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Assessment of Biological Responses of EpiAirway™ 3-D Cell Constructs vs. A549 Cells for Determining Toxicity of Ambient Air Pollution

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Abstract

Context—EpiAirway™ 3-D constructs are human-derived cell cultures of differentiated airway epithelial cells that may represent a more biologically relevant model of the human lung. However, limited information is available of its utility for exposures to air pollutants at the air-liquid interface (ALI).

Objective—To assess the biological responses of EpiAirway™ cells in comparison to the responses of A549 human alveolar epithelial cells after exposure to air pollutants at ALI.

Methods—Cells were exposed to filtered air, 400ppb of ozone (O_3) or a photochemically-aged Synthetic Urban Mixture (SynUrb54) consisting of hydrocarbons, nitrogen oxides, O_3, and other secondary oxidation products for 4 h. Basolateral supernatants and apical washes were collected at 9 and 24 h post-exposure. We assessed cytotoxicity by measuring lactate dehydrogenase (LDH) release into the culture medium and apical surface. Interleukin 6 (IL-6) and interleukin 8 (IL-8) proteins were measured in the culture medium and in the apical washes to determine the inflammatory response after exposure.

Results—Both O_3 and SynUrb54 significantly increased basolateral levels of LDH and IL-8 in A549 cells. No significant changes in LDH and IL-8 levels were observed in the EpiAirway™ cells, however, IL-6 in the apical surface was significantly elevated at 24 h after O_3 exposure.

Conclusion—LDH and IL-8 are robust endpoints for assessing toxicity in A549 cells. The EpiAirway™ cells show minimal adverse effects after exposure suggesting that they are more toxicologically resistant compared to A549 cells. Higher concentrations or longer exposure times are needed to induce effects on EpiAirway™ cells.

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Declaration of Interest

The authors report no declarations of interest.

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1. Introduction

Inhalation toxicology studies have relied on *in vivo* and *in vitro* testing to investigate the toxicity of air pollutants. These inhalation exposure studies have been used to provide insights on the interaction of an airborne substance with a biological model. While animal models for inhalation exposure studies have been considered the “gold standard,” (Akhtar et al., 2011, Paur et al., 2011) there has been a demand to find alternative models. In a 2007 report from the National Academy of Sciences entitled “Toxicity Testing in the 21st Century: A vision and Strategy” the authors called for eliminating animal usage with a combination of human cell-based tissue models, advanced analytical methods, and computational toxicology (National Research Council, 2007). In addition, the European Union has banned the use of animals in the cosmetic industry (McKim, 2014).

To meet the increased demand for alternative biological models for toxicology studies, several advances have been made to *in vitro* exposure technologies. These new exposure technologies allow cell cultures to be exposed to air-pollutant mixtures at the air-liquid interface (ALI). Under submerged conditions, the airborne pollutant is added to the culture medium and then directly applied to the cells. It is now widely accepted that the ALI exposure is a more realistic approach than exposing cells under submerged conditions. In ALI conditions, the apical surface of the cells is exposed to the air while the basolateral surface of the cells is fed with culture medium through a porous membrane (Akhtar et al., 2011, Maier et al., 2008), similar to what occurs *in vivo*. To conduct cell exposures at ALI conditions, new *in vitro* exposure technologies were developed both at research universities and commercially (Blank et al., 2006, de Bruijne et al., 2009, Aufderheide and Mohr, 1999, Aufderheide and Mohr, 2004, Lenz et al., 2009, Tippe et al., 2002, Cooney and Hickey, 2011, Aufderheide et al., 2013, Savi et al., 2008, Volckens et al., 2009, Ning et al., 2008, Zavala et al., 2014).

In parallel to the advancement of *in vitro* technology, the advances and development of new cell culture models has been significant. A large portion of published studies have used immortalized or transformed cell lines such as A549, BEAS-2B, 16HBE14o-, and Calu-3. These cell lines have received criticism as their biological functions can differ from those of primary passage, differentiated human airway epithelial cells (Akhtar et al., 2011). For this reason, the use of primary normal human bronchial epithelial (NHBE) cells is highly desired. In addition to NHBE cells grown in tissue culture plates similarly to cell lines, primary cultured cell models in 3-D matrices grown on porous membrane inserts have been developed and are commercially available. Two different human airway 3-D culture models were developed by MatTek Corp and Epithelix Sarl. MatTek developed the EpiAirway™ tissue model, while Epithelix developed the MucilAir™ model (McKim, 2014). Studies have shown that these 3-D cell constructs represent more physiologically relevant conditions than conventional immortalized or transformed cell lines (Rothen-Rutishauser et al., 2008). These 3-D culture models, in addition to conventionally grown NHBE cells, may represent appropriate replacements of *in vivo* models and replace the current use of cell lines as a more biologically relevant model of the human lung.

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Although the use of 3-D cell models is promising, minimal information is available in the peer reviewed literature evaluating their biological responses in ALI exposure conditions. There are 7 studies where EpiAirway™ cells were exposed at ALI conditions (Balharry et al., 2008, Iskandar et al., 2013, Kelly et al., 2014, Künzi et al., 2013, Mathis et al., 2013, Seagrave et al., 2007, Seagrave et al., 2010, Sexton et al., 2011). Only one study that used diesel exhaust (DE) was relevant to ambient air pollution (Seagrave et al., 2007). Similarly, only one study using the MucilAir 3-D cell model at ALI conditions that is relevant to air pollution has been published (Anderson et al., 2013). While the EpiAirway™ and MucilAir 3-D models have shown to better mimic biological responses in humans, it is difficult to assess the benefits of these models for air pollution studies due to the lack of peer reviewed studies comparing responses induced at ALI conditions in different cell culture models. In addition, various culture models can express biomarkers differently and therefore each cell model needs to be characterized to determine which biomarkers are robustly expressed and at what time-point following exposure. More studies are needed to begin to understand how 3-D culture models can be beneficial to future ALI exposure studies.

