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An improved method for the isolation of rat alveolar type II lung cells: Use in the Comet assay to determine DNA damage induced by cigarette smoke

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ABSTRACT

Smoking is a cause of serious diseases, including lung cancer, emphysema, chronic bronchitis and heart disease. DNA damage is thought to be one of the mechanisms by which cigarette smoke (CS) initiates disease in the lung. Indeed, CS induced DNA damage can be measured *in vitro* and *in vivo*. The potential of the Comet assay to measure DNA damage in isolated rat lung alveolar type II epithelial cells (AEC II) was explored as a means to include a genotoxicity end-point in rodent sub-chronic inhalation studies. In this study, published AEC II isolation methods were improved to yield viable cells suitable for use in the Comet assay. The improved method reduced the level of basal DNA damage and DNA repair in isolated AEC II. CS induced DNA damage could also be quantified in isolated cells following a single or 5 days CS exposure. In conclusion, the Comet assay has the potential to determine CS or other aerosol induced DNA damage in AEC II isolated from rodents used in sub-chronic inhalation studies.

1. Introduction

Smoking is a cause of serious diseases, including lung cancer, emphysema, chronic bronchitis and heart diseases (Institute of Medicine, 2001; Aoshiba and Nagai, 2003; DeMarini, 2004). Alveolar epithelium cells (AEC), the lung's first line of defence against the external environment, are thought to be the target cells of cigarette smoke (CS) exposure (Kamp et al., 1998; Aoshiba and Nagai, 2003). Alveolar cell injury is also proposed to be a factor in lung damage that precedes CS induced lung disease (Kamp et al., 1998).

In the lung, two types of AEC are present; AEC I and AEC II. AEC I are squamous, occupy approximately 95% of the lung surface area and function in gas exchange, water permeability and alveolar fluid homeostasis. AEC II are cuboidal, occupy 5% of the lung surface area, function in fluid transport and are involved in producing, secreting and recycling lung surfactant. AEC II also maintain the alveolar epithelium by proliferating and differentiating into AEC I, an essential function, as AEC I are easily injured due to their shape and inability to perform mitogenesis (Chen et al., 2004, 2007). Cellular differences have enabled AEC I and AEC II cells to be isolated from lung tissue and identified *in vitro* (Richards et al., 1987; Witherden and Tetley, 2001; Chen et al., 2004). Recently, DNA damage has been measured by the Comet assay in isolated mouse AEC II (Lindberg et al., 2013).

CS is a complex and dynamic mixture of more than 6000 chemicals (Perfetti and Rodgman, 2013). Studies have demonstrated that one of the mechanisms by which CS induces an effect in AEC II is via oxidative stress and subsequent DNA damage (Aoshiba and Nagai, 2003; Rennard et al., 2006). Isolated rat AEC II have been used to measure oxidative damage (Liu et al., 2014) and the pre-treatment of mice with α-tocopherol acetate and acetyl salicylic acid, known antioxidants, significantly reduced DNA damage in the lung (Tsuda et al., 2000; Ueno et al., 2011). The oxidative effect of CS extract can also be reduced by the antioxidant compounds

Abbreviations: AEC I, alveolar type I cells; AEC II, alveolar type II cells; AP, apurinic/pyrimidinic sites; BSA, Bovine serum albumin; CS, cigarette smoke; DNA, deoxyribonucleic acid; DAPI, 4′,6-diamidino-2-phenylindole; DMSO, dimethyl sulphoxide; DMEM, Dulbecco's modified Eagle medium; EDTA, ethylenediaminetetraacetic acid; FBS, fetal bovine serum; FPG, formamidopyrimidine DNA-glycosylase; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IP, intraperitoneal; ISO, International Organization for Standardization; $KBrO_3$, Potassium Bromate; MMS, methyl methanesulfonate; NaCl, sodium chloride; NaOH, sodium hydroxide; OECD, Organisation for Economic Co-operation and Development; PBS, phosphate buffered saline; RT, room temperature; RPMI, Roswell Park Memorial Institute medium; SD, standard deviation; TI, tail intensity; WTPM, wet tobacco particulate matter.

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N-acetylcysteine and Trolox in isolated human alveolar type I like cells, AEC II that are undergoing differentiation into AEC I *in vitro* (Kosmider et al., 2011).

The Comet assay is a commonly used method for assessing DNA damage *in vitro* and *in vivo* (Tice et al., 2000; Hartmann et al., 2003; Brendler-Schwaab et al., 2005; Burlinson et al., 2007; Dhawan et al., 2009). Developed in the 1980s (Ostling and Johanson, 1984; Singh et al., 1988), the Comet assay has been used by numerous researchers and various methods/protocols exist, which makes data comparisons difficult. Recent interest in the development of a regulatory standard protocol has resulted in the publication of the OECD 489 Guideline in September 2014 (OECD, 2014). The analysis and interpretation of Comet assay data has also been a long-standing debate. The OECD 489 Guideline (OECD, 2014) recommends that the median percentage tail DNA is determined and highlights a number of statistical analysis methods for data analysis. The use of this guideline should enable data comparison between laboratories and encourage the use of comparable statistical methods.

The alkaline version of the Comet assay (pH > 13) is used to analyse direct acting genotoxic compounds. The Modified Alkaline Comet assay, which utilises lesion-specific endonucleases that recognise and cleave DNA at specific base alterations, has increased sensitivity and specificity and can measure indirect DNA damage such as oxidative damage, apurinic/pyrimidinic (AP) sites or DNA repair (Smith et al., 2006). A number of publications detail the use of the Comet assay to quantify CS induced DNA damage *in vitro* and *in vivo* (Tsuda et al., 2000; Thorne et al., 2009; Ueno et al., 2011; Weber et al., 2013). However, to our knowledge, CS induced DNA damage has not been measured in isolated rat lung cells collected as part of a sub-chronic inhalation protocol. Micronucleus frequency has been measured following cigarette smoke *in vivo* exposure, however the micronucleus assay could not detect differences between peripheral blood or bone marrow samples collected from sham air or cigarette exposed animals (Van Miert et al., 2008).

