significant OSA [2]. CPAP is a distending mechanical split pressure applied at a continuous level throughout the respiratory cycle to maintain an open airway, preventing airway collapse during sleep [3, 4]. OSA is associated with significant excess cardiovascular risk [3, 4].

Despite its beneficial effects on airway patency, CPAP treatment is associated with a high prevalence of side effects [5, 6]. Some patients adapt to the treatment within a few weeks, others struggle for longer periods, and some discontinue treatment entirely with consequent detriment to their health. Although the long-term compliance rate is generally good, 8-15% of OSA patients refuse treatment after a single night of use in the laboratory setting [6-9]. There are reports of many adverse symptoms occurring with CPAP use, including nasal congestion, sneezing, anosmia, itchy nose, dry nose, mouth, throat and eyes, blocked ears, and dizziness [5]. Thus, initial experiences of the patient with CPAP may be of great importance in long-term treatment compliance.

The development of nasal symptoms with CPAP treatment may be related to the induction of nasal inflammation. Several clinical and experimental studies have reported on local and systemic inflammatory outcomes with ventilatory support [10-16], but little is known about the early induction of nasal inflammation with CPAP and how this relates to changes in nasal physiology, symptoms and therefore compliance. In addition, reported symptoms and inflammatory changes may be influenced by pre-existing conditions, such as OSA. Cell culture studies with CPAP are even scarcer and have mainly focused on stretch injury.
(barotrauma) rather than air pressure. We hypothesised that examining early symptoms and inflammatory changes after a short period of CPAP in CPAP-naïve healthy individuals in vivo, and in epithelial cell cultures in vitro would provide complementary insights into the mechanisms associated with the development of adverse symptoms and inflammation.

This study aimed (1) to investigate the short-term, dose-response effects of CPAP on airway and systemic inflammation, nasal symptoms and airway obstruction in CPAP-naïve healthy individuals in vivo and (2) to examine the dose-response secretion of two key interleukins by bronchial epithelial cell cultures (BEAS-2B) with CPAP during several hours of application in vitro.

MATERIALS AND METHODS

In vivo study

Study subjects and protocol

Thirty-one healthy non-smokers (21 male and 10 female) with no prior history of nasal symptoms or disease were recruited for the study. The protocol was approved by the Research Ethics Committee at Royal Free Hampstead NHS Trust (study reference 09/H0720/24) and was conducted in accordance with the Declaration of Helsinki. Informed written consent was obtained from all subjects prior to their inclusion in the study.

One higher and one lower CPAP pressure (within the range of clinical use) was selected for the in vivo component of the study: 7.5 cm H₂O and 12.5 cm H₂O. Twenty-two subjects received three hours of standard CPAP (REMstar® Auto M Series with A-Flex™) at 7.5 cm H₂O pressure, without humidification, through a nasal mask. Thirty-one subjects (11 of whom had received the 7.5 cm H₂O protocol six months previously) received three hours of
CPAP treatment at 12.5 cm H₂O, also without humidification. The subjects’ mouth was closed to prevent leakage. Assessments were performed before and after intervention; thus the baseline measurement of each individual served as their own control. The following assessments were made: (i) nasal and systemic inflammation (interleukin (IL)-6, IL-8 and myeloperoxidase (MPO) concentration in serum and nasal wash samples, and nasal wash leukocyte count, (ii) functional assessments (spirometry, acoustic rhinometry, and nasal mucociliary clearance), and (iii) nasal symptoms.

**Measurements**

*Nasal and systemic inflammation*

Nasal wash samples were obtained and processed according to a technique that we have previously reported [17], modified from that described by Hilding [18]. In brief, a paediatric tracheostomy tube (Bivona Fome-Cuf, size I.D 2.5 mm; Smiths Medical, Kent, UK) was used to collect the nasal lavage. The recovered lavage from the two nostrils was pooled for analysis. To ensure standard conditions, all sampling procedures were performed by the same investigator.

Peripheral venous blood samples were obtained for serum measurements. A 5 ml sample of venous blood was collected into a sterile vacutainer, centrifuged at 2,000 rpm for 10 minutes at 4°C, and the supernatant was stored at -80°C for later analysis of inflammatory mediators.

