Assessment of Acute *In Vitro* Human Cellular Responses to Smoke Extracts from a Reduced Toxicant Prototype Cigarette

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Abstract

Assessment of novel tobacco and nicotine products for their harm reduction potential compared with cigarettes is of significance for regulators and public health. The National Research Council's report, "Toxicity Testing in the 21st Century," outlines recent advances in molecular biology and the utility of human *in vitro* systems in toxicity testing. Such approaches could enable efficient, ethical, and economical risk assessment of chemicals and chemical mixtures. We investigated the potential of a simple *in vitro* lung model to discriminate between a commercially available cigarette (CC) and a reduced toxicant prototype (RTP) cigarette, which yielded significantly reduced toxicant emissions compared with the CC. The investigation compared cytotoxicity, oxidative stress-related and proinflammatory endpoints, associated with smoking-related disease development. Furthermore, we assessed the discriminatory potential of the model upon exposure to a selection of test matrices, which captured different fractions of the cigarette smoke. RTP whole smoke (WS) was significantly less cytotoxic than CC; however, there was no significant difference with respect to cytokine expression, namely interleukin (IL)-6, IL-8, and matrix-metalloproteinase-1 secretion. The RTP aqueous extract significantly reduced intracellular oxidant production and antioxidant depletion compared with CC. Dose-related responses were observed with some RTP and CC particulate matter (PM) exposures, but they were shown to not be significantly different from each other except for antioxidant depletion. RTP PM induced significantly less depletion of GSH compared with PM from the CC cigarette. The ability of this model to differentiate between CC and RTP WS cytotoxicity and oxidative stress suggests that it could be a useful tool to support regulatory submissions for novel tobacco and nicotine delivery products.

Keywords: cigarette smoke, cigarette smoke aqueous extract, cytotoxicity, inflammation, *in vitro*, oxidative stress, particulate matter

Introduction

CHRONIC EXPOSURE TO CIGARETTE SMOKE has the potential to cause the development of diseases such as chronic obstructive pulmonary disease (COPD) and cancer.¹ Cigarette smoking induces oxidative stress and proinflammatory responses in the lungs, which consequently drive the development of smoking-related diseases.^{2–5} Subsequent attraction of inflammatory cells, such as neutrophils, macrophages, and lymphocytes contributes to lung tissue injury and tissue remodeling, through the release of reactive oxygen species

(ROS), cytokines, and proteases, which in turn, can also drive the development of COPD.^{2–5}

Cigarette smoke contains oxidative species and other toxicants, which can induce intracellular ROS production in lung epithelial cells, leading to depletion of antioxidants and oxidative stress. Furthermore, oxidative stress causes DNA damage and activation of signaling cascades that stimulate cell proliferation and transformation; key events leading to the initiation of lung cancer.^{2,4,5}

The US Food and Drug Administration has identified a list of 93 toxicants present in tobacco smoke that are considered

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to be harmful or potentially harmful.^{6,7} Previously, British American Tobacco developed a reduced toxicant prototype (RTP) cigarette, which incorporated several toxicant reduction technologies and subsequently yielded lower emission levels of a number of these toxic constituents, compared with a commercially available cigarette.⁸ Inclusion of a tobacco substitute sheet released glycerol into the smoke upon burning, which diluted the tar and reduced the overall toxicant level.⁹ Treated tobacco reduced the yields of phenolics, aromatic amines, hydrogen cyanide, and other nitrogenous toxicants within smoke,¹⁰ and the inclusion of amine-functionalized resin within the filter reduced hydrogen cyanide, volatile acids, and carbonyl compounds.¹¹

Further details of the technologies used to reduce cigarette smoke toxicant yields are described in Supplementary Table S1 (Supplementary Data are available online at www.liebertpub.com/aivt).

Toxicological assessments that employed both computational methods and regulatory approved *in vitro* toxicology assays, (e.g., Ames and *in vitro* micronucleus assays) to compare the mutagenicity and genotoxicity of the RTP cigarette and commercial control (CC) cigarettes have been previously reported.^{12,13} In the present study, we built upon this work by evaluating smoke from an RTP cigarette using a simple *in vitro* lung model. The model enabled the investigation of biological endpoints relevant to smoking-related disease processes involved in lung cancer and COPD namely cytotoxicity, inflammation, and induction of oxidative stress. Different methods of smoke exposure were used depending on the endpoint, to facilitate a biologically relevant assessment of the effects of cigarette smoke.

