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Evaluation of an air–liquid interface cell culture model for studies on the inflammatory and cytotoxic responses to tobacco smoke aerosols

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ABSTRACT

In vitro toxicological studies for tobacco product assessment have traditionally been undertaken using the particulate phase of tobacco smoke. However, this does not truly reflect exposure conditions that occur in smokers. Thus *in vitro* cell culture systems are required in which cells are exposed to tobacco whole smoke (WS) at the air–liquid interface (ALI). In this study bronchial epithelial cells were cultured on semi-permeable membranes, transitioned to the ALI and the robustness and sensitivity of the cells to tobacco WS and vapour phase (VP) assessed. Although no effect of air exposure was observed on cell viability, IL-6 and IL-8 release was increased. Exposure to WS resulted in a significant dose dependent decrease in cell viability and a significant non-dose dependent increase in inflammatory mediator secretion. The VP was found to contribute approximately 90% of the total cytotoxicity derived from WS. The cell culture system was also able to differentiate between two smoking regimens and was sensitive to passage number with increased inflammatory mediator secretion and lower cell viability observed in cell cultures of low passage number following WS exposure. This simple cell culture system may facilitate studies on the toxicological impact of future tobacco products and nicotine delivery devices.

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1. Introduction

Tobacco smoke is a complex, dynamic, mixture of more than 6000 identified constituents (Rodgman and Perfetti, 2013), of which approximately 150 have been documented as toxicants (Fowles and Dybing, 2003). Long-term exposure to these and other smoke chemicals is thought to drive the development of many smoking-related diseases (US Department of Health and Human Services, 2004) including chronic obstructive pulmonary disease (COPD). COPD is a major public health issue and is the fourth leading cause of death in the United States and Canada and by the year 2020 it is predicted to become the third most frequent cause of death and the fifth most frequent cause of chronic disability (Lopez and Murray, 1998). Although the precise etiology of COPD is unknown, the condition appears to be associated with long-term exposure to toxic aerosols and particles, however, the vast majority of cases are due to tobacco smoking (Sethi and Rochester, 2000). There is therefore a need to gain a better understanding of the mechanisms associated with the development of this disease, particularly as a consequence of tobacco smoke exposure. This knowledge may potentially aid in more effective disease specific treatments and in the development of new, tobacco related, harm reduction technologies.

Traditionally, the toxicological evaluation of tobacco whole smoke (WS) primarily focused on the particulate fraction and to date this fraction continues to be investigated both for regulatory and research purposes. Particulate matter is often collected by passing WS through a Cambridge filter pad, eluting the trapped particulates with an organic solvent and then applying this solution to submerged cell culture systems to assess cellular responses. As this fraction only constitutes 5–10% of the WS generated from burning tobacco (Keith and Tesh, 1965), the remaining fraction is not assessed by this approach. To overcome this problem and thus to capture components from both the vapour and particulate phases, WS can either be bubbled through a biological buffer or cell culture medium. The resulting 'aqueous extract' can then be used in a variety of *in vitro* test systems (Cantrall et al., 1995; Bernhard et al., 2004; Carnevali et al., 2006; Cervellati et al., 2014), in particular those tests in which endothelial cell functions are being assessed. However, many of the non-aqueous soluble chemicals in smoke will not be trapped and as many lung cell culture systems are submerged, the results of these exposure studies may not truly

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reflect the effect of WS exposure on the lung. Therefore to fully assess the biological effects of WS on the lung, an *in vitro* cell culture system is required that can deliver WS to lung cells exposed at the air–liquid interface (ALI) (CORESTA, 2005).

Over the last few years there has been an increase in the development of both *in vitro* ALI cultures (Kreft et al., 2014; Carson et al., 2014; Mathis et al., 2013) and WS generation and delivery systems (Thorne et al., 2013; Thorne and Adamson, 2013; Kaur et al., 2010; Aufderheide, 2008). The combination of these two allow for well-defined aerosols to be delivered to lung cells in culture that more closely represents what happens in the lungs of smokers. One way in which this has been achieved is through the use of the Borgwaldt RM20S smoking machine coupled to an ALI cell culture system using either cell lines or primary cell cultures (Phillips et al., 2005; Maunders et al., 2007; Adamson et al., 2011). However, to ensure that these ALI culture systems are fit for the assessment of conventional and next generation tobacco products, electronic cigarettes and tobacco heating devices, their robustness and sensitivity requires further assessment. The robustness of an assay can be defined as the measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage. Sensitivity is defined as the ability of the model to differentiate between different tobacco aerosols.

In the current study, the stability of a simple cell culture system, utilising a bronchial epithelial cell line exposed at the ALI was assessed. Robustness was measured by addressing the cytotoxic and inflammatory response of the culture to WS and vapour phase (VP) exposure at different cell seeding densities and passage numbers. Sensitivity was assessed by measuring the cell cultures response to different fractions of tobacco smoke and following exposure of WS generated under two smoking regimens.

2. Materials and methods

2.1. Chemicals and reagents

Chemicals and reagents were obtained from the following sources: Dulbecco's Modified Eagle Medium, RPMI-1640 medium, Hanks' Balanced Salt Solution, Penicillin–Streptomycin solution, L-glutamine, phosphate buffered saline (PBS), Transwell® culture inserts (12 mm or 24 mm diameter, 0.4 μM pore size) and trypsin–EDTA from Fisher Scientific (Loughborough, UK); glacial acetic acid and neutral red solution from Sigma–Aldrich™ (Poole, UK); fetal bovine serum (FBS) and UltraCULTURE™ from Lonza (Basel, Switzerland); interleukin-6 and -8 (IL-6 and IL-8) and matrix metalloproteinase-1 (MMP-1) single plex electrochemiluminescence assays from Meso Scale Discovery (Gaithersburg, USA); NCI-H292 human bronchial epithelial cells from the American Type Culture Collection (Middlesex, UK: cell no. CRL-1848); 3R4F reference cigarettes from the University of Kentucky (Kentucky, USA).

2.2. Cell culture

A bronchial epithelial cell line (NCI-H292) was cultured in RPMI-1640 cell culture medium supplemented with 10% FBS, 2 mM L-glutamine, 50 U/mL penicillin and 50 μg/mL streptomycin in an incubator at 37 °C, 5% CO_2. Forty eight hours prior to WS exposure, cells were seeded on the apical side of 12 mm Transwell® culture inserts, in sterile 12 well plates, at a density of 3×10^5 cells/mL in 0.5 mL of supplemented RPMI-1640. For each culture insert, 1 mL of supplemented RPMI-1640 was also placed in the basal compartment of each well. During the robustness assessment, cells were recovered from liquid nitrogen at passage 83, some were expanded until passage 89 (Low-P) and others to passage 99 (High-P). The cells were then seeded at either 2.85 (Low-SD) or 3.15 (High-SD) $\times 10^5$ cells/mL (±5% of the standard seeding density) to give four cell culture groups (1. Low-SD, Low-P; 2. Low-SD, High-P; 3. High-SD, Low-P and 4. High-SD, High-P). All cell cultures were maintained at 37 °C, 5% CO_2 for 24 h until a confluent monolayer was achieved. Twenty four hours before WS exposure the basal and apical cell culture media were replaced with 1 mL and 0.5 mL respectively of UltraCULTURE™ containing 2 mM glutamine, 50 U/mL penicillin and 50 μg/mL streptomycin.