The study presented here provides the first use of the EpiAirway™ 3-D cell culture model at ALI conditions exposed to filtered air, O_3 and a particle-free air pollutant mixture. The particle-free air pollutant mixture is a Synthetic Urban Mixture (SynUrb54) consisting of 54 hydrocarbons and NO_X that have been photochemically aged. Photochemically aged atmospheres have been shown to induce cytotoxicity and elevated levels of interleukin 8 (IL-8), a pro-inflammatory cytokine, on A549 cells under similar conditions to those presented here and therefore served as a positive control atmosphere (Doyle et al., 2004, Doyle et al., 2007, Ebersviller et al., 2012a, Ebersviller et al., 2012b, Rager et al., 2011, Sexton et al., 2004). A549 cell cultures were simultaneously exposed in an effort to compare the magnitudes of various endpoints expressed by a widely used immortalized cell line with the EpiAirway™ model. Through this study we can begin to understand the sensitivity of EpiAirway™ cells relative to a well-known immortalized cell line for their implementation in future ALI exposure studies.

2. Methods

2.1 A549 immortalized cell line

A549 cells were grown on collagen-coated 12 mm diameter Snapwell membranes in complete F12-K medium. The basolateral side received 2 mL of medium whereas the apical side received 0.5 mL of medium. Cells were plated at a density of 2.0×10^5 cells per insert 48 h prior to exposure and maintained under submerged conditions in an incubator at 5% CO_2. Four hours prior to exposure, complete medium was replaced with serum-free F-12K medium. Immediately before exposures, the serum-free medium was removed in the apical side while the basolateral side was replaced with 2 mL of fresh medium to obtain the desired ALI condition during exposure.

2.2 EpiAirway™ primary cultures

The primary cells used were commercially available EpiAirway™ 3-D human-derived tracheal/bronchial epithelial cells (AIR-100-SNP, MatTek Corporation, Ashland, MA). All

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cells used were from the same donor who was a 23 year old Caucasian male, non-smoker, and had no known airway diseases. Cultures were ordered from the manufacturer one month in advanced and were grown on 12 mm diameter Snapwell membranes. These ready-to-use cultures were shipped overnight, incubated upon arrival, and maintained following the manufacturer's instructions. The basolateral side of these membranes received 2 mL of medium supplied by the manufacturer. Apical washes to remove any mucus product were conducted with Dulbecco's phosphate-buffered saline (DPBS) when replacing the basolateral medium. A final apical wash was conducted prior to exposure.

2.3 Environmental irradiation chamber and test atmospheres

The 120 m^3 (triangular-cross-section; 7.4 m by 6.0 m by 5.4 m high) Gillings Outdoor Irradiation Chamber was used in this study to generate the different test atmospheres for conducting cell exposures (see Figure 1). This chamber is located on the roof of the Gillings School of Global Public Health at UNC-Chapel Hill and is enclosed in Teflon film walls. The outdoor irradiation chamber has been previously described in detail (Ebersviller et al., 2012a, Ebersviller et al., 2012b, Lichtveld et al., 2012).

To evaluate whether the type of atmosphere contributed to differences in biological response in two cell models, we exposed cells to a single pollutant or a complex gaseous mixture. *In vitro* cultures were exposed to three different test atmospheres over the course of 2 days: 1) filtered chamber air (negative control atmosphere), 2) 400ppb of O_3 and 3) photochemically aged SynUrb54 – mixture of hydrocarbons, nitrogen oxides, ozone, and other secondary products. The composition of SynUrb54 has been previously described in detail (Sexton et al., 2004, Ebersviller et al., 2012b). Briefly, Photochemical degradation of SynUrb54 lead to the production of multiple generations of (oxidized) daughter compounds (Ebersviller et al., 2012b). Toxicity studies using A549 cells have shown that atmospheric transformation products resulting from photochemical degradation induce higher toxicity than single compounds or unreacted chemical mixtures (Ebersviller et al., 2012b, McIntosh-Kastrinsky et al., 2013, Sexton et al., 2004). An O_3 concentration for 400ppb was selected since it was previously shown (Ebersviller et al., 2012a) that this concentration produces a robust IL-8 protein increase in the culture medium similar to that observed in the photochemcially aged atmospheres.

Exposures for each condition were conducted for 4 h. Different sets of cells were used for each exposure condition. Two independent sets of exposures were conducted two weeks apart. Each week cells were exposed to air, O_3, and SynUrb54. Air and O_3 exposures were conducted back-to-back during each week. As shown in the timeline in Figure 2A, the irradiation chamber was vented with HEPA-filtered ambient air before the start of each exposure to remove any background contents. The cells were exposed to the filtered chamber air after sunset (in the dark) where no toxicological effects should be observed post-exposure. Immediately after the air exposure ended and the cells were removed from the exposure system, O_3 was injected to the chamber using an ozone generator (model OL80A; Ozone Services, Yanco Industries, Burton, British Columbia, Canada) located in the analytical laboratory below the chamber. O_3 was injected for ~40 minutes to achieve a

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concentration of 400ppb. While still in the dark, the new cells were exposed to the 400ppb of O_3.

On a separate day, the photochemically aged SynUrb54 exposures were conducted and followed the timeline shown in Figure 2B. After venting the chamber before sunrise, the SynUrb54 mix was injected into the chamber via a mixture of gas cylinder injections and liquid injections. The SynUrb54 mix remained in the irradiation chamber during the entire day (from sunrise to sunset) to allow for photochemical aging. After sunset, the cells were exposed to the photochemically aged mixture containing atmospheric transformation products; aged SynUrb54 is a particle-free atmosphere. During the first set of exposures, the SynUrb54 exposure was conducted the day after air/O_3 exposures. During the second set of exposures, the SynUrb54 exposure was conducted the day before air/O_3 exposures. Aging of the SynUrb54 mixture in the outdoor smog chamber is dependent on the weather being sunny with no/minimal clouds for photochemistry to be optimal. For this reason, the SynUrb54 exposure was conducted either a day before or after the air/O_3 exposures, when the predicted weather indicated a sunny day. After each exposure, the cells were placed in the incubator for either 9 or 24 h, as shown in Figure 2C.