Our aim was to determine whether this simple *in vitro* lung model was sufficiently sensitive to differentiate between cigarette products that generate smoke with different toxicant profiles, using a selection of endpoints relevant to smoking-related disease development. Data from such *in vitro* studies could be used as part of a weight-of-evidence approach when assessing the disease risk reduction potential of future novel tobacco and nicotine products.

Materials and Methods

Chemicals and reagents

All chemicals and reagents were obtained from Gibco[®] and ThermoFisher Scientific (Loughborough, United Kingdom) unless otherwise stated.

Test products

The RTP cigarette had a circumference of 21 mm, a tobacco rod length of 46 mm, and a 37 mm triple cellulose acetate filter. The rod comprised a blend of 50% washed, extracted, and enzyme-treated tobacco, 15% tobacco substitute sheet, and 35% conventional tobaccos, such as Burley and Virginia. The machine-smoked International Organization for Standardization (ISO) tar delivery¹⁴ of the RTP cigarette was 7 mg. The CC cigarette, (Lucky Strike Silver), had a circumference of 25 mm, a tobacco rod length of 56 mm, and a 27 mm mono cellulose acetate filter, comprising conventional cigarette components. The machine-smoked ISO tar delivery¹⁴ of CC was also 7 mg. Full details of the design features and smoke chemistry of each cigarette are provided in Supplementary Tables S2 and S3.

Generation of cigarette smoke

Traditionally, *in vitro* studies have primarily employed particulate matter (PM) exposures. In this study, we utilized whole smoke (WS) and cigarette smoke aqueous extract (AqE) exposures in addition to PM, to expose cells to both the vapor and particulate phases of the cigarette smoke, respectively (Table 1). For all exposures, cigarettes were conditioned according to ISO 3402¹⁵ and smoked under ISO standard puffing conditions (ISO 3308; 35 mL puff volume drawn over 2 seconds, once every minute).¹⁶

Cell culture

Human bronchial epithelial cells (NCI-H292; ATCC, Manassas, VA) were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (BioWhittaker[®]; Lonza, Vervier, Belgium), 2 mM L-glutamine, 50 U/mL penicillin, and 50 µg/mL streptomycin. Before their use in the study, a vial of cells from each cell bank was sent externally for authentication (Human STR Testing Service; LGC Standards, Middlesex, United Kingdom). Following on, from the receipt of the report confirming their authentication, the cells were used in this study.

Cells were maintained in an incubator at 37°C in a humidified 5% CO₂ atmosphere. All experiments using NCI-H292 cells were performed on cells of the same passage number (P89).

Assessment of responses following exposure to cigarette smoke

A summary of the assays employed in this study can be found in Table 2.

ROS measurement (DCF assay). NCI-H292 cells were seeded at a density of 1×10^4 cells/well in 96-well plates and left to incubate at 37°C/5% CO₂. After 72 hours, the culture medium was replaced with Phenol Red-free (PRF) DMEM/F12 medium supplemented with 2 mM L-glutamine, 50 µg/mL streptomycin, and 50 U/mL penicillin and returned to the incubator for 24 hours.

The medium was washed off and cells were incubated with 10 µM CM-H₂DCF-DA for 1 hour at 37°C. Cells were washed with PRF DMEM/F12 and exposed to test products in the form of 5.3–200 µg/mL PM (dimethyl sulfoxide [DMSO] normalized to 0.83%), or 3.125–100% AqE for 1 hour at 37°C. The following experimental controls were included in the experiments: PRF DMEM/F12 medium, 0.83% DMSO (for PM experiments), 0.1–0.2% Triton X-100 (for cell viability experiments only; Sigma-Aldrich), 28 mM potassium bromate (KBrO₃; Sigma Aldrich), or 15.6 µM tert-butyl hydroperoxide (t-BHP; Sigma-Aldrich). After exposure, the cells were washed twice with PBS and then replaced with 100 µL PRF DMEM/F12.

To ensure that any increases in intracellular ROS were not due to cytotoxicity, cell viability was assessed using the CellTiter-Glo[®] Luminescent Cell Viability Assay (Promega), according to the manufacturer's instructions. Luminescence was read using a SpectraMax[®] M3 Multi-Mode Plate Reader (Molecular Devices, Wokingham, United Kingdom) and treatment responses were normalized to control values and expressed as a percentage change relative to the control. ROS production was assessed by measuring DCF fluorescence. Plates were read using a SpectraMax M3 Multi-Mode Plate Reader set to scan at an excitation wavelength