Measurements of the inflammatory cytokines (IL-6, IL-8 and MPO) in nasal wash supernatants and sera were performed by a standard enzyme-linked immunosorbent assay (ELISA) technique. The ELISA kits were obtained from R&D Systems, Abingdon, UK. The detection limits were 0.70 pg/ml for IL-6, 3.5 pg/ml for IL-8, and 1.5 ng/ml for MPO.
Physiological assessments

Acoustic rhinometry measurements were performed in accordance with a previously published protocol [19]. The device used was an A1 Acoustic rhinometry equipment with software version 0.5 (GM Instruments, Kilwinning, UK). All measurements were performed by the same operator in the same air-conditioned room to provide similar conditions with regard to temperature, humidity and ambient noise levels. The following five important acoustic rhinometry variables were assessed and examined separately: (i) outermost minimum cross-sectional area (MCA1), (ii) the distance of the MCA1 from the nasal orifice (D-MCA1), (iii) innermost minimum cross-sectional area (MCA2), (iv) the distance of the MCA2 from the nasal orifice (D-MCA2), and (v) the volume of the nasal segment between the 2nd and 5th cm from the nasal orifice (V2-5). Area and volume values from the right and left nostrils were averaged to account for variations with the nasal cycle.

The best of three attempts at spirometry was recorded using a Vitalograph 2160 (Maids Moreton, Buckingham, UK). A bronchodilator was not administered. We recorded forced expiratory volume in 1 second (FEV1), forced vital capacity (FVC), FEV1/FVC ratio and peak expiratory flow rate (PEFR).

Nasal mucociliary clearance was measured using the modified in vivo saccharin transit time (STT) technique described by Rutland and Cole [20]. Saccharin was applied on the inferior turbinate of the nasal cavity under direct visualisation, and the time at which the subject perceived a sweet taste on the tongue was recorded in seconds using a stopwatch.

Assessment of nasopharyngeal symptoms
The presence or absence of nasopharyngeal symptoms (including rhinorrhoea, postnasal drip (PND)), nasal congestion, sneezing, reduced smell, and itchy nose) was assessed before and after CPAP intervention.

*In vitro study*

All chemicals and reagents were of tissue culture grade and were obtained from Sigma-Aldrich Chemical Co. (Poole, UK) unless otherwise stated. ELISA kits were obtained as above for the *in vivo* work.

*Culture of bronchial epithelial cells*

BEAS-2B cells, a virus-transformed human bronchial epithelial cell line, were obtained from the American Type Culture Collection (Manassas, VA, USA) [21]. After culturing, when the cells became fully confluent, the culture medium was removed, and the cells were washed with 10 ml of sterile PBS. The PBS was then discarded, after which 2-3 ml Trypsin/EDTA (0.25%, w/v) was added for 3 to 5 minutes to disperse the cells so that they could be transferred to 6 cm Falcon ‘Primera’ culture dishes (Becton Dickinson, Oxford, UK). Cultures were then incubated (Galaxy R; Wolf Laboratories, York, UK) in 2 ml fresh, sterile, complete culture medium containing 10% foetal calf serum (Sigma-Aldrich), 5 ml antibiotic/antimycotic solution, and 4 ml of each of the following: bovine pancreatic insulin (2.5 mcg/ml), human transferrin (2.5 mcg/ml), hydrocortisone (0.36 mcg/ml) and L-glutamine (0.02 mg/ml) made up to a final volume of 500 ml in Medium 199 and filter-sterilised through a 0.22 μm syringe filter. The antibiotic/antimycotic solution contained 10,000 units penicillin G, 10mg streptomycin and 25mcg amphotericin B per millilitre. Cells were then incubated for one to three days at 37°C in 95% air and 5% CO₂.
In vitro CPAP exposure

Cultured BEAS-2B cells were exposed in vitro to CPAP pressure in a chamber at 0, 4, and 7 cm H2O for 1, 2, 3, or 4 hours, after which the release of cytokine concentration was measured in the culture medium. The protocol is summarised in Figure 1A.

Ten tissue culture dishes (60 x 15 mm) were used at each time-point. The day prior to the experiment, the complete medium was removed and replaced with 2 ml of 199 medium/antibiotics and incubated at 37°C in 95% air and 5% CO2 overnight. This was replaced the next morning before the experiment with 5 ml of fresh 199 medium/antibiotics. The cells were then incubated for 5 minutes, and 125 µl aliquots of cell culture supernatant were removed at zero time (before any pressure application) and stored at -80°C for later analysis.

