The suitability of the *ex vivo* Comet assay to determine DNA damage induced by cigarette smoke in isolated rat lung cells

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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]**
- **As part of a longer term project to incorporate *ex vivo* end-points into sub-chronic inhalation studies in rodents, 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 for this study as they are hypothesised to be a target of cigarette smoke exposure in the lung**

METHODS

Isolation of AEC II

- ✓ Sprague Dawley rats (9 wk, female, 201-225 g) were supplied by Charles River Laboratories
- ✓ AEC II were isolated from the rat lung using published methods[2-4] with some modifications **Table 1**
- ✓ Cell viability was determined by trypan blue dye exclusion using a Neubauer chamber

Table 1: Stages for AEC II isolation

Step	Procedure
1. Bronchoalveolar lavage (BAL)	Lungs perfused 6 x 0.15 M NaCl (3 x 4ºC & 3 x at 37ºC)
2. Trypsinisation	1. Trypsin concentration prepared according to body weight (1.25 %/kg) 2. Lungs filled with Trypsin solution & incubated at 37ºC for 15 min 3. Lung parenchyma cut into 1 mm pieces 4. FBS added to inactivate trypsin
3. DNAse I	Lung explants incubated with DNAse I, 37ºC for 5 min*
4. Filtration - 150 µm	Lysate filtered through a150 µm nylon filter at 4ºC
5. Filtration - 30 µm	Lysate filtered through a 30 µm nylon filter at 4ºC
6. Percoll® gradient centrifugation (PGC)	1. Filtrate placed on Percoll® gradients (1.09 + 1.04 g/ml) 2. Centrifugation (250 x *g,* 20 min, 4ºC) 3. Epithelial cells recovered (4th layer) 4. Cells washed with PBS & centrifuged (2 x 300 x *g,* 5 min, 4ºC) 5. Pellet resuspended in DMEM supplemented with 1% Penicillin/Streptomycin & 1% L-Glutamine
7. Culture	Cells were cultured at 37ºC, 5% CO_2 for 1 hour & supernatant containing non-attached cells collected

* Omitted from 1 wk inhalation study

Alkaline phosphatase staining

- ✓ Isolated AEC II 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 (AP) as previously described[4,5], washed with dH_2O & subsequently counterstained with 1% Methylene green
- ✓ Cells were counted using light microscopy (x1000) & percentage AEC II calculated

Cell population determination

- ✓ Cells were isolated from n = 6 rats as detailed in **Table 1**, & samples collected at stages 4, 5, 6 & 7
- ✓ To identify Leukocytes,1.0x10^6 cells were incubated with a FITC-CD45 antibody (554877; BD Biosciences). Non-stained cells and cells incubated with a FITC mouse IgG1,κ isotype antibody (550616, BD Biosciences) were used as controls
- ✓ Cell were analysed at 535 nm using a FACSCalibur Flow Cytometer (BD Biosciences).
- ✓ AEC II within samples collected at stages 4, 5, 6 & 7 were determined by AP staining[4,5]

Basal DNA damage

- ✓ Cells were isolated from n = 10 rats as detailed in **Table 1,** samples collected at stages 4, 5, 6 & 7 & the level of DNA damage quantified by the Alkaline Comet assay (detailed below)

1 week inhalation study

- ✓ n = 12 rats were exposed to 0.8 mg/L cigarette smoke (CS) from 3R4F reference cigarettes (University of Kentucky) or sham air (1 h per day, 5 days)
- ✓ AEC II were isolated as described in stages 1 to 4 of **Table 1** & the level of DNA damage quantified by the Alkaline & Modified Alkaline Comet assay (detailed below)

Alkaline and Modified Comet assay procedure

- ✓ **Comet positive control:** AEC II isolated from sham exposed rats were incubated with 750 µM methyl methanesulfonate (MMS) for 1 hour at 37ºC
- ✓ **Cell sample preparation**: ~20,000-100,000 cells were resuspended in 0.6% low melting-point agarose at 37ºC & 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,10%, 0.2 M NaOH, DMSO & 1% Triton X-100, pH 10) for 24h at 5ºC ± 3
- ✓ **Wash:** Slides were rinsed (3x 5 min) with 4ºC Enzyme reaction buffer (ERB, 40 mM HEPES, 0.1 M KCl, 0.5 mM EDTA, 0.2 mg/mL BSA, pH 8) at room temperature (RT)
- ✓ **Enzyme incubation (Modified Alkaline Comet assay only):** Slides were incubated for 45 min, at 37ºC 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, 4ºC) within the electrophoresis tank (Thistle Scientific, pre-cooled, 4ºC) 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) added 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