During all exposures, NO and NO_2 levels were measured using a Teledyne model 9841 NOx analyzer (Teledyne Monitor Labs, Englewood, CO) while O_3 was measured with a Teledyne model 9811 monitor. For the SynUrb54 exposures, carbonyl-specific analyses with o-(2,3,4,5,6-pentafluorobenzyl) hydroxylamine chloride (PFBHA) was conducted using the previously described protocol (Ebersviller et al., 2012b, Lichtveld et al., 2012). Modified mister samplers, similar to those described by Seaman *et al.* (Seaman et al., 2006) were used to collect samples in the morning soon after SynUrb54 injection was made into the chamber and again during the cell exposures. Morning samples were obtained to observe the background carbonyl content in the chamber prior to photochemical degradation, while night samples allow us to observe the production of carbonyl-specific compounds that the cells are being exposed to as a result of atmospheric transformations. The PFBHA derivatives were then analyzed by gas chromatography/mass spectrometry (GC-MS) on a Varian 3800 GC/ Saturn 2200 Ion Trap MS.

2.4 In vitro exposure apparatus

Two identical Gas in Vitro Exposure Systems (GIVES) were used to expose cells to the test atmospheres described above. The GIVES is an 8-Liter polycarbonate chamber (Billups-Rothenberg, MIC-101TM, Del Mar, CA) and has been previously described in detail (Ebersviller et al., 2012a). They were maintained at 37°C and 5% CO_2 levels were regulated during exposure. The dew point in the environmental irradiation chamber was maintained at >17.5°C during exposures, allowing for sufficient humidity to maintain cell viability intact. A vacuum pump was used to draw air from the outdoor irradiation chamber into the GIVES at a flow rate of 1 L/min per exposure system. Immediately after exposure, cells were allowed to incubate for 9 or 24 h. A different set of cells was used for each exposure condition and time-point in this 2-day set of exposures. A second set of exposures under the same conditions and time-points was repeated.

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2.5 Biological analysis

Basolateral supernatants and apical washes with 1 mL of DPBS were collected at 9 and 24 h post-exposure for each exposure condition. For all EpiAirway™ exposures, 3 replicates per exposure condition and time-point were obtained. For all A549 exposures, 2–4 replicates per exposure condition and time-point were obtained. Data from independent sets of exposures were combined and analyzed using ANOVA followed by Tukey post-test where differences were considered significant if p 0.05. Data for all endpoints are presented as the mean ± standard error from the mean.

2.5.1 Cytotoxicity—Cytotoxicity was assessed via LDH concentrations measured in the basolateral medium and the apical washes using a cytotoxicity kit (LDH Cytotoxicity, Takara) according to the manufacturer's instructions. At each time-point, 1 mL of basolateral medium was removed from each insert, transferred to 1.5 mL Eppendorf tube, and frozen until analysis was conducted. Similarly, 1 mL of DPBS used for apical washes was frozen until analysis was conducted.

2.5.2 Cytokines—Interleukin-6 (IL-6) and IL-8 protein release in the basolateral medium and apical surfaces were measured to determine the inflammatory response via enzyme-linked immunosorbent assay kits (ELISA; BD Biosciences) according to the manufacturer's instructions.

3. Results

3.1 Chemical characterization of exposure atmospheres

Chemical measurements were taken for all test atmospheres. The NO, NO_2, and O_3 concentrations during the various exposure conditions are presented in Figure 3. During the filtered air exposure, the average concentrations measured were 0.005 ppm, 0.003 ppm, and 0.031 ppm of NO, NO_2, and O_3, respectively. The average concentrations measured during the O_3 exposure condition were 0.004 ppm, 0.004 ppm, and 0.37 ppm of NO, NO_2, and O_3, respectively. For the photochemically aged SynUrb54, cells were exposed to an average concentration of 0.001 ppm, 0.0365 ppm, and 0.266 ppm of NO, NO_2, and O_3, respectively. The chromatograms in Figure 4 show the measured carbonyl-specific products of the unreacted and photochemically aged SynUrb54 using a GC-MS.

Several carbonyl compounds produced due to photochemical oxidation were identified and they included compounds such as formaldehyde, acetone, hydroxyacetone, butanal, glycolaldehyde, glyoxal, and methylglyoxal.

3.2 Cell model responses

The cell line A549 and EpiAirway™ cells were exposed to filtered chamber air, 400ppb O_3, and photochemically aged SynUrb54. After exposures, biological samples were obtained for measurement of LDH, IL-6 protein, and IL-8 protein levels. These endpoints were measured in cells exposed to O_3 or aged SynUrb54 compared to endpoint levels expressed after filtered air exposure. To ensure that filtered chamber air did not induce adverse effects during exposure, A549 cells exposed to chamber air for 4 h were compared to unexposed

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cells that remained in the incubator. No significant changes were observed (data not shown), therefore chamber air served as the negative control atmosphere.

3.2.1 LDH—Cytotoxicity results (Figure 5) show statistically significant increases in LDH levels at both time-points in the basolateral medium after A549 cells were exposed to O_3 or aged SynUrb54. Conversely, no increases in LDH levels in the basolateral medium was observed in EpiAirway™ cells. No elevated LDH levels were observed in the apical washes for both A549 and EpiAirway™ cells. These results indicate that both positive control test atmospheres produced similar effects in A549 cells as measured via its basolateral medium.