The experimental equipment is shown in Figure 1B. CPAP (REMstar® Auto M Series with A-Flex™), at pressures of 4 or 7 cm H2O, was applied by incubating the cells in an airtight chamber (4.6 L) for the appropriate time, with 10 replicates per time point. The machine leak alarm was monitored to ensure that pressure was being delivered to the cells. The air-tight chamber was placed inside a 60 L acrylic SI.60 incubator (Stuart Scientific, Redhill, England, UK) to ensure conditions of controlled temperature reflecting the temperature in the nasal airway (31-33°C); humidity was maintained at approximately 98% by placing a 150 mm tissue culture dish with 20 ml of sterile water in the chamber. Humidity was monitored using a hygrothermometer. During each experiment, the modified chamber was tilted gently to an angle of 10° from the horizontal in each quarter of the horizontal plane on a Luckham 4RT rocking table (Luckham Ltd., Burgess Hill, England, UK), thereby momentarily displacing approximately half of the medium covering the surface of the culture plate during each tilt to
directly expose the cells to pressurised, humidified air, mimicking intranasal physiological conditions.

At the end of each time-interval for the allocated pressure, and at which point the experiment was terminated, 1 ml aliquots of cell culture supernatant were removed. The remaining medium was then removed, and the cells were harvested by scraping them in 1 ml of 199 medium only. All experimental media and cell scrapes were stored at -80°C for later analysis. Control experiments that were not exposed to CPAP were run for each of the four time points.

_Measurements of IL-6 and IL-8 concentration_

The cell culture supernatant samples were analysed for IL-6 and IL-8 by ELISA kits as described above.

To account for differences in the sizes of the cultures, cytokine concentrations were expressed corrected for total cellular protein concentration. Total cellular protein concentration was measured by a modified Lowry assay (Bio-Rad Laboratories, Inc., Hercules, CA, USA) [22]. All results were expressed as pg cytokine/µg cellular protein.

_Statistical analysis_

Data were analysed using GraphPad Prism 5 software (GraphPad Software Inc., La Jolla, CA, USA). The Kolmogorov–Smirnov test of normality was applied. For the _in vivo_ study, paired t-tests were used to examine differences between baseline and post-CPAP therapy measurements. A one-way ANOVA was run to examine dose-response differences between treatments, followed by post-hoc Tukey’s multiple comparison tests. For the subgroup analysis, one-way repeated measures ANOVA for parametric data and Friedman test for
nonparametric data were run to examine dose-response differences between treatments as appropriate, followed by post-hoc Tukey’s multiple comparison tests. Pearson (r) and Spearman (rho) correlations were conducted as appropriate to examine relationships between variables. A Chi-squared ($x^2$) test was used to compare nasopharyngeal symptoms at baseline and after CPAP therapy. For in vitro study, one-way ANOVAs were run to examine dose-response differences between cell culture responses to CPAP over several hours of application, followed by post-hoc Tukey’s multiple comparison tests. Multiple linear regression analyses were performed to determine a set of independent variables (time and pressure) that predicted in vitro cytokine productions. A P value < 0.05 was considered statistically significant.

**Ethics statement**

Written informed consent was received from participants prior to inclusion in the study, and the study had institutional approval as detailed above.

**RESULTS**

*In vivo studies*

The baseline characteristics of the subjects enrolled in the study are reported in Table 1. Both groups were healthy non-smokers, with a mean age between 33 and 34 years.

**Table 1**

Baseline information of subjects enrolled in the study. Eleven patients took part in both protocols.