The OECD 413 Guideline, a rodent nose only sub-chronic inhalation toxicity guideline (OECD, 2009) is available and is used, with minor modifications, for the testing of cigarettes (Baker et al., 2004; Gaworski et al., 2009; Potts et al., 2010; Werley et al., 2013). This study was initiated to prepare for future regulatory needs and also in response to genotoxicity assay regulatory guideline changes. In 2011, the International Conference on Harmonisation Guidelines (International Conference On Harmonisation, 2011) and UK Committee for Mutagenicity test guidelines UK (Committee on Mutagenicity, 2011) identified 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. Therefore, this study aimed to develop a suitable method to isolate AEC II from the rat lung which could then be used in the Comet assay to measure CS induce DNA damage. Here we demonstrate that the AEC II isolation method described in this study, isolated viable cells with low levels of basal DNA damage in control animals and that CS induced DNA damage could be measured by the Comet assay following a single or repeated exposure to CS.

2. Materials and methods

2.1. Chemicals and reagents

All chemicals and reagents were purchased from Sigma Aldrich (Madrid, Spain) unless otherwise stated. Formamidopyrimidine DNA glycosylase (FPG) was purchased from New England Biolabs (Hitchin, UK).

2.2. Animals

Sprague Dawley Rats (9–11 weeks) were supplied by Charles River Laboratories (Barcelona, Spain). Animals were acclimatised for at least 2 days before AEC II isolation method development experiments or for 8 days prior to use in a smoke inhalation study in accordance with OECD 413 (OECD, 2009). Animal housing and procedures used were in compliance with Spanish Law RD 53/2013 (Boletín Oficial Del Estado, 2013) and European Directive 2010/63/UE (European Union, 2010).

2.3. Reference cigarettes

3R4F reference cigarettes were obtained from the University of Kentucky (Kentucky, USA). Prior to use, 3R4F cigarettes were conditioned for a minimum of 48 h and a maximum of 10 days at 22 ± 1 °C and 58 ± 3% relative humidity, according to International Organisation for Standardisation 3402 (International Organization for Standardization, 1999).

2.4. Alveolar type II cell isolation procedure

AEC II were isolated using published methods (Richards et al., 1987; Witherden and Tetley, 2001; Knaapen et al., 2002) with some modifications. For the experiments that determined DNA damage during the isolation process and the effect of slide time in lysis buffer (detailed below), animals were exsanguinated via the abdominal aorta under intraperitoneal sodium pentobarbital anaesthesia (75 mg/kg). For the gradation in DNA damage, DNA repair and 1 week inhalation study (see below), to reduce the time for AEC II isolation, animals were exsanguinated via the abdominal aorta and a ketamine/medetomidine cocktail anaesthesia (100 mg/kg and 0.5 mg/kg, respectively) intramuscularly administered. The anaesthesia modification ensured that cells could be isolated and Comet slides placed in lysis buffer within a set time of 45 ± 3 min. Following anaesthesia, lungs were subsequently dissected from the animals together with the trachea. Lung artificial ventilation was achieved by perfusion of the pulmonary artery with 0.15 M NaCl and bronchoalveolar lavage was performed with 0.15 M NaCl (3 × 4 °C and 3 × 37 °C). Immediately thereafter, lung lobes were filled with trypsin solution (prepared according to animal body weight, 1.25%/kg) and incubated at 37 °C for 15 min. Afterwards, lung parenchyma was cut to 1 mm pieces on ice and fetal bovine serum (FBS) added to inactivate trypsin. The volume of lung homogenate was adjusted to 20 mL with phosphate buffered saline (PBS) and then incubated for 5 min at 37 °C with DNase I (250 μg/mL; Roche Diagnostics, S.L., Madrid, Spain). The resulting suspension was then filtered on ice through 150 μm and 30 μm nylon filters (Laborat S.L., Madrid, Spain) to remove larger cells and cell debris. The filtrate was then laid onto a discontinuous Percoll® gradient of heavy and light density, 1.089 and 1.040 respectively (Richards et al., 1987) and centrifuged (20 min, 250×*g*, 4 °C). Following centrifugation, the 4th layer from the top was collected, washed with PBS and centrifuged (5 min, 300×*g*, 4 °C). The resulting pellet was resuspended in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS, 1% Penicillin/Streptomycin solution and 2 mM L-glutamine and cultured (1 h, 37 ± 0.5 °C, 5 ± 0.2% CO_2, >85% relative humidity) in 100 mm Petri dishes. The culture supernatant was then centrifuged (5 min, 300×*g*, 4 °C) to collect non-attached epithelial cells. The cell pellet was resuspended in an appropriate volume of 4 °C DMEM medium to enable cell counting (approximately 0.1 × 10^6 cells in 3.0 mL of supplemented DMEM), AEC II purity determination and the Comet assay (detailed below). The full AEC II isolation procedure detailed above was used for the DNA damage during the AEC II isolation process experiments (detailed below).

For all other experiments, AEC II was collected after the 150 μm filtration stage and used for further analysis. For the gradation in DNA damage, DNA repair and 1 week inhalation experiments (detailed below), the DNase I incubation stage was omitted to ensure cells could be isolated and Comet slides placed in lysis buffer (detailed below) within a set time of 45 ± 3 min.

2.5. Identification of alveolar type II cells by alkaline phosphatase staining

In order to determine isolated AEC II purity, cells were centrifuged for 5 min (105×*g*) using a Cytospin™ (Thermo Scientific, Loughborough, UK), slides dried at room temperature (RT) overnight (approx. 15 h) and then incubated with alkaline phosphatase as previously described (Bingle et al., 1990; Witherden and Tetley, 2001). AEC II purity was calculated as the percentage of alkaline phosphatase positive AEC II with respect to the total number of cells present on a slide.