TABLE 1. FORMATS OF CIGARETTE SMOKE EXPOSURE AND THEIR GENERATION

	<i>Cigarette exposure</i>		
	<i>Particulate matter</i>	<i>Cigarette smoke aqueous extract</i>	<i>Whole smoke</i>
Smoking machine	Borgwaldt RM200 (Borgwaldt-KC, Hamburg, Germany)	Borgwaldt RM20H (Borgwaldt-KC)	Borgwaldt RM20S (Borgwaldt-KC)
Procedure	PM was: Collected on a Cambridge filter pad during the smoking of the cigarettes. Eluted with DMSO (Sigma-Aldrich, Dorset, United Kingdom) to a concentration of 24 mg/mL. ^{14,15} Further diluted with cell culture medium, to a range of concentrations for use in experiments.	Smoke from a single cigarette was bubbled through 20 mL of PRF DMEM/F12 medium in a glass impinger. ¹⁶ AqE was: Made fresh for each individual experiment Diluted in the appropriate culture medium when used in experiments.	Cigarette smoke was diluted with air and delivered to purpose-built Perspex TM exposure chambers for exposure of cells at the ALI. ^{17,18}
Experimental controls	Cells exposed to DMSO diluted in media	Cells exposed to PRF DMEM/F12 media alone	Cells exposed to air alone in the smoke chambers (AIR) Cells kept in the incubator (INC)
Quality control	Measurement of nicotine, water, and nicotine-free dry PM. ¹¹	Measurement of carbon monoxide passing through the smoke generation system ¹⁶ The mean of six individual measurements of the optical density of each batch of AqE at 320 nm (OD ^{320nm}). ¹⁶ Measurement of nicotine in each AqE sample.	Daily checks on airflow and puff volume before use of the machine

ALI, air-liquid interface; AqE, aqueous extract; DMSO, dimethyl sulfoxide; PM, particulate matter.

of 485 nm and an emission wavelength of 530 nm. DCF fluorescence was expressed in relative fluorescence units (RFU).

Measurement of reduced glutathione (GSH). Three days before PM or AqE exposure, NCI-H292 cells were seeded at a density of 1×10^4 cells/well in 96-well plates. Following incubation for 72 hours, the medium was replaced with PRF DMEM/F12 medium supplemented with 2 mM L-glutamine, 50 μ g/mL streptomycin, and 50 U/mL penicillin, and the plates were incubated for 24 hours. Following 1 hour incubation with the test products, the level of reduced glutathione (GSH) was determined using the GSH-GloTM Glutathione Assay (Promega), according to the manufacturer's instructions. Luminescence was read using a SpectraMax M3 Multi-Mode Plate Reader and expressed as relative luminescence units (RLU). RLU values from each test plate were normalized to the control values (untreated cells) and expressed as a percentage change relative to the untreated control.

To ensure that any GSH measured was not due to the membrane of the cells being compromised, the presence of the cytoplasmic enzyme lactate dehydrogenase (LDH) was measured in the exposure medium. LDH was measured using

the CytoTox-ONETM Homogenous Membrane Integrity Assay (Promega) according to the manufacturer's instructions. Fluorescence was read using a SpectraMax M3 Multi-Mode Plate Reader, set to scan at an excitation wavelength of 560 nm and an emission wavelength of 590 nm. RFU readings were normalized to control values and cytotoxicity was expressed as a percentage change relative to the Triton X-100-treated cells.

Measurement of cytotoxicity and mediator release

For experiments with WS, NCI-H292 cells were seeded on to the apical region of 12 mm TranswellTM (Corning) inserts at a density of 1.5×10^5 cells/well. One milliliter per well of complete RPMI medium was added to the basal region (containing the cell culture inserts). For experiments with PM, NCI-H292 cells were seeded at a density of 2×10^4 cells/well in 96-well plates for cytotoxicity assessments, and 2×10^5 cells/well in 12-well plates for the assessment of mediator release. Following incubation for 18–24 hours, the media was replaced with UltraCULTURETM medium (BioWhittaker) supplemented with 2 mM L-glutamine, 50 μ g/mL streptomycin, and 50 U/mL penicillin. At this stage the cells had reached confluency. The plates were incubated for a further 18–24 hours.