3.2.2 IL-6 and IL-8—Statistically significant changes were observed for IL-8 (Figure 6) at 9 and 24 h post-exposure in the basolateral medium, which show a similar trend to those seen with basolateral LDH levels in A549 cells. The only statistically significant increase in the apical washes for IL-8 was observed at the 9 h time-point after O_3 exposure. No changes were observed in both the basolateral medium and apical washes for EpiAirway™ cells. It is worth noting that background levels (from air exposures) of IL-8 were ~100× higher in EpiAirway™ cells compared to A549 cells, suggesting significantly greater baseline production of this cytokine. No changes were observed in basolateral or apical IL-6 (Figure 7) in A549 cells. No significant changes were seen with EpiAirway™ cells with basolateral IL-6. However, analysis of the apical washes indicates that levels of IL-6 were significantly elevated after O_3 exposure at the 24 h time-point only. It is worth noting that the cytokine concentrations are similar in both the apical and basolateral sides; contrary to what was observed with IL-8.

Discussion

The study presented here aimed to provide insights on the biological response of EpiAirway™ cells and how they compare to the conventional A549 cell line following ALI exposure. Based on our results, LDH and IL-8 protein are sensitive markers of cytotoxicity and inflammation, respectively, for A549 cells as they are robust and easily detected at different time-points following exposure. This agrees with previous studies conducted with the UNC environmental irradiation chambers (Doyle et al., 2004, Doyle et al., 2007, Ebersviller et al., 2012a, Ebersviller et al., 2012b, Lichtveld et al., 2012, Sexton et al., 2004). The EpiAirway™ cells showed no changes in LDH and IL-8 after exposure to O_3 and SynUrb54 at any time-point. It is worth noting that the background levels of LDH, IL-6, and IL-8 proteins in the basolateral medium of EpiAirway™ cells were much higher than those found in A549 cells, indicating much greater baseline release of these mediators. Baseline secretion of LDH was 2–3 times higher in the EpiAirway™ cells as compared to A549 cells. For the cytokines, the EpiAirway™ cells secreted about 16× more IL-6 and 100× more IL-8 than A549 cells. Thus, baseline metabolic activity and production of specific cytokines appears to be much higher in EpiAirway™ cells, potentially making pollutant-induced increases in these markers more difficult to assess.

The lack of cytokine and LDH response of the EpiAirway™ cells from these types of exposures was not surprising as these models contain ciliated and mucus-secreting goblet cells and basal cells that likely make these models more resistant to air pollutants. Although

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apical washes to remove any mucus prior to exposures were conducted, if any mucus was left behind or produced during the 4 h exposure period, it provided a protective layer containing protective mediators such as glutathione and other antioxidants. The interactions between its mixed-cell phenotype makes the EpiAirway™ cells a more biologically relevant model compared to a mono-layer culture (BéruBé et al., 2010, Balharry et al., 2008). It has been shown in 16HBE14o-1 cells, a human bronchial epithelial cell line, that IL-6 and IL-8 are secreted apically and secretion is always greater in the apical side (Chow et al., 2010). Apical washes on EpiAirway™ cells were conducted and analyzed in an effort to determine if proteins remained in the apical surface due to tight junctions preventing the proteins from diffusing into the basolateral medium. IL-8 was measured in the basolateral medium at levels ~100× higher than the apical surface. For IL-6, the levels measured in the basolateral medium and apical surface were similar. However, the only significant change in EpiAirway™ cells occurred by measuring secretion of IL-6 in the apical surface, suggesting that analysis of apical washes can be a suitable method to evaluate cytokine secretion.

Our results showing that EpiAirway™ cells are more toxicologically resistant also agree with the findings by Balharry et al., 2008 where it was concluded that EpiAirway™ cells exposed to various tobacco smoke components were 10–100× more toxicologically resistant than conventional immortalized or transformed monolayer cells. This is in contrast to other studies which have shown O_3-induced cytokine responses in differentiated NHBE cells at O_3 concentrations comparable to what was used in this study (Ahmad et al., 2011, Damera et al., 2009, McCullough et al., 2014). It is important to note that in these other studies the amount of time the NHBE cells were maintained at ALI conditions to differentiate prior to exposure varied from a couple of days to 2 weeks. In contrast, the EpiAirway™ cells were maintained in ALI conditions for 30+ days prior to their use.

Our findings with the EpiAirway™ cells are similar to those where EpiAirway™ cells were exposed to DE at ALI conditions (Seagrave et al., 2007). In that study, a comprehensive panel of endpoints were evaluated after cells were exposed under ALI conditions to 3 mg/m^3 of DE using a Cultex® exposure sytem. While Seagrave et al. (2007) observed minimal changes 1 h post-exposure, cells ultimately recovered after 21 h post-exposure. It was also noted that baseline levels of IL-6 in the basolateral medium ranged from 100–350 pg/ml, whereas IL-8 baseline levels in the basolateral medium were very high at 14,500 ± 3,000 pg/mL. The cytokine baseline levels observed with our EpiAirway™ cells were lower, which could be due to our inserts requiring a volume 4 fold higher. We measured IL-6 baseline levels at 91±40 pg/ml and IL-8 baseline levels 5,300±2200 pg/ml. The baseline levels we observed here with our EpiAirway™ cells are comparable to those observed in previously published studies where differentiated human airway epithelial cells were used (Becker et al., 2004, Fulcher et al., 2009, Jeannet et al., 2015, Schneider et al., 2010). As there is only 1 other study with EpiAirway™ cells looking at the effects of ambient air pollutants (i.e., DE) at ALI conditions, there is no conclusive evidence that a particular endpoint or time-point after exposure has been identified.

The use of EpiAirway™ cells in ALI conditions for air pollution toxicity studies is promising. Future studies, however, should aim to develop dose-response curves using various ambient air pollutants and other assays that can extrapolate the effects observed *in*

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vitro to those observed in humans, similar to those presented by Hatch et al. (2014). The advantage of purchasing these ready-to-use cells and their relatively long shelf life will allow researchers to conduct studies that were otherwise impractical with immortalized or transformed cell lines. With these cells, repeated exposures to pollutants at atmospherically relevant concentrations can be conducted. The use of EpiAirway™ cells, like any other cell culture model, has some limitations. The appropriate ALI exposure conditions for these cells have not been established, while the purchasing lead time can be an initial factor deterring researchers from their use. In addition, large sample sizes or prolonged studies could hinder the ability to test cells from matched donors. In contrast, an advantage to these EpiAirway™ cells can be cultured from diseased donors to study the effects from exposure to air pollutants in diseased populations (e.g., COPD patients, asthmatics, etc.). Nevertheless, EpiAirway™ cells have the potential to provide new insights into the toxic effects of air pollution in humans.

