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Study of Gaseous Benzene Effects Upon A549 Lung Epithelial Cells Using a Novel Exposure System

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ABSTRACT

Volatile organic compounds (VOCs) are ubiquitous pollutants known to be present in both indoor and outdoor air arising from various sources. Indoor exposure has increasingly become a major cause of concern due to the effects that such pollutants can have on health. Benzene, along with toluene, is one of the main components of the VOC mixture and is a known carcinogen due to its genotoxic effects. The aim of this study was to test the feasibility of an *in-vitro* model to study the short-term effects of exposure of lung cells to airborne benzene. We studied the effects of exposure on DNA and the production of reactive oxygen species (ROS) in A549 cells, exposed to various concentrations of benzene (0.03; 0.1; 0.3 ppm) in gaseous form using a custom designed cell exposure chamber. Results showed a concentration-dependent increase of DNA breaks and an increase of ROS production, confirming the feasibility of the experimental procedure and validating the model for further *in-vitro* studies of exposure to other VOCs.

Key words: *in vitro*; A549; benzene exposure; volatile organic compounds; comet assay; ROS;

1. INTRODUCTION

People spend much of their time indoors where they are continuously exposed to low concentrations of a wide variety of chemicals including volatile organic compounds (VOCs). It has been demonstrated that building materials, furniture and consumer products are a source of low level VOC exposure contributing together with the outdoor air to the exposure of general population (Delgado-Saborit et al., 2011; Wolkoff, 2012). Indoor exposure to VOCs has been reported to cause a number of symptoms ranging from irritation of the respiratory system to sick building syndrome (Brinke et al., 2004; Wang et al., 2013). In addition, VOCs are also reported to contribute to oxidative stress induction via production of reactive metabolites and hence have the potential to damage DNA (Knaapen et al., 2006; Lu et al., 2007; Saint-Georges et al., 2008; Snyder and Hedli, 1996; Sørensen et al., 2003a; Wang et al., 2013). Exposure to VOCs such as styrene can also lead to an inflammatory response in the lungs that can then result in oxidative stress (Bönisch et al., 2012; Fubini and Hubbard, 2003; Röder-Stolinski et al., 2008; van Berlo et al., 2010). It has been suggested that other compounds such as benzene and toluene can trigger an inflammatory response, but DNA damage caused by such response has not been supported by solid evidence (Pariselli et al., 2009; Wang et al., 2013). The relationship between exposure to benzene and DNA damage has been extensively studied, especially for occupational exposures, demonstrating that benzene is involved in DNA damage through reactive metabolic intermediates (Hartwig, 2010; Snyder and Hedli, 1996) and generation of reactive oxygen species (ROS) (Barreto et al., 2009). Because of the detailed knowledge of its metabolism, benzene serves as an ideal positive control test compound for the development of new toxicological assays and tests. Furthermore, in several studies of human exposure to benzene at medium and low concentrations (0.001 ppm – 0.03 ppm), a correlation was found between levels of urinary benzene, benzene metabolites and urinary 8-oxo-2'-deoxyguanosine (8-oxo-dG) a biomarker that is widely considered to be associated with ROS related DNA damage and repair (Andreoli et al., 2012; Bagryantseva et al., 2010; Manini et al., 2010).

A recent review (Hartwig, 2010) summarised the possible mechanisms through which benzene and its metabolites can cause DNA damage. *In vivo*, after absorption, benzene is metabolized by cytochrome P450 2E1 (CYP2E1) in the liver to produce metabolites including: phenol, catechol, hydroquinone and 1,2,4-trihydroxybenzene. These closed ring metabolites then undergo further metabolism in the bone marrow to form *o*- and *p*-benzoquinone. *p*-Benzoquinone is thought to be the toxic metabolite responsible for myelotoxicity due to its high reactivity and is known to form adducts with proteins and DNA (Linhart et al., 2011; Rappaport et al., 2005). Bone marrow, due to the high rate of cell mitosis and lower DNA repair capacity (Buschfort-Papewalis et al., 2002) is a sensitive tissue for DNA instability, resulting in possible health effects sooner than other tissues.

All of the phenolic benzene metabolites are chemically active in cells, increasing the oxidative DNA damage, directly or through further metabolization. Therefore, although the main target tissues and the metabolism of benzene have been studied and understood, it is important to develop models to assess the toxicity caused by VOC exposure of other metabolically competent tissues including the lung epithelium, which is a principal route of exposure to benzene and other VOCs. Such models could also be useful to study other VOCs, as currently there is little evidence of any linkage between exposure and DNA damage for other VOC species, even if it has been suggested from studies in animal models and *in-vitro* studies (Sarma et al., 2011a; Singh et al., 2009).

Currently there are only few detailed studies on the development of *in vitro* models to examine the effects of such pollutants on the lung. Pariselli et al. (2009) developed a method to expose lung cells to various concentrations of benzene and toluene, evaluating some of the effects caused by the pollutants on lung cells growing on permeable supports that allow exposure of cells to gas mixtures, modelling a real life situation of exposure.

Although Pariselli et al. (2009) reported interesting results on inflammatory response triggered by VOC exposure and cell viability, no further studies on DNA damage or ROS production were reported deriving from lung epithelium models. We therefore aimed to progress by exploring the feasibility of an air-liquid interface (ALI) *in-vitro* model using lung epithelia cells grown on a permeable membrane. The main focus of this study was on the short-term effects of exposure of lung cells to VOCs with two main endpoints: oxidative stress and DNA damage.