2.6. Comet assay in vivo and in vitro positive controls

To demonstrate that DNA damage could be measured in isolated AEC II following *in vivo* exposure to a genotoxic compound, 7 female rats were injected intraperitoneal (IP) with 80 mg/kg Methyl methanesulfonate (MMS), 6 control rats were injected with PBS. Treatment dose was based on a previous study using this compound (Hashimoto et al., 2007). Rats were sacrificed 24 h after treatment and AEC II isolated as detailed above. To ensure AEC II isolated from control animals were responsive to an exogenous agents, AEC II isolated from three PBS treated animals were incubated with 750 μM of MMS for 1 h at 37 °C. Comet slides were prepared as detailed below for the Alkaline Comet assay. Two Comet slides containing NCI-H292 cells (American Type Culture Collection, ATCC, Barcelona, Spain, catalogue number CRL-1848™) were included in each Comet tank to control for the Comet assay procedure and to prepare historical control charts for assay acceptance (20% of historical control data). NCI-H292 cells were seeded in 6-well plates (Cultek, S.L:U. Madrid, Spain), 1.2×10^6 cells/well in 4.0 mL of complete Roswell Park Memorial Institute medium (RPMI, Life Technologies, S.A., Spain) supplemented with 10% heated-inactivated FBS, 100 units/mL penicillin and 100 μg/mL streptomycin (Invitrogen by Life Technologies). After seeding for 16 h, approximately 80% confluent cells were incubated independently for 1 h with 2.0 mM $KBrO_3$ or PBS. Cells were subsequently trypsinised, cell number plus viability determined and slides prepared for the Comet assay as detailed below. Prior to use, cell cultures were screened to confirm the absence of mycoplasma.

2.7. Cell viability

AEC II viability and total cell number was determined by trypan blue dye exclusion (Life Technologies, S.A., Spain) and a Neubauer chamber. NCI-H292 cell viability was determined by trypan blue exclusion using an automated Vi-CELL® cell viability analyser (Beckman-Coulter, Madrid, Spain). Approximately 500 μL of NCI-H292 cell lysate were collected from each culture condition at the trypsinisation stage and analysed.

2.8. Alkaline Comet assay

The Comet assay was performed under alkaline conditions (pH > 13) as described by Tice et al. (2000) and the Comet Assay Interest Group (Comet Assay Interest Group website, 2013) with some modifications. Cells (20,000–100,000 isolated AEC II or NCI-H292 cells, detailed above) were centrifuged (1 min, 300×*g*) and pellets resuspended in 0.6% low melting-point agarose (37 °C). The cell/agarose suspension was placed on Superfrost® slides (Menzel-Glaser, Thermo Fisher Scientific, Braunschweig, Germany) that were pre-coated with 1.0% collagen (PureCol®, NUTACON B.V., Holland) and 1.5% normal melting-point agarose. At least two slides per cell culture treatment/animal were prepared. Cover-slips were subsequently applied and slides placed on a pre-chilled metallic plate (4 °C) for 2–3 min. The cover-slips were removed and the slides immersed in lysis buffer (2.5 M NaCl, 100 mM Ethylenediaminetetraacetic acid [EDTA],10 mM Trizma, 0.2 M NaOH, 10% Dimethyl sulphoxide [DMSO] and 1% Triton X-100, pH 10) for 24 h at 5 °C ± 3. Following lysis, slides were gently rinsed (3 × 5 min each) at RT with enzyme reaction solution (40 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid [HEPES], 0.1 M KCl, 0.5 mM EDTA, 0.2 mg/mL Bovine serum albumin [BSA], pH 8) and then incubated for 5 min in electrophoresis buffer (1 mM EDTA, 300 mM NaOH, pH > 13, 4 °C) within a pre-cooled electrophoresis tank (Thistle Scientific, Glasgow, UK). Electrophoresis (20 min, 25 constant volts) was followed by incubation (3 × 5 min each, RT) in neutralisation buffer (0.4 M Trizma, pH 7.5), air drying for approximately 12 h at RT and then staining with Vectashield Mounting Medium containing 4′,6-diamidino-2-phenylindole (DAPI, Vector Laboratories Inc., supplied by Palex Medical, S.A., Barcelona, Spain).

2.9. Modified Alkaline Comet assay

The Modified Alkaline Comet assay was performed as described above for the Alkaline Comet assay, with an additional step after the 24 h slide storage in lysis buffer stage. For isolated AEC II, two additional slides were prepared per animal and then incubated with 500 μL of FPG (1:5000) diluted in enzyme reaction solution. NCI-H292 cells cultures exposed to 2.0 mM $KBrO_3$ were incubated with 200 μL of FPG (1:5000) diluted in enzyme reaction solution. Slides were covered with a Parafilm® cover-slip and incubated at 37 °C for 45 min under humidified conditions. Cover-slips were removed, slides washed in enzyme reaction solution and electrophoresis performed as described above. The Modified Comet assay was only performed for the following experiments: gradation in DNA damage, DNA repair and 1 week inhalation study (detailed below).

2.10. Comet visualisation and statistical analysis

Nuclei were visualised using a fluorescence microscope (20× magnification). Where possible, 100 nuclei per slide were scored and DNA damage determined using Comet Assay IV image analysis software (Perceptive Instruments, Harverhill, UK). Percentage tail intensity (TI) was recorded. Mean TI and standard deviation (SD) values were subsequently calculated using Microsoft Excel. All data was interpreted using a parametric statistical approach published by Bright et al. (2011) and Minitab® 16 Statistical Software. A value of $p \leqslant 0.05$ was considered statistically significant.

2.11. Evaluation of DNA damage levels during AEC II isolation process

AEC II were isolated from 13 female Sprague Dawley rats (3–4 rats per isolation stage) and collected at the 150 and 30 μm filtration, Percoll® gradient and 1 h culture stages. At least two slides per animal were prepared and the level of DNA damage was assessed by the Alkaline Comet assay. Cell viability and AEC II purity were determined as detailed above for each sample. On review of the data obtained it was decided that the 150 μm filtrate should be used for further experiments.