Conclusion

EpiAirway™ cells, under the conditions tested in this study, are more resistant to pollutant-induced inflammation and cytotoxicity than A549 cells. More studies, however, are needed to fully characterize the EpiAirway™ models for use at ALI conditions with atmospherically relevant pollutants at various concentrations. It is also important to emphasize the need for future studies to be conducted with repeated daily doses (i.e. 2 h per day for 5 days during multiple weeks). This would be possible as the EpiAirway™ models have been maintained for more than 40 days after they are received from the manufacturer (Dabin and Dayle A, 2011). By doing so, we can begin to compare these repeated, multi-day exposures to the multi-hour exposures that are typically conducted with immortalized or transformed cell lines.

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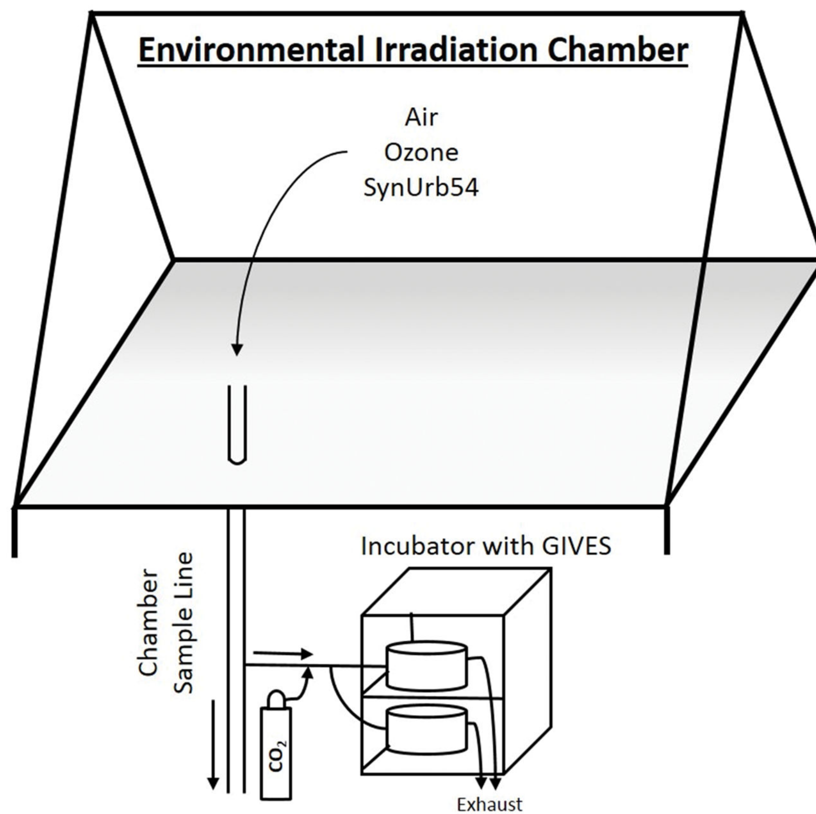

Figure 1.

Schematic of environmental irradiation chamber with *in vitro* exposure system. The environmental irradiation chamber is located on the rooftop of the Gillings School of Global Public Health at UNC-Chapel Hill. Directly below the chamber an analytical chemistry laboratory allows for direct sampling of the chamber contents via sample lines connected through the rooftop. The *in vitro* exposure system is located inside the laboratory.

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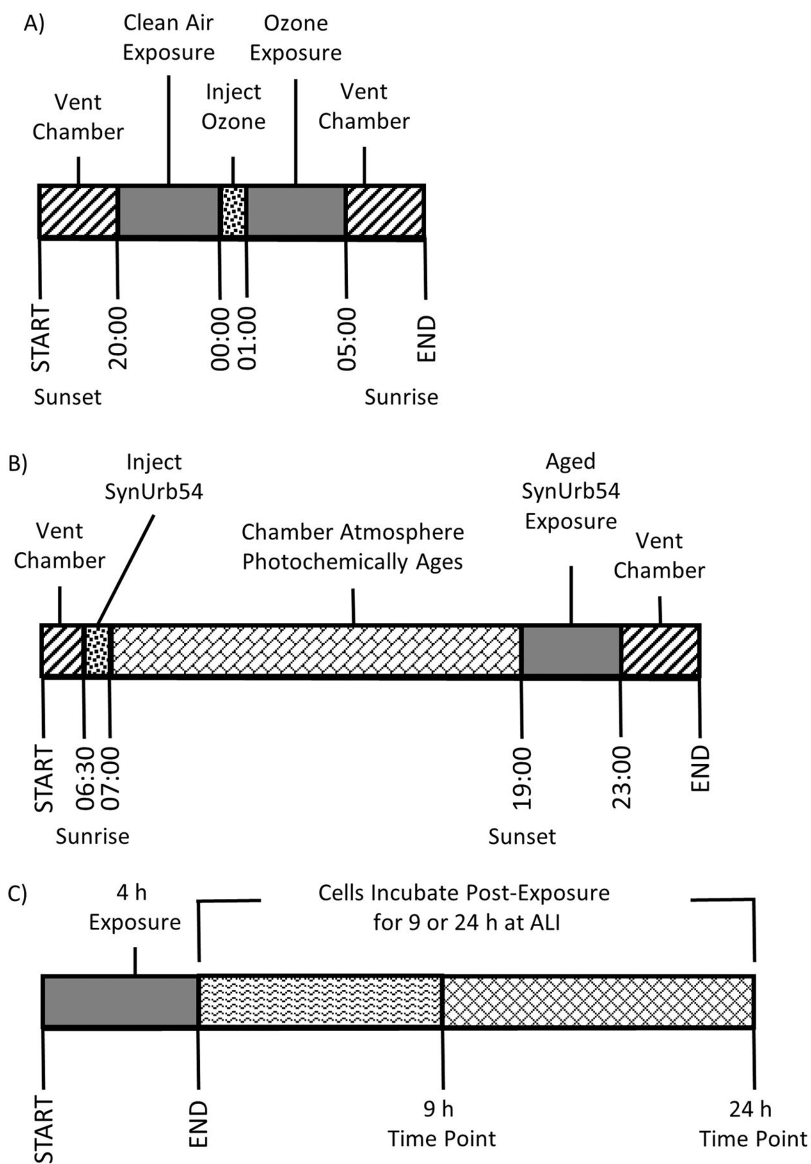

Figure 2.

