Acute inhalation toxicity: *in vitro* and *ex vivo* systems

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Alternative approaches for acute inhalation toxicity to address global regulatory and non-regulatory data requirements. Webinar: 26th April 2016
Outline

- Introduction to the lung
  - Anatomy
  - Deposition
  - *In vitro* toolbox

- Case studies
  - Products
  - Generating aerosols
  - Example approaches

- Summary
## Role of the respiratory tract

Cells in the upper and lower respiratory tract, and particle deposition

<table>
<thead>
<tr>
<th>Respiratory tract region</th>
<th>Cell types</th>
<th>Deposition (particle size)</th>
</tr>
</thead>
<tbody>
<tr>
<td>extrathoracic</td>
<td>Ciliated cells - sweep debris</td>
<td>&gt;10 μm</td>
</tr>
<tr>
<td>bronchial bronchiolar</td>
<td>Goblet cells - secrete mucus</td>
<td>&lt;10 μm, &gt;3 μm</td>
</tr>
<tr>
<td>alveolar</td>
<td>Clara cells - divide and differentiate</td>
<td>PM10s penetrate the bronchiolar and alveolar regions of the lung</td>
</tr>
<tr>
<td></td>
<td>Alveolar - AT1, AT2 and macrophages</td>
<td>&lt;3 μm, PM2.5s can enter the bloodstream</td>
</tr>
</tbody>
</table>
In vitro toolbox

- ESC/iPSC
  - **Tumour derived cell lines** (e.g. H292)
    - Easy to culture and consistent results
  - **Immortalised cell lines** (e.g. BEAS-2B)
    - Genetically more ‘normal’ than tumour-derived cells
- **Primary cells** (e.g. NHBE)
  - Retain metabolic capability and physiological characteristics
  - Donor variation
  - Limited lifespan in culture
- **3D organotypic tissue systems**
  - Retain metabolic capability and physiological characteristics
  - Donor variation
- **Lung slices**
  - Cells retain spatial orientation and intercellular interactions
  - Donor variation
  - Short lifespan after slice preparation
- **Lung-on-a-chip, microtissues**
### Chronic Obstructive Pulmonary Disease (COPD)

Adverse Outcome Pathway (AOP)

<table>
<thead>
<tr>
<th>Initiating event:</th>
<th>Tissue Response:</th>
<th>Tissue Effects:</th>
<th>Pulmonary Effects:</th>
<th>Clinical manifestations:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tobacco exposure or other toxic insult to lung epithelium</td>
<td><strong>3. Oxidative stress</strong></td>
<td>1. Cytokines/chemokines</td>
<td>1. <strong>Ciliary dysfunction</strong></td>
<td>1. Chronic bronchitis</td>
</tr>
<tr>
<td>1. Ligand-receptor interactions</td>
<td>2. Increased integrin and adhesion molecule expression</td>
<td>2. Increased mucous secretion</td>
<td>2. Reduced lung elasticity</td>
<td></td>
</tr>
<tr>
<td>2. Intracellular response</td>
<td>3. Monocyte recruitment (persistent influx of neutrophils)</td>
<td>3. Fibroblast activation</td>
<td>3. Reduced airflow</td>
<td></td>
</tr>
<tr>
<td>8. <strong>Fibrosis</strong></td>
<td></td>
<td>9. Injury/repair cycling</td>
<td>7. Chronic inflammation</td>
<td></td>
</tr>
</tbody>
</table>

COPD:
- Progressive (usually) airflow limitation in airways/lungs due to noxious particles or gases and associated with inflammatory response
Product Diversity

The era of next generation nicotine delivery products
How we expose cells for biological testing

Products and aerosols

<table>
<thead>
<tr>
<th>Product</th>
<th>Aerosol type</th>
<th>Key</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cigarette</td>
<td>Vapour/gas</td>
<td></td>
</tr>
<tr>
<td>THP</td>
<td>Particles</td>
<td></td>
</tr>
<tr>
<td>e-Cigarette</td>
<td>Droplets</td>
<td></td>
</tr>
</tbody>
</table>