2. MATERIALS AND METHODS

2.1 Exposure System

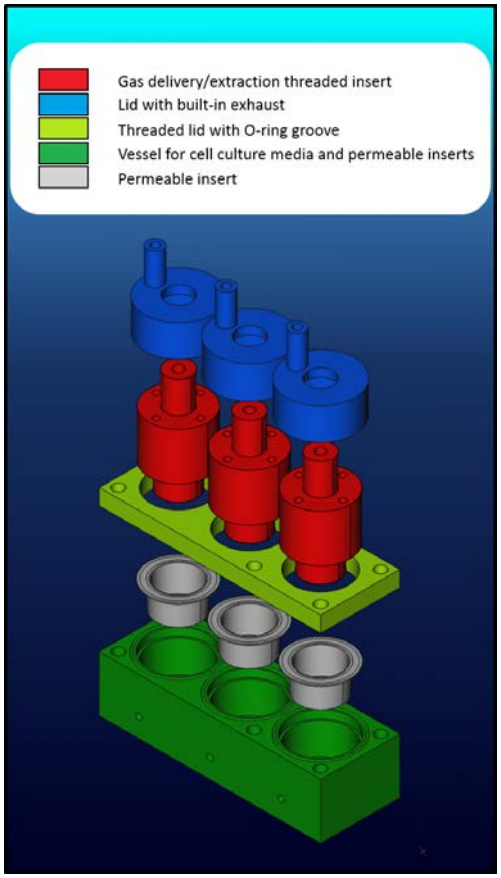

Figure 1a: Exploded representation of the exposure vessel with all the part of the assembly.

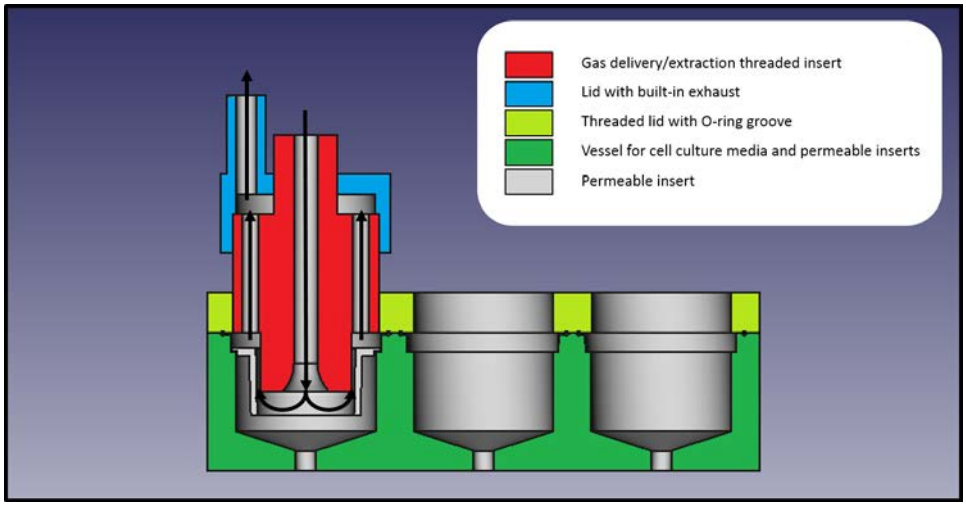

Figure 1b: Cross section of the assembled exposure vessel, arrows indicate the gas flow. In use, the two vessels to the right are also fitted with the gas delivery system and permeable insert.

An exposure vessel was custom designed based on the CULTEXs system (Vitrocell, Germany) described by Pariselli et al. (2009). The system consisted of three wells milled into a polymethyl methacrylate (PMMA) block, suitable to fit a permeable 6-well insert (Corning Inc., NL) into each one, and 5ml of media under the inserts (Figure 1a and 1b). The capacity to circulate cell culture media was included by adding two 5mm holes at the bottom and on the side of each well. These could be capped if the circulation of the media was not needed.

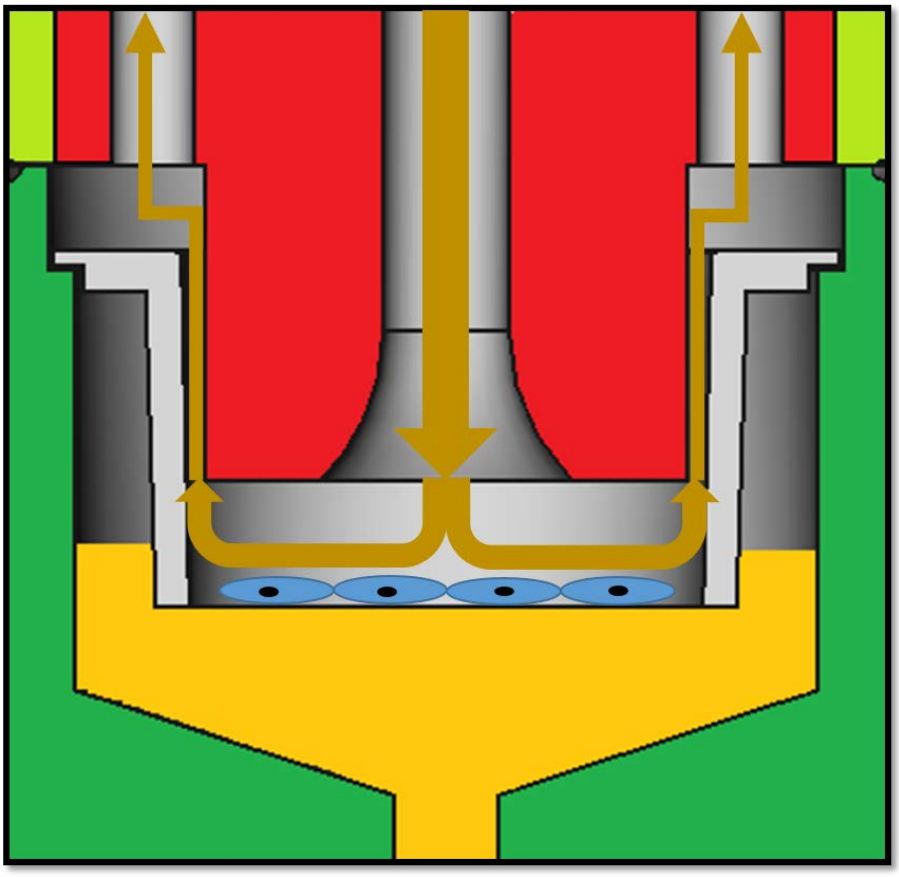

Figure 2: Detailed cross-section of the gas-cell interface, with the level of the cell culture media indicated in yellow.