2.12. Extended slide storage time in lysis buffer

AEC II were isolated from 9 female rats, the resulting 150 μm filtrate from each animal was split and incubated at 37 °C for 1 h with 750 μM MMS or PBS. A total of 16 Comet slides were prepared from each animal/treatment and two of each slides stored in lysis buffer for 24 h (standard protocol parameter), 3, 7 or 14 days. After the specified slide time in lysis buffer (24 h, 3, 7 or 14 days) slides were processed as detailed above for the Alkaline Comet assay. Cell viability and AEC II purity were determined as detailed above.

2.13. Smoke inhalation protocol

3R4F reference cigarettes were smoked to International Organization for Standardization (ISO) standards 3308 and 4387 (International Organization for Standardization, 2000a,b) on 30-port smoking machines with an active sidestream exhaust (type SM85i). Some minor deviations from ISO standard 3308 were required for technical reasons. Deviations included; the counting of whole puffs rather than 1/10 of a puff, humidity and temperature were not measured within the smoking machine as it was not possible, the use of free smoking mode rather than restricted and 1 mm less distance between cigarettes. 3R4F smoke was diluted with filtered, conditioned fresh air (21 °C, 65% relative humidity) to obtain the required concentration of Wet Total Particulate Matter (WTPM, detailed below). Exposure chambers type EC-FPC-232 (Borgwaldt Körber Solutions GmBH, Germany) equipped with individual glass exposure tubes were used. Sham exposed rats were exposed to filtered conditioned fresh air (21 °C, 65% relative humidity) in a separate exposure chamber. Body weight, behaviour and clinical observations were recorded directly after exposure.

2.14. Gradation in DNA damage

Rats (3 animals/group) were exposed to 3R4F CS (0.80 mg WTPM/L) for 15 min, 30 min, 1 h or 1.5 h. Animals from the sham air group were exposed to filtered conditioned fresh air for 1.5 h. All animals were sacrificed directly after the corresponding exposure period, AEC II were isolated as detailed above and the 150 μm filtrate collected for further analysis. The Alkaline Comet assay, Modified Alkaline Comet assay, cell viability and AEC II purity were determined as detailed above. AEC II isolated from sham air exposed animals were incubated MMS as detailed above and used as a positive control.

2.15. DNA repair

Rats (3 animals/group) and were exposed to filtered conditioned fresh air (sham air) or diluted CS from 3R4F cigarettes (0.80 mg WTPM/L) for 1 h. Animals were then sacrificed immediately or 15 min, 30 min, 1 h or 1.5 h following CS exposure. Sham air exposed animals were sacrificed immediately following filtered air exposure. AEC II were isolated as detailed above and the 150 μm filtrate collected for further analysis. The Alkaline Comet assay, Modified Alkaline Comet assay, cell viability and AEC II purity were determined as detailed above.

2.16. 1 week inhalation experiment

Rats (6 animals/group) were exposed to 3R4F CS (0.8 mg WTPM/L) or filtered conditioned fresh air (sham air) for 1 h per day for 5 consecutive days. Body weight was recorded on the day of distribution to groups, before the first exposure and prior to sacrifice. Behaviour and clinical observations were recorded daily. Animals were sacrificed following day 5 exposure, AEC II were isolated as detailed above and the 150 μm filtrate collected for further analysis. The Alkaline Comet assay, Modified Alkaline Comet assay, cell viability and AEC II purity were determined as detailed above. Animals were sacrificed and slides placed in lysis buffer within 45 ± 3 min. AEC II isolated from sham air exposed animals were incubated with MMS as detailed above and used as a positive control.

3. Results

3.1. Cell viability and AEC II isolation

The AEC II isolation method isolated cells with >95% viability from untreated, sham air or CS exposed rats. The average purity of the AEC II 150 μM filtrate, from all studies detailed herein was 63%.

3.2. Comet assay in vivo and in vitro positive controls

The AEC II isolated (150 μm filtrate) from the 13 animals treated IP with PBS or MMS had approximately 98% cell viability and 51.8 ± 9.8% AEC II purity. The level of DNA damage from animals treated IP with PBS was 8.24 ± 22.56% and 37.45 ± 21.77% following IP treatment with 80 mg/kg MMS. The values obtained were significantly different (Fig. 1). MMS *in vitro* treatment (750 μM) also induced DNA damage, a value of 83.63% ± 17.64 was obtained (Fig. 1). The mean DNA damage levels, from all experiments detailed, for MMS treatment *in vitro* in isolated AEC II cells and NCI-H292 cells exposed to 2.0 mM Potassium Bromate were 76.62 ± 20.78% and 65.17 ± 17.01%, respectively.

3.3. Evaluation of DNA damage levels during AEC II isolation process

Table 1 details data collected from 13 animals and analysis of isolated AEC II by the Alkaline Comet assay. Cell viability for all experiments was greater than 98%. AEC II purity progressively increased with each stage of the AEC II isolation method (Table 1), values obtained were 49.2 ± 10.73%, 57.4 ± 20.07%, 63.5 ± 10.61% and 73.2 ± 12.33%, for the 150 μm filtration, 30 μm filtration, Percoll® and 1 h culture stage. Basal DNA damage also increased with each stage and was 8.47 ± 17.41%, 9.42 ± 19.23%, 15.65 ± 21.93% and 27.36 ± 31.27% for the 150 μm filtration, 30 μm filtration, Percoll® and 1 h culture stage (Table 1 and Fig. 2). No statistically significant differences in basal DNA damage was observed between 150 μm and 30 μm filtration stages. However, the levels of basal DNA damage were statistically

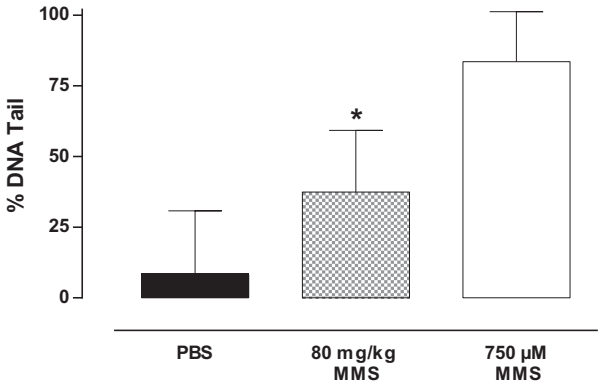

Fig. 1. The level of DNA damage (single strand breaks), as identified by the Alkaline Comet assay, induced *in vivo* by PBS and MMS intraperitoneal treatment and also by MMS treatment *in vitro*. Values plotted are Mean ± SD of TI. $^{*}p \leqslant 0.001$ when compared to PBS treated animals.