Aerosol capture

- Filter pad:
  - Passes through (not captured) x
  - Trapped ✓
- Pad washed in solvent
- Aqueous extract (AaE)
- Particulate matter

Cell exposure

- Membrane
- Cells
- Media
- Submerged cultures
Modes of biological exposure to the test article

- **PM**: Submerged exposure to filter trapped particles washed in solvent
- **AqE**: Submerged exposure to aerosol bubbled media or buffer
- **ALI**: Whole aerosol or VP only exposure at the air-liquid (air-agar) interface
- **e-liquid**: Submerged exposure to unaltered e-liquid or its ingredients

What are the associated challenges and dose implications with each mode of exposure?
In vitro air-liquid interface (ALLI) exposure systems

BAT's approach

A

RM20S Smoking Machine

B

VC 10 Smoking Robot

6/4 module with QCMs
TPM increases mucous secreting cell numbers *in vitro*

A potential model of goblet cell hyperplasia

TPM affects cell viability (●) and TEER (▲)

* p<0.005 and ** p<0.001

TPM increases % of MUC5AC positive cells as measured by flow cytometry (A) and immunocytochemistry (B).

* p<0.005 and ** p<0.001

Transmission (A and B) and scanning (C) electron micrographs of HBEC ALI cultures at day 1 (A) and day 28 (B and C)
Continuous cell lines exposed to whole aerosol

Key events modelled *in vitro*

![Diagram showing untreated and treated cells](image)

**Whole smoke induces DNA damage**

- **Untreated cell**
- **Treated cell**

![Graph showing whole smoke dilution factor](image)

**Whole smoke dilution factor**

- AC
- 1:50
- 1:40
- 1:30
- 1:20

![Graph showing DNA damage](image)

**% DNA Damage**

- Untreated cell
- Treated cell

Cytotoxicity and DNA damage observed with 3R4F reference cigarette exposures compared to Vype ePen

![Graph showing aerosol dilution](image)

**Aerosol dilution (aerosol:air, 1:x)**

- Reference Cigarette
- Vype ePen

![Graph showing average intensity](image)

**Average intensity**

- Mass (µg/cm²)

D Thorne, J Wilson, T-S Kumaravel, ED Massey, M McEwan
Mutation Research 2008, 673(1):3-8
Commercially available 3D organotypic models for inhalation toxicology

- MucilAir™
- SmallAir™
- EpiAirway™
- EpiAlveolar™

www.epithelix.com
www.mattek.com
Functional endpoint analysis

Ciliary beat frequency (CBF)

Microscope with digital high speed image recording

Analysis software measures CBF
EpiAirway$^\text{TM}$ and e-cigarette testing

Comparison of cytotoxicity after cigarette and e-cigarette exposure

Development of an \textit{in vitro} cytotoxicity model for aerosol exposure using 3D reconstructed human airway tissue; application for assessment of e-cigarette aerosol

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b Vital Link Corporation, 230 Waverly Avenue, Waltham, MA 02451, United States

Comparison of cytotoxicity after cigarette and e-cigarette exposure

Fig. 1. Schematic representation of the VITROCELL$^\text{TM}$ VC-31 Smoking System, maximun 135 L/min stainless steel exposure module, EpiAirway$^\text{TM}$ tissue model. (A) VC-31 single port smoking system, enclosed in a ventilation hood with a piston/syringe that draws and delivers smoke or aerosol to the dilution box (B) Dilution box, where smoke or aerosol is diluted, mixed, and delivered to the exposure module. Diluted smoke/aerosol within the dilution box transits to exhaust. (C) 12.6 inL stainless steel exposure module, where EpiAirway$^\text{TM}$ tissue is handled during exposure. (D1) Culture insert on which EpiAirway$^\text{TM}$ tissue culture is supported at the air-liquid interface with monolayer aerosol delivered "trumpet" sitting 2 mm above the surface of the tissue. (D2) EpiAirway$^\text{TM}$ human airways epithelium, (D3) fresh culture media. (A1) (B) images show media basally feeding human airway epithelium. Transmission electron microscope (magnification x 20,000) showing (E1) cilia and (E2) tight junctions. Hematoxylin and eosin stained cross sections (magnification x 300) of (E3) pseudostratified mucociliary epithelium of EpiAirway$^\text{TM}$ tissue and (E4) endodermal human bronchial epithelium in comparison.
Comparing perturbations in MucilAir™