<table>
<thead>
<tr>
<th>Details of subjects studied</th>
<th>7.5 cm H₂O (n=22)</th>
<th>12.5 cm H₂O (n=31)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>SD</td>
<td>Mean</td>
</tr>
</tbody>
</table>

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Changes in nasal and systemic inflammation with CPAP

The changes in serum and nasal wash inflammatory markers in response to three hours of nasal CPAP are reported in Table 2. Both CPAP pressures resulted in significant increases in nasal inflammation as assessed by nasal wash leukocyte and MPO measurements. The increase in nasal IL-6 and IL-8 concentrations following CPAP was only statistically significant at the higher pressure. Both pressures also resulted in changes in systemic inflammatory markers, with significant increases in serum IL-6 concentrations and decreases in serum IL-8 concentrations following CPAP. There was no change in serum MPO concentration. The ANOVA test highlights the observed dose (pressure) responses for changes in inflammation with CPAP, illustrated in Figure 2. When one-way repeated measures ANOVA was conducted for the subset of subjects (n=11) that underwent both experiments (7.5 and 12.5 cm H₂O), the changes in nasal wash IL-6 (p=0.038) and nasal wash MPO (p=0.027) remained statistically significantly.

Table 2

Changes in inflammatory markers in serum and nasal wash fluid from baseline to post-three hours of CPAP treatment.

<table>
<thead>
<tr>
<th>Marker</th>
<th>7.5 cm H₂O (n = 22)</th>
<th>12.5 cm H₂O (n = 31)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>After CPAP</td>
</tr>
<tr>
<td>Inflammatory markers in serum</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-6, pg/ml</td>
<td>4.6 (3.5)</td>
<td>6.3 (3.5)</td>
</tr>
<tr>
<td>IL-8, pg/ml</td>
<td>20.9 (7.8)</td>
<td>16.5 (7.3)</td>
</tr>
</tbody>
</table>
Data are expressed as the geometric mean (SD). *versus baseline, paired samples t-test. **ANOVA: compares the baseline (mean of the two baselines in the 11 subjects who had both pressures) with the 22 results at 7.5 cm H₂O and the 31 subjects at 12.5 cm H₂O nCPAP. IL, interleukin; MPO, myeloperoxidase; CPAP, continuous positive airway pressure.

Changes in physiology with CPAP

The changes in spirometry, rhinometry and nasal mucociliary clearance are reported in Table 3. At both pressures, three hours of nasal CPAP treatment resulted in a significant slowing of nasal clearance (i.e., increased saccharin transit time) without significant changes in rhinometry variables. At both pressures, CPAP was associated with small but significant changes in FVC and PEFR (but not FEV₁).

Table 3
Changes in lung function, rhinometry findings and nasal mucociliary clearance from baseline to after three hours of nCPAP treatment. ANOVA

<table>
<thead>
<tr>
<th>Parameter</th>
<th>7.5 cm H₂O (n = 22)</th>
<th>12.5 cm H₂O (n = 31)</th>
<th>P value*</th>
<th>ANOVA**</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lung function test</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FEV (l)</td>
<td>3.03 (0.52)</td>
<td>3.05 (0.50)</td>
<td>0.093</td>
<td>0.596</td>
</tr>
<tr>
<td>FEV1 %</td>
<td>86.31 (5.94)</td>
<td>86.22 (5.75)</td>
<td>0.506</td>
<td>0.091</td>
</tr>
<tr>
<td>FVC (l)</td>
<td>3.54 (0.63)</td>
<td>3.58 (0.63)</td>
<td>0.030</td>
<td>0.026</td>
</tr>
<tr>
<td>FVC %</td>
<td>83.06 (5.62)</td>
<td>83.71 (5.53)</td>
<td>0.052</td>
<td>0.071</td>
</tr>
<tr>
<td>FEV1/FVC ratio</td>
<td>85.99 (6.00)</td>
<td>85.76 (6.10)</td>
<td>0.615</td>
<td>0.354</td>
</tr>
<tr>
<td>PEFR (l)</td>
<td>519.00 (71.53)</td>
<td>527.27 (62.79)</td>
<td>0.024</td>
<td>0.625</td>
</tr>
<tr>
<td>Acoustic rhinometry</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MCA1</td>
<td>0.57 (0.31)</td>
<td>0.60 (0.38)</td>
<td>0.527</td>
<td>0.175</td>
</tr>
<tr>
<td>DMCA1</td>
<td>2.15 (0.38)</td>
<td>2.15 (0.34)</td>
<td>0.947</td>
<td>0.529</td>
</tr>
<tr>
<td>MCA2</td>
<td>1.62 (0.54)</td>
<td>1.58 (0.45)</td>
<td>0.488</td>
<td>0.531</td>
</tr>
<tr>
<td>DMCA2</td>
<td>4.23 (0.39)</td>
<td>4.24 (0.32)</td>
<td>0.884</td>
<td>0.568</td>
</tr>
<tr>
<td>V2-5</td>
<td>4.43 (1.05)</td>
<td>4.51 (1.08)</td>
<td>0.482</td>
<td>0.113</td>
</tr>
<tr>
<td>Nasal mucociliary clearance</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>STT, minutes</td>
<td>16.31 (2.71)</td>
<td>17.41 (3.31)</td>
<td>0.035</td>
<td>0.045</td>
</tr>
</tbody>
</table>

