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Short communication

An *in vitro* investigation of the inflammatory response to the strain amplitudes which occur during high frequency oscillation ventilation and conventional mechanical ventilation

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ABSTRACT

Children randomised in the neonatal period to high frequency oscillatory ventilation (HFOV) or conventional mechanical ventilation (CMV) in the United Kingdom Oscillation study (UKOS) had superior lung function at 11 to 14 years of age. During HFOV, much smaller tidal volumes, but a higher mean airway distending pressure is delivered, hence, a possible explanation for a volume dependent effect on long term lung function could be an increase in inflammation in response to higher tidal volumes and strains. We tested that hypothesis by assessing interleukin-6 (IL-6) and -8 (IL-8) release from A549 alveolar analogue cells following biaxial mechanical strain applied at 0.5 Hz occurring during conditions mimicking strain during CMV (5%-20% strain) and conditions mimicking strain during HFOV (17.5% ± 2.5% strain) for up to 4 hours. Cyclic strain of 5%-20%, occurring during CMV, increased levels of both IL-6 and IL-8 compared to unstrained controls, while 17.5% ± 2.5% strain, occurring during HFOV, was associated with significantly lower levels of IL-6 (46.31 ± 2.66 versus 56.79 ± 3.73 pg/mL) and IL-8 (1340.2 ± 74.9 versus 2522 ± 248 pg/mL) secretion compared to conditions occurring during CMV at four hours. These results may provide a possible explanation for the superior lung function in 11 to 14-year-old children who had been supported in the neonatal period by HFOV.

1. Introduction

Very prematurely born infants less than 29 weeks of gestational age entered into the United Kingdom Oscillation Study (UKOS) were randomised in the first hour after birth to either conventional mechanical ventilation (CMV) or high frequency oscillatory ventilation (HFOV) (Johnson et al., 2002). At 11-14 years, the UKOS cohort was re-examined and those supported by HFOV in the neonatal period had superior lung function (Zivanovic et al., 2014). During HFOV, much smaller tidal volumes are delivered than during CMV (Dimitriou et al., 2004). Hence, we postulated that this may have resulted in the difference in school age respiratory outcomes. Indeed, results of animal studies have shown that it is the level of volumetric distension and resulting epithelial basement membrane strain, not the pressures used in mechanical ventilation, which results in lung damage (Dreyfuss et al., 1988, Hernandez et al., 1989).

A possible explanation for the volume dependent effect on lung damage may be an increase in inflammation. *In vitro* studies have shown that the amount of interleukin released from alveolar cell analogues is proportional to the degree of cyclical mechanical strain (Iwaki et al., 2009, Vlahakis et al., 1999). Interleukin-8 (IL-8) release from A549 alveolar epithelial cells was greater in cells subjected to 30% cyclic strain compared to unstrained controls (Vlahakis et al., 1999). IL-6 and -8 levels were also elevated at 20% strain in human pulmonary microvascular endothelial cells, but not significantly so at 5% strain suggesting the response occurs only above a threshold level of strain (Iwaki et al., 2009). During HFOV as used in UKOS, the lungs were inflated to an appropriate volume by increasing the mean airway pressure (MAP), resulting in a high level of constant strain. The lungs were then oscillated with small tidal volumes. During CMV, there is a low level of resting strain resulting from the positive end expiratory pressure (PEEP) and then, in comparison to HFOV, higher tidal volumes were used.

We, therefore, hypothesised that applying strain to alveolar cell analogues in conditions mimicking the strain characteristics of HFOV compared to CMV would be associated with lower levels of cytokine release. To test our hypothesis, we assessed IL-6 and IL-8 release from A549 human alveolar basal epithelial cells in conditions mimicking HFOV and CMV.

