A 6 WEEK INHALATION PROTOCOL TO MEASURE CIGARETTE SMOKE INDUCED DAMAGE IN THE RAT LUNG BY *EX VIVO* COMET AND HISTOPATHOLOGICAL ANALYSIS

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AIM

To develop a 6 week sub-chronic rat inhalation protocol, with an ex vivo genotoxicity endpoint, which aims to reduce & refine animal use.

INTRODUCTION

- ***Guidelines for the provision of data for genotoxicity in mammalian cells have been re-defined identifying that an ex vivo study undertaken at the same time as in vivo work is preferable to a second in vitro study in mammalian cells[1-2]***
- ***We have explored the potential of the Comet assay to monitor DNA damage in isolated rat lung alveolar type II epithelial cells (AEC II). AEC II were selected as they are hypothesised to be a target of cigarette smoke (CS) exposure in the lung***
- ***A 6 week inhalation study protocol will enable data to be available more quickly and may provide sufficient information for British American Tobacco (BAT) to support the use of an ingredient or cigarette technology***
- ***90 day inhalation studies would be required when needed for regulatory purposes***

METHODS

Inhalation protocol

- Sprague Dawley rats (9 wk) were supplied by Charles River Laboratories
- N = 10 female & 10 male rats were exposed to 0.6 mg/L CS from 3R4F reference cigarettes (University of Kentucky) or sham air for 6 weeks
- Exposure (Monday to Friday) was for 1 or 2 h (1 h interval between CS exposure)
- Lung lobes were processed for AEC II isolation (right) or histopathology (left)

Isolation of AEC II

- AEC II were isolated from the right lung lobe using published methods[3-5] with some modifications:
  - perfused 6 x with 0.15 M NaCl (3 x 4°C & 3 x 37°C)
  - filled with Trypsin solution (prepared according to body weight, 1.25 %/kg) & incubated at 37°C for 15 min
  - parenchyma were cut to 1 mm and filtered through a 150 µm nylon filter (4°C)
  - trypan blue dye exclusion with a Neubauer chamber determined cell viability

Identification of AEC II by Alkaline phosphatase staining

- Isolated cells were centrifuged for 5 minutes (105 x *g*) using a Cytospin (Thermo Scientific) & slides dried at room temperature (RT) for ~15 hours
- Slides were incubated with Alkaline phosphatase as previously described[5,6], washed with dH_2O & subsequently counterstained with 1% Methylene green
- Cells were counted using light microscopy (x1000) & percentage AEC II calculated

Histopathology

- Left lung lobes were instilled and stored submerged in ethanol glycerol acetic acid formaldehyde solution (EGAFS) for 48 h, followed by 70 % ethanol until processing
- Longitudinal paraffin sections at main bronchus were prepared & stained with hematoxylin-eosin or alcian blue/periodic acid Schiff's reagent

Alkaline and Modified Alkaline Comet assay procedure

- **Comet positive control:** AEC II isolated from sham exposed rats were incubated with 750 µM methyl methanesulfonate (MMS) for 1 h at 37°C
- **Cell sample preparation:** ~20,000-100,000 cells were resuspended in 0.6 % low melting-point agarose at 37°C & placed on Superfrost slides pre-coated with 1.0 % collagen & 1.5 % normal melting-point agarose
- **Lysis:** Slides were placed in Lysis buffer (2.5 M NaCl, 100 mM EDTA,10 mM Trizma, 0.2 M NaOH, DMSO & 1% Triton X-100, pH 10) for 24 h at 5°C ± 3
- **Wash:** Slides were rinsed (3 x 5 min) with 4°C Enzyme reaction buffer (ERB, 40 mM HEPES, 0.1 M KCl, 0.5 mM EDTA, 0.2 mg/mL BSA, pH 8) at RT
- **Enzyme incubation (Modified Alkaline Comet assay only):** Slides were incubated for 45 min, at 37°C with FPG (New England BioLabs®) diluted 1:5,000 in ERB
- **Alkaline unwinding & electrophoresis:** Incubation of slides with Electrophoresis buffer (1 mM EDTA, 300 mM NaOH, pH >13, 4°C) within the electrophoresis tank (Thistle Scientific, pre-cooled, 4°C) for 5 min at RT. Electrophoresis, 20 min, 25 constant volts at RT
- **Neutralisation:** ~3.5 mL of Neutralisation buffer (0.4 M Trizma, pH 7.5) per slide
- **Fixation & visualisation:** Slides were air dried for approximately 24h at RT & 20 µL of VECTASHIELD Mounting Medium containing DAPI (Vector Laboratories Inc.) applied