Table 1
Cell viability, AEC II purity and the levels of basal DNA damage (single strand breaks) at different stages of the AEC II isolation method as determined by the Alkaline Comet assay. Values are Mean ± SD.

AEC II isolation step	% Cell viability	% AEC II Purity	DNA damage % DNA tail
Filtration – 150 μm	98.2 ± 2.28	49.2 ± 10.73	8.47 ± 17.41
Filtration – 30 μm	99.0 ± 1.35	57.4 ± 20.07	9.42 ± 19.23
Percoll® gradient centrifugation	99.3 ± 0.85	63.5 ± 10.61	15.65 ± 21.93
1 h culture	99.8 ± 0.51	73.2 ± 12.33	27.36 ± 31.27

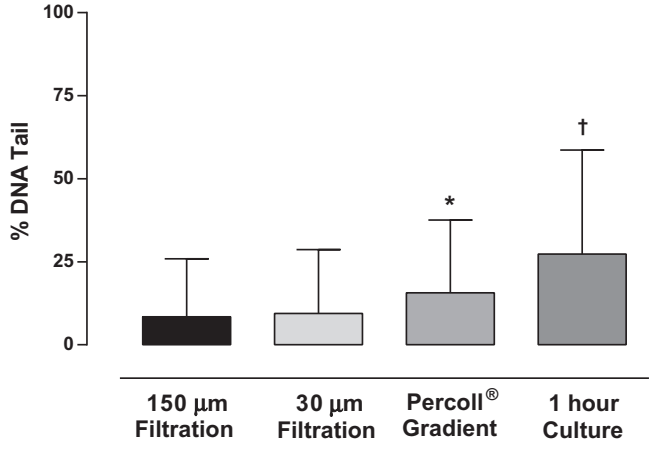

Fig. 2. The level of basal DNA damage (single strand breaks), as identified by the Alkaline Comet assay, at the different stages of the AEC II isolation method. Values plotted are Mean ± SD of TI. $^{*}p \leqslant 0.001$ when compared to 150 μm filtration and 30 μm filtration stages. $^{\dagger}p \leqslant 0.001$ when compared to 150 μm filtration and 30 μm filtration and Percoll® gradient stages.

significantly higher at Percoll® and culture stages when compared to those observed at the 150 μm and 30 μm filtration stages. The DNA damage increases observed may not only be related to increased cell manipulation, but also the additional time required to complete the method. On review of this data, it was decided that the 150 μm filtrate should be used for all future experiments.

3.4. Extended slide storage time in lysis buffer

The AEC II isolated (150 μm filtrate) from the 9 animals used for these experiments had approximately 99.4% cell viability and 73.8 ± 7.33% AEC II purity. The levels of basal DNA damage in AEC II that were treated with PBS and stored for 24 h, 3, 7 and 14 days in lysis buffer were within the expected range obtained previously for AEC II cells (approx. 15–21%). Values obtained were 15.11 ± 28.64%, 14.60 ± 27.75%, 21.27 ± 31.94% and 16.61 ± 28.82% for storage in lysis buffer for 24 h, 3, 7 and 14 days (Fig. 3). Statistical analysis revealed that the levels of DNA strand breaks in slides stored for 3 or 14 days were similar to the control (24 h time point). The 7 day time point had a DNA damage value of 21.27% ± 31.94, which was significantly different from the 24 h time point, however when 3, 7 and 14 days data were compared, no statistically significant differences were detected. The levels of DNA damage obtained for AEC II treated with 750 μM MMS and stored in lysis buffer for 24 h, 3, 7 and 14 days were 81.48 ± 19.06%, 91.12 ± 10.76%, 89.61 ± 11.53%, 89.48 ± 13.94%, respectively (Fig. 3). Statistical analysis indicated that DNA damage levels were statistically higher in the slides stored for 3, 7 and 14 days when compared to the 24 h time point. However, the levels observed were within the historical range obtained for AEC II incubated with 750 μM MMS.

3.5. Gradation in DNA damage

The AEC II isolated for these experiments had approximately 97.4 ± 1.80% cell viability and 65.1 ± 7.1% purity. No statistically significant differences in the levels of DNA strand breaks were observed between sham air and CS exposed animals, regardless of the smoke exposure period (15 min, 30 min, 1 h or 1.5 h, Fig. 4A). In all cases, DNA damage ranged from 1% to 6%, within the basal DNA damage levels observed previously for the Alkaline Comet assay with AEC II. Values obtained were 1.05 ± 2.63%, 2.23 ± 5.93%, 1.38 ± 3.17%, 1.60 ± 5.05%, 6.16% ± 13.64 for 0, 15 min, 30 min, 1 h or 1.5 h 3R4F smoke exposure (Fig. 4A). In the case of oxidative DNA, values obtained were 23.38 ± 14.78%, 21.94 ± 17.18%, 27.78 ± 14.54%, 36.52 ± 12.65%, 37.23 ± 16.91% for 0, 15 min, 30 min, 1 h or 1.5 h 3R4F smoke exposure (Fig. 4B). Although no statistically significant differences in the levels of oxidative DNA damage were observed, possibly due to low animal numbers per group, DNA damage did appear to increase after 1 h of exposure to 3R4F CS (Fig. 4B).

