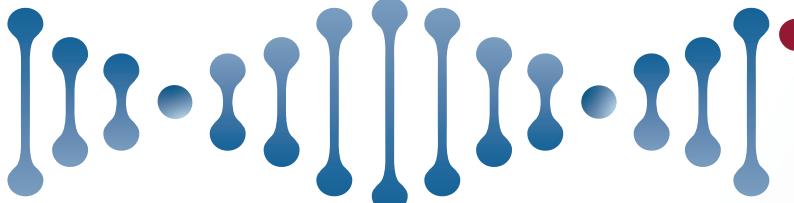


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In this issue, Jaclyn Goodrich and colleagues report on the impact of wildland-urban
interface firefighting on miRNA and DNA methylation changes

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RESEARCH ARTICLE

Characterizing the Pulmonary Toxicity and Potential Mutagenicity of Formaldehyde Fumes in a Human Bronchial Epithelial Tissue Model

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Keywords: duplex sequencing | formaldehyde (FA) | in vitro fume generation and exposure | in vitro human air-liquid-interface (ALI) bronchial epithelial tissue model

ABSTRACT

Formaldehyde (FA) is a highly reactive aldehyde that is regarded as an inhalation hazard and human carcinogen. Herein, we report a follow-up study evaluating the effects of exposure duration on the toxicity and mutagenicity of FA using a human in vitro air-liquid-interface (ALI) airway tissue model. Previously we exposed ALI cultures to 7.5, 15 and 30-ppm FA fumes 4 h/day for 5 days; currently, we have increased the exposure duration of cultures exposed to 7.5 and 15 ppm FA to 5 days/week for 4 weeks, followed by a 28-day recovery. Due to its toxicity, cultures exposed to 30 ppm FA were treated for 5 days, followed by the recovery. Tissue responses were evaluated following the treatment and recovery. DNA damage was measured using the Comet-Chip assay after 3 days of exposure, and mutagenesis was evaluated by duplex sequencing following the recovery. The toxicity detected following the 4-week exposure was similar to that seen previously with the 5-day exposures: both 7.5 and 15 ppm FA induced moderate decreases in tissue integrity, FANCD2 DNA-repair enzyme expression and IL-6 release, and moderate increases in IL-1RA release. Effects on cell proliferation, ciliary function and tissue structure were minimal. Additionally, neither the 4-week exposure to 7.5 and 15 ppm FA nor the 5-day exposure to 30 ppm FA induced DNA damage or mutations. Using this experimental design, exposure of human ALI airway cultures to FA fumes does not produce genotoxicity or mutagenicity, even when exposures are conducted over a 28-day period.

1 | Introduction

Formaldehyde (FA) is a highly reactive, water-soluble aldehyde that is ubiquitously present in the human body and the

environment at very low concentrations. FA is synthesized endogenously in the human body through several metabolic pathways, including via amino acid and methanol metabolism, lipid peroxidation, and P450-dependent demethylation (Chen

Yuan Le and Baiping Ren contributed equally to this work.

et al. 2024). In addition, FA gas and vapor have long been used for area decontamination and sterilization (Luecken et al. 2018) and as a monomer or additive in the manufacture of various products, for example, textiles, flooring, and plastics, as well as a preservative in antiseptics and cosmetics (Salthammer et al. 2010; NTP 2021). Furthermore, FA fumes are produced as a byproduct of fires, cigarette smoke, and automotive exhaust (Luecken et al. 2018). Occupational exposure to FA is a health concern for over 2 million people (USDL 2009). Research to comprehensively understand the dose-response relationships for the adverse health outcomes from FA fume exposure has a critical role in finding ways to mitigate its toxicological effects on humans.

The sensitizing and irritating effects of inhaled FA fumes produce a multitude of toxic responses in the human respiratory system, such as rhinitis and obstruction in the upper respiratory tract, as well as chest pain and dyspnea in the lower respiratory tract (Solomons and Cochrane 1984). The carcinogenicity of inhaled FA was demonstrated by its association with nasopharyngeal cancer in rodent experiments and in epidemiological studies on industry workers (Olsen and Asnaes 1986; Boysen et al. 1990; IARC 2006). Long-term exposure to FA induced leukemia in rats, but there is insufficient evidence for FA causing lymphohematopoietic cancers in humans (Vaughan et al. 2000; Beane Freeman et al. 2009; NTP 2021). In addition, forestomach and gastrointestinal tract tumors were observed in rats treated with FA orally, but the results were variable (IARC 2006). Based on sufficient evidence from both rodent and human studies, FA is classified as a Group I carcinogen by the International Agency for Research on Cancer (IARC) and a known carcinogen by the National Toxicology Program (NTP) (IARC 2006; NTP 2021).