Timeline of A) Air and ozone exposures, B) Photochemically aged SynUrb54 exposures and C) Cell exposure and post-exposure procedures. All exposures were conducted for 4 h.

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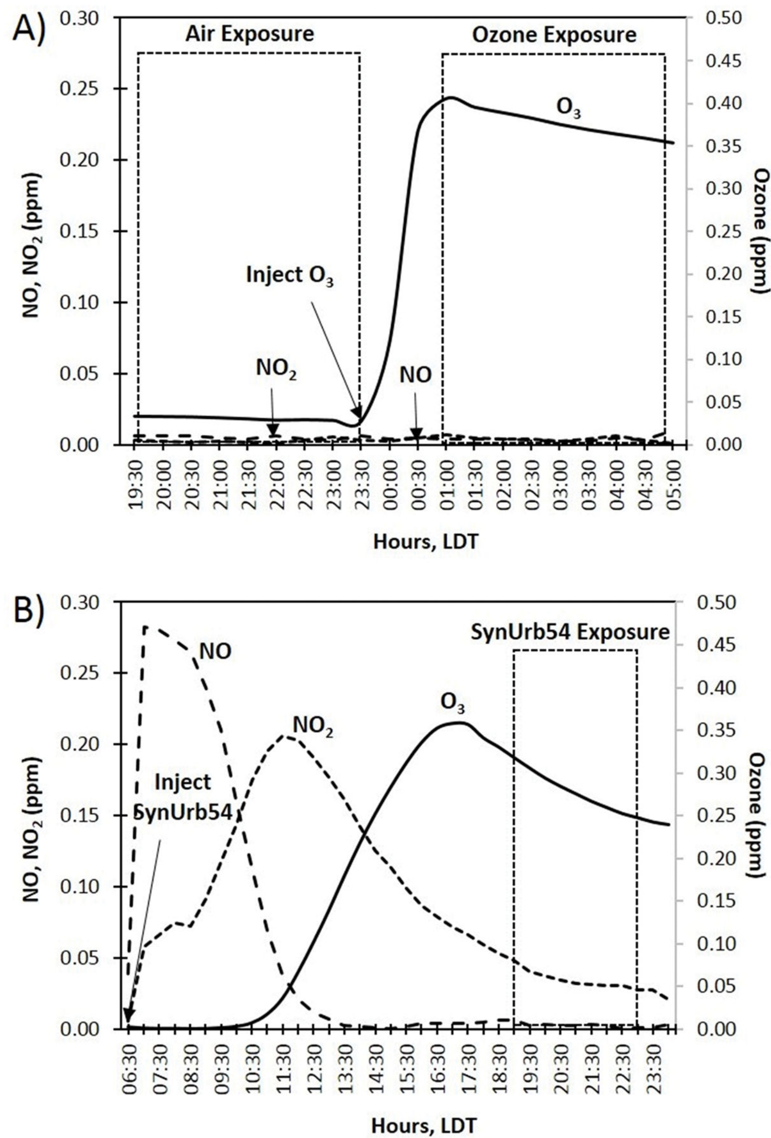

Figure 3.

NO, NO_2, and O_3 measurements during A) air and ozone exposures and B) SynUrb54 exposures. Concentrations and exposure times presented correspond to exposures conducted in week 1. Concentrations and exposure times were similar in week 2.

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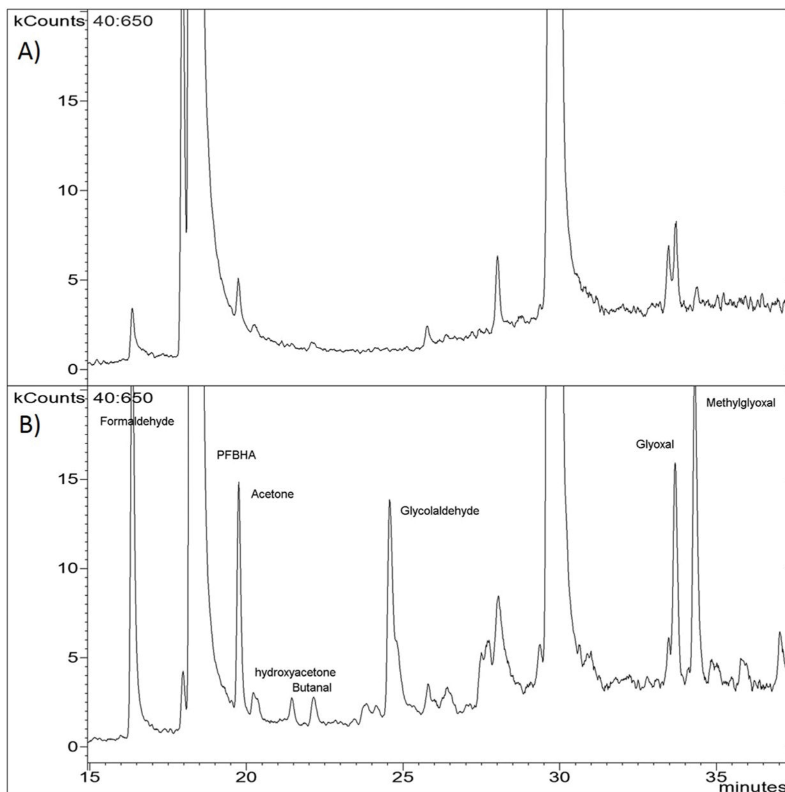

Figure 4.