The lid was constructed with threaded holes to fit gas delivery inserts, and the wells were sealed using nitrile O-rings. The gas delivery tubes were designed with a trumpet shape in order to reduce the linear velocity of the delivered gas to reduce mechanical stress to the cells, and to allow maximum mixing and even delivery of the gas along each insert (Figure 2). Each tube was threaded externally to screw into the lid allowing fine adjustments of the distance between the nozzle and the cell layer. The tubes were provided with holes to allow the delivered gas to flow out to the exhaust, providing a continuous flow. The vessel was kept in a gas chromatograph (GC) oven at 37°C during the exposure to maintain the cells at an optimal temperature. The vessel was kept in an incubator at 37°C between the exposures. The gas delivery to the cells was regulated by mass flow controllers

(MFCs) (Brooks Instrument, NL). One 500 sccm (standard cubic centimeters/minute) MFC was used to regulate the synthetic air (BOC gases, UK) flow, and a 100 sccm MFC was used to regulate the benzene flow (1ppm balanced in nitrogen, BOC gases, UK). A total flow rate of 30 ml/min was used, split between the three wells, with a theoretical flow rate of 10 ml/min in each well.

The synthetic air passed through a bubbler heated at 37°C to humidify the air to prevent desiccation of cells, and the pollutant delivery (benzene 1ppm balanced in nitrogen, BOC, UK) was regulated by a second MFC. The two gases were then mixed in a glass mixing chamber hosted inside the GC oven, filtered through a 0.45µm filter and delivered to the cells. The system worked with positive pressure of the exposure gases to ensure sterility.

2.2 Cell Culture

A549, epithelial lung carcinoma cells (HPA, 86012804) have been previously used as a model for lung epithelium exposure (Pariselli et al., 2009; Saint-Georges et al., 2008; Shang et al., 2013). Furthermore, this cell line has been reported to express CYP2E1, the cytochrome P450 involved in the metabolism of benzene (Foster et al., 1998; Medinsky et al., 1994; Mögel et al., 2011; Snyder and Hedli, 1996; Sørensen et al., 2003a), and therefore represents a suitable model of study for benzene and VOC toxicology.

A549 cells were cultured in 6-well plates using RPMI 1640 medium (2.5 ml), containing 25mM HEPES and $NaHCO_3$ to maintain the pH constant during the exposure period (CO_2 was not contained in the synthetic air). The medium was further supplemented with glutamine (2mM), penicillin (100 U/ml), streptomycin (100 µg/ml), amphotericin B (0.25 µg/ml) and 10% foetal bovine serum (FBS). Cells were passaged regularly using a standard trypsin-EDTA protocol in 6-well plates and split regularly once they reached approximately 90% confluence. For the exposure experiments, approximately 0.3×10^6 cells were seeded into 1.5 ml RPMI 1640 on the collagen coated Transwell inserts with pores of 1µm (Corning, NL) and 2 to 2.5 ml RPMI 1640 were added

at the bottom of the well, and the plate was placed in an incubator. Once the cells reached 90% confluence (24–36 hours) they were serum starved for 12 hours by exchanging the medium containing FBS with FBS free medium, to synchronize the cell cycle prior exposure (Campisi et al., 1984). After the exposure, the cells were washed with phosphate buffer solution (PBS) for 5 minutes before trypsinization.

2.3 Synthetic Air and Benzene Exposure

For each exposure experiment, 3 Transwell inserts cells from the same 6-well plate were exposed for 2 hours to synthetic air (controls), the exposure vessel was then rinsed, washed with 70% ethanol and allowed to dry. ~~and~~ ~~s~~Subsequently, the remaining 3 Transwell inserts were exposed to one of three different concentrations of benzene (0.03 ppm; 0.1 ppm; 0.3 ppm), which were reported to have cytotoxic effects (Pariselli et al., 2009). A first pilot experiment was performed with 3 technical replicates simultaneously exposed to each benzene concentration accompanied by an air exposed control. The obtained results were then confirmed by an independent replicate. The low (0.03 ppm) and high (0.3 ppm) benzene concentrations were further tested by a third independent replicate exposure. Each exposure experiment was accompanied by a set of incubator controls, which consisted in a 6-well plate left for 2 hours in the incubator, where 3 wells contained cells with media (Incubator Media), and 3 wells contained cells with the media removed (Incubator Dry). The removal of the media from the 3 wells was considered the start point of the 2 hours incubation of the incubator controls.

Comment [d1]: You need to explain how many replicates have you done in the first (old) experiment and second (new) experiment.

When you report the data in the Tables and Figures, you need state how many replicated from which of the two sets of experiments are you reporting information from.

The results that you are not showing in this manuscript (mainly the old data), you have to add it in the Supporting Information, and cross-reference accordingly across the manuscript. This refers to cases like line 252-253.

Comment [d2]: How do you define the incubator control pre- and post-exposure that you report in Figure 3.