FA damages DNA directly through the formation of DNA adducts and DNA-protein-crosslinks (DPX), and also increases oxidative stress that potentially can damage DNA indirectly (NTP 2021; Umansky et al. 2022; Bernardini et al. 2020). Continuous oxidative damage stimulates cell turnover, facilitating the fixation of DNA damage that leads to mutations (Kerns et al. 1983). Mechanisms of actions (MoAs) for FA genotoxicity are well-documented in bacteria, yeast, and mammalian cells (Kawanishi et al. 2014). In general, FA induces point mutations and frameshifts in *E. coli* and mammalian cells, as well as chromosome aberration (CA) in mammalian cells (Kawanishi et al. 2014; Crosby et al. 1988; Merk and Speit 1998; Ma and Harris 1988). However, in vivo studies have generated inconsistent results for FA-induced genotoxicity, possibly due to the effects of the route of exposure on tissue sensitivity and species-specific effects. For example, inhalation of 15 ppm FA for 8 weeks induced CA in rat pulmonary lavage cells, but not in bone marrow (Dallas et al. 1992). Micronucleus (MN) formation also was observed in the GI tract of rats after the oral administration of FA, but not in mice (Migliore et al. 1989).

Most rodent studies evaluating the cytotoxicity and genotoxicity of FA fumes have been conducted at relatively high doses (e.g., a 2-year study by Kerns et al. employed a dose of 14.3 ppm FA), whereas studies of occupationally exposed human populations found that moderate to severe nose and throat irritation were observed at airborne concentrations of 2.0–3.0 ppm FA (Kerns et al. 1983; Solomons and Cochrane 1984; Heck et al. 1990; Paustenbach et al. 1997; Vaughan et al. 2000). In addition to

effects on the respiratory system, long-term FA exposures affect distant locations: both MN and sister chromatid exchange (SCE) induction was observed in blood samples of workers exposed occupationally to FA (Costa et al. 2008, 2015). Thus, it appears relevant to investigate FA toxicity in tissues other than those of the nasal cavity.

One disadvantage of rodent models is their poor predictivity for human responses, particularly when applying OECD guidelines to access acute inhalation toxicity (Movia et al. 2020). Due to the differences in the anatomy and physiology of their respiratory systems, rats and mice appear to be hyper- and hyporesponsive to FA exposure, respectively (Kane and Alarie 1977; Chang et al. 1981; Kerns et al. 1983; Casanova et al. 1991). As obligate nose breathers, rats receive higher concentrations of FA and consequently higher levels of DPX in the nasal cavity than species breathing through both the mouth and nose, including mice and humans (Chang et al. 1981; Casanova et al. 1991). In addition, higher respiratory rates may increase nasal mucosal exposure to FA, further enhancing the toxic response in rats (Casanova et al. 1991; Kane and Alarie 1977).

Recently, human in vitro cultures have been used as an alternative for investigating FA toxicity. Exposures to FA increased the expression of genes related to DNA damage, including *GADD45A*, *GADD45B*, and *ATF3* in primary human nasal epithelial cells (HNECs) (Neuss et al. 2010). One hour exposure to FA also induced DPX in both HNECs and human lung A549 cells, and this DNA damage was completely repaired after 24 h via uncharacterized DNA repair mechanisms (Zhong and Hee 2004; Speit et al. 2008; Neuss et al. 2010).

In this present study, we have evaluated FA toxicity in organotypic cultures derived from human large airway tissue. The well-differentiated primary human air-liquid-interface (ALI) tissue model possesses the tissue structure and many of the functional responses of the human airway epithelium and thus may emulate *in vivo* human tissue responses better than traditional monolayer cell cultures (Upadhyay and Palmberg 2018). Disease-relevant responses, such as cytokine release, ciliary beating frequency (CBF), mucin homeostasis, and tissue morphological changes can be measured in human ALI airway cultures (Cao et al. 2021).