Statistical analysis

Comet

- ~100 cells/slide were assessed at 20x magnification & % tail intensity (TI) recorded using Comet Assay IV image analysis software (Perceptive Instruments)
- Mean TI & standard deviation (SD) were calculated & values analysed using a published parametric statistical analysis approach[7]

Histopathology

- Semi-quantitative severity scores (1-5, 0 not observed) were determined blind by light microscopy
- Data were analysed using the Cochran-Mantel-Haenzel (CMH) test

RESULTS

AEC II purity & Comet analysis

- **The % AEC II in lung lysate was 56.3 - 63.0 %**
- **AEC II viability was 99.2 - 99.8 %**
- **Mean % tail DNA (± SD) were increased following 1 h and 2 h 3R4F CS exposure (Table 1 and Figure 1).**
- **There was no difference in % tail DNA between 1 and 2 h CS exposure**

Histopathology

- **Goblet cells, unpigmented macrophages & pigmented macrophages (Figure 2) were increased following exposure to 1h 3R4F CS or 2h 3R4F CS (Table 2)**
- **Unpigmented macrophages and pigmented macrophages were significantly higher following 2h 3R4F CS exposure when compared to 1h 3R4F CS (Table 2)**

Exposure	Alkaline Comet assay DNA damage - % DNA tail	Modified Alkaline Comet assay DNA damage - % DNA tail
Sham air	3.19 % ± 7.29	18.58 % ± 15.52
3R4F CS – 1 h	13.43 % ± 14.54	37.75 % ± 16.17
3R4F CS – 2 h	13.83 % ± 16.20	39.84 % ± 17.56

Table 1: The level of sham air and CS induced DNA damage as measured by the Alkaline Comet assay & Modified Alkaline Comet assay. Values: Mean ± SD.

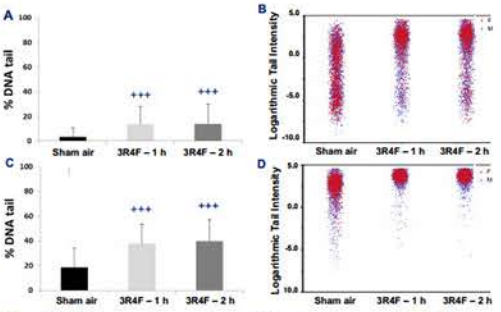

Figure 1: DNA damage, as measure by the Alkaline Comet assay and Modified Alkaline Comet assay in AEC II isolated from the right lung lobe after 6 week sham air or CS exposure (1 or 2 h 3R4F). A & C: Bar charts (Mean ± SD of TI), B & D: Scatter plots (Individual log–transformed TI). +++ p<0.001

Table 2: Histopathological endpoints evaluated in the left lung

Endpoint	Statistical parameter	Sham air	3R4F - 1 h	3R4F - 2 h
Goblet cell hyperplasia, main bronchus	Mean ± SEM Incidence p-value	0.4 ± 0.16 6/16	1.5 ± 0.30 12/17 0.012	1.4 ± 0.26 13/15 0.011
Unpigmented macrophages	Mean ± SEM Incidence p-value	0.5 ± 0.15 7/20	1.9 ± 0.18 19/20 ≤0.001	2.4 ± 0.19 18/19 ≤0.001
Pigmented macrophages	Mean ± SEM Incidence p-value	0.0 ± 0.00 0/20	0.5 ± 0.14 8/20 0.002	1.1 ± 0.17 15/19 ≤0.001

Figure 2: Left lung tissue section from Sham air (A) & 2h CS (B) exposed rats. PM: pigmented macrophages. Magnification 20x

CONCLUSIONS

- ***We have developed methods for AEC II isolation & the ex vivo Comet assay which may have potential use to determine DNA damage resulting from CS exposure***
- ***The protocol developed reduced animal number by up to 50%, as separate lobes were used for Comet or histopathological analysis***
- ***This supports a strategy for the reduction & refinement of animal use in product testing***

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- Histopathological analysis & imaging was performed at Histovia GmbH

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