Development of a gaseous exposure system to assess *in vitro* cytotoxicity of MucilAir™ tissues following exposure to airway irritants/non-irritants

M. Hollings, A. Seymour, E. Rothwell and R. Bedford
Labcorp Early Development Laboratories Ltd., *In Vitro* Inhalation & *In Vitro* Toxicology, Harrogate, UK

Introduction

This study was initiated to investigate a continuous flow gaseous exposure system to three gaseous compounds. There is a current push to investigate products in a more physiologically relevant environment (continuous flow versus static). 3D airway tissue models are a relevant method for analysis of toxicity for these gaseous products. These tissues have increased physiological relevance over monolayer cell cultures as they have attributes that represent the respective regions of the human respiratory tract that are generally only seen *in vivo*.

MucilAir™ (Epithelix Sarl, Switzerland) tissues were exposed using a gaseous exposure system with Vitrocell® 24/4 modules to ethylene oxide (EtO), nitric oxide (NO) and sulphur hexafluoride (SF6) for a total of 60 minutes. Gas was diluted at varying concentration with flowing air and presented as parts per million (ppm).

In this study, we used the lactate dehydrogenase (LDH) release, WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt), trans-epithelial electrical resistance (TEER), cilia beat frequency and a pro-inflammatory cytokine panel were used to assess cytotoxicity. Post-exposure tissues were left for 24hr recovery after treatment prior to analysis.

EtO decreases greater than 50% versus concurrent control were observed for WST-8 and LDH, but not for NO or SF6. Significant changes were observed for CBF and TEER, but not for SF6. Significant increases/decreases were observed in cytokine release for all test articles.

This study demonstrates the potential of the gaseous exposure system to differentiate between test articles exposed in a physiologically relevant environment and to provide insight into the different biological pathways affected.

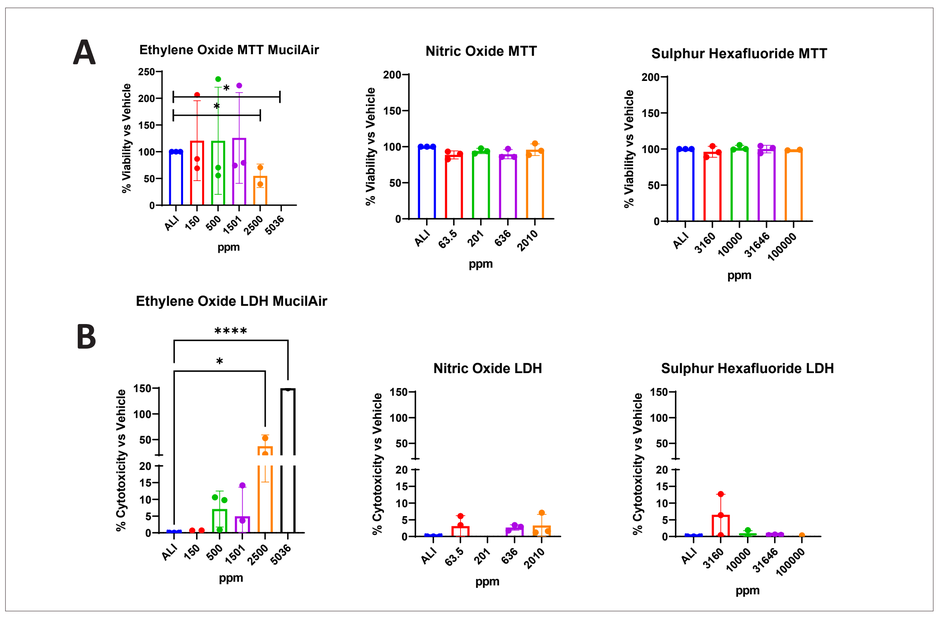

Figure 1. Image A shows MTT results as a %viability of ALI, for each dose, for all 3 test articles. Image B shows LDH release presented as %Cytotoxicity against ALI, each dose, for all 3 test articles. Noting statistical significance between doses. Statistical difference was noted for EO between 2500 and 5036 ppm against ALI control (Two-way ANOVA with multiple comparisons. * P < 0.05).