GCMS chromatograms of carbonyl-specific analysis of A) fresh (morning) and B) aged (night) SynUrb54. Several peaks representing specific carbonyl compounds have been identified in the aged sample. Chromatograms shown are from exposures conducted in week 1. Similar chromatograms were obtained for week 2.

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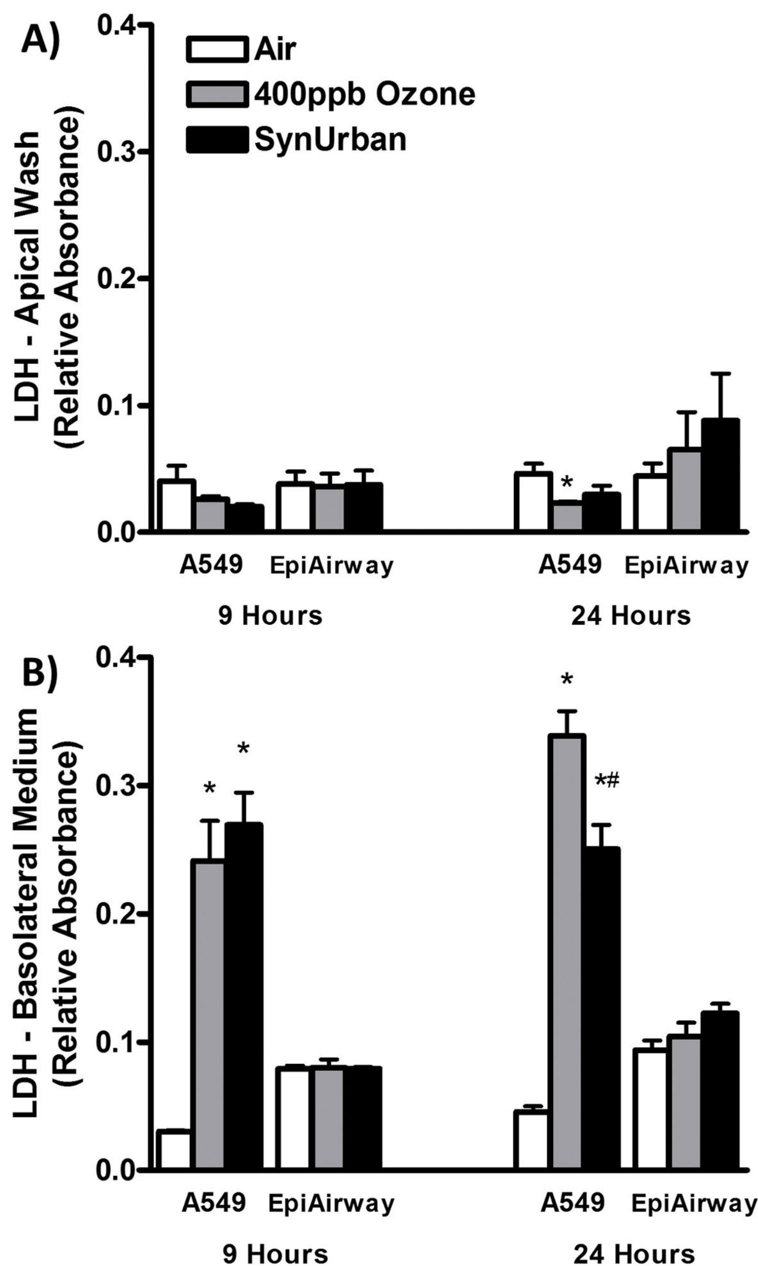

Figure 5.

Cytotoxicity results at 9 and 24 h post-exposure for A) apical washes and B) basolateral medium. O_3 and aged SynUrb54 induced statistically significant increases in LDH compared to air in A549 cells in the basolateral medium. In the apical wash, a significant decrease in LDH is observed at 24 h only after O_3 exposure when compared to clean air exposures but not SynUrb54. No statistically significant changes were observed with EpiAirway™ cells. An asterik (*) represents significant difference over air exposure. A pound sign (#) represents significant difference in SynUrb54 compared to O_3.

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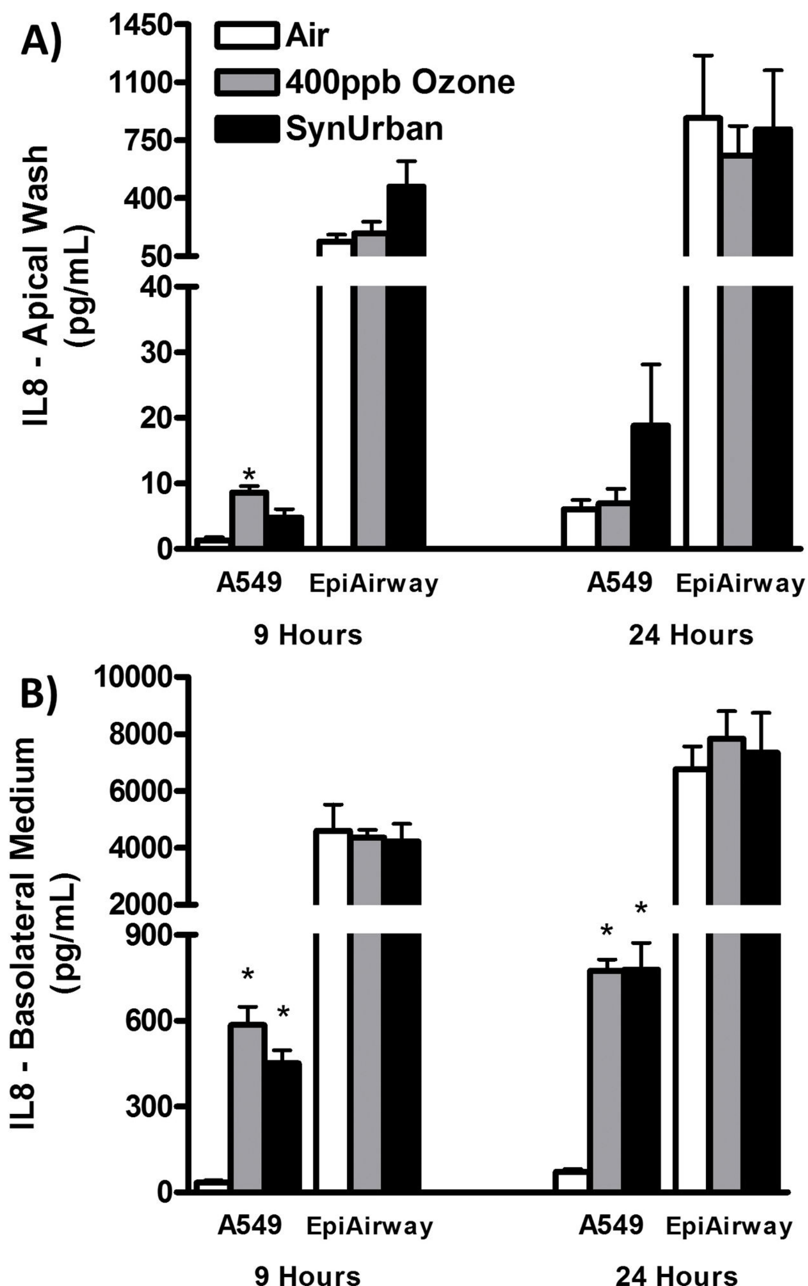