In a previous study, we exposed human ALI airway cultures to 7.5, 15, and 30 ppm FA 4 h per day for 5 consecutive days (Ren et al. 2022). Even though our human ALI airway cultures are not derived from nasal tissue, we found that FA elicited a dose-responsive array of toxic responses that were similar to those reported for nasopharyngeal cancer, including the induction of squamous cell differentiation in cultures treated with 30 ppm FA (Ren et al. 2022).

In this current study, we extended the 7.5 and 15 ppm FA exposure duration from 5 days to a total of 4 weeks (5 exposures/week for a total of 20 days of exposure) and explored the effect of this longer exposure period on the toxicity of FA fumes that elicited marginal to moderate toxicity in our prior study. In addition to the cellular and tissue responses investigated by Ren et al. (2022), we used the Comet-Chip assay and error-corrected duplex sequencing (DS) analysis to assess the genotoxicity and

mutagenicity of the extended treatments to 7.5 and 15 ppm FA and 5 days exposure to 30 ppm FA. Given that mutations produced by ethyl methanesulfonate (EMS) accumulate with repeated dosing of human ALI airway cultures (Wang et al. 2024), we hypothesized that the extended repeated dose exposure at 7.5 and 15 ppm would potentiate any mutagenicity produced by FA at these levels of exposure.

2 | Materials and Methods

2.1 | FA Fume Generation and Exposure of the ALI Cultures

Formaldehyde liquid (formalin), stabilized with 10%–15% methanol, was purchased from Thermo Fisher Scientific (Waltham, MA, USA). FA fumes were generated using the VITROCELL Spiking System (Walldkirch, Germany) and cell exposures were conducted using the VITROCELL 24/48 in vitro Exposure System (consisting of the Dilution System and Exposure Module). This system was validated for FA fume exposure in our previous study (Ren et al. 2022); identical methods and reagents

were used in this current study. After evaporation from the liquid solution, FA vapors or fumes (30 ppm; hereinafter referred to as the high concentration) were serially diluted by ultra-zero synthetic air (hereinafter referred to as clean air; NexAir, Memphis, TN, USA), first to 15 ppm (mid concentration) and then to 7.5 ppm (low concentration). FA fumes were guided by vacuum through the aerosol trumpet of the Exposure Module to the apical surface of ALI cultures at a flow rate of 2.0 mL/min.

During the exposure, the human airway ALI cultures were maintained in PneumaCult-ALI Maintenance Medium (STEMCELL Technologies, Seattle, WA, USA), pre-warmed to 37°C. Cultures were exposed to FA fumes 4 h every day, 5 days per week for 4 weeks (a total of 20 exposure days over a 28-day period), followed by a 28-day recovery period for the low and mid concentration groups (Figure 1A) or 5 consecutive days for the high concentration group, followed by a 28-day recovery period (Figure 1B). Key tissue responses were assessed at the indicated timepoints for the low and mid concentration groups during the exposure phase. Note that endpoints analyzed by nondestructive methods (e.g., TEER, CBF, LDH release) were performed after 5 days of exposure (T5) in this study for conformation of our