2. Materials and Methods

2.1 Cell culture

A549 cells (obtained from ATCC-LCG, Teddington, UK) were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 1,000 g/L D-glucose, 1 mM sodium pyruvate and 4 mM L-glutamine, with 10% fetal bovine serum and penicillin (100 U/mL)-streptomycin (100 µg/mL) (all Thermo Fisher Scientific, Paisley, UK). Culture media was exchanged every two to three days and cultures were maintained at 37°C and 5% carbon dioxide (CO_2). A549 cells were sub-cultured a maximum of 30 times with cell subcultures randomly assigned to experimental conditions. Prior to strain application, cells were plated at an initial density of 10×10^3 cells/cm^2 in 2 mL culture media on six-well collagen type I-coated BioFlex culture plates (Flexcell International Corporation, Dunn Labortechnik GmbH, Asbach, Germany). Cells were cultured for 72 hours to achieve approximately 75% confluence assessed by phase contrast microscopy. Higher levels of confluence resulted in growth inhibition and apoptosis. To ensure all cells experienced uniform strain, cells at the circumferential edge of the BioFlex wells, the region where the membrane is strained at an undefined level, were scraped away and culture media exchanged one hour prior to strain application.

2.2 Biaxial mechanical strain application

The A549 cells on collagen type I-coated BioFlex plates were subjected to biaxial tensile strain for up to 4 hours using a Flexcell 4000 tension system (FX-4000, Flexcell International Corporation, Dunn Labortechnik GmbH, Asbach, Germany) within a standard incubator at 37°C and 5% CO_2. Some cells were subjected to static strains of 20% representing a high mean airway pressure during HFOV and others to 5% representing the low positive end expiratory pressure during CMV (Fig. 1A). To simulate the strain levels experienced during CMV, a 15% cyclic strain was superimposed on a baseline strain of 5% resulting in 5%-20% applied peak-to-peak strain, while to simulate HFOV, a 5% cyclic strain was superimposed on a mean strain of 17.5% resulting in 15%-20% applied peak-to-peak strain (Fig. 1B). In both models, the maximum strain magnitude was 20% and the cycle frequency was 0.5 Hz. Additional A549 cells cultured on BioFlex plates placed in the incubator next to the FX-4000 for the duration of each experiment provided unstrained control (Ctrl) samples.

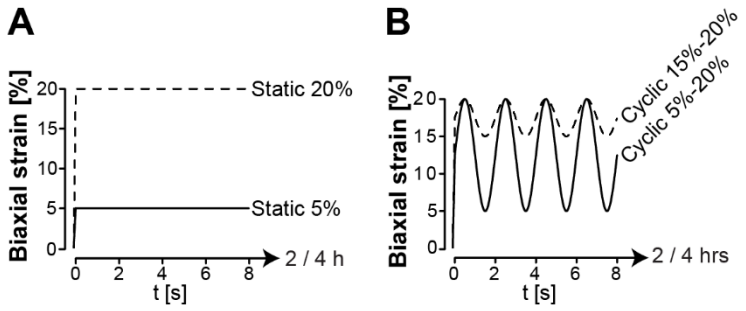

Fig. 1: Diagrammatic representation of the experimental models. Figure A shows the biaxial strain of a high mean airway pressure as seen during HFOV (20%) and a low-level strain (5%) representing PEEP during CMV. Figure. B shows the cyclical strain which occurs during CMV (5-20%) and which occurs during HFOV (15-20%).

2.3 Cell viability analysis

Cell viability of both strained and control samples was assessed post strain application for each condition using propidium iodide staining and flow cytometry. Cells were detached from

BioFlex plates using trypsin-EDTA (Thermo Fisher Scientific) and suspended in phosphate buffered saline (PBS) containing propidium iodide. Cell viability was analysed using flow cytometry. In all conditions, over 99% of the cells were viable post strain application (data not shown).

2.4 Assessment of interleukin secretion to culture media

IL-6 and IL-8 levels were assessed from aliquots of culture media supernatant frozen at -20°C directly after strain application using enzyme-linked immunosorbent assays (ELISAs). Commercially available kits to assess human IL-6 and IL-8 levels were used according to manufacturer's instructions (DuoSet ELISA Kit, R&D Systems, Abingdon, UK). Samples were defrosted, centrifuged briefly to remove particulates, diluted in 1% bovine serum albumin (BSA)-PBS for IL-6, or 0.1% BSA in tris buffered saline (TBS) with 0.005% Tween 20 for IL-8, and 50 µL added to each well of the provided ELISA microplate. Recombinant IL-6 and IL-8 were used to create standard curves for quantification.

2.5 Statistical analysis

Each experimental condition was repeated three times with two to three biological replicates per replicate experiment, that is, six to nine biological replicates per condition. As absolute values secreted varied between replicate experiments, data were normalised to the two-hour unstrained control within each replicate experiment. All datasets adhered to a normal distribution. A general linear model for analysis of variance with Tukey tests for multiple comparisons was used. Time point and applied strain were fixed factors and replicate experiment was included as a random factor. Statistical analyses were performed using Minitab 18 software (Minitab, Coventry, UK).