MPO, ng/ml 10.3 (8.4) 9.3 (9.4) 0.805 11.5 (8.5) 9.1 (7.2) 0.138 0.858

IL-6, pg/ml 2.4 (2.8) 2.4 (3.1) 0.670 3.7 (1.5) 4.9 (1.8) 0.001 0.012

IL-8, pg/ml 86 (114) 81 (98) 0.689 164 (90) 206 (164) 0.281 0.006

MPO, ng/ml 1.9 (5.2) 3.4 (6.6) 0.006 2.0 (4.3) 3.8 (5.5) 0.002 0.006
Data are expressed as the means (SD). *versus baseline, paired samples t-test. **ANOVA: compares the baseline (mean of the two baselines in the 11 subjects who had both pressures) with the 22 results at 7.5 cm H₂O and the 31 subjects at 12.5 cm H₂O CPAP. FEV, forced expiratory volume; FVC, forced vital capacity; PEFR, peak expiratory flow rate; MCA1, outermost minimum cross-sectional area; DMCA1, the distance of the MCA1 from the nasal orifice; MCA2, innermost minimum cross-sectional area; DMCA2, the distance of the MCA2 from the nasal orifice; V2-5, the volume of the nasal segment between the 2nd and 5th cm from the nasal orifice; STT, saccharin transit time

Changes in nasopharyngeal symptoms with CPAP

Nasopharyngeal symptoms before and after nasal CPAP treatment are presented in Table 4. None of the subjects had any upper airway symptoms before CPAP. The median number of nasopharyngeal symptoms increased significantly from 0 at baseline to 1 (0-3) after 7.5 cm H₂O CPAP (P = 0.002) and to 2 (1-3) after 12.5 cm H₂O CPAP (P < 0.001). After CPAP at 7.5 cm H₂O, 12/22 subjects (55%) experienced at least one nasal symptom; after CPAP at 12.5 cm H₂O, 21/31 subjects (68%) experienced at least one nasal symptom. There was an overall increase in the frequency of all of the symptoms during nasal CPAP treatment; the most common nasal symptom at both pressures was itchy nose. The higher the pressure, the more symptoms were recorded: χ² (P = 0.041).

<table>
<thead>
<tr>
<th>CPAP pressure</th>
<th>Nasopharyngeal symptoms</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rhino-rhoea</td>
</tr>
<tr>
<td>Baseline (before treatment)</td>
<td>0</td>
</tr>
<tr>
<td>7.5 cm H₂O (n = 22)</td>
<td>2 (9%)</td>
</tr>
<tr>
<td>12.5 cm H₂O (n = 31)</td>
<td>5 (16%)</td>
</tr>
</tbody>
</table>

* Data are expressed as numbers (%) of subjects.
Relationships between symptoms and changes in nasal physiology and inflammation

The data demonstrate relationships between the development of nasal symptoms, nasal inflammation and impaired nasal function in vivo. The greater the nasal symptoms with CPAP, the slower the nasal mucociliary clearance (r=0.40 p=0.025) and the greater the nasal inflammation as assessed by nasal wash IL-6 (r= 0.43 p=0.045), Figures 3-A and 3-B respectively. The significant slowing in nasal clearance was also associated with the degree of nasal inflammation, as assessed by nasal MPO concentrations (r = 0.42, P = 0.049), illustrated in Figure 3C.

In vitro studies

Changes in IL-6 and IL-8 levels over time

CPAP was associated with both time- and pressure (dose)-dependent release of the inflammatory cytokines IL-6 and IL-8 from cultured BEAS-2B cells in vitro. These data are reported in Table 5 and illustrated in Figure 4.
Table 5

CPAP is associated with time- and pressure-dependent releases of IL-6 and IL-8 from cultured BEAS-2B cells in vitro.