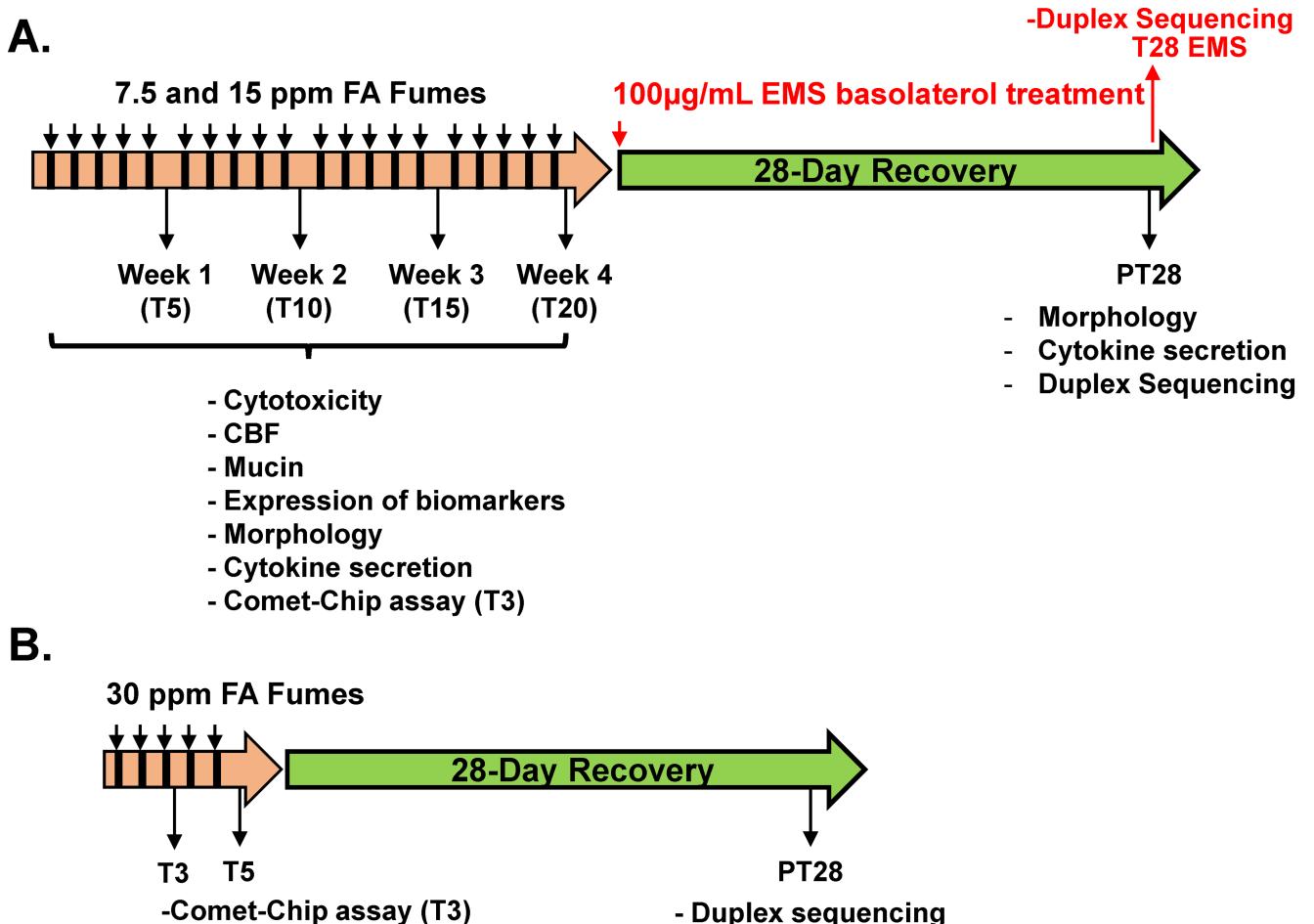


FIGURE 1 | Schematic of ALI airway model exposure to FA fumes. (A) The ALI cultures were exposed to the low (7.5 ppm) and mid (15 ppm) concentrations of FA fumes 4 h per day for 5 days for a total of 20 exposure days over a 28-day period, followed by a 28-day recovery. (B) The ALI cultures were exposed to the high concentration of FA fumes, that is, 30 ppm, for 5 consecutive days, followed by a 28-day recovery. Comet-Chip assay was processed after 3-day of exposure, while duplex sequencing was performed at the end of the recovery. Cell and tissue responses were measured at various times where feasible throughout the study. “T” denotes the number of treatments administered.

previous observations (Ren et al. 2022); while measurements at T5 that required consuming cultures (e.g., immunohistochemistry staining) were not performed in this current study. Gene mutations were assayed at the end of the 28-day recovery phase (PT28) for all three concentration groups. Concurrent clean air-exposed ALI cultures were included as the vehicle control.