REFERENCES

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RESULTS

Statistical analysis

- ✓ ~100 cells/slide were assessed at 20x magnification & percentage tail intensity (TI) recorded using Comet Assay IV image analysis software
- ✓ Mean & standard deviation (SD) of TI were calculated
- ✓ Data were analysed by using a published parametric statistical analysis approach[6]

Cell populations

- ✓ Flow cytometry & AP staining results are detailed in **Table 2**
- ✓ Differences in Leukocyte & AEC II number can be observed at the different stages of the AEC II isolation method

Table 2: Leukocyte & AEC II at different stages of the isolation method as determined by flow cytometry & AP staining

AEC II isolation Step	Leukocytes	% AEC II
4. Filtration - 150 µm	17.6% ± 4.28	62.6% ± 8.84
5. Filtration - 30 µm	17.6% ± 3.37	60.6% ± 6.26
6. Percoll® gradient centrifugation	36.6% ± 7.55	59.2% ± 5.64
7. Culture	22.9% ± 9.55	70.1% ± 8.97

Basal DNA damage

- ✓ The levels of basal DNA damage, as determined by the Alkaline Comet assay are detailed in **Table 3 & Figure 1**
- ✓ Increasing the stages of the AEC II isolation method affects the level of basal DNA damage observed

Table 3: The levels of basal DNA damage at different stages of the AEC II isolation method as determined by the Alkaline Comet assay

AEC II Isolation Step	DNA damage - % DNA tail	% Cell viability	% AEC II
4. Filtration - 150 µm	8.47 ±17.41	98.2 ± 2.28	49.2 ± 10.73
5. Filtration - 30 µm	9.42 ±19.23	99.0 ± 1.35	57.4 ± 20.07
6. Percoll® gradient centrifugation	15.65 ±21.93	99.3 ± 0.85	63.5 ± 10.61
7. Culture	27.36 ± 31.27	99.8 ± 0.51	73.2 ± 12.33

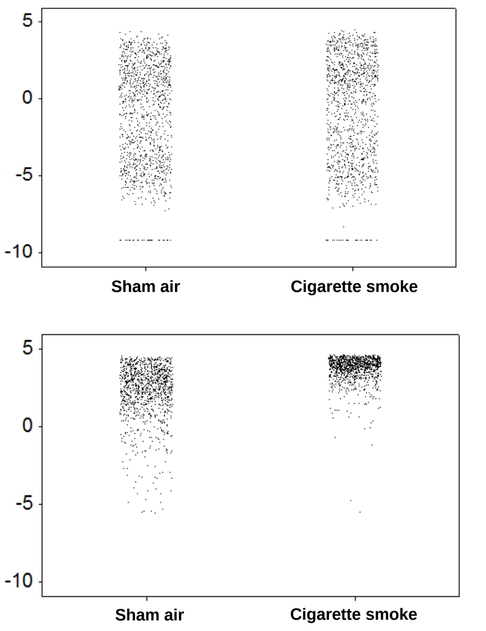

Figure 1: Basal DNA damage at different stages of the AEC II isolation method. Mean ± SD of TI

1 week inhalation study

- ✓ DNA damage, as determined by the Alkaline Comet assay, was 5.80 ± 11.22% compared to 8.31 ±14.94% for sham air & CS exposed animals respectively **Figure 2A & B**
- ✓ The level of DNA damage, as determined by the Modified Alkaline Comet assay was significantly different for sham air exposed animals when compared to CS, 21.81 ± 21.55% vs. 49.60 ± 23.02%, p<0.01 **Figure 2C & D**

Figure 2: DNA damage, as measure by the Alkaline Comet assay and Modified Alkaline Comet assay, in AEC II after 5 days sham air or CS exposure. Positive control 750 µM MMS treatment *ex vivo*. A & C: Bar charts (Mean ± SD of TI), B & D: Scatter plots (Individual log–transformed TI). * p<0.01

CONCLUSIONS

- **We have developed methods for AEC II isolation & the *ex vivo* Comet assay**
- **These methods may have potential use to determine DNA damage resulting from CS exposure**

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