TABLE 2. SUMMARY OF ASSAYS USED TO ASSESS THE BIOLOGICAL RESPONSES OF THE COMMERCIAL CONTROL AND REDUCED TOXICANT PROTOTYPE CIGARETTES

Endpoint	Techniques	Cigarette exposure method
Cytotoxicity Calculation of the smoke concentration that reduced the viability of NCI-H292 cells by 50% (EC ₅₀ concentration)	Neutral red uptake assay to measure EC ₅₀ .	<ul style="list-style-type: none"> • 24 hour exposure to particulate matter (PM)
Mediator release Changes in the secreted levels of inflammatory and tissue remodeling mediators	(MSD, Maryland, USA) multiplexing platform to measure IL-6, IL-8, and MMP-1	<ul style="list-style-type: none"> • 30 minute exposure to whole mainstream smoke followed by 24 hour recovery
Oxidative stress Detection of the production of endogenous ROS	Use of 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate acetyl ester (CM-H ₂ DCF-DA), an intracellular probe, which fluoresces upon contact with ROS	<ul style="list-style-type: none"> • 1 hour exposure to particulate matter (PM)
Oxidative stress Depletion of the levels of the antioxidant glutathione	GSH-Glo™ assay kit, (Promega, Southampton, UK) to measure levels of the antioxidant glutathione in reduced form (GSH)	<ul style="list-style-type: none"> • 1 hour exposure to cigarette aqueous smoke extract (AqE)

IL-6, interleukin 6; IL-8, interleukin 8; MMP-1, matrix metalloproteinase 1; MSD, Meso Scale Discovery; ROS, reactive oxygen species.

Cytotoxicity assessment. For WS experiments, NCI-H292 cells were exposed for ~30 minutes to WS from the test cigarettes at smoke:air dilutions ranging from 1:2.5–1:80 to 1:2.5–1:200 for RTP and CC, respectively. Additional treatment conditions included cells kept in the incubator (INC control), and cells exposed to air alone (AIR control), as described previously.¹⁷ Following exposure, the cells were resubmerged in supplemented UltraCULTURE™ media and left to recover for 24 hours, after which cytotoxicity was assessed using the NRU assay.

For PM experiments, cells were exposed for 24 hours to PM diluted in supplemented UltraCULTURE™ at concentrations ranging from 10 to 1000 µg/mL, or to control solutions (unconditioned medium, DMSO vehicle control, or sodium dodecyl sulfate positive control). Following exposure, cytotoxicity was assessed using the NRU assay.

NRU assay. The NRU assay was performed as described previously.^{17,18} Briefly, following exposure to the WS or PM, cells were washed with PBS and incubated for 3 hours with neutral red reagent (Sigma-Aldrich) diluted 1:65 in UltraCULTURE™. Unincorporated dye was removed by washing cells in PBS, and the neutral red was eluted from the cells with a destain solution (50% ethanol, 49% distilled water, and 1% glacial acetic acid (Sigma-Aldrich) for 10 minutes. Eluted dye was read on a SpectraMax M3 Multi-Mode Plate Reader at 540 nm, with a reference filter of 630 nm. Background measurements from blank wells were subtracted from the values for treated and untreated cells. Cell viability values were expressed as a percentage of the untreated controls (i.e., air exposed cells for WS exposures or media-only exposed cells for PM-exposed cells).

Measurement of mediator release. Using the cell viability data generated from the NRU assays, subcytotoxic

concentrations of WS (smoke:air dilution, vol:vol: RTP cigarette: 1:4000–1:20; CC cigarette: 1:4000–1:140) and PM (10–50 µg/mL; normalized to the highest DMSO concentration 0.208%), were identified for use in assessment of the secretion of proinflammatory mediators, including interleukin (IL)-6, IL-8, and matrix metalloproteinase (MMP)-1. 10 µg/mL lipopolysaccharide (LPS) was used as a positive control in the PM experiments.

Following exposure, the medium from each well was removed and stored at –80°C for quantification of mediators at a later date (≤2 weeks following exposure). Stored media were defrosted and assessed for levels of IL-6, IL-8, and MMP-1 using the Meso Scale Discovery (MSD) Human IL-6, IL-8, and MMP-1 MULTI-ARRAY® 96-well small spot plates and MSD Sector Imager 2400 Model 1250 according to the manufacturer's instructions. Mediator responses were quantified against a standard curve and log transformed. For PM experiments, the solvent controls were subtracted from the mediator responses before transformation.

Statistical analysis. For the DCF and GSH experiments, the General Linear Model (GLM) and Dunnett's comparison tests were used to assess the responses of the control, PM, and AqE treatments. For PM experiments, each treatment was compared against the response from the DMSO-treated cells; for AqE the comparator was the PRF DMEM/F12 control. GLM and Tukey's pairwise comparison tests were used to compare the responses between cigarette types using data obtained from the DCF, GSH, and mediator release experiments.

Curves for cell viability data generated from the NRU assay were fitted using a four-parameter logistic curve. Differences between EC₅₀ values for cigarette types were determined using an *F*-test. For all statistical analyses *p* ≤ 0.05 were considered to be significant. GraphPad Prism® and Minitab® were used to carry out all statistical analyses.