3. Results

Levels of IL-6 secretion from A549 cells did not increase with culture duration over four hours and were low compared to levels of IL-8 secretion which increased 1.57-fold ($p < 0.0005$) with culture duration and were 17 and 26-fold that of IL-6 at 2 and 4 hours respectively.

Static strain at both 5% and 20% did not significantly increase IL-6 secretion, which remained constant with culture duration (Fig. 2A and 2B). IL-8 levels in all strained and unstrained conditions increased with culture duration (Fig. 2C and 2D).

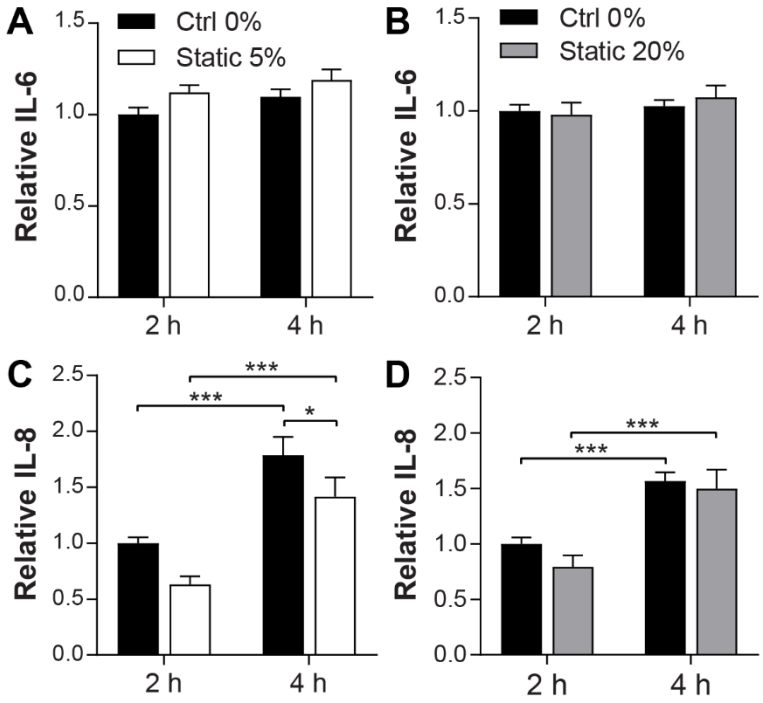

Fig. 2: Schematic depicting IL-6 and IL-8 secretion from A549 cells relative to unstrained controls (Ctrl 0%) following an applied static strain level of 5% (which occurs during PEEP) (Figures A and C) and following an applied static strain of 20% (representative of a high mean airway pressure which occurs during HFOV) (Figures B and D), at two and four hours. Mean ± s.e.m., $6 \leq n \leq 9$, General linear model with Tukey pairwise comparisons: *$p < 0.05$, ***$p < 0.001$.

High amplitude cyclic strain which occurs during CMV (5%-20%) significantly increased IL-6 levels (Fig. 3A) from 40.01 ± 2.60 pg/mL to 50.02 ± 4.16 pg/mL (25.01%) at two hours, and from 40.92 ± 1.64 pg/mL to 56.79 pg/mL (38.8%) at four hours compared to unstimulated controls ($p < 0.0005$). IL-8 levels were similarly increased in response to 5%-20% strain (Fig. 3D) from 1026.6 ± 67.3 pg/mL to 2522 ± 248 pg/mL (145.7%) at 4 hours ($p < 0.0005$). While a cyclical strain of 17.5% ± 2.5% which occurs during HFOV did increase IL-6 (Fig. 3B) and IL-8 (Fig. 3E) secretion at four hours, the levels of both were significantly lower than those secreted in response to 5%-20% strain which occurs during CMV, with IL-6 levels of 56.79±3.73 pg/mL versus 46.31±2.66 pg/mL ($p = 0.003$, Fig. 3C) and IL-8 levels of 2533±248 pg/mL versus 1340±74.9 pg/mL ($p < 0.0005$, Fig. 3F) for 5%-20% compared to 15%-20%.