2.4 ROS Assay

Reactive oxygen species were assessed by measuring the oxidation of the redox sensitive dye 2',7'-dichlorodihydrofluorescein diacetate (H2DCF-DA, Sigma-Aldrich, UK). Following hydrolysis by intracellular esterases, the resultant H2DCF is unable to leave the cell and is then oxidised by ROS, to form the fluorescent DCF (dichlorofluorescein) molecule. The level of fluorescence is

proportional to the degree of oxidative stress in the cells. Briefly, the cells were preloaded with H2DCF-DA dissolved in the growth medium (10 µM, final concentration) for 30 minutes. After the loading of the cells, both in the exposed samples and in the incubator controls a first fluorescence reading was taken and marked as pre-exposure. After the treatment (air/benzene exposure or 2 hours in the incubator), a second fluorescence reading was taken, marked as post-exposure. DCF fluorescence was quantified before and after exposure using a Tecan Ifinite F200 pro plate reader (excitation wavelength: 485 nm, emission wavelength: 535 nm) using I-control V.1.9 software. To minimise the exposure of the cells to air, quantification of the fluorescence before the exposure was performed without removing the apical medium from the inserts. Three controls and three exposed samples were analysed. Cells that had not been loaded with H2DCF-DA were used as a negative control and to take into account background levels of fluorescence.

2.5 Comet Assay

DNA strand breaks were assessed by the alkaline comet assay (Singh et al., 1988). Briefly, after trypsinization, the cells were re-suspended in 150 µl PBS. 15 µl of the suspension were added to 150 µl of 0.5% (v/v) warm low melting point agarose (LMPA) and the cell suspension was added to a microscope slide that had been previously coated with 0.5% (v/v) normal melting point agarose (NMPA). After a coverslip was added, the slides were left on an ice-cold tray to allow the agarose to solidify. Once the agarose had solidified, the coverslip was removed and the slides were incubated for 1 hour at 4° C in lysis buffer (2.5 M NaCl, 0.1 M Na_2EDTA, 10 mM Tris base, 1% sodium N-lauryl sarcosinate, 10% DMSO and 1% Triton X-100, pH 10). Next, the slides were then transferred to a horizontal electrophoresis tank containing electrophoresis buffer (NaOH 300 mM, EDTA 1 mM, pH 13.0) and DNA allowed to unwind for 20 minutes. DNA was then subjected to electrophoresis (32 V, 0.8 V/cm) for 20 minutes. The slides after the electrophoresis were transferred to the neutralization buffer (Tris HCl 0.4 M, pH 7.5) and washed 3 times for 5 minutes,

followed by 1 wash in H_2O for 5 minutes. Finally, slides were stained with 50 µl of Sybr gold (Invitrogen, 10× solution in neutralisation buffer, UK). Slides were examined at 320× magnification using a fluorescence microscope (Zeiss axiovert 10, Zeiss, UK) fitted with a 515–560 nm excitation filter and a barrier filter of 590 nm. A USB digital camera (Merlin, Allied Vision Technologies, UK) received the images, which were analysed using a personal computer-based image analysis system Comet Assay IV (Perceptive instruments, UK).

A random sample of at least 300 nuclei on each slide was assessed and categorised into quantifiable or not quantifiable. Unquantifiable nuclei consisted largely of "hedgehogs", indicative of nuclei where most of the DNA was in the tail and probably represent nuclei from apoptotic and necrotic cells. A minimum of 60 nuclei were quantified per slide (N = 64 - 200). Percent tail DNA (TD %) was chosen to assess the extent of DNA damage in quantifiable cells, as this has been shown to suffer much less from inter-run variation than other Comet parameters because it is largely independent of electrophoresis voltage and run time (Olive and Durand, 2005).

2.6 Data Analysis

Data analysis was performed using SPSS Statistics 21 and Microsoft Excel 2013.

3. RESULTS

Light microscopy analysis of the cells before and after the exposure demonstrated little mechanical stress and slight drying sustained by the cells due to the airflow, but the following analyses showed that this did not result in overt cell toxicity (data not shown).

3.1 Induction of Oxidative Stress

~~Cells exposed to the highest concentration of benzene (0.3 ppm)~~Cells -were analysed for levels of ROS before and after the exposure together with a control that was exposed only to synthetic air

Comment [d3]: Update this sentence, since you have sample ROS in all exposure levels now

and a set of incubator controls both with and without apical medium. The results presented in Figure 3 clearly indicate that cells exposed to 0.3 ppm benzene had elevated levels of intracellular ROS compared to controls as assessed by the fluorescein oxidation assay. The presented results are based on two independent replicates (each including three technical replicates) for 0.03 ppm and 0.3 ppm benzene, and a single measurement of three technical replicates for 0.1 ppm benzene. Incubator control results are based on 5 independent replicates. The means were compared using ANOVA with Bonferroni post-hoc comparisons. The only statistically significant difference between the pre and post exposure was observed for the 0.3 ppm benzene exposed cells (p<0.001).