3.6. DNA repair

Regardless of the time of sacrifice, cell viability exceeded 97% and AEC II purity was over 50%. When the animals were sacrificed immediately (0 min) or 15 min, 30 min, 1 h and 1.5 h following 1 h CS exposure, most smoke exposed groups were statistically different from the sham air control group in terms of DNA single strand

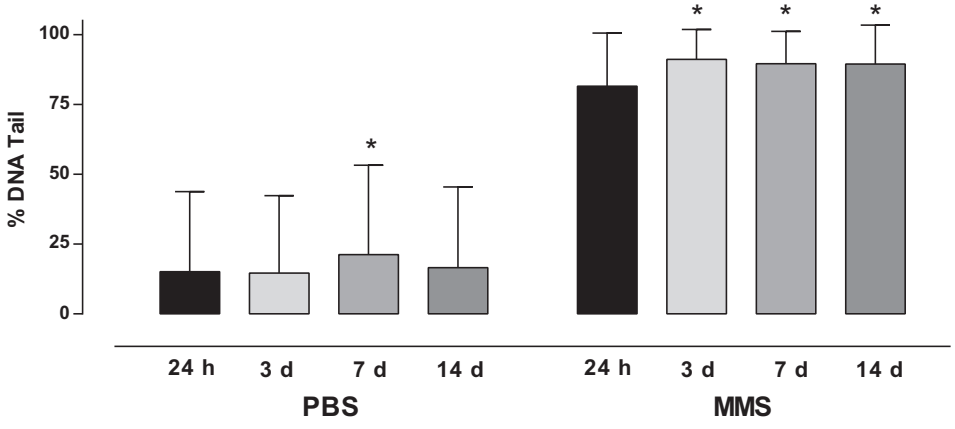

Fig. 3. PBS and MMS induced DNA damage (single strand breaks), measured by the Alkaline Comet assay following the storage of slides in lysis buffer for 24 h, 3, 7 and 14 days. Values plotted are Mean ± SD of TI. $^{*}p \leqslant 0.05$ when compared to 24 h time point.

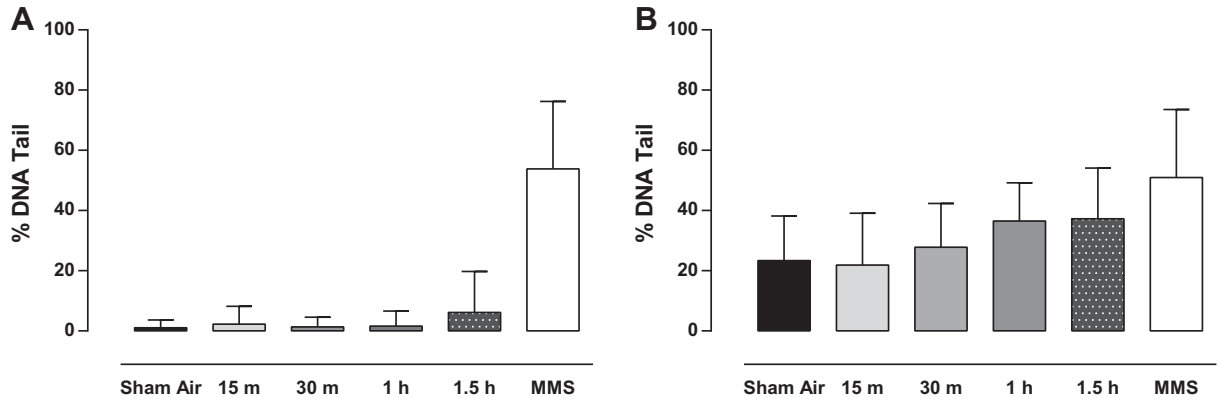

Fig. 4. Influence of 3R4F cigarette smoke exposure time (0, 15 min, 30 min, 1 h, and 1.5 h) on (A) levels of single strand DNA breaks and (B) oxidative DNA damage. MMS was used as an *in vitro* positive control. Values plotted are Mean ± SD of TI.

breaks (Fig. 5A). The only exception to this was the animals sacrificed 15 min after exposure. Values obtained were 3.57 ± 8.15%, 7.14 ± 12.46%, 3.31 ± 7.96%, 6.51 ± 11.34%, 5.31 ± 9.59%, 5.07 ± 8.81% for sham air and animals sacrificed immediately or 15 min, 30 min, 1 h and 1.5 h following 1 h 3R4F CS exposure. The 15 min time point was also significantly less than all the other smoke exposed groups, which is possibly due to animal variability. Although statistical analysis revealed that most smoke exposed groups were statistically different from the sham air control group in terms of DNA strand breaks, all values were within the expected range for control/sham air exposed animals (i.e. up to ~10%). The levels of oxidative DNA damage were significantly higher in all smoke exposure groups when compared to the sham air group (Fig. 5B). Values obtained were 23.48 ± 16.76%, 46.17 ± 16.88%, 36.78 ± 18.17%, 38.99 ± 17.92%, 31.47 ± 16.09%, 27.64 ± 15.91% for sham air and animals sacrificed immediately or 15 min, 30 min, 1 h and 1.5 h following 1 h 3R4F CS exposure. The levels of oxidative DNA damage differed statistically depending on the time of sacrifice after exposure (Fig. 5B). The highest levels were observed in the animals sacrificed immediately after exposure followed by sacrifice 15 and 30 min post exposure, which were significantly higher than animals sacrificed 1 and 1.5 h after CS exposure.

3.7. 1 week inhalation experiment

No animals died following 5 days sham air or cigarette smoke exposure and normal behaviour was observed. As routinely observed in rat inhalation studies, chromodacryorrhea (red eye secretion or Harderian gland secretion), chromorhinorrea and wet fur were observed in sham air and cigarette smoke animals. No differences in body weight were observed between sham air or cigarette smoke exposed animals. The AEC II isolated for these experiments had approximately 98% cell viability and 66% purity. Single strand DNA damage, as determined by the Alkaline Comet assay, was 5.80 ± 11.21% compared to 8.31 ± 14.94% for sham air and 1 h 3R4F CS exposed animals (Fig. 6A), no statistical difference was observed. The level of oxidative DNA, as measured by the Modified Alkaline Comet assay, was significantly different for sham air exposed animals when compared to 1 h 3R4F CS exposure, 21.81 ± 21.55% vs. 49.60 ± 23.02% (Fig. 6B).