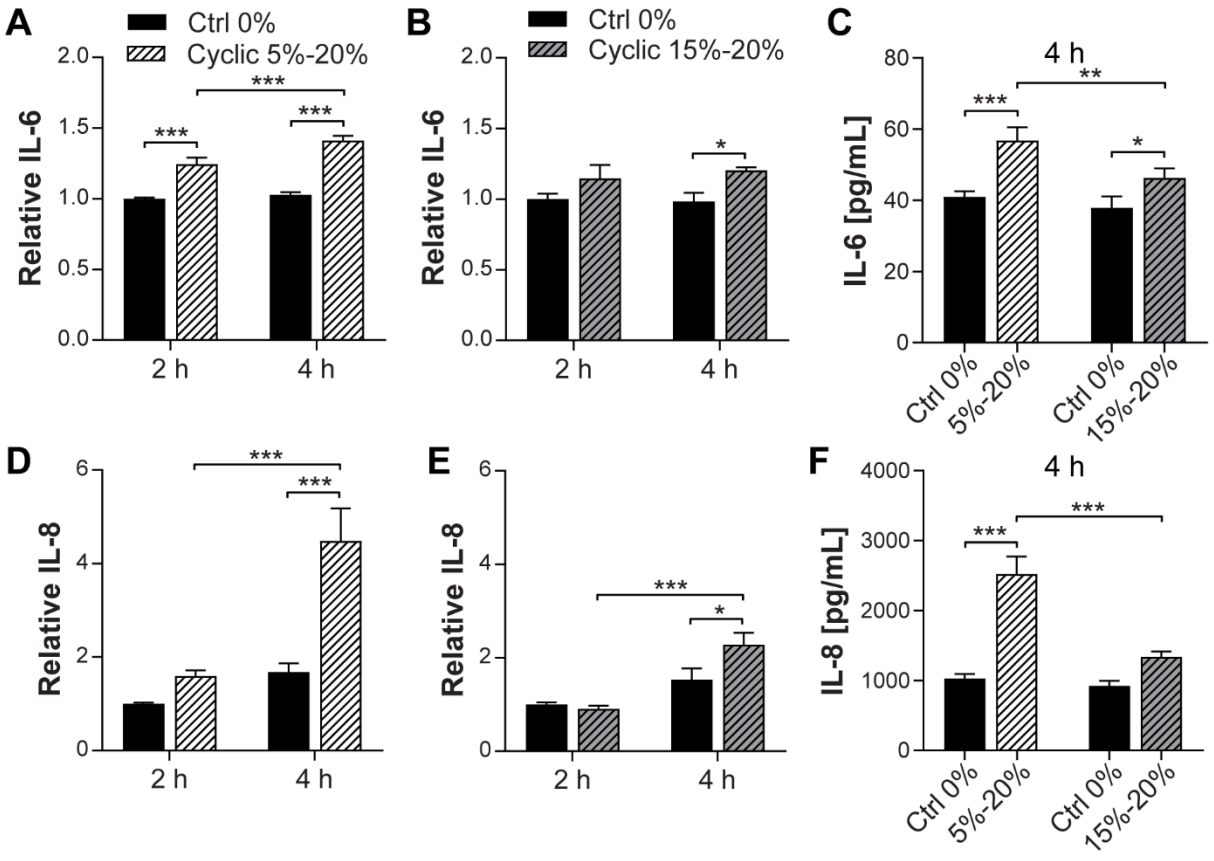

Fig. 3: Schematic depicting IL-6 and IL-8 secretion from A549 cells relative to unstrained control (Ctrl 0%), following applied cyclical strain from 5% to 20% (which occurs during CMV) (Figures A and D) and following applied cyclic strain from 15% to 20% (which occurs

during HFOV) (Figures B and E), at two and four hours. Secretion of IL-6 (C) and IL-8 (F) at four hours in pg/mL showing lower secretion in response to 15%-20% versus 5%-20% cyclic strain. Mean ± s.e.m., $6 \leq n \leq 9$, General linear model with Tukey pairwise comparisons: *$p <$ 0.05, **$p<0.01$, ***$p < 0.001$.