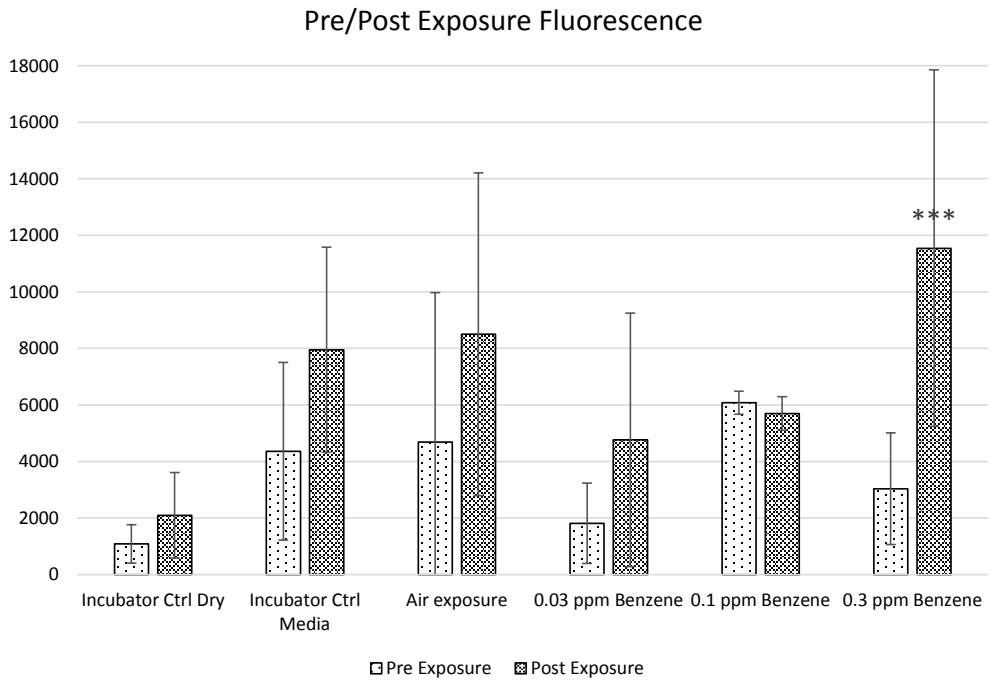

Figure 3: Summary of the DCF fluorescence measurements before and after exposure. Error bars represent standard deviation. (***) indicates a statistically significant difference between the pre and the post exposure.

A generalized increase of fluorescence was observed in all the treatments, including the incubator controls, suggesting a baseline production of ROS in normal cell metabolism. Unfortunately, in the

Comment [d4]: Something seems rather odd in the exposure of 0.1 ppm. It doesn't follow the patter of higher ROS in the post exposure, which is clearly observed, although not statistically significant in all the test, except for 0.3 ppm, where it is statistically signifi

Comment [d5]: How many replicates this figure represents?

case of 0.1 ppm benzene exposure, the pre-exposure condition showed a high reading, most likely due to media background fluorescence. These findings are comparable with previously published data (Pariselli et al., 2009). Although further tests need to be performed, treatment with benzene shows an oxidative effect in the cells, which could be related to either an inflammatory response to benzene itself, or more probably to oxidising metabolites such as benzoquinones (Snyder and Hedli, 1996).

Comment [d6]: Add a reference supporting this statement

3.2 Induction of DNA Strand Breaks

Following exposure of cells to both low (0.03 ppm) and medium (0.1 ppm) benzene concentrations, the incidence of highly damaged "hedgehog" cells was below 15% of the total number of cells counted. In contrast the number of not quantifiable nuclei was increased at higher benzene concentrations (23–60% of unquantifiable nuclei, N=300). The high frequency of "hedgehogs" at the highest concentration of benzene exposure tested (0.3 ppm) made DNA damage quantification difficult and is indicative of direct benzene cytotoxicity to cells at this concentration of benzene exposure.

Table 1: Descriptive statistics of the Comet assay data (% tail intensity).
* indicates a statistically significant difference with the control.

Comment [d7]: How many replicates of which experiment are present here?

	N (Cells)	Mean	Median	Standard Deviation	Variance	*H* (Variance/Mean)
Incubator Controls (Media)	1550	4.52	0.74	9.40	70.98	20.61
Incubator Controls (Dry)	1376	6.73	3.40	11.80	135.26	18.98
Controls	1028	7.40	3.15	11.60	147.82	20.77
0.03 ppm benzene	314	11.49	3.21	15.58	243.83	21.26
0.1 ppm benzene	318	16.02*	5.53*	21.28	456.49	28.37
0.3 ppm benzene	375	12.27*	5.13*	16.90	303.75	23.29

The % tail intensity was used as a metric to quantify DNA damage in the cells. Experimental data were pooled from 5~~9~~ controls and ~~all of the~~ one of the independent~~3~~ replicates of each exposure, which consisted of 3 technical replicates for each exposure, and the findings are summarised in

Comment [d8]: Do you mean the technical replicate or the exposure replicate?

Table 1 and Figures 4 and 5. Each exposure event was compared with the relative control. The reported data refers to a single exposure experiment and the relative controls. Two independent replicates were performed for the ~~experiment~~0.03 ppm and 0.3 ppm exposure, obtaining similar results (~~data not shown~~Supplementary material, Table 1). Differences were observed between the absolute values of the tail intensities, however the ratios between the exposed and the control cells were found to be similar between the replicates.~~.~~ ~~A paired samples t test showed a high coefficient of correlation between the ratios (R=0.821-0.951), although due to the limited amount of replicates and data points the test was not statistically significant (*p*=0.200-0.387)~~The ratios were compared by an Kruskal-Wallis independent samples test, which retained the null hypothesis of the samples having the same distribution (*p*=0.102). An independent samples median test also retained the null hypothesis, suggesting that the medians of the ratios are similar (*p*=0.354). The observed differences of the absolute values between the independent replicates are likely due to different starting conditions of the cells. Tail intensities of each exposed sample were compared with its relative air exposed control using Friedman's 2-way ANOVA by ranks. The only statistically significant differences were observed between the samples exposed to 0.1 ppm and 0.3 ppm benzene and their relative controls. No statistically significant difference was observed between air exposed controls and incubator controls under any condition.

A clear concentration-dependent induction of DNA strand breaks following the exposure to benzene (0.03–0.3 ppm) was observed as assessed by the exposed/control ratio of mean tail intensity (Figure 6). In general, due to the high amount of "hedgehogs", the cells exposed to 0.3 ppm benzene, showed a decreased amount of DNA damage compared to the cells exposed to 0.1 ppm benzene. Linear regression analysis showed a significant correlation between the level of benzene exposure and the extent of DNA damage when the value was normalized against the relative controls (R^2=0.718~~914~~). An increase of DNA damage was observed in all the exposures, however a statistically significant difference with the control was observed only for 0.1 ppm and 0.3 ppm benzene concentrations (Figure 4). Figure 5 represents the pooled data of each exposure experiment

Comment [d9]: Add a cross-reference of where this data can be found in the SI

Comment [d10]: Between the replicates of the same exposure, or between exposures?

in a scatter plot. Data was plotted to evaluate the increase of dispersion of the readings with higher benzene exposure, indicating a generalized increase in DNA damage related to the concentration of airborne benzene. Importantly, we observed a statistically significant increase in levels of DNA-strand breaks in cells exposed to 0.1 ppm benzene were there was no evidence of direct cytotoxicity to the cells.