2.3. Study design

The study was designed in three stages and at every stage measurements of cell viability and the concentration of secreted inflammatory and tissue remodelling mediators were measured. Firstly, to assess stability, cultures were maintained at the ALI, without WS exposure, for up to 3 h. The cytotoxic and inflammatory mediator response following a 30 min exposure to WS and VP were also undertaken. Secondly, to determine the robustness of the cell culture system, the effect of cell seeding density and passage number on the cytotoxic and inflammatory response to WS and VP exposure were measured. Thirdly, the sensitivity of the model was assessed by measuring the responses of the cell culture system to WS and VP, and from WS derived from two smoking regimens; International Organization for Standardization (ISO) and Health Canada Intense (HCI). In this third stage the initial seeding density and passage number remained constant at 3×10^5 cells/mL and 89 respectively.

2.4. Cigarettes and smoking

3R4F cigarettes were conditioned for a minimum of 48 h prior to use (60 ± 3% relative humidity, 22 ± 1 °C) and smoked in a test atmosphere of 60 ± 5% relative humidity, 22 ± 2 °C in accordance with ISO 3402:1999. Cigarettes were either smoked according to ISO 3308:2000 (35 mL puff volume, drawn over 2 s, once every minute with ventilation holes unblocked) or to the HCI smoking regimen (55 mL puff volume, drawn over 2 s, twice a minute with ventilation holes blocked). For the first two stages of the study, six puffs were taken from each of five cigarettes using the ISO 3308:2000 smoking regimen to give a total exposure time of 30 min. For the third stage, in which cultures were exposed to WS generated from the two smoking regimens, cells were exposed to WS generated according to ISO 4387:2000 (whereby cigarettes were smoked to the length of the filter +8 mm).

2.5. Tobacco whole smoke, vapour phase and air exposure

The WS exposure system used in this study has been described previously (Adamson et al., 2011; Phillips et al., 2005; Thorne et al., 2009; Maunders et al., 2007). Cells, prepared 24 h prior to exposure, were transitioned to the ALI by removal of the covering apical cell culture medium, transferred into a perspex aerosol exposure chamber (Fig. 1) and then exposed for 30 min to dilutions (1:500–1:2.5, smoke:air, vol:vol) of mainstream WS or VP using a Borgwaldt RM20S smoking machine (Hamburg, Germany; Fig. 2). VP exposure was achieved by the addition of an in-line Cambridge filter pad to remove WS particulates.

During exposure, cells were fed basally with DMEM at a flow rate of 2 mL/min containing 50 U/mL penicillin and 50 μg/mL streptomycin. Throughout WS and VP exposure, cell cultures were maintained at 37 °C. Following exposure, the culture inserts were

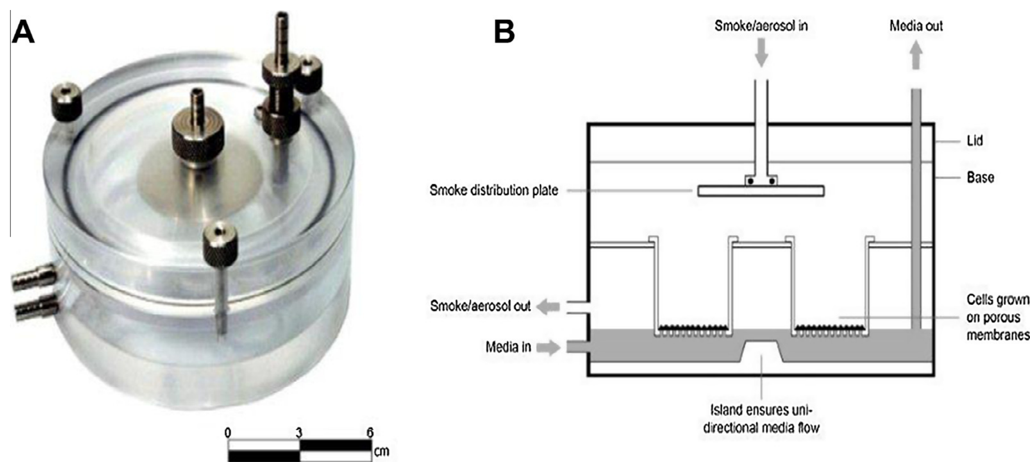


Fig. 1. Aerosol exposure chamber (A) and a schematic cross-section (B). The exposure chamber introduces the test aerosol through a single gas inlet. A symmetrical smoke distribution plate ensures uniform cellular exposure (Thorne et al., 2013).

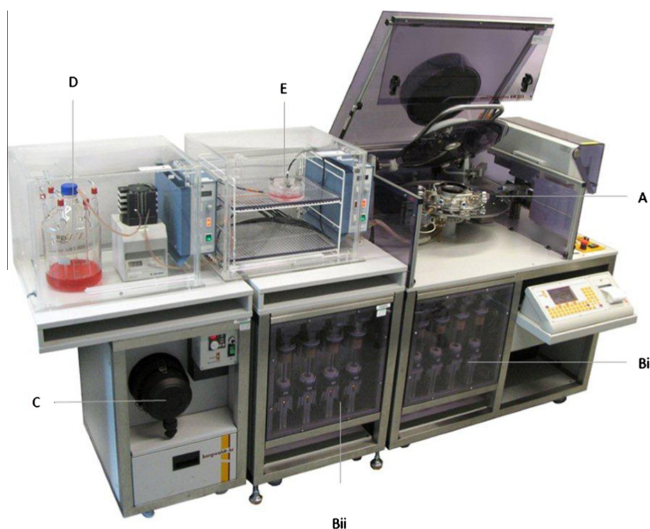


Fig. 2. A Borgwaldt RM20S smoking machine with 8 syringes. (A) Tobacco smoke generator. (Bi and Bii) A syringe based dilution system with 4 syringes that can be combined to give a total of 8. (C) Air flow controller. (D) Cell culture medium maintained at 37 °C feeding exposure chambers with fresh cell culture medium. (E) BAT exposure chamber housed at 37 °C, attached to the smoke diluter and cell culture medium (Adamson et al., 2011).

transferred back to fresh 12-well culture plates containing 1 mL supplemented UltraCULTURE™ pre-warmed at 37 °C. 0.5 mL of supplemented UltraCULTURE™ was added to the apical surface of each culture insert and the cells incubated for a further 24 h at 37 °C, 5% CO_2 in a humidified atmosphere. Control cultures, in which culture medium covering the apical surface of the cells was removed, were either returned to the incubator (ALI) for between 0.5 and 3 h, or exposed to a flow of sterile laboratory air at 200 cm^3/min (AIR) for 30 min. An incubator control (INC) was included in which cells were maintained submerged in culture medium at 37 °C and 5% CO_2 throughout the exposure and 24 h recovery period. Following the recovery period, culture medium from the apical and basal compartments of each culture insert and from all exposure studies were individually combined and stored at −80 °C until the secreted inflammatory and tissue remodelling mediators were measured. Cell viability was measured using the neutral red uptake assay. For comparison purposes cytotoxicity curves were expressed either as a function of WS and VP dilution (smoke:air, vol:vol) or deposited total particulate matter (μg PM/cm^2).

2.6. Tobacco whole smoke deposition

A chemical spectrofluorescence method was used to determine the dose of WS cells received during the ISO and HCI smoking regimen exposures (Adamson et al., 2011).