Methods

3D cell model: MucilAir™ tissues (donor MD067001) were from Epithelix Sarl, Switzerland cultured following manufacturer's guidelines.

Test articles: EtO (0.65%, 6500 ppm), NO (0.2%, 2000 ppm) and SF6 (10%, 100,000 ppm), were purchased from BOC (Linde PLC, Pullach, Germany)

Controls: ALI control for treatment was exposed to 0.2L/min flowing air. Untreated tissues were used as INC controls. Blank transwells were used as negative controls for the transepithelial electrical resistance (TEER), WST-8 (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) and LDH (Takara-Bio, Göteborg, Sweden) assays. Triton X-100 at 1% treated basolaterally used as a positive control.

Gaseous exposure: Vitrocell® 24/4 exposure modules were utilized for gaseous exposure. Test article cylinders were set to 1 Bar before being regulated at 0.1 L/min for each dilution bar. Test article was then diluted with different flow rates of flowing air with mass flow controllers to achieve target ppm concentrations. A vacuum rate of 5 mL/min was used for all exposures. Exposure duration was 60 min.

TEER measurements were conducted pre- and post-exposure with EVOM2 epithelial voltohmmeter (World Precision Instruments Ltd., Hitchin, United Kingdom).

Cilia Beat Frequency (CBF) measurements were performed using the Sisson-Ammons Video Analysis (SAVA) system (Ammons Engineering, Mt. Morris, MI, USA) and normalized to ALI control.

BD® Cytometric Bead Array (CBA) human inflammatory cytokines kit (BD Biosciences, San Jose, CA, USA) was used to measure cytokine levels in tissue recovery media.

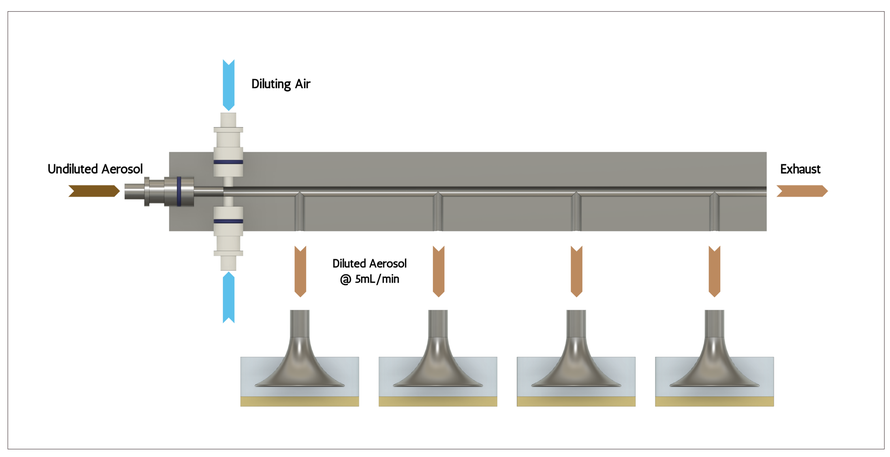

Image 1. Dilution schematic of Vitrocell® 24/4 module.