<table>
<thead>
<tr>
<th>IL-6 pg/μg of protein</th>
<th>Mean (SD)</th>
<th>ANOVA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 hour</td>
<td>2 hours</td>
</tr>
<tr>
<td>Baseline</td>
<td>0.01 (0.001)</td>
<td>0.01 (0.002)</td>
</tr>
<tr>
<td>Control (no CPAP)</td>
<td>0.01 (0.002)</td>
<td>0.01 (0.003)</td>
</tr>
<tr>
<td>CPAP 4 cm H2O</td>
<td>0.01 (0.005)</td>
<td>0.02 (0.005)</td>
</tr>
<tr>
<td>CPAP 7 cm H2O</td>
<td>0.03 (0.005)</td>
<td>0.07 (0.014)</td>
</tr>
<tr>
<td>ANOVA P=</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Posthoc 4 vs control†</td>
<td>0.012</td>
<td>0.016</td>
</tr>
<tr>
<td>Posthoc 7 vs control†</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Posthoc 4 vs 7 control†</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Data are expressed as the means (SD). One-way ANOVA test. † Post hoc Tukey’s multiple comparison tests. * P value < 0.05 was considered statistically significant.

In linear regression analysis, both pressure and time independently contributed to release of IL-6 and IL-8 (IL-6 adjusted R²=0.55, p<0.001; IL-8 adjusted R²=0.60, p<0.001 time β 0.278 and 0.399, pressure β 0.694 and 0.671 respectively).

**DISCUSSION**

We report that CPAP results in release of inflammatory mediators from cultured human bronchial epithelial cells in vitro, in a time- and pressure-dependent manner. In addition, in healthy control subjects, CPAP was associated with dose (pressure)-response changes in nasal and systemic inflammatory markers, reduced nasal function and the development of nasal symptoms. The development of nasal symptoms related to the degree of functional impairment and nasal inflammatory response. To the best of our knowledge, this is the first
report to examine the *in vitro* and *in vivo* effects of CPAP in this way, providing new data on the mechanisms of CPAP intolerance in the crucial early phase of therapy.

Findings from the *in vitro* component of this study showed cytokine (IL-6 and IL-8) secretion by bronchial epithelial cells in response to CPAP in a pressure- and time-dependent manner. IL-6 is an important pro-inflammatory cytokine. IL-8 is a chemokine that attracts neutrophils to the site of inflammation. Our *in vivo* findings are in line with the *in vitro* findings, particularly with regard to the neutrophilic nature of inflammation. Neutrophil chemotaxis following epithelial IL-8 release is the likely explanation of increased leukocyte count and elevated myeloperoxidase (MPO) activity in nasal wash fluid samples since MPO is an enzyme abundantly present in neutrophils. The neutrophilic nature of CPAP-induced local inflammation has also been shown in a rat model in which early nasal inflammation was mediated by macrophage induced inflammatory protein-2 and manifested as neutrophil extravasation following five hours of 10 cm H₂O CPAP [10]. Paradoxically, in nasal wash fluid samples, IL-8 levels remained unchanged in this study at both pressures and an increase in IL-6 levels was only evident in response to the higher pressure. This may arise from rapid consumption and binding of interleukins before they cross the nasal epithelium. Our study therefore suggests that CPAP itself may be pro-inflammatory and that this effect occurs early after initiation of therapy.

In this study, even a brief period of CPAP application resulted in increased IL-6 levels in serum suggesting a systemic inflammatory response. However, we did not observe an increased systemic IL-8 concentration or MPO activity. The decrease in serum IL-8 levels may be due to local recruitment of leukocytes and increased consumption. Unaltered systemic MPO activity is plausible since MPO may predominately increase at the site of
inflammation. Studies in OSA are even more complex as the condition itself is associated with up-regulated upper airway inflammation[23, 24] and in such circumstances CPAP may not up-regulate this further [25]. The work complements previous findings in patients with OSA, where CPAP is known to increase nasal inflammation [11].