2.7. Smoke chemistry analysis

Chemical analysis of mainstream WS was carried out as previously described (McAdam et al., 2012) and according to the smoking conditions specified in ISO 4387:2000 with a modification to the smoking parameters as described by Gregg et al., 2004. In brief, cigarettes were smoked to ISO or HCI regimens using a Borgwaldt RM20 D smoking machine and the mainstream WS passed through a Cambridge filter pad to remove the particulate phase. The resultant VP was collected into a 3L Tedlar bag. For particulate smoke chemistry, trapped particulates were analysed in two ways; headspace analysis for the volatile or semi-volatile compounds and solvent extraction for analysis of constituents with a low vapour pressure. VP analysis was undertaken according to the method described by Dong et al. (2000).

2.8. Cell viability assay

The neutral red uptake protocol was based on guidelines set out by the National Institute of Health (National Institute of Health, 2001). Culture medium was removed from exposed cells whilst on culture inserts and washed twice with PBS. 0.5 mL and 1.0 mL of neutral red (0.05 g/L in UltraCULTURE™) was added apically and basally respectively to the culture inserts and incubated for 3 h at 37 °C, 5% CO_2 in a humidified atmosphere to allow active uptake of the dye into viable cell lysosomes. To remove unincorporated dye, the cells were washed twice with PBS. Neutral red was eluted from cells by incubation with 500 μL of destain solution (50% ethanol, 49% distilled water, 1% glacial acetic acid (vol:vol:vol)) and shaken gently for 10 min at ~300 rpm. 100 μL aliquots of the eluates were read on a microplate spectrophotometer at 540 nm using a reference filter of 630 nm. Background measurements from blank culture inserts were subtracted from the neutral red uptake values of both untreated and treated cells. Neutral red uptake levels of treated

cultures were expressed as a percentage of air exposed controls (AIR).

2.9. Inflammatory and tissue remodelling mediator secretion

The concentrations of IL-6, IL-8 and MMP-1 in the cell culture medium were measured as a single plex using MSD, a highly sensitive electrochemiluminescence array method (Meso Scale Discovery) in accordance with the manufacturers' instructions.

2.10. Statistical analysis

Data are reported either as means ± standard deviations (SD) or boxplots ± range with each experiment conducted 3–6 times and with three culture inserts per experiment.

Control culture comparisons were analysed using a one-way analysis of variance (ANOVA) with differences between groups determined using Tukey's adjustment for multiple comparisons.

Neutral red cell viability data for WS and VP studies were modelled using a 4-parameter sigmoid dose response curve with a variable slope and the EC_{50} values determined. Comparisons between groups were undertaken using the F-test. All analyses were conducted using GraphPad Prism version 6.00 for Windows, GraphPad Software, CA, USA, www.graphpad.com.

For secreted inflammatory and tissue remodelling mediators, comparisons between groups were made using the general linear model ANOVA with analysis of treated samples versus controls performed using Tukey's adjustment for multiple comparisons. All analyses were conducted with Minitab® version 16. An unpaired t-test was performed on comparisons between HCI and ISO at a single equivalent dose of 0.19 μg/cm^2.

A 2 sample t-test was undertaken between HCI and ISO smoking on each chemical constituent. A comparison of slopes and Y intercepts from WS derived from the $\log_{10}$ transformed smoke dilution – particulate deposition curves following ISO and HCI smoking was undertaken using a linear regression analysis.

A p value of less than 0.05 was considered significant.

3. Results

3.1. The stability of NCI-H292 cells at the air–liquid interface

No significant reduction in cell viability was observed for cells cultured at the ALI for up to 3 h when compared to the INC controls (Fig. 3). Remaining above 90%, the viability of cultures exposed to AIR for 30 min was similar ($p > 0.05$) to the ALI (0.5 h) control, but significantly ($p < 0.05$) lower than that observed for the INC controls.

No significant differences in the concentrations of secreted inflammatory mediators was observed between the INC and ALI controls (Fig. 4). Exposure to a flow of sterile air for 30 min resulted in a significant ($p < 0.05$) three to four-fold increase in IL-6 (ALI; 27 ± 7 pg/mL, INC; 26 ± 7 pg/mL, AIR; 104 ± 31 pg/mL) and IL-8 (ALI; 100 ± 78 pg/mL, INC; 97 ± 73 pg/mL, AIR; 335 ± 170 pg/mL) secretion but not MMP-1 when compared to both the INC and ALI controls.

3.2. The effects of air, WS and VP exposure on NCI-H292 cell cytotoxicity and inflammatory mediator secretion

The EC_{50} for WS exposure was 1:54 (smoke:air, vol:vol), a significantly greater dilution ($p < 0.005$) than that required following exposure to VP alone (1:46) (Fig. 5). The average difference in cell viability between the two dose response curves was 11%, and thus VP constitutes 89% of the total toxicity of WS.

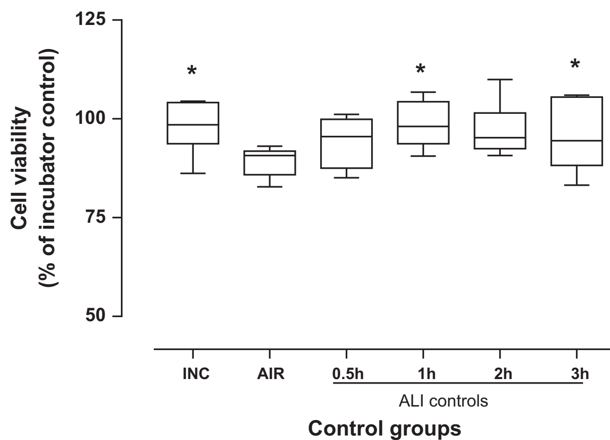


Fig. 3. Viability of NCI-H292 ALI cultures exposed for up to 3 h. Data are expressed as boxplots ± range derived from three replicate cell culture inserts and from six independent experiments. INC: Incubator control, AIR: cultures exposed at the ALI to a flow (200 cm^3/min) of sterile laboratory air for 30 min, ALI: Cell cultures transitioned to the air–liquid interface for between 30 min and 3 h, $^*p < 0.05$ compared to AIR.

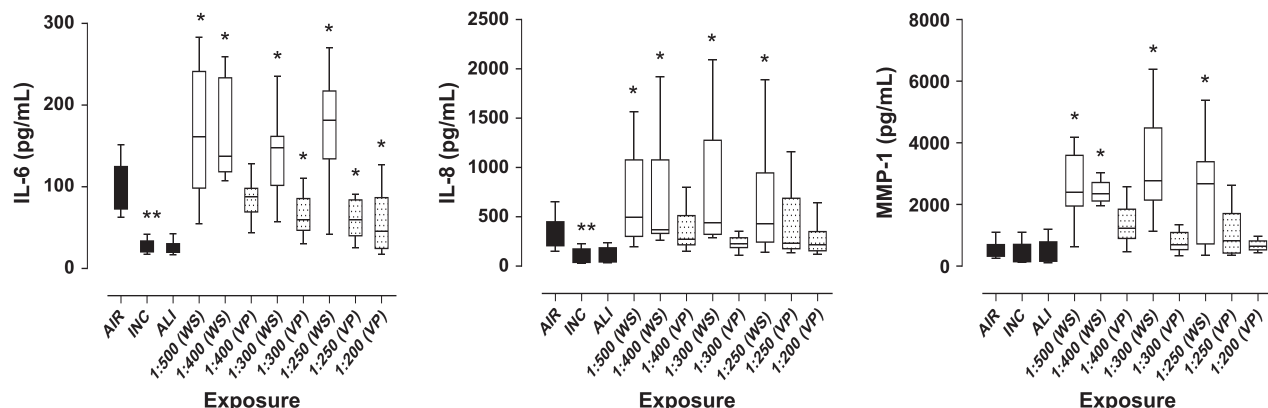


Fig. 4. Inflammatory mediator secretion following WS and VP exposure. Data are expressed as boxplots ± range for five independent runs each performed in triplicate. AIR: Air control, INC: Incubator control, ALI: Air–liquid interface control, WS: Whole smoke, VP: Vapour phase, ■: Control cultures, □: WS exposure, (▒): VP exposure. WS and VP dilutions (smoke:air, vol:vol). $^*p < 0.05$ compared to AIR controls.