The real-time concentration of FA fumes generated during the daily 4 h exposure was monitored using FITR (Gasmet, Vantaa, Finland), with a 0.5 L/min flow as previously described (Ren et al. 2022); results from the FITR analysis are shown in Supplementary Table 1. To quantify the FA fumes delivered to the apical surface of ALI cultures, FA fumes were dissolved into 110 µL biology-grade H₂O in a stainless-steel insert (VITROCELL) located in the exposure module in place of an ALI culture insert. Following 30 min of exposure, 85 µL residual H₂O was collected, quantified as described below, and the amount used in the following formula to estimate the amount of FA delivered to the culture inserts during 4 h of FA exposure:

$$\text{FA 4 h } (\mu\text{g}) = \frac{[\text{FA}] (\mu\text{g}/\text{mL}) \times 85 \mu\text{L} \times 240 \text{ min}}{30 \text{ min} \times 100 \text{ min}}$$

2.2 | Quantification of FA Fumes Using HPLC-PDA

The quantification protocol was established and validated, and data had been reported in our previous study (Ren et al. 2022). In brief, the 85 µL residual H₂O containing FA fumes were collected and analyzed using a high-performance liquid chromatography-photodiode array (HPLC-PDA) detection method. For sample preparation, FA was derivatized with 2,4-dinitrophenyl-hydrazine (DNPH; Sigma-Aldrich, St. Louis, MO, USA) in a pH 5.0 phthalate buffer, generating a stable Schiff base product after a 2-h incubation at room temperature. The reaction mixture then was injected into a Waters Alliance 2695 HPLC system equipped with a 2998 PDA detector (Milford, MA, USA), and FA derivatives were eluted through a Waters Atlantis T3 column (4 × 150 mm, 5 µm). The column setup included a mobile phase consisting of water (Mobile A) and acetonitrile (Mobile B) at a flow rate of 1.0 mL/min. Initially, the mobile phase was 40% Mobile B, followed by a linear increase up to 100% Mobile B within 4.5 min, which was maintained for 30 s. The composition of the eluting buffer was later restored to 40% Mobile B for another 30 s, and equilibrated for an additional 4 min. The elution of the derivatized FA product was monitored at a wavelength of 360 nm. Finally, the amount of FA was quantified using a quadratic regression calibration curve ranging from 1.25 to 20 µg/mL with Waters Empower 3 software.

2.3 | Human ALI Airway Tissue Model

Human ALI airway tissue models were established as previously described (Cao et al. 2018). Briefly, normal human primary tracheobronchial epithelial cells (obtained from MatTek, Ashland, MA, USA) were expanded in PneumaCult-Ex Medium (STEMCELL Technologies). Expanded cells were seeded onto 24-well PET Transwell cell culture inserts (Corning, New York, NY, USA) at a seeding density of 1.2 × 10⁵ cells/cm² and allowed

to continue proliferating in the Ex Medium. When 100% confluence was reached, the cells were fed only from the basolateral side with PneumaCult-ALI Maintenance Medium (STEMCELL Technologies) for 4 weeks, with the medium refreshed every 2 or 3 days.

2.4 | Lactate Dehydrogenase (LDH) Cytotoxicity Assay

The cytotoxicity of FA fumes was assessed by measuring the activity of LDH released into the basolateral medium using an LDH Activity Assay Kit (Roche, Indianapolis, IN, USA). The reaction mixture (100 µL) was freshly prepared by mixing Catalyst and Dye Solution at a 1:40 (v/v) ratio and incubated with an equal volume of the basolateral medium for 15 min at room temperature in the dark. The reaction was terminated by adding 50 µL of the Stop Solution. Absorbance was measured at a wavelength of 490 nm using a Synergy H4 microplate reader (BioTek, Winooski, VT, USA).

2.5 | Transepithelial Electrical Resistance (TEER)

Changes in the tissue barrier were evaluated 24 h after 5 (T5), 10 (T10), 15 (T15), and 20 (T20) repeated exposures by measuring TEER using an EVOM2 epithelial volt-ohmmeter equipped with STX2 double electrodes (World Precision Instruments, Sarasota, FL, USA). Maintenance Medium, pre-warmed to room temperature, was added to both the apical (200 µL) and basolateral compartments (400 µL) of the culture plate. TEER was measured for each culture insert at three positions spaced at 120° and an average was calculated for data analysis.