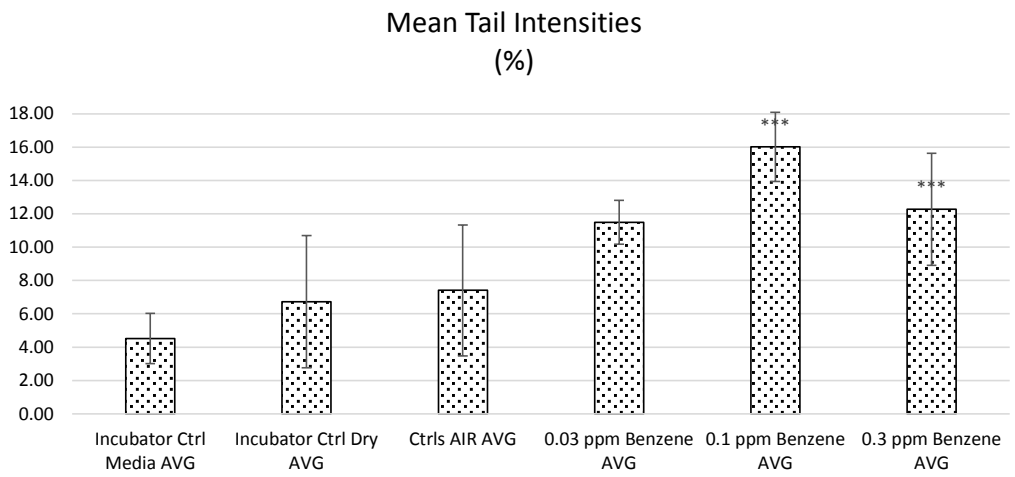

Figure 4: Mean tail intensity (%) of the control and exposed cells. Error bars represent the standard deviation of the means.
(***) indicates a statistically significant difference between the exposed sample and their relative controls.

Comment [d11]: How many replicates of which experiment are present here?

The coefficient of dispersion (*H*) (Tice et al., 2000), expressed as the ratio of variance/mean, was also calculated to further evaluate the data (Table 1). *H* is an accepted parameter used to express the dispersion of the Comet data and is sensitive to a large DNA damage response in a small proportion of cells and is also less affected by inter-experimental variability. All of these parameters were consistent with a concentration-dependent increase in DNA strand breaks following exposure of cells to benzene.

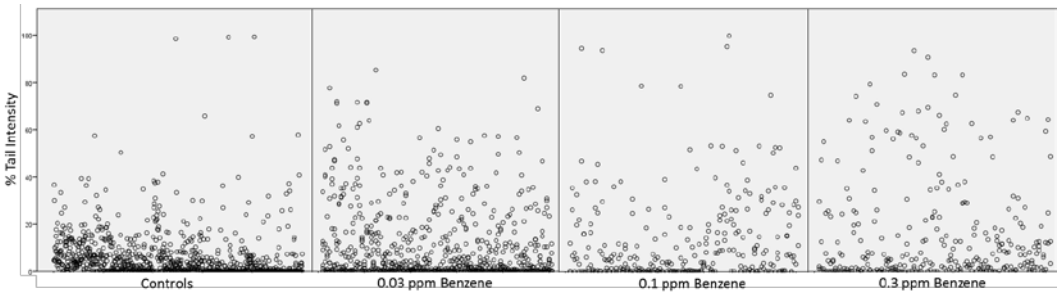

Figure 5: Graphical representation of the % tail intensities measured in the Comet assay as a function of benzene concentration. The figure includes 5 independent replicates for the controls, 2 independent replicates for 0.03 and 0.3ppm benzene, and a single independent replicate for 0.1ppm benzene.

Comment [d12]: How many replicates of which experiment are present here?

To minimise the effects of inter-experiment variability, the ratio of mean % tail intensity between the treated cells and the controls of the same exposure experiment were compared. This enhanced the difference between the low level exposure (2h 0.03 ppm) and the higher levels. The reported ratios (Figure 6) are obtained by averaging the ratio of exposed/control mean tail intensity (%) of each independent replicate. Interestingly, the difference between the ratios of the two higher conditions was smaller than the difference in the ratios observed between the 0.03 ppm and 0.1 ppm benzene, as shown in Figure 6. Moreover, the higher concentration showed a lower ratio than 0.1 ppm exposed cells, with a higher variability. This is likely due to the higher amount of unquantifiable cells in the highest benzene ~~concentration~~concentration, which made the quantification difficult.