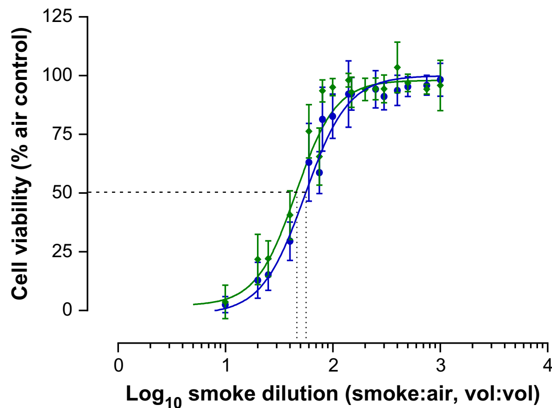


Fig. 5. Cytotoxicity of NCI-H292 cells exposed to 3R4F WS (●) and VP (♦). Data points are mean ± SD from three replicates cell culture inserts in five independent experiments. There was a significant ($p < 0.005$) increase in EC_{50} for WS (1:54, smoke:air, vol:vol) compared to VP following exposure to VP (1:44, smoke:air, vol:vol).

Following exposure to a range of WS dilutions, the lowest of which was equivalent to 95% cell viability, all secreted inflammatory and tissue remodelling mediator concentrations were significantly ($p < 0.05$) increased when compared to the AIR controls (Fig. 4). Exposure to the VP of 3R4F resulted in lower concentrations of all inflammatory and tissue remodelling mediators when compared to equivalent WS dilutions. IL-6 concentrations at the three lowest dilutions of VP were significantly ($p < 0.05$) lower than those seen in the AIR controls.

3.3. The effects of passage number and cell density on cytotoxicity and inflammatory mediator secretion following WS exposure

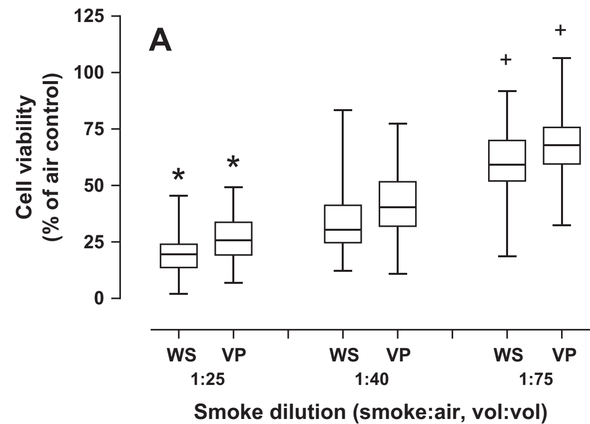
Cell viability of the INC and ALI control cultures, at different cell densities and passage numbers, were not significantly different from each other (data not shown). There was a significant ($p < 0.001$) increase in cell viability with decreasing concentrations of WS and VP (Fig. 6A) when data from all seeding densities and passage numbers were combined. Cell viability was significantly higher in cultures exposed to VP ($p < 0.001$) and lower in low passage numbered cultures (Fig. 6C). No effect of seeding density was observed (Fig. 6B).

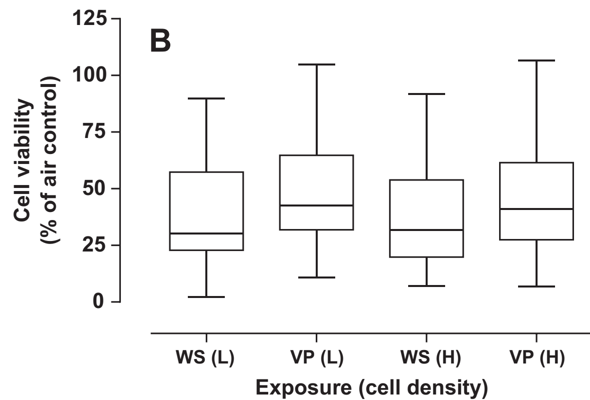
Although no significant effect of seeding density or passage number was observed on control cultures (INC and AIR) for IL-6 secretion, there was a significant effect of passage number on constitutive IL-8 secretion. Cultures at higher seeding densities and those derived from lower passaged numbered cells secreted more IL-8 than equivalent cultures with lower seeding densities and higher passage numbers following WS and VP exposure (Table 1).

3.4. Smoke chemistry

The results of standard smoke chemistry analyses for 3R4F generated under the ISO and HCI smoking regimens are shown in Table 2 and Fig. 7.

HCI smoking resulted in statistically significant ($p < 0.001$) higher toxicant yields per tobacco for all constituents when compared to ISO smoking (Fig. 7). When yields were expressed as a function of TPM, per mg tar or per mg nicotine (data not shown) the particulate based toxicants were generally lower, and the aldehydes higher when generated at HCI, compared to ISO. The WS water and hydrogen cyanide content were also elevated following HCI smoking and remained significantly elevated regardless of the normalisation of the data.





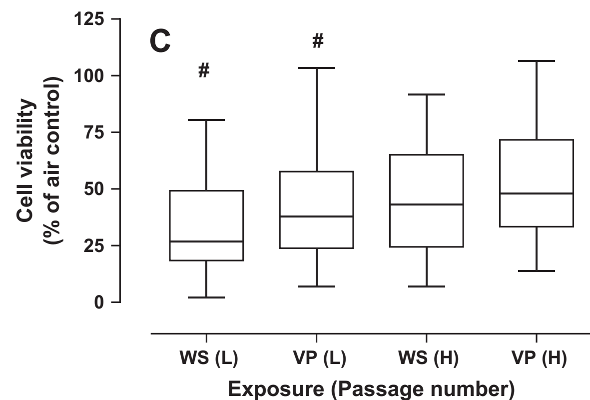


Fig. 6. The effect of WS and VP exposure (A), cell density (B) and passage number (C) on the cell viability. Data represented as boxplots ± range for three replicate cell culture inserts and six independent experiments. WS: Whole smoke, VP: Vapour phase, H: High seeding density or passage number, L: Low seeding density or passage number. There was a significant ($p < 0.001$) effect of smoke dilution and passage number ($p < 0.001$) on cell viability. $^{*}p < 0.05$ compared to equivalent exposures at 1:40 and 1:75, $^{+}p < 0.05$ compared to equivalent exposure at 1:40, $^{\#}p < 0.05$ compared to equivalent exposure.

3.5. Production of ISO and HCI particulate deposition graphs

No significant effect of smoking regimen was observed either on the slope or *Y* intercept (Fig. 8).