4. Discussion

We have demonstrated that *in vitro* release of IL-6 and IL-8 from alveolar analogue cells was lower following low amplitude cyclical mechanical strain which occurs during HFOV when compared to high amplitude cyclical strain which occurs during CMV. To our knowledge, this is the first study to assess interleukin release *in vitro* using biaxial strain regimes which occur during CMV and HFOV.

Uniquely in UKOS, infants were put on their randomised mode of ventilation in the first hour after birth (Johnson et al., 2002) and thus infants were exposed to different tidal volumes and levels of stretch from that early age. Over-distension of the lungs or cyclical opening and closing of lung units causes disruption of structural elements and release of pro-inflammatory mediators (Speer, 2006). During HFOV, as used in UKOS, cyclical lung opening and closing is minimised as the infant's lungs receive oscillations around a mean airway pressure chosen to optimise lung volume.

The Flexcell FX4000 cell strain system was unable to apply the required strain amplitudes at frequencies higher than 0.5 Hz, so to aid comparison of the two cyclic regimes, we used 0.5 Hz for our simulations of both CMV and HFOV. Although cells were strained at a frequency of 0.5 Hz, whereas a frequency of 1 Hz is typical in CMV and 10 to 15 Hz during HFOV, previous studies, have shown that an increase in frequency of strain from 0.2Hz to 1 Hz, a

fivefold increase, did not influence the magnitude of IL-8 release from A549 cells (Vlahakis et al., 1999, Ning and Wang., 2007). Whilst the frequency used in HFOV was not matched in our experiment, importantly the strain amplitude in the CMV model (5 to 20%) was higher than that of the HFOV model (15 to 20%), simulating what happens during HFOV or CMV.

We recognise that the strain magnitude does not directly correlate with a change in lung volume, as it has been shown that inflation of the lung, as a percentage of total lung capacity, does not correspond linearly to the degree of stretch on the epithelial basement membrane (Bachofen et al., 1987). An increase of 50% in the alveolar surface area corresponded to a basement membrane stretch of only 22%, as part of this increase in alveolar surface area is formed by the unfolding of the alveolar septal plates (Bachofen et al., 1987, Vlahakis et al., 1999). A similar relationship between lung expansion and basement membrane stretch has also been described in rat lungs (Gil and Weibel, 1972, Tschumperlin and Margulies, 1999).

There are strengths and some limitations to our study. Four different methods of stretch were used and compared to each other and non-stretched controls. We used A549 cells originating from an adult lung adenocarcinoma specimen as an analogue for type II alveolar cells. Such cells have been used in previous studies simulating the effects of mechanical ventilation *in vitro* (Vlahakis et al., 1999, Ning and Wang, 2007). Both A549 cells and primary cell cultures of human type II alveolar cells have been shown to release IL-8 in response to cyclical mechanical stretch (Vlahakis et al., 1999, Ning and Wang, 2007, Stucky et al., 2015). In addition to their similar response to mechanical stretch, A549 cells secrete surfactant in culture (Cooper et al., 2016), are similar in morphology to primary type II alveolar cell cultures (Lieber et al., 1976) and produce IL-6 and IL-8 in response to other inflammatory stimuli (Arnold et al., 1994). A549 cells were used in preference to human alveolar type II cells as the latter

exhibit issues with purity of isolation (Vlahakis et al., 1999, Tschumperlin and Margulies, 1998) and are fragile when stretched.

It has been demonstrated that IL-6 and IL-8 have non-inflammatory effects, such as inhibiting angiogenesis and increasing proliferation of fibroblasts (Antoniou et al., 2006, Feugate et al., 2002). Increased inflammatory cytokines disrupt VEGF signalling, reducing angiogenesis and alveolarization (O'Reilly et al., 2013). This may explain the difference in long term respiratory outcomes of the two randomised groups from the UKOS study.

In conclusion, we have demonstrated that low amplitude cyclical mechanical strain which occurs during HFOV, rather than high amplitude strain which occurs during CMV, resulted in lower levels of IL-6 and IL-8 secretion from A549 alveolar basal epithelial cells. These results may explain the superior lung function of the children who in the neonatal period had been supported by HFOV in the UKOS trial.

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Conflict of interest statement

The authors have no conflicts of interest relevant to this article to disclose.

Contributors' statement

All authors have made substantial contributions to the conception and design of the study, or acquisition of data or analysis and interpretation of the data, drafting the article and revising it critically and the final approval of the version submitted.

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