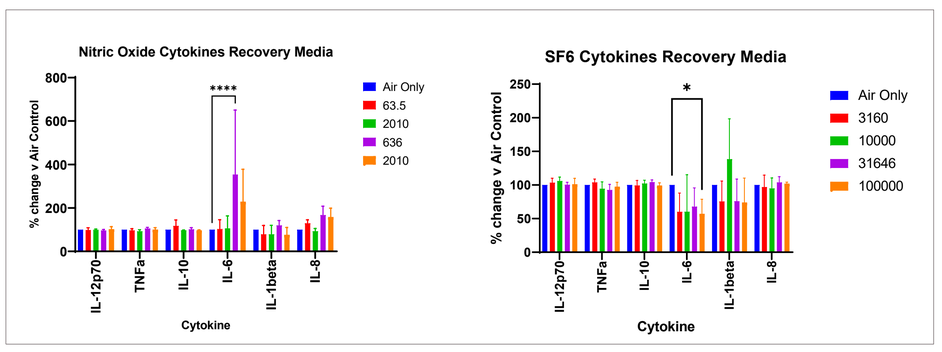

Figure 2. Cytokine release was measured in the 24-hour recovery media following exposure aerosol and normalized to the ALI control. Increases in IL-6 and IL-1β were for NO with significance for IL-6. Decrease was observed for IL-6 with SF6 with significance. Statistics run with two-way ANOVA with multiple comparisons. * P < 0.05. **** P <0.0001).

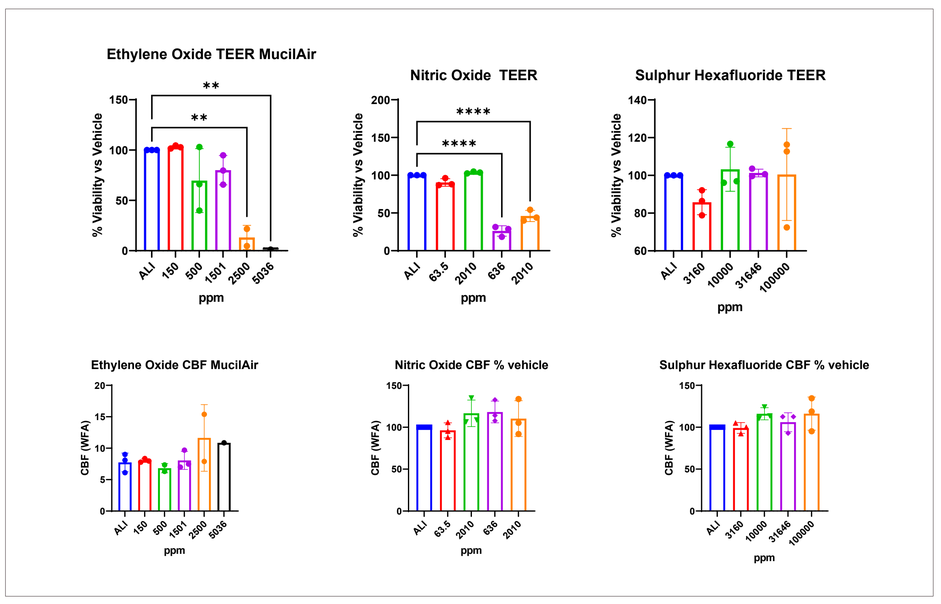

Figure 3. Barrier integrity and CBF measurements exposure to combustible cigarette, HTP and ENDS. Barrier integrity following exposure was measured by transepithelial electrical resistance (TEER). Mean ± SEM. Two-way ANOVA with multiple comparisons. *** P < 0.001; **** P < 0.0001. CBF measurements were performed using the Sisson-Ammons Video Analysis (SAVA) system and normalized to ALI control.

Results

- Exposure of MucilAir™ to EtO, NO and SF6 aerosols resulted in cytotoxicity for EtO only at highest two concentrations. No cytotoxicity was observed for NO or SF6 with either MTT or LDH assays.
- Increased release of various pro-inflammatory cytokines was observed for NO, IL-6 and IL-1β. A decrease was observed for SF6 for IL-6. These cytokines have been shown to be involved in the NLRP3 inflammasome pathway. Cytokine analysis is being conducted for EtO.
- Tight junctions were decreased in a concentration related fashion as demonstrated by TEER for both EtO and NO. No tight junction decrease was observed for SF6, although some variability was observed at the highest concentration. The results for EtO and NO did not correlate with a reduction in CBF, instead an increase was observed for EtO (although not significant) and no increase observed for NO or SF6.

Conclusions

- MucilAir™ tissues can be used alongside the MTT and LDH assay to expose to various gaseous test articles, results can be used to differentiate statistically between doses.
- Furthermore, additional endpoints can be included to further elucidate mechanistic endpoints.

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