4. Discussion

In this study, a method is described, which can be used to measure DNA damage in isolated rat lung cells following single or repeated CS exposure. This study was initiated in response to regulatory guideline modifications (International Conference On Harmonisation, 2011; UK Committee on Mutagenicity, 2011) which identified that an *ex vivo* study undertaken at the same time as *in vivo* work is preferable to a second mammalian cell *in vitro* study. When a rat inhalation study is required for regulatory requirements, AEC II analysis by the Comet assay could be a potential endpoint to measure the genotoxicity of CS and data submitted as part of a regulatory dossier. Refining an *in vivo* approach with the addition of a genotoxicity endpoint will enable DNA damage to be quantified at the proposed site CS of exposure, AEC II cells. Future studies will investigate whether the right lung lobe can be

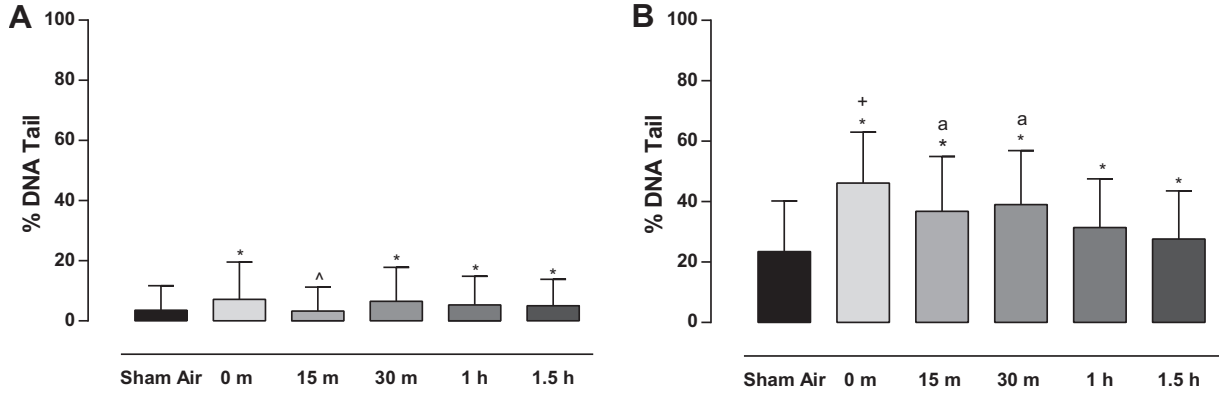

Fig. 5. Influence of sacrifice time (0, 15 min, 30 min, 1 h, and 1.5 h) and DNA repair following 1 h 3R4F cigarette smoke exposure on (A) levels of single strand DNA breaks and (B) oxidative DNA damage. $^{*}p \leqslant 0.01$ when compared to sham air control group. $^{\wedge}p \leqslant 0.05$ when compared to sacrifice time 0 min, 30 min, 1 h and 1.5 h following exposure. $^{+}p \leqslant 0.05$ when compared to sacrifice time 15 min, 30 min, 1 h and 1.5 h following exposure. $^{a}p \leqslant 0.001$ when compared to sacrifice time 1 h and 1.5 h following exposure. Values plotted are Mean ± SD of TI.

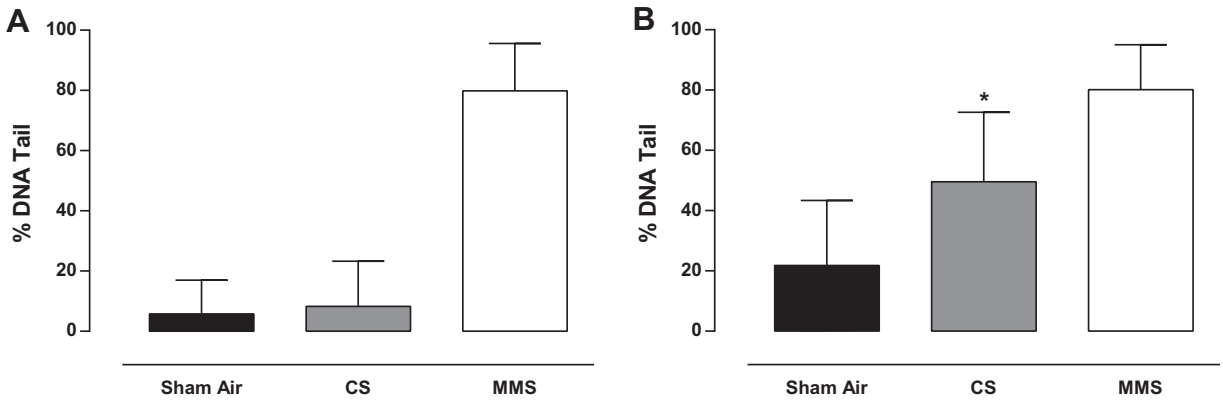

Fig. 6. The level of DNA damage as measure by the (A) Alkaline Comet assay (single strand breaks) and (B) modified Alkaline Comet assay (oxidative DNA damage) in isolated AEC II after 5 days sham air or 3R4F CS exposure. MMS was used as an *in vitro* positive control. Values plotted are Mean ± SD of TI. *$p \leqslant 0.05$ when compared to sham air control group.

used for the Comet assay and the left lung lobe for histopathology, potentially reducing animal numbers by up to 50%, which will be in accordance with the 3R principles (Reduction, Replacement and Refinement).

Selecting the appropriate lung cell isolate was a focus of the experiments detailed within this manuscript. This was to ensure that the AEC II isolation method developed, when used with the Comet assay, would have the potential resolving power to differentiate between sham air and CS. AEC II isolated in all studies had approximately 95% viability and DNA damage could be induced *in vitro* by MMS, demonstrating that cells were viable, responsive to genotoxic agents and not excessively damaged during the isolation process. The published AEC II isolation methods (Witherden and Tetley, 2001; Knaapen et al., 2002) used AEC II following one hour culture. Concerns over DNA repair during the one hour culture stage and the increased levels of basal DNA damage observed as each stage of the method progressed (Fig. 2), led us to use select the 150 μm filtrate that had approximately 63% AEC II purity.