This in vivo nasal inflammatory response was associated with clinical and functional consequences in that we also demonstrated CPAP reduced nasal clearance and was associated with a high prevalence of new nasal symptoms. It has been suggested that the presence of nasal inflammation predicts patients at greater risk of discontinuing CPAP therapy [26] and IL-8, a potent neutrophil chemoattractant that we have shown is up-regulated by CPAP in vitro, causes rhinorrhoea when directly instilled to the nose [27]. Three hours of CPAP decreased mucociliary clearance at both 7.5 and 12.5 cm H₂O in healthy individuals. Our findings are contrary to those reported by Oliviera [28], who found significantly decreased STT after 20 minutes of CPAP in healthy individuals. This may be attributed to differences in the duration of CPAP treatment and suggests that CPAP may provide an initial improvement in nasal clearance that is followed by impairment due to inflammation. These inflammatory and functional changes may contribute to the high incidence of symptoms and adverse effects associated with CPAP treatment. In this study, more than half of the subjects experienced at least one nasal symptom after a single session. Previous studies have reported high incidences of side effects during long-term therapy, which approached 97% in a large series [5]. To assess the duration of symptom changes, in a subsequent pilot experiment we assessed nasal symptoms prior to and after 3 hours of CPAP at 12cm H₂O, then again at three, six, nine and 24 hours later. None of the subjects had nasal symptoms prior to CPAP and all had one or more symptom after. A three, six, nine and 24 hours post CPAP the numbers remaining
symptomatic were 4/5, 1/5, 1/5 and 0/5 respectively suggesting that acute nasal symptoms typically last for between three and six hours following initiation of CPAP.

In this study, CPAP treatment did not result in any change in acoustic rhinometry parameters in healthy individuals; thus, it did not alter nasal patency. This was unexpected given the apparent nasal inflammatory response. Nasal geometry has been reported to affect CPAP tolerability [29]. Although the effect of short- or long-term CPAP on acoustic rhinometry parameters has not been investigated previously, several studies have reported unaltered rhinomanometry results after long-term CPAP therapy [29, 30]. One study reported a reduction in airway resistance after an acute exposure to nasal CPAP for 6 hours in healthy CPAP-naïve individuals [31]. In this study, a small but statistically significant improvement was identified in lung function parameters (i.e., FVC and PEFR). This suggests that the CPAP applied in our subjects had a demonstrable biological effect.

OSA is associated with increased cardiovascular risk [32, 33], as is increased systemic inflammation [34]. Whether CPAP reduces cardiovascular risk remains controversial [35-40], but the finding that CPAP itself, at least in the acute setting, is pro-inflammatory is potentially important regarding the timing of the initiation of therapy. In the long-term, Steiropoulos [41] reported significant improvements in systemic inflammatory markers, including total lymphocyte counts, CD4+ cells, TNF-α levels and uric acid levels after six months in patients with good compliance to CPAP therapy; however, no such improvements were identified in patients with poor compliance. In contrast, Kohler [35] did not find any differences in systemic inflammatory markers, high-sensitive C-reactive protein (CRP), plasma IL-6, IFN-γ, and adiponectin levels between patients receiving therapeutic and sub-therapeutic CPAP treatments for four weeks. Thus, the relationships between CPAP, systemic
inflammation, and the duration of therapy are complex. We report small but statistically significant elevation in systemic IL-6 with CPAP and it is known that even small changes in long-term IL-6 concentration can be associated with excess cardiovascular risk [42]. The effects of acute changes are less well studied.

The mechanisms by which CPAP may be pro-inflammatory include airway drying (i.e., not using humidification) or direct distension. The possible benefits of humidification have been controversial, and our in vitro work, in which cells were exposed to high-humidity, demonstrates that drying or the absence of humidification alone cannot be solely responsible for the pro-inflammatory changes observed. An experimental study in rats failed to demonstrate any beneficial effects of heated humidification on nasal inflammation [43], whereas clinical and experimental studies have reported conflicting results on the benefits of humidification [44, 45]. Most previous in vitro work has used direct distension [46-48], and a mouse model of airway stretch for ventilator-associated lung injury was associated with increased expression of the murine equivalent of IL-8 [13]. Stretch may affect inflammation via oxidative stress, as stretch-induced IL-6 and IL-8 production can be reduced by the use of anti-oxidants to increase intra-cellular glutathione; production can be increased with glutathione depletion [49]. Our data add to the literature by reporting a direct effect of pressure rather than stretch. It is unlikely that the nasal epithelium is able to accommodate stretch given the confines of the nose within the bony structures of the skull.