The differential level of 33 secreted cytokines in the culture media of MucilAir™ exposed to 3R4F (A) and Vype ePen (B)

Differential expression of 44,184 sequenced RNAs (Log2 fold change - X axis and the Log10 pFDR - Y axis) Air vs 3R4F (A) and air vs Vype® ePen (B) over a period of 48hrs post exposure recovery

3R4F exposure gene ontology enrichment identified perturbations in oxidative stress response, inflammation and xenobiotics metabolism response pathways

<table>
<thead>
<tr>
<th>GO ID</th>
<th>Term</th>
</tr>
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<tbody>
<tr>
<td>GO:0005149</td>
<td>Interleukin-1 receptor binding</td>
</tr>
<tr>
<td>GO:0016491</td>
<td>Oxidoreductase activity</td>
</tr>
<tr>
<td>GO:0055114</td>
<td>Oxidation-reduction process</td>
</tr>
<tr>
<td>GO:0051186</td>
<td>Cofactor metabolic process</td>
</tr>
<tr>
<td>GO:0044283</td>
<td>Small molecule biosynthetic process</td>
</tr>
<tr>
<td>GO:0018119</td>
<td>Peptidyl-cysteine S-nitrosylation</td>
</tr>
<tr>
<td>GO:0017014</td>
<td>Protein nitrosylation</td>
</tr>
<tr>
<td>GO:0061036</td>
<td>Positive regulation of cartilage development</td>
</tr>
<tr>
<td>GO:0005506</td>
<td>Iron ion binding</td>
</tr>
<tr>
<td>GO:0046872</td>
<td>Metal ion binding</td>
</tr>
</tbody>
</table>
*In vitro* airway surface liquid (ASL) provides a rich source of information to study tissue homeostasis

Good similarity between protein profiles from clinical samples and 3-D *in vitro* lung models

Characterisation of proteins in *in vitro* airway surface liquid

Comparison of *in vitro* ASL samples with healthy sputum

A strong overlap of 112 common proteins

Haswell et al. Society of Toxicology meeting 2014 (Abstract # 1530)
Haswell et al Society of Toxicology meeting 2016 (Abstract # 3041)
Precision cut lung slices (PCLS)

Tissue is stabilised by perfusion of agarose through the airways, prior to slicing.

Cores are taken through the tissue.

Slices are placed on a mesh in culture medium.

Precision-cut slices generated using a Krumdieck tissue slicer.
An *ex vivo* approach to the differential parenchymal responses induced by cigarette whole smoke and its vapour phase

Toxicity of cigarette smoke to the lung slices. Rat lung slices were subjected to a 30 min/day exposure of diluted cigarette whole smoke, (WS) or vapor phase, (VP) for 3 consecutive days. Lung slices were harvested for MTT assay 24 h post last exposure. Relative survival rates of smoke-exposed groups were obtained by comparing to the air-exposed group.

Fig. 2. Alveolar structure of cultured rat lung slices exposed to cigarette smoke. Lung slice left untreated (A), exposed to air (B), 4, 10, 20% whole smoke, WS (C, E, and F) or to 4% vapor phase, VP (D) were harvested for histology processing 24 h post last exposure. Three consecutive days of exposure were performed as described earlier. Bar represents 20 nm and circles indicate alveolar septum damage.

Acute inhalation toxicity: 

*in vitro* and *ex vivo* systems - considerations

- Different complexity - model specific aspects of disease processes
- Dosimetry - what are the cells exposed to?
- Exposure systems - acute/short term exposure - longer term
- Validation and qualification - fit for purpose?
- Support biomarker discovery *in vitro* and in the clinic-AOPs
Contributions and thanks

**BAT**
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