Results

Responses following exposure with PM

Increase in ROS generation in lung epithelial cells. Endogenous ROS production was assessed in cells exposed to PM from the RTP and CC cigarettes. Cells were exposed to doses of PM ranging from 0 to 200 $\mu\text{g/mL}$. Doses of 5.5–96.9 $\mu\text{g/mL}$ of PM did not have a cytotoxic effect on the cells following a 1 hour exposure; however, at 200 $\mu\text{g/mL}$ cell viability decreased to $\sim 80.0\%$ (Supplementary Table S4). Based on these observations 5.3, 11, 22.3, 47, 96.9, and 200 $\mu\text{g/mL}$ PM were selected for use in the DCF assay.

Media and DMSO controls did not elicit a response, indicating no significant differences in DCF fluorescence. While treatment with KBrO_3 positive control induced an increase in fluorescence, this was shown to not be significantly higher than the negative controls. For future experiments, the positive control was changed to t-BHP. PM from both cigarette types induced a dose-dependent increase in DCF fluorescence, indicating an increase in intracellular ROS (Fig. 1). However, no significant differences were observed in ROS production between cigarette types (Fig. 1).

Additional analysis was carried out to assess the impact of nicotine levels and number of cigarettes required to prepare the stock PM solutions for each product. No differences were observed between products when these factors were taken into consideration (data not shown).

Depletion in antioxidant (GSH) levels in lung epithelial cells. To ensure that any observed depletion in the level of the antioxidant GSH was caused as a response to neutralization of oxidative species and not due to leakage through a compromised cell membrane, the viability of NCI-H292 cells following exposure to PM was assessed by measuring LDH (usually found in the cytoplasm) in

FIG. 1. ROS generation in NCI-H292 cells following 1 hour exposure to cigarette smoke particulate matter from RTP and CC cigarettes. Detection of intracellular ROS production in NCI-H292 cells following 1 hour exposure to 0, 5.3, 11, 22.3, 47, 96.9, and 200 $\mu\text{g/mL}$ PM, or 28 mM potassium bromate (KBrO_3). Data are represented as the mean $\pm$ standard deviation from six experiments. CC, commercial control; DMSO, dimethyl sulfoxide; PM, particulate matter; ROS, reactive oxygen species; RTP, reduced toxicant prototype.

FIG. 2. GSH depletion and cytotoxicity in NCI-H292 cells following 1 hour exposure to cigarette smoke PM from RTP and CC cigarettes. Detection of GSH and cytotoxicity in NCI-H292 cells following 1 hour exposure to 0, 66, 100, and 200 $\mu\text{g/mL}$ PM, 0.83% DMSO, 28 mM potassium bromate (KBrO_3) or 0.2% Triton X-100. Cytotoxicity was assessed through the measurement of LDH in the supernatant. Data are represented as the mean $\pm$ standard deviation of six experiments. GSH, reduced glutathione; LDH, lactate dehydrogenase.

the cell exposure media. LDH levels were $\leq 5\%$ of the maximum response for the Triton X-100-positive control for all PM experiments.

No significant differences in GSH levels were observed in cells treated with 0.83% DMSO (vehicle control) versus untreated cells, indicating that DMSO did not affect GSH levels. KBrO_3 , a known oxidant and the positive control for GSH depletion, and Triton X-100, the positive control for cell death and thus LDH release; both significantly depleted GSH levels compared with untreated cells, ($\sim 75\%$ and 1%, respectively compared with untreated cells, $p \leq 0.05$).

A dose-dependent decrease in GSH levels was observed following exposure of the cells to PM from both cigarette types. Statistical analysis comparing the GSH response from both cigarettes at each concentration showed no significant differences observed in GSH depletion at each concentration (Fig. 2). However, analysis comparing the overall depletion of GSH from both cigarette treatments showed the RTP PM to induce a significantly lower response than that induced by PM from the CC ($p \leq 0.05$).

Cytotoxic response in lung epithelial cells. A dose-dependent increase in cytotoxicity was observed following exposure to PM from both cigarette types (Fig. 3). No significant differences were observed between the EC_{50} values for PM from the RTP cigarette versus the CC cigarette (238.4 $\mu\text{g/mL}$ vs. 239.4 $\mu\text{g/mL}$, respectively; $p > 0.05$). Based on these observations, the following concentrations were selected for use in the mediator release experiments: PM: 10, 20, 30, 40, and 50 $\mu\text{g/mL}$.