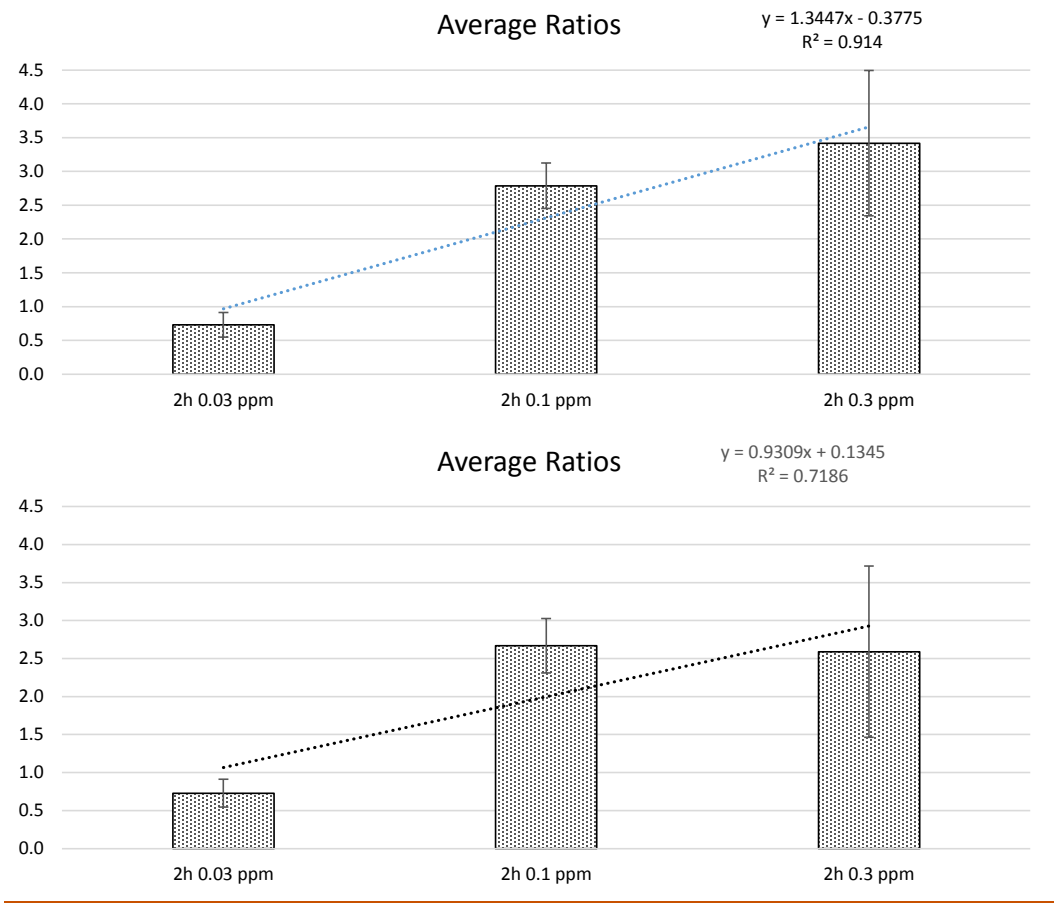

Figure 6: Average ratio between mean % tail intensities of treated cells and each of the relative controls of 3 independent replicates for 0.03 ppm and 0.3ppm and of 2 independent replicates for 0.1 ppm. Error bars represent standard deviation between the ratios of each replicate.

Comment [d13]: How many replicates of which experiment are present here?

4. DISCUSSION

The aim of this pilot study was to develop an ~~ALI~~ *in-vitro* system to study the short-term effects of airborne exposure of lung cells to toxic chemicals in a physiologically relevant model. Although carcinoma cells were used instead of primary cells, A549 cells are commonly used as a toxicological model for lung tissue. Moreover, they represent a valuable model as they can reproduce at cellular level the aromatic VOCs metabolic pathway through the ~~cytochrome P450 2E1~~CYP2E1, which is a multi-organ pathway in a complete organism (Bois et al., 1996; Hartwig,

Comment [d14]: Define

2010; Snyder and Hedli, 1996). Benzene was chosen as a test pollutant due to its known genotoxic effects. The benzene concentrations used in this work lie between typical ambient air and occupational exposure levels. Delgado-Saborit et al. (2009; 2011) report personal exposure concentrations measured in the UK of 0.15-30.2 µg m^{-3} (0.05-9.3 ppb) while in Saudi Arabia, ambient air benzene concentrations ranged from 0.09-1.1 ppb (Alghamdi et al., 2014) and in 43 Chinese cities varied between 0.7-10.4 ppb (Barletta et al., 2005). These compare with concentrations in this study of 0.03-0.3 ppm (30-300 ppb), and the European Union ambient air quality standard of 5 µg m^{-3} (1.5 ppb). On the other hand, the occupational exposure limit for benzene recommended by both NIOSH (US) and HSE (UK) is 1 ppm time-weighted average over an 8-hour shift.

As previously reported, a limitation of many *in vitro* cellular models of lung toxicity is that cells are exposed to test chemicals dissolved in cell culture media (Pariselli et al., 2009). This is physiologically unrealistic, and furthermore the media itself can have a matrix effect, and the uptake and the interaction of the pollutants with the cells does not model a real life situation (Ritter et al., 2001). The exposure vessel custom designed and used in this study proved to be a useful and reliable tool to evaluate the effect of benzene on cultured lung A549 cells. It also represents a more physiologically relevant model than exposure of cells to benzene dissolved in cell culture media. Development of permeable cell culture inserts has allowed the development of models whereby lung epithelial cells can be grown in a monolayer and the apical medium can be removed during the exposure to airborne chemicals, thus better modelling a lung epithelium and allowing exposure of cells to controlled atmospheres. This method of exposure is much closer to an in-vivo situation, providing a more realistic model for exposure studies. For logistical reasons, the gas flow rate was higher than previously reported in the literature (10 ml/min vs. 2 ml/min). However, this variable did not affect the performance of the exposure vessel or the cells negatively, as shown by the comparisons between incubator controls and synthetic air exposed controls (Figure 3 and 4).