The strengths of our study include the careful and comprehensive assessment of symptoms, upper and lower airway function, and nasal and systemic inflammation, which demonstrated a dose response in healthy subjects. A further strength is the use of both in vivo and in vitro approaches to address the clinical problem. The pressures we selected for the in vitro work
were necessarily different from the *in vivo* work, as higher pressures *in vitro* resulted in excessive evaporation of the cell culture fluid. Our results have important implications for clinical practice. In particular, by demonstrating a relationship between nasal symptoms, mucociliary clearance and inflammation, it should be possible to investigate strategies to reduce the nasal inflammation associated with CPAP treatment, which may reduce symptoms and, therefore, aid compliance. Approaches include anti-inflammatory agents or humidification (discussed above), and we have provided further rationale for the development of strategies to mitigate nasal inflammation during CPAP therapy. This is particularly important as existing nasal corticosteroids appear clinically ineffective [50]. An alternative strategy might involve dose titration at the beginning of the therapy (i.e., a gradual increase of the pressure until attaining optimal clinical benefits with minimal side effects), as we have provided evidence that suggests that the inflammatory effects are dose (pressure)-dependent.

This study also has several limitations that need to be considered. Ours was a relatively small sample. Associations observed in the *in vivo* study do not provide direct evidence for a causal relationship between CPAP and airway inflammation, though this is why we included the complementary *in vitro* work. The design of the study may have been more robust with the inclusion of a sham CPAP arm but we were concerned that even sham CPAP might affect nasal inflammation. We cannot comment on the timing of resolution of inflammation associated with CPAP as we were interested primarily in induction of this response. Whilst we elected to measure IL-6 and IL-8 to provide consistency across the *in vitro* and *in vivo* work, there are alternative markers including CRP and TNF-alpha that may have provided additional insight into cardiovascular risks associated with CPAP. Finally, as many of our analyses are hypothesis-generating we have not attempted to correct analyses for multiplicity and the results should be interpreted in the light of this.
In conclusion, we report a high prevalence of nasal symptoms following CPAP therapy in healthy subjects associated with changes in nasal function and an inflammatory response in the nasal and systemic compartments. This study also suggests that CPAP triggers an early pressure-dependent inflammatory reaction, as evidenced by the increased secretion of inflammatory markers by cultured bronchial epithelial cells. These findings have implications for the adherence of patients to CPAP therapy, especially during the important initiation phase. Strategies to combat the initial side effects of this treatment modality and to improve compliance and retention might target the epithelial lining of the respiratory system in an attempt to address the origin of the inflammatory response.

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REFERENCES


**FIGURE LEGENDS**

**Figure 1.** *In vitro* experimental protocol (A) and a diagrammatic representation of the experimental equipment used to expose BEAS-2B cells to CPAP (B).
**Figure 2.** CPAP is associated with a pressure-dependent alteration in nasal and systemic inflammatory markers, and nasal mucociliary clearance. Lines represent mean and standard error. Significant differences illustrated using ANOVA with post-hoc analysis.

**Figure 3.** Relationship between nasal mucociliary clearance (saccharin transit time) and nasopharyngeal symptoms from 31 healthy subjects after 3 hours of CPAP treatment at 12.5 cm H$_2$O (r = 0.40; P = 0.025) (A), relationship between nasopharyngeal symptoms and nasal wash IL-6 concentration in 22 healthy subjects after 3 hours of CPAP treatment at 7.5 cm H$_2$O CPAP treatment (r = 0.43; P = 0.045) (B) and relationship between nasal mucociliary clearance (saccharin transit time) and nasal wash MPO concentration in 22 healthy subjects after three hours 7.5 cm H$_2$O CPAP application (r = 0.42; P = 0.049) (C).
Figure 4. Dose (pressure) dependent release of IL-6 and IL-8 from cultured human BEAS-2B cells *in vitro* with no pressure, and CPAP at 4 cm H$_2$O, and 7 cm H$_2$O.