3.6. The effect of smoking regimen on cell viability and inflammatory mediator release

The EC_{50} values for WS derived from the ISO and HCI regimens were 2.3 and 4.0 $\mu g/cm^2$ respectively with exposure to WS derived from the ISO regimen significantly ($p < 0.001$) more toxic than that

Table 1
The effect of passage number (P) and seeding density (SD) on interleukin-6 (IL-6) and 8 (IL-8) secretion from bronchial cell cultures exposed to WS and VP at a dose equivalent to 95% cell viability.

Inflammatory mediator	Exposure group	Seeding density and passage number			
		High-SD, High-P	High-SD, Low-P	Low-SD, High-P	Low-SD, Low-P
		pg/mL			
IL-6	AIR	78.7±31.2	73.0±26.0	67.0±30.8	65.4±21.0
	INC	12.9±4.6	16.3±8.1	18.5±5.3	20.2±8.0
	WS	87.2±46.8#	109±38.9#+	93.1±36.0#+	89.5±27.3+
	VP	35.3±14.5#	36.2±16.3#	35.0±10.4#	35.6±11.2#
IL-8	AIR	169.7±141.3	280.7±127.3*	113.8±74.2	215.8±117.1*
	INC	29.7±12.2	64.4±41.8*	38.0±16.2	71.3±43.6*
	WS	267.1±127.8#	447±217*,+	222±116#	393±168*,+
	VP	128.6±77.4	208.0±120.4*	109.2±60.6	200.5±86.5*

Data represented as means and standard deviation for three replicate cell cultures and six independent experiments. AIR: Air control, INC: Incubator control, ALI: Air-liquid interface control, WS: Whole smoke, VP: Vapour phase, High-SD: high seeding density, Low-SD: Low seeding density, High-P: High passage number, Low-P: Low passage number. There was a significant effect of exposure ($p < 0.001$) on both IL-6 and IL-8 secretion, and a significant ($p < 0.001$) effect of cell density and passage number on IL-8 secretion.
* $p < 0.05$ compared to equivalent cell density.
+ $p < 0.05$ compared to VP and AIR.
# $p < 0.05$ compaired to AIR.

Table 2
Smoke yields for 3R4F generated under ISO and HCI machine smoking conditions.

Smoke constituent	Unit	Toxicant yield/tobacco			
		ISO	±SD	HCI	±SD
NFDPM (Tar)	mg/cig	8.00	0.48	27.69**	1.57
TPM	mg/cig	9.46	0.63	49.13**	3.75
Nicotine	mg/cig	0.69	0.04	1.98**	0.09
Carbon monoxide	mg/cig	10.83	0.79	31.92**	1.41
Water	mg/cig	0.77	0.19	19.46**	2.49

Results are displayed as the mean ± SD of 25 separate measurements. Data was analysed using 2 sample t-test. NFDPM; Nicotine free dry particulate matter, TPM; total particulate matter.
** $p < 0.001$ compared to the ISO smoking regimen.

derived under the HCI regimen (Fig. 9). Cell cultures were exposed to a range of subtoxic WS concentrations (ISO: 0.15–0.19 μg/cm^2, HCI: 0.19–0.41 μg/cm^2) and the levels of secreted inflammatory and tissue remodelling mediators were measured and compared to the AIR control. Regardless of regimen there was a significant increase in all inflammatory and tissue remodelling mediator secretion at all doses, except for IL-6 at 0.15 μg/cm^2, when compared to the AIR control. To compare the secretion of inflammatory mediators at ISO and HCI, comparisons were made at an equivalent particulate dose of 0.19 μg/cm^2. WS generated under the HCI regimen induced a significantly ($p < 0.0001$) greater IL-8 response (HCI; 1139 ± 632 pg/mL, ISO; 661 ± 95 pg/mL) than WS generated under the ISO smoking regimen (Fig. 10).

4. Discussion

The conducting airway is made up of many cell types that constitute the first line of defence against the inhalation of external environmental pollutants. It acts both as a physical and a

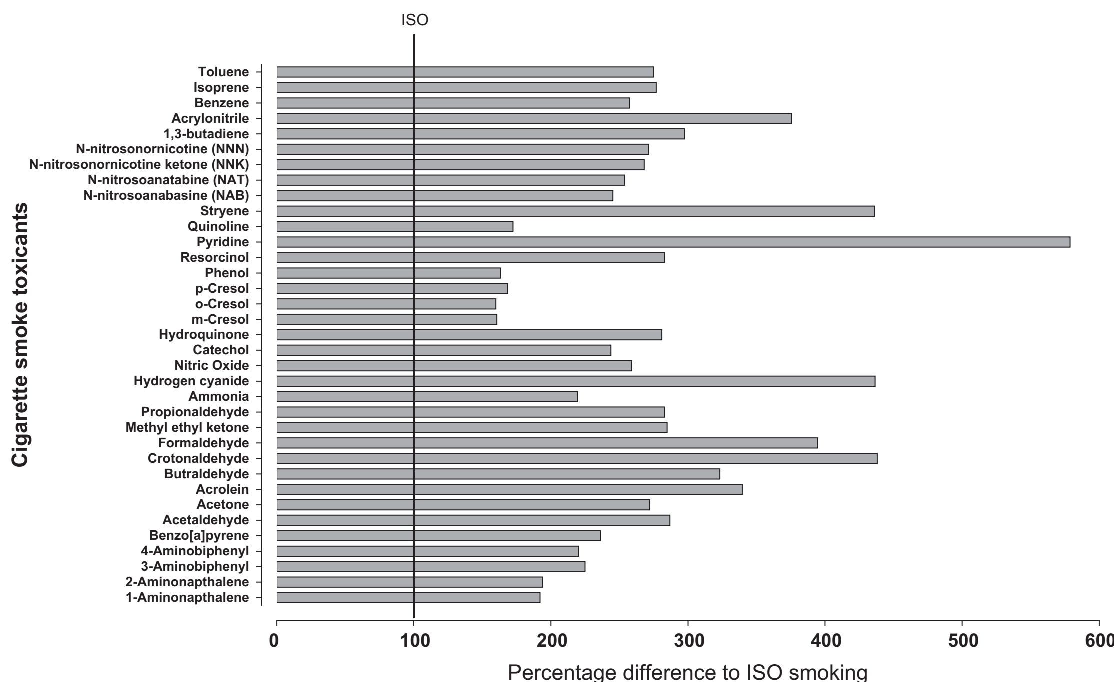


Fig. 7. Percentage difference in toxicant yields following generation using the HCI smoking regimen. Results are displayed as percentage difference to ISO from 25 separate measurements. Data were analysed using 2 sample t-test. All toxicants derived from the HCI smoking regimen were significantly ($p < 0.001$) increased compared to the ISO smoking regimen.