Figure 6.

IL-8 expression results at 9 and 24 h post-exposure for A) apical washes and B) basolateral medium. O_3 and aged SynUrb54 induced statistically significant increases in IL-8 measured in the basolateral medium compared to air in A549 cells. The only significant increase in IL-8 in the apical wash occurred at 9 h after O_3 exposure. No statistical significant changes were observed with EpiAirway™ cells after O_3 and SynUrb54 exposures when compared to air exposures. Background (Air) levels in EpiAirway™ cells were ~100× compared to A549 cells. An asterik (*) represents significant difference over air exposure. A pound sign (#) represents significant difference in SynUrb54 compared to O_3.

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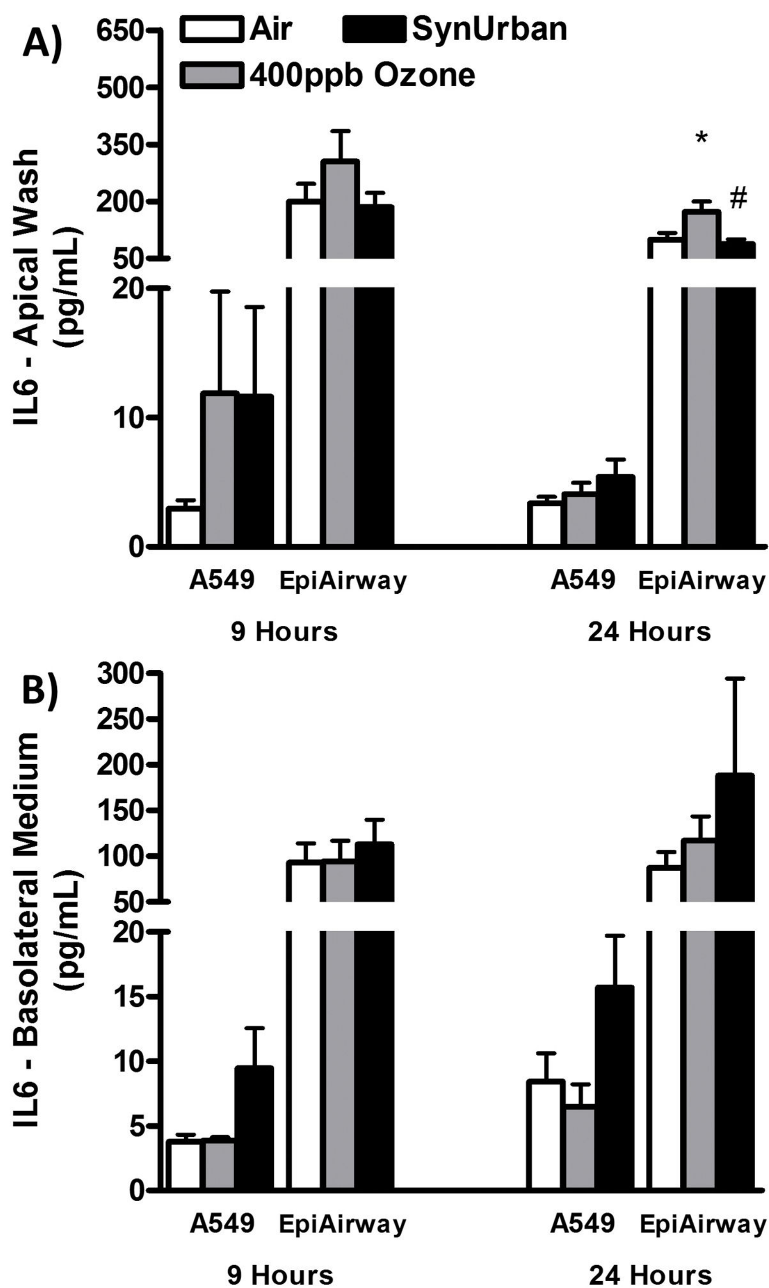

Figure 7.

IL-6 expression results at 9 and 24 h post-exposure for A) apical washes and B) basolateral medium. No IL-6 changes were observed in A549 cells after exposures in either the basolateral medium or apical washes. Analysis of the apical washes of EpiAirway™ cells indicate that levels of IL-6 were significantly elevated after O_3 exposure at the 24 h time-point only. An asterik (*) represents significant difference over air exposure. A pound sign (#) represents significant difference in SynUrb54 compared to O_3.