Prior to performing a CS inhalation study, an *in vivo* positive control study was performed to determine if the AEC II isolation method could measure DNA damage induced by a known genotoxic compound. In agreement with others (Hashimoto et al., 2007; Ulutas et al., 2011), MMS administered IP induced DNA damage and the AEC II isolation method developed with the Alkaline Comet assay could quantity the level of DNA damage induced *in vivo*.

The lysis buffer experiments demonstrated that slides could be stored in lysis buffer for up to 14 days without adverse outcomes. Our standard Comet method, in accordance with the OECD 489 guideline (OECD, 2014), will detail 24 h slide storage in lysis buffer. From the data obtained, it can be assumed that slides can be stored in lysis buffer for up to 14 days. Storage periods longer than 14 days are not recommended as agarose will possibly detach from the slides.

The method developed for AEC II isolation was optimised for the Alkaline Comet assay. Higher levels of basal DNA damage were observed in control/sham air animal samples used for the Modified Alkaline Comet assay. However, statistical differences were observed between sham air and CS exposure samples collected from the DNA repair experiment and the 1 week inhalation study, demonstrating that the Modified Alkaline Comet assay is sensitive enough (even with small animal numbers per group) to differentiate between sham air and CS exposure. Further optimisation experiments could be conducted to resolve this.

Female rats were used in this study, however the AEC II isolation method detailed was used to isolate cells from 9 to 10 weeks males and 6 months female rats (data not shown). AEC II isolated from males and older female rats had equivalent viability, levels of basal DNA damage and responses *in vitro* to MMS when compared to 9–11 week old females used in this study. This data confirms that the AEC II isolation method detailed could be used in studies where male and female rats of an older age would be used, a requirement for a 90 day inhalation study.

The Comet method used was comparable to the OECD 489 guideline (OECD, 2014) and data was analysed using one of the statistical method recommended in this guideline (Bright et al., 2011). The Bright et al. method was selected as it is recommended for Comet assay data where abnormally distributed data is obtained due to biological variation, slide-to-slide and treatment variation, plus variations in assay conditions when data is analysed from a number of Comet experiments.

In agreement with others (Tsuda et al., 2000; Ueno et al., 2011), the studies performed demonstrated that DNA damage induced by a single exposure to CS could be quantified in the lung by the Comet assay. The exception was the gradation in the DNA damage experiment, where no significant increase in DNA damage was observed following CS exposure, possibly due to the time required to isolate AEC II. The studies published by Tsuda et al. (2000) and Ueno et al. (2011), both used the Alkaline Comet assay to detect single strand breaks and whole mouse tissue homogenates, which are quick to prepare. In this study, CS induced increases in single strand DNA breaks were only observed in the DNA repair experiment, again this was possibly due to animal variability, a species difference or even DNA repair. Tsuda et al. (2000) have demonstrated that DNA damage could be measured in mouse tissues 15 min after CS exposure and that the level of DNA damage returned to the level of control animals 60 min after exposure, if the animals were not sacrificed immediately. This suggests that DNA repair mechanisms are rapidly activated following CS exposure. In whole mouse lung homogenates, Ueno et al. (2011) have detected DNA repair 20 min following CS exposure (Ueno et al., 2011). The AEC II isolation method detailed takes approximately 43 min, considerably longer than preparing whole lung homogenates, therefore DNA repair could be the reason that a significant increase in DNA single strand breaks was only observed in one experiment. Therefore, the Alkaline Comet assay may not be a suitable method for detecting single stand DNA breaks following single/short term CS exposure in isolated rat AEC II. A future study with increased animal numbers per group will confirm the suitability of the Alkaline Comet assay to measure CS induced single strand breaks.

The Modified Alkaline Comet assay detected CS induced oxidative DNA damage in most experiments, the exception being

the gradation in DNA damage experiment, possibly due to animal variation. The DNA repair experiments also detailed a significant reduction in the level of oxidative DNA damage if the animals were not sacrificed immediately, the lowest levels were observed in animals sacrificed 1 h and 1.5 h post exposure. On review of the data, animals were sacrificed immediately following CS exposure and the time for cell isolation to the placing of slides for the Comet assay in lysis buffer was standardised to 45 ± 3 min.

The 1 week inhalation study was performed to investigate whether DNA damage induced by repeated CS exposure could be measured by the Comet assay. The level of oxidative DNA damage was significantly higher in CS exposed animals when compared to sham air exposed animals. Additional studies are required to determine if the Comet assay is a suitable endpoint to measure DNA damage following 45 or 90 days repeated CS exposure. Further studies would also be needed to assess whether the Comet assay can differentiate between different cigarettes and new potentially reduced risk product categories such e-cigarettes or products that heat rather than combust tobacco.

In conclusion, this study has demonstrated that AEC II are a site of CS induced DNA damage in the rat lung and that CS induced DNA damage could be measured in isolated AEC II by the Comet assay after a single or repeated (5 days) CS exposure. We propose that AEC II analysis by the Comet assay has a potential use in future regulatory rodent 90 day inhalation studies to monitor CS induced DNA damage alongside the characteristic histopathological respiratory tract lesions that develop following long term CS exposure.

Authors contributions

Annette Dalrymple, Patricia Ordoñez, David Thorne, Debbie Dillon and Clive Meredith designed the study. Patricia Ordoñez managed all experimental work at Vivotecnia. Annette Dalrymple drafted the manuscript. Patricia Ordoñez analysed the data and prepared the figures. All authors approved the final manuscript.

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Conflict of interest

The authors report no conflicts of interest and are employees of British American Tobacco or contracted by British American Tobacco.

Transparency Document

The Transparency document associated with this article can be found in the online version.

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