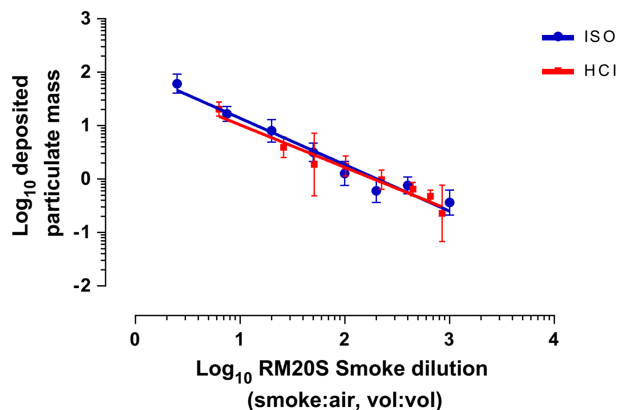


Fig. 8. Log_{10} transformation of deposited particulate mass and WS dilution (smoke:air, vol:vol) for ISO and HCI generated 3R4F WS.

biochemical barrier and although it is thought to play a role in the development of tobacco smoke-induced COPD (Patel et al., 2003; Thorley and Tetley, 2007; Chen et al., 2010) the exact mechanism(s) that lead to this condition are not fully understood. An approach to investigate the early cytotoxic and inflammatory responses of the bronchial epithelium to tobacco smoke exposure is the use of bronchial epithelial cells transitioned to and exposed at the ALI. This approach has previously shown promise in a number of cell culture systems (Beisswenger et al., 2004; Fukano et al., 2004; Phillips et al., 2005; Maunders et al., 2007; Aufderheide, 2008; Mathis et al., 2013). However, for these cell cultures to be used in the assessment of conventional tobacco products and next generation tobacco and nicotine products, robustness and sensitivity studies are required to ensure that such cell culture systems are fit for purpose.

There have been many studies that have investigated the effects of tobacco smoke exposure on lung epithelial cells. These have used both cell lines (Weber et al., 2013) and primary cell cultures (Mathis et al., 2013) in conjunction with various smoking machines to deliver aerosols from combustible and non-combustible products. In the present study we used the NCI-H292 bronchial cell culture system (Phillips et al., 2005) because of its ease of cell culture, its high throughput relative to primary cell cultures and because it allows for the standardisation of the complete cell culture and exposure system. The advantage of this system is that it is flexible with the potential to incorporate different ALI cultures, both cell lines and primary cultures, when assessing a range of tobacco products and nicotine delivery devices.

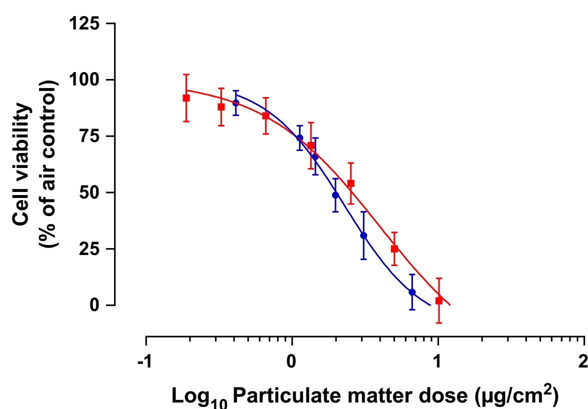


Fig. 9. Cytotoxicity sigmoidal dose response curves for NCI-H292 cells exposed to 3R4F tobacco smoke generated at the ISO and HCI smoking regimens. There was a significant effect of smoking regimen on the EC_{50} ((●): ISO EC_{50} was 2.3 μg/cm^2 and the: (■): HCI was 4.0; μg/cm^2 $p < 0.001$).

At the ALI, the bronchial cells were found to be stable for up to 3 h with cellular viability similar to that of the INC controls. Exposure of cells to a relatively low flow rate of sterile laboratory air for 30 min had no significant effect on viability when compared to the ALI control (0.5 h). There was a small, but significant, reduction in viability when compared to the INC control, even though viability levels remained above 90%. We speculate that at the flow rates used in the current study, sufficient humidity may be achieved above the cells by the evaporation of culture medium. This may help preserve a high degree of cell viability even during a 30 min exposure to a flow of continuous, 60% humid, sterile laboratory air. Although cell viability was relatively unaffected by air exposure, IL-6 and IL-8, mediators identified as important in the pathogenesis of a number of lung diseases (Chen et al., 2010; Patel et al., 2003; Thorley and Tetley, 2007), were both significantly increased compared to the INC and ALI cultures. These effects on cell viability and inflammatory mediator release have been observed previously (Knebel et al., 1998; Beisswenger et al., 2004; Steinritz et al., 2013) and may limit the sensitivity and robustness of the assay. Thus careful consideration of the cell lines and experimental conditions (exposure time, dose, sampling time) used in the study is required to ensure meaningful data is generated.

The *in vitro* cytotoxicity test used in this study utilises neutral red which has previously been reported as the most sensitive assay for assessing the cytotoxic potential of tobacco smoke (Putnam et al., 2002). Using this assay we demonstrated that 89% of the observed cytotoxicity of WS is derived from the VP and confirms previous results utilising WS in acute exposure models (Bombick et al., 1997; Saladino et al., 1985; Fukano et al., 2004;). WS exposure also induced an increase in the secretion of all three inflammatory mediators at all smoke doses which is in line with previous observations made with this and other cell culture systems (Phillips et al., 2005; Beisswenger et al., 2004). Although the inflammatory and tissue remodelling response following VP exposure was lower when compared to WS exposure, IL-6 concentrations were significantly lower than that observed for the AIR control. This would indicate that the constituents of tobacco smoke can both induce and potentially inhibit the secretion of some inflammatory mediators following exposure. Thus to fully understand the mechanism(s) that drive disease a knowledge of the response of each constituent in tobacco smoke alone and in combination will be required.

The robustness of the model was assessed by determining the cytotoxic and inflammatory response to WS and VP exposures following changes to the seeding density and cell passage number. Passage number had a significant effect on both cell viability and inflammation, regardless of which fraction of smoke was used. In AIR controls, IL-8 secretion was significantly increased in cultures derived from low passage numbered cells, an observation similar to that seen following WS and VP exposure. This corroborates studies that have addressed the effect of passage number on protein expression in adenocarcinoma cells (Esquenet et al., 1997) and on cell morphology in Caco-2 cells (Briske-Anderson et al., 1997). Thus in any future studies experimental parameters, such as passage number will also need to be considered to ensure data variability is minimised prior to product assessment.

Whole smoke generated under the HCI regimen was found to be less cytotoxic than equivalent doses expressed as μg PM/cm^2 of smoke derived from the ISO smoking regimen. In this model, the cytotoxic effect of WS was primarily driven by VP components and on HCI smoking there was a general reduction in the majority of the particulate and VP toxicants when expressed per mg of TPM, tar or nicotine (data not shown), an observation in agreement with published studies (Roemer et al., 2012). All ISO and HCI exposures resulted in a significant increase in mediator release compared to air controls and on comparison at a common dose of 0.19 μg/cm^2,