366 However, some mechanical stress was observed in the cells at the centre of the airflow by optical
367 microscopy analyses, which can be solved in future experiments by reducing the flow rate as
368 suggested by Pariselli et al. (2006). A longer exposure (4h) of cells to synthetic air was also tested,
369 but the majority of the cells did not survive during the experiment. Overall, our findings confirmed
370 the feasibility of the experiments and the reliability of the model.
371
372 Measurement of ROS levels using the DCF assay showed a significant increase of oxidised
373 fluorescein in cells exposed to 0.3 ppm benzene for 2 hours. This finding is in accordance to
374 previously reported data that has linked benzene exposure with inflammation and ROS production
375 (Barreto et al., 2009; Mögel et al., 2011; Pariselli et al., 2009; Sørensen et al., 2003b; Wang et al.,
376 2013). Furthermore, this data is consistent with a previous report by Mögel et al. (2011) that A459
377 cells express ~~cytochrome P450~~CYP ~~-~~2E1, that is involved in the benzene metabolism and ROS
378 production. Intracellular ROS production could be related to the production of oxidising metabolites
379 of benzene such as benzoquinone as suggested by Hartwig (2010).
380
381 Metabolism of benzene has also been linked to DNA damage due to reactive benzene metabolites
382 and ROS generation, which was confirmed in the current study by the results of the Comet assay.
383 Figure 5 shows the direct correlation between the benzene concentration and DNA damage, and
384 importantly there is evidence of DNA strand breaks at concentrations that were not directly toxic to
385 the cells. One of the possible mechanisms of action could be through the known reactive benzene
386 metabolite *p*-benzoquinone. One of its cellular targets is topoisomerase-II, which is inhibited,
387 generating DNA double strand breaks (DSB) which are then repaired by homologous recombination
388 (HR) or non-homologous end-joining (NHEJ). NHEJ is thought to be more error prone than HR,
389 however DSB damage can lead to DNA aberrations by base deletions or translocations.
390 Furthermore, the metabolism of benzene through redox cycling and generation of ROS is proposed
391 as another main source of DNA damage. When hydroquinone or catechol are oxidised in the

presence of oxygen, superoxide (O_2^-) is formed, which is then transformed into H_2O_2 by superoxide dismutase. H_2O_2 is not responsible for DNA damage directly, but it increases the oxidative potential of the cell, and in the presence of transition metals ions, hydroxyl radical (•OH) is formed, which reacts with lipids, proteins and DNA. •OH is responsible for the formation of 8-oxo-dG and other nucleobase alterations due to oxidation (Halliwell and Aruoma, 1991). H_2O_2 production is also linked to an inflammatory response of which, as stated above, VOC exposure has been reported to be a trigger.

In addition to non-toxic concentrations, relatively high concentrations of benzene (compared to the concentrations that can be found in everyday life), which have been reported to have cytotoxic effects as well as genotoxic effects, were also studied. In the current study, we saw evidence of benzene cytotoxicity following exposure of cells for 2h at a concentration of 0.3 ppm as indicated by the fact that a large number of cells nuclei were not quantifiable by the Comet assay, but appeared instead as “hedgehogs”. Further evidence of that effect is represented by the tail intensity data normalised against the controls (Figure 6) and the *H* value decreasing at the higher concentration. In the 0.3 ppm exposure a significant fraction of nuclei in the Comet assay appeared as “hedgehogs” and were classified as unquantifiable (23-60% of unquantifiable nuclei, N=300 per slide), while at lower levels of benzene, the number of “hedgehogs” was negligible. Although the significance of hedgehog nuclei in the Comet assay is not entirely clear, they may represent nuclei derived from either apoptotic or necrotic cells, although other studies have shown that cells sustaining extensive DNA damage can survive and repair the damage taken (Collins, 2004). These results could provide a further insight on the interpretation of the data reported by Pariselli et al. (2009). In that study, the tests were performed after incubating the cells in complete medium for 24h after the exposure, allowing the cells to respond to the pollutants and their effects. It is known that large amount of DNA damage and bulky adducts can trigger apoptotic response (Roos and Kaina, 2006), and furthermore benzene has been shown to be capable of inducing apoptosis by

production of ROS and DNA damage in leukaemia cells (Sarma et al., 2011b). In our study, cells were analysed immediately after 2h exposure, not allowing sufficient time for the apoptotic response to happen, therefore there is the possibility that most of the "hedgehogs" we observed in the higher benzene exposure could eventually undergo apoptosis.

The Comet assay data confirmed the genotoxic activity of benzene not only in its widely acknowledged target organs (bone marrow), but also in metabolically competent cells derived from lung epithelial cells. Although the mechanism of action was not fully elucidated, generation of ROS arising from benzene exposure was observed, as well as an association between exposure to benzene and DNA damage.

In conclusion, with this pilot study we have confirmed that A549 cell line represents a valid model to test exposure and DNA damage arising from airborne exposure to benzene. Although the metabolism of benzene *in vivo* is more complex and involves different tissues, A549 cells appear to produce a response that is compatible to the one found in the target organs of those compounds *in vivo*. Further metabolomic and proteomic studies would be needed to confirm the theory, and further studies with different endpoints will be needed in order to have a better understanding of benzene metabolism and its correlation with DNA damage. Furthermore, the results obtained by studying a well known carcinogen confirmed the validity of this model for analysing not only the effects of exposure of lung cells to benzene, but also opens the way for analysing other common VOC with the same procedure, to assess the potential DNA damage caused by exposure.

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TABLE LEGEND

Table 1: Descriptive statistics of the Comet assay data (% tail intensity).
* indicates a statistically significant difference with the control.

FIGURE LEGENDS

Figure 1a: Exploded representation of the exposure vessel with all the part of the assembly.

Figure 1b: Cross section of the assembled exposure vessel, arrows indicate the gas flow. In use, the two vessels to the right are also fitted with the gas delivery system and permeable insert.

Figure 2: Detailed cross-section of the gas-cell interface, with the level of the cell culture media indicated in yellow.

Figure 3: Summary of the DCF fluorescence measurements before and after exposure. Error bars represent standard deviation
*** indicates a statistically significant difference between the pre and the post exposure.

Figure 4: Mean tail intensity (%) of the control and exposed cells. Error bars represent the standard deviation.
*** indicates a statistically significant difference between the exposed sample and their relative controls.

Figure 5: Graphical representation of the % tail intensities measured in the Comet assay as a function of benzene concentration.

Figure 6: Average ratio between mean % tail intensities of treated cells and each of the relative controls of 3 independent replicates. Error bars represent standard deviation.