Secretion of proinflammatory/tissue remodeling cytokines in lung epithelial cells. A dose-dependent increase in the secretion of MMP-1 was observed following exposure to subtoxic concentrations of PM ($>80\%$ Viability, Fig. 4a) from both cigarettes. However, no significant differences in

FIG. 3. Cytotoxic response in NCI-H292 cells exposed to cigarette smoke PM from RTP and CC cigarettes. Cytotoxicity response of NCI-H292 cells following 24 hour exposure to 0–1000 $\mu\text{g/mL}$ PM from each cigarette type. The NRU assay was used to measure cytotoxicity. Data are represented as the mean $\pm$ standard deviation from six experiments.

MMP-1 secretion were observed between cigarette types. Exposure to PM from RTP or CC cigarettes resulted in no clear dose-dependent responses or significant differences in the secretion of either IL-6 or IL-8 (Fig. 4b, c).

Responses following exposure to cigarette smoke AqE

Increase in ROS generation in lung epithelial cells. Experimental data showed that AqE from both cigarettes induced little or no cytotoxic effect on the cells, $\geq 96.9\%$ viability at all concentrations for each cigarette type as measured by CellTiter-Glo (Supplementary Table S5). Based on these observations, the following concentrations were selected for use in the DCF assay: 5%, 15%, 30%, 45%, 60%, 75%, and 100% AqE.

Treatment with the t-BHP-positive control induced a significant increase in DCF fluorescence over vehicle controls (~ 200 RFU, $p < 0.05$), indicating the assay was performing as expected. Similar to the response with PM, exposure of cells to AqE from both cigarette types induced a dose-

FIG. 4. Secretion of MMP-1, IL-6, and IL-8 by NCI-H292 cells exposed to cigarette smoke PM from RTP and CC cigarettes for 24 hours. Comparison of secretion of (a) MMP-1, (b) IL-6, and (c) IL-8 by NCI-H292 cells following 24 hour exposure to 0–50 $\mu\text{g/mL}$ PM or 10 $\mu\text{g/mL}$ lipopolysaccharide (LPS). Data are represented as the mean $\pm$ standard deviation from five experiments. IL, interleukin; MMP, matrix metalloproteinase.

FIG. 5. ROS generation in NCI-H292 cells following 1 hour exposure to cigarette smoke AqE from RTP and CC cigarettes. Detection of intracellular ROS production in NCI-H292 cells following 1 hour exposure to 0%, 5%, 15%, 30%, 45%, 60%, 75%, and 100% AqE or 15.6 μ M t-BHP. Data are represented as the mean $\pm$ standard deviation from six experiments. *Concentrations at which there was a significant difference in ROS generation between cells exposed to AqE from RTP versus CC cigarettes ($p < 0.05$). AqE, aqueous extract.

dependent increase in DCF fluorescence, indicating an increase in intracellular ROS. Significantly lower levels of ROS were generated following exposure to AqE from RTP cigarettes ($p < 0.05$; Fig. 5).

Depletion in antioxidant (GSH) levels in lung epithelial cells. As with the PM experiments, LDH levels were $\leq 5\%$ of the maximum response for the positive control (Triton

FIG. 6. GSH depletion and cytotoxicity in NCI-H292 cells following 1 hour exposure to cigarette smoke AqE from RTP and CC cigarettes. Detection of GSH and cytotoxicity in NCI-H292 cells following 1 hour exposure to 0%, 6.25%, 12.5%, 25%, 50%, and 100% AqE, 20 mM KBrO₃ or 0.2% Triton X-100. Cytotoxicity was assessed through the measurement of LDH in the supernatant. Data are represented as the mean $\pm$ standard deviation of six experiments. *Concentrations at which there was a significant difference in GSH depletion between cells exposed to AqE from RTP versus CC cigarettes ($p < 0.05$).

FIG. 7. Cytotoxic response in NCI-H292 cells exposed to WS from RTP and CC cigarettes. Cytotoxicity response of NCI-H292 cells following 0.5 hour exposure to WS followed by a 24 hour recovery. The smoke dilutions (smoke:air, vol: vol) used ranged from 1:2.5–1:80 for the RTP cigarettes to 1:2.5–1:200 for CC cigarettes. The NRU assay was used to measure cytotoxicity. Data are represented as the mean $\pm$ standard deviation from nine experiments. WS, whole smoke.