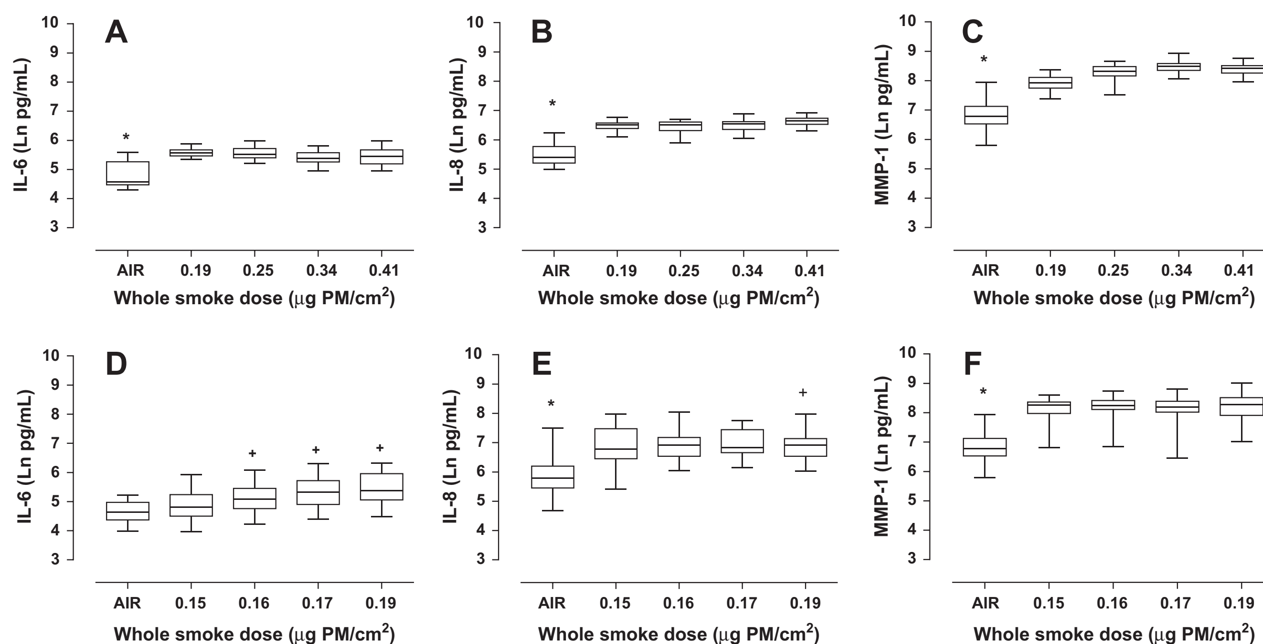


Fig. 10. The effect of WS exposure on NCI-H292 IL-6 (A and D), IL-8 (B and E) and MMP-1 (C and F) secretion following ISO (A, B and C) and HCI (D, E and F) smoking. $^{*}p < 0.001$ compared to all smoke doses, $^{+}p < 0.05$ compared to the equivalent dose (0.19 μg PM/cm^2) generated under ISO smoking.

there was a trend towards increased IL-8 secretion following HCI exposure. The reason for this is unclear. However, a number of volatile aldehydes (crotonaldehyde and formaldehyde) are increased relative to ISO smoking and as these compounds are known to play a role in pulmonary inflammation (van der Toorn et al., 2013), increased mediator secretion may be driven in part by these constituents of the smoke. Further studies are required to fully determine the effect of these aldehydes in the response of lung cells to WS exposure.

In this study a simple ALI bronchial cell culture system has been assessed following a 24 h recovery period as a potential tool for future tobacco product testing. To fully explore the potential of this cell culture system will also require potential changes in exposure time, WS and VP dose and additional assessments at earlier (<24 h) and possibly later time points following exposure. The cell culture system was able to differentiate between WS and VP with respect to cytotoxicity and inflammatory response. In addition, it was also able to differentiate between smoking regimens with regard to cytotoxicity. Due to the inhibitory action of VP on IL-6 release and the increased inflammatory response of smoke generated under the HCI smoking regime, further studies are needed to understand the role of individual smoke toxicant and toxicant mixtures on this process and the subsequent potential for inducing disease. Finally, although this cell culture exposure system enables rapid screening of aerosols from different combustible tobacco products generated under different exposure regimens, to fully understand the mechanisms that lead to tobacco smoke induced injury and disease, further studies will require a combined approach using more traditional exposure matrices such as cigarette smoke extracts and TPM. We have demonstrated the current cell culture exposure system is flexible, sensitive, robust and future studies with conventional tobacco products and next generation tobacco and nicotine products and more physiologically relevant ALI cell culture models will be undertaken.

Conflict of Interest

The authors report no declarations of interest and are employees of British American Tobacco.

Transparency Document

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

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References

Adamson, J., Azzopardi, D., Errington, G., Dickens, C., McAughey, J., Gaca, M.D., 2011. Assessment of an *in vitro* whole tobacco smoke exposure system: the Borgwaldt RM20S 8-syringe smoking machine. Chem. Cent. J. 5, 50.

Aufderheide, M., 2008. An efficient approach to study the toxicological effects of complex mixtures. Exp. Toxicol. Pathol. 60 (2–3), 163–180.

Beisswenger, C., Platz, J., Seifart, C., Vogelmeier, C., Bals, R., 2004. Exposure of differentiated airway epithelial cells to volatile smoke *in vitro*. Respiration 71, 402–409.

Bernhard, D., Huck, C.W., Jakschitz, T., Pfister, G., Henderson, B., Bonn, G.K., Wick, G., 2004. Development and evaluation of an *in vitro* model for the analysis of tobacco smoke effects on cultured cells and tissues. J. Pharmacol. Toxicol. Methods 50, 45–51.

Bombick, D.W., Bombick, B.R., Ayres, P.H., Putnam, K., Avalos, J., Borgerding, M.F., Doolittle, D.J., 1997. Evaluation of the genotoxic and cytotoxic potential of mainstream whole smoke and smoke condensate from a tobacco containing a novel carbon filter. Fundam. Appl. Toxicol. 39, 11–17.

Briske-Anderson, M.J., Finley, J.W., Newman, S.M., 1997. Influence of culture time and passage number on morphological and physiological development of Caco-2 cells. Proc. Soc. Exp. Biol. Med. 214 (3), 248–257.

Cantrall, D.E., Sisson, J.H., Veys, T., Rennard, S.I., Spurzem, J.R., 1995. Effects of tobacco smoke extract on bovine bronchial epithelial cell attachment and migration. Am. J. Physiol. 268, L723–L728.

Carnevali, S., Luppi, F., D'Arca, D., Caporali, A., Ruggieri, M.P., Vettori, M.V., Caglieri, A., Astancolle, S., Panico, F., Davalli, P., Mutti, A., Fabbri, L.M., Corti, A., 2006. Clusterin decreases oxidative stress in lung fibroblasts exposed to cigarette smoke. Am. J. Respir. Crit. Care Med. 174 (4), 393–399.

Carson, J.L., Brighton, L.E., Jaspers, I., 2014. Phenotypic modification of human airway epithelial cells in air–liquid interface culture induced by exposure to the tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK). Ultrastruct. Pathol. 2, 1–6.

Cervellati, F., Muresan, X.M., Sticozzi, C., Gambari, R., Montagner, G., Forman, H.J., Torricelli, C., Maioli, E., Valacchi, G., 2014. Comparative effects between electronic and cigarette smoke in human keratinocytes and epithelial lung cells. Toxicol. In Vitro 28 (5), 999–1005.

Chen, H., Wang, D., Wang, X., 2010. Role of airway epithelium-origin chemokines and their receptors in COPD. J. Epithelial. Biol. Pharm. 3, 26–33.

CORESTA, 2005. *In vitro* exposure of cells at the air liquid interface – Interlaboratory Study of the *In Vitro* Toxicity of Particulate Matter from Four Tobaccos. Reports of the Task Force *In Vitro* Toxicity Testing of Tobacco Smoke.

Dong, J.Z., Glass, J.N., Moldoveanu, S.C., 2000. A simple GC–MS technique for the analysis of VP mainstream tobacco smoke. J. Microcol. Sep. 12 (3), 142–152.

Esquenet, M., Swinnen, J.V., Heyns, W., Verhoeven, G., 1997. LNCaP prostatic adenocarcinoma cells derived from high and low passage numbers display divergent responses not only to androgens but also to retinoids. J. Steroid Biochem. Mol. Biol. 62, 391–399.

Fowles, J., Dybing, E., 2003. Application of toxicological risk assessment principles to the chemical constituents of tobacco smoke. Tob. Control 12, 424–430.