X-100) indicating little or no cytotoxicity across all doses (Fig. 6). Exposure to AqE from both cigarettes induced a dose-dependent decrease in GSH levels. Significantly less GSH depletion was observed following exposure of the cells to AqE from the RTP cigarette versus the CC cigarettes ($p < 0.05$; Fig. 6). Furthermore, GSH levels in cells exposed to AqE from the RTP cigarette did not fall below 92% and were not significantly different from untreated cells.

Responses following exposure to whole mainstream smoke

Cytotoxic response in lung epithelial cells. A dose-dependent increase in cytotoxicity was observed following exposure to WS from both cigarette types. A significantly lower dilution and thus more concentrated amount of smoke from the RTP cigarette was needed to achieve an EC₅₀ when compared with the CC cigarette. (smoke:air dilution 1:7.6 vs. 1:58.6, respectively; $p < 0.05$; Fig. 7). Based on these observations, the following concentrations were selected for use in the mediator release experiments: WS (smoke:air dilution, vol:vol): RTP cigarette: 1:4000, 1:2000, 1:1000, 1:500, 1:300, 1:160, 1:80, 1:40 and 1:20; CC cigarette: 1:4000, 1:2000, 1:1000, 1:500, and 1:140).

Secretion of proinflammatory/tissue remodeling cytokines in lung epithelial cells. A dose-dependent increase in the secretion of MMP-1 was observed following exposure of cells to WS (Fig. 8a). However, no significant differences in MMP-1 secretion were observed between cigarette types. No clear dose-dependent responses or significant differences were observed in the secretion of IL-6 or IL-8 (Fig. 8b, c) following exposure of cells to RTP or CC cigarettes.

Discussion

As a first step to determine whether this simple *in vitro* model of the lung epithelium could discriminate between

FIG. 8. Secretion of MMP-1, IL-6, and IL-8 by NCI-H292 cells exposed to whole smoke from RTP and CC cigarettes. Comparison of secretion of (a) MMP-1, (b) IL-6, and (c) IL-8 by NCI-H292 cells following 0.5 hour exposure to WS followed by a 24 hour recovery. Data are represented as the mean $\pm$ standard deviation from eight experiments.

products of varying toxicant yields, we compared the effects of smoke derived from a novel RTP cigarette with that from a CC cigarette, using a selection of endpoints relevant to smoking-related disease.

Cigarette smoke is a complex and dynamic mixture of more than 6000 individual chemical constituents¹⁹ and these can be found partitioned between the vapor and particulate phases of the smoke. In this study, exposure to WS, PM, and AqE enabled the comparison of the whole aerosol, particulate, and (water soluble) vapor phases of cigarette smoke, respectively. The majority of the toxicant reductions in the RTP cigarette resided in the gaseous/vapor phase (Supplementary Table S3¹⁹), which was reflected in the results of this study, where differences between the cigarettes were generally observed in the experiments using WS or AqE (which captured the vapor phase components of the aerosol). Further experiments using gas vapor phase as the exposure medium would provide further confirmation of this.

Oxidative stress is a key mediator of both lung cancer and COPD.^{3-5,20-22} Cigarette smoke has an abundance of free radicals, with the vapor phase tending to contain short-lived species that exert their damaging effects locally, whereas the longer-lived species tend to act systemically and are found in the particulate phase.²³ We compared oxidative stress levels indirectly by measuring both ROS pro-

duction and the antioxidant status of lung epithelial cells in response to smoke from RTP or CC cigarettes. Exposure to PM induced a dose-dependent increase in ROS production, however no differences were observed between cigarettes.

In contrast, AqE from the RTP cigarette induced significantly lower levels of ROS production compared with the CC cigarette. It is possible that the significantly lower level of intracellular ROS production induced by AqE from RTP cigarettes indicates that intracellular ROS production may be linked to toxicants in the vapor phase, which are reduced in the RTP cigarette. Carbonyls such as acrolein have been linked to the induction of cellular oxidative stress²⁴ and it has been reported that carbonyls are captured in AqE.^{23,25-28} Furthermore, the ratio of carbonyl components of AqE are similar to that observed in cigarette smoke, although the absolute levels were lower.²⁹

The antioxidant status of NCI-H292 lung epithelial cells was assessed using the GSH-GloTM assay to measure changes in the level of the reduced form of the antioxidant glutathione. The dose-dependent depletion of GSH observed with PM and AqE from RTP or CC cigarettes suggests that intracellular GSH was oxidized in response to elevated ROS in the cytosol. Cell viability data confirmed that observed decreases in GSH levels were not due to leakage from damaged

or dying cells. AqE from the RTP cigarette induced significantly less GSH depletion when compared with that of the CC cigarette. In contrast, PM from the RTP cigarette caused a significantly greater depletion of GSH compared with the CC cigarette. However, this observation may not necessarily indicate that the RTP cigarette was more potent with respect to antioxidant depletion on a cigarette-by-cigarette basis, due to the greater number of RTP cigarettes required to generate the stock 24 mg/mL concentration of PM compared with CC cigarettes.

Cytotoxicity of lung epithelial cells was assessed to gain a measure of the overall cellular health to show that data from other endpoints were representative of a response from a healthy cell, as opposed to a cell functioning under highly abnormal or stressful conditions. In parallel with the assessment of mediator release, a dose-dependent increase in cytotoxicity was observed with PM and WS from both cigarettes. However, a difference in the cytotoxic responses induced by the different cigarette types was only observed when WS was used as the exposure medium. Literature shows that the vapor phase is primarily responsible for the cytotoxic effects observed in this assay.^{25,30–32} The RTP cigarette had significant reductions in acetaldehyde and acrolein (Supplementary Table S3) compared with the CC cigarette, both of which have been shown to inhibit cell proliferation and/or induce cytotoxicity *in vitro*.^{12,33–37} This may explain why WS from RTP cigarettes induced significantly less cytotoxicity in lung epithelial cells versus the CC cigarettes. Given the clear discrimination in WS-induced cytotoxicity between the test products in this study, this endpoint may be useful for the future assessment of cytotoxic effects derived from novel tobacco and nicotine delivery product aerosols.

Although WS from the RTP cigarette was less cytotoxic than the CC cigarette, changes in the toxicant profile of the RTP cigarette did not appear to induce a difference in the proinflammatory/tissue remodeling response when compared with the CC cigarette, although levels did appear to be elevated compared with controls. Exposure to WS and PM from both cigarettes resulted in a dose-related increase in MMP-1 secretion, but no significant difference between the products. Given that we measured a very limited number of endpoints in a single cell type, other cellular proinflammatory and repair/remodeling endpoints in conjunction with the presence of other key players (such as immune cells) may be required to investigate this complex process in greater depth. Furthermore, 3D tissues derived from primary cells, which maintain a more physiologically relevant tissue structure could model also these processes more effectively.

In vitro genotoxicity studies are widely used for the comparative assessment of the potential health risks of different tobacco products.^{38–40} However, the use of *in vitro* systems to model the potential for development of smoking-related diseases has some specific limitations. For example, smoking-related diseases are complex, without a clear linear path of progression. Furthermore, disease occurrence often follows chronic exposure, which can be difficult to represent in *in vitro* models, which utilize acute, or short-term repeat-acute exposure regimens. Additionally, it is important to identify the most relevant level and route of cigarette smoke exposure that is repre-

sentative of the biological situation. This is a challenge which exists across all toxicological assessments.⁴¹ While it is not possible to extrapolate data from *in vitro* models alone to estimate the impact on human disease risk, *in vitro* data could be utilized in a weight-of-evidence package alongside existing animal data and human studies for a more comprehensive assessment of novel tobacco and nicotine delivery products, relative to conventional cigarettes.

Conclusions

As a first step toward “fitness-for-purpose” evaluation, we utilized a simple model of the lung epithelium *in vitro* to detect differences in responses between cell cultures exposed to WS, and smoke extracts derived from a RTP cigarette, compared with a CC cigarette using a selection of biological endpoints. The results suggest that the *in vitro* model may be suitable for comparative toxicological assessment of novel tobacco and nicotine delivery products, relative to conventional cigarettes, in the areas of whole aerosol cytotoxicity and cellular oxidative stress. The assessment of proinflammatory and tissue remodeling responses may require more sophisticated *in vitro* systems combined with more specific endpoints and human clinical data to fully understand the toxicological impact in these important areas. Furthermore, this study highlights the importance of relating test product emissions to toxicological responses derived from exposure to test aerosol fractions, which may favor the capture of certain toxicants over others.

While data generated using *in vitro* models alone cannot predict human disease risk, they may be employed alongside aerosol chemistry, *in vitro* genotoxicity, and human studies to support a weight of evidence package for the purposes of risk substantiation of novel tobacco and nicotine products.

Acknowledgments

The authors would like to thank Ermioni Papadopoulou, Oscar M. Camacho, and Graham Errington for their advice on study design and for performing statistical analyses. Editorial assistance with the preparation of this article was provided by JEM Comms Ltd. and funded by British American Tobacco.

Author Disclosure Statement

All authors are full-time employees of British American Tobacco (Investments) Ltd. This work was fully funded by British American Tobacco (Investments) Ltd.

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