Fukano, Y., Ogura, M., Eguchi, K., Shibagaki, M., Suzuki, M., 2004. Modified procedure of a direct *in vitro* exposure system for mammalian cells to whole tobacco smoke. Exp. Toxicol. Pathol. 55, 317–323.

Gregg, E., Hill, C., Hollywood, M., Kearney, M., McAdam, K., McLaughlin, D., Purkis, S., Williams, M., 2004. The UK smoke constituents testing study. Summary of results and comparison with other studies. Beit Tabakforsch Instl. 21, 117–118.

ISO 3308, 2000. Tobacco and Tobacco Products – Routine analytical tobacco-smoking machine. International Organisation for Standardisation, Geneva.

ISO 3402, 1999. Tobacco and tobacco products – atmosphere for conditioning and testing. International Organisation for Standardisation, Geneva, Switzerland.

ISO 4387, 2000. Determination of total and nicotine-free dry particulate matter using a routine analytical smoking machine. International Organisation for Standardisation, Geneva, Switzerland.

Kaur, N., Lacasse, M., Roy, J.P., Cabral, J.L., Adamson, J., Errington, G., Waldron, K.C., Gaça, M., Morin, A., 2010. Evaluation of precision and accuracy of the Borgwaldt RM20S(®) smoking machine designed for in vitro exposure. Inhal. Toxicol. 22 (14).

Keith, C.H., Tesh, P.G., 1965. Measurement of the total smoke issuing from a burning tobacco. Tob. Sci. 9, 61–64.

Knebel, J.W., Ritter, D., Aufderheide, M., 1998. Development of an in vitro system for studying effects of native and photochemically transformed vaporous compounds using an air/liquid culture technique. Toxicol. Lett. 96–97, 1–11.

Kreft, M.E., Jerman, U.D., Lasič, E., Lanišnik Rižner, T., Hevir-Kene, N., Peternel, L., Kristan, K., 2014. The characterization of the human nasal epithelial cell line RPMI 2650 under different culture conditions and their optimization for an appropriate in vitro nasal model. Pharm. Res. 22.

Lopez, A.D., Murray, A.C., 1998. The global burden of disease 1990–2020. Nat. Med. 4, 1241–1243.

Mathis, C., Poussin, C., Weisensee, D., Gebel, S., Hengstermann, A., Sewer, A., Belcastro, V., Xiang, Y., Ansari, S., Wagner, S., Hoeng, J., Peitsch, MC., 2013. Human bronchial epithelial cells exposed in vitro to tobacco smoke at the air–liquid interface resemble bronchial epithelium from human smokers. Am. J. Physiol. Lung Cell Mol. Physiol. 304 (7), L489–L503.

Maunders, H., Patwardhan, S., Phillips, J., Clack, A., Richter, A., 2007. Human bronchial epithelial cell transcriptome: gene expression changes following acute exposure to whole tobacco smoke *in vitro*. Am. J. Physiol. 292, L1248–L1256.

McAdam, K.G., Gregg, E.O., Bevan, M., Dittrich, D.J., Hemsley, S., Liu, C., Proctor, C.J., 2012. Design and chemical evaluation of reduced machine-yield tobaccos. Regul. Toxicol. Pharmacol. 62 (1), 138–150.

NIH, 2001. Guidance document on using in vitro data to estimate in vivo starting doses for acute toxicity. Publication 01-4500. National Institute of Environmental Health Sciences, National Institutes of Health, US Department of Health and Human Services, Public Health Service, Washington, D.C.

Patel, I.S., Roberts, N.J., Lloyd-Owen, S.J., Sapsford, R.J., Wedzicha, J.A., 2003. Airway epithelial inflammatory responses and clinical parameters in COPD. Eur. Respir. J. 22, 94–99.

Phillips, J., Kluss, B., Richter, A., Massey, E., 2005. Exposure of bronchial epithelial cells to whole tobacco smoke: assessment of cellular responses. Altern. Lab. Anim. 33, 239–248.

Putnam, K.P., Bombick, D.W., Doolittle, D.J., 2002. Evaluation of eight in vitro assays for assessing the cytotoxicity of tobacco smoke condensate. Toxicol. In Vitro 16, 599–607.

Rodgman, A., Perfetti, T.A., 2013. The Chemical Components of Tobacco and Tobacco Smoke, second ed. CRC Press, ISBN 978-1-4665-1548-2.

Roemer, E., Schramke, H., Weiler, H., Buettner, A., Kausche, S., Weber, S., Berges, A., Stueber, M., Muench, M., Trelles-Sticken, E., Pype, J., Kohlgrueber, K., Voelkel, H., Wittke, S., 2012. Mainstream smoke chemistry and in vitro and in vivo toxicity of the reference tobaccos 3R4F and 2R4F. Beitr Tabakforsch Int. 25, 316–335.

Saladino, A.J., Willey, J.C., Lechner, J.F., Grafstrom, R.C., LaVeck, M., Harris, C.C., 1985. Effects of formaldehyde, acetaldehyde, benzoyl peroxide, and hydrogen peroxide on cultured normal human bronchial epithelial cells. Cancer Res. 45, 2522–2526.

Sethi, J.M., Rochester, C.L., 2000. Smoking and chronic obstructive pulmonary disease. Clin. Chest Med. 21, 67–86.

Steinritz, D., Mohle, N., Pohl, C., Papritz, M., Stenger, B., Schmidt, A., Kirkpatrick, C., Thiermann, H., Vogel, R., Hoffmann, S., Aufderheide, M., 2013. Use of the Cultex Radial Flow System as an in vitro exposure method to assess acute pulmonary toxicity of fine dusts and nanoparticles with special focus on the intra- and inter-laboratory reproducibility. Chem. Biol. Interact. 206, 479–490.

Thorley, A.J., Tetley, T.D., 2007. Pulmonary epithelium, tobacco smoke, and chronic obstructive pulmonary disease. Int. J. Chron. Obstruct. Pulmon. Dis., 409–428

Thorne, D., Adamson, J., 2013. A review of in vitro tobacco smoke exposure systems. Exp. Toxicol. Pathol. 65 (7–8), 1183–1193.

Thorne, D., Wilson, J., Kumaravel, T.S., Massey, E.D., McEwan, M., 2009. Measurement of oxidative DNA damage induced by mainstream tobacco smoke in cultured NCI-H292 human pulmonary carcinoma cells. Mutat. Res. 673 (1), 3–8.

Thorne, D., Kilford, J., Payne, R., Adamson, J., Scott, K., Dalrymple, A., Meredith, C., Dillon, D., 2013. Characterisation of a Vitrocell® VC 10 in vitro smoke exposure system using dose tools and biological analysis. Chem. Cent. J. 7 (1), 146.

The health consequences of smoking: A report of the Surgeon General. Washington, DC: Office on Smoking and Health. US department of health and human services, 2004.

van der Toorn, M., Slebos, D.J., de Bruin, H.G., Gras, R., Rezayat, D., Jorge, L., Sandra, K., van Oosterhout, A.J., 2013. Critical role of aldehydes in tobacco smoke-induced acute airway inflammation. Respir. Res. 14, 45.

Weber, S., Hebestreit, M., Wilms, T., Conroy, L.L., Rodrigo, G., 2013. Comet assay and air–liquid interface exposure system: a new combination to evaluate genotoxic effects of tobacco whole smoke in human lung cell lines. Toxicol. In Vitro. 27 (6), 1987–1991.