2.6 | Cilia Beating Frequency (CBF)

Cultures were equilibrated to 30°C on a heated plate before CBF measurement. Cilia movement was recorded using a high-speed camera (Ammons Engineering, Clio, MI, USA) and a Leica DMI4000B microscope (Buffalo Grove, IL, USA). Four random areas devoid of mucus clumps were captured for each culture, and CBF was automatically analyzed using the Sisson-Ammons Video Analysis System (SAVA System; Ammons Engineering). Gaussian-whole field analysis (G-WFA), an integrated feature of the SAVA System, was used for analyzing the CBF.

2.7 | Immunoblotting

Expression of select protein markers was measured using immunoblotting as described previously (Ren et al. 2022). Briefly, denatured proteins were separated on a 4%–12% NuPage Novex Bis-Tris gradient gel (Life Technologies, Carlsbad, CA, USA) and transferred onto a nitrocellulose membrane (LI-COR, Lincoln, NE, USA) using an XCell SureLock Blot Module (Life Technologies). Proteins were detected by incubating first with primary antibodies (mouse anti-acetylated α-tubulin, mouse anti-AKR1B10, rabbit anti-DNAI1, and mouse anti-CDC20B obtained from Sigma-Aldrich; goat anti-MGMT and mouse anti-β-actin purchased from Santa Cruz, Dallas, TX, USA; and rabbit

anti-ADH1C and rabbit anti-FANCD2 from Abcam, Cambridge, MA, USA) and then with the IRDye-conjugated secondary antibodies (LI-COR). Images were captured using a LI-COR Odyssey CLx Imaging System, and densitometry was conducted using Image Studio software.

2.8 | Mucin Secretion

Secretion of MUC5AC, MUC5B, and CCSP into the apical compartment of the culture inserts was measured by ELISA as described previously (Xiong et al. 2018). Briefly, 50 µL of an apical wash from each cell culture were coated onto a high-protein-binding ELISA plate (Corning), and the plate was dried overnight at 37°C. The plates then were blocked with bovine serum albumin (BSA; 5 mg/mL) for 1 h at room temperature. Mucins were detected by first incubating with primary antibodies (mouse anti-MUC5AC, Pierce, Rockford, IL, USA; mouse anti-MUC5B, Abcam; mouse anti-CCSP, Sigma-Aldrich) and then incubated with horseradish-peroxidase-conjugated goat anti-mouse antibody (Rockland, Limerick, PA, USA). Color was developed by adding 3,3',5,5'-tetramethylbenzidine (TMB; Thermo Fisher Scientific) and reactions were terminated by adding an equal volume of 2 N HCl. Absorbance was measured at a wavelength of 450 nm using a Synergy H4 microplate reader.

2.9 | Histology and Immunohistochemistry (IHC)

Morphological changes caused by FA were assessed at T20 and PT28 as described previously (Ren et al. 2022). Three individual ALI cultures per group were collected and incubated with 10% formalin for 48 h; the cultures then were transferred to 70% ethanol for processing to paraffin-embedded blocks and histological staining. Cell apoptosis was evaluated using cleaved caspase-3 antibody, as described in our previous study (Ren et al. 2022). To access percentages of proliferating cells or basal cells, Ki-67- and p63-stained nuclei (brown nuclei) were respectively counted using the Scanscope System and its integrated nuclear algorithm (Leica Biosystems, Vista, CA, USA). The percentage of periodic acid-Schiff (PAS)-stained goblet cells was quantified semi-automatically, with the total number of nuclei being counted automatically and PAS-stained goblet cells being counted manually.

2.10 | Secretion of Cytokines

Secretion of cytokines into the basolateral medium was quantified using a Bio-Rad Bio-plex Pro Human Cytokine 27-plex assay kit (Hercules, CA, USA) and by following the manufacturer's manual. As previously described (Ren et al. 2022), 50 µL basolateral medium was collected separately from replicate cultures at T1, T5, T15, T20, and PT28. The experimental samples and analyte standards were incubated with fluorescent magnetic beads for 1 h with vigorous shaking (850 ± 50 rpm), followed by three washes with kit-supplied wash buffer. Next, the beads were incubated with a mixture of 39 cytokine detection antibodies for 30-min with vigorous shaking to form protein-antibody conjugates for each cytokine analyte. Streptavidin (SA)-PE was then added and incubated for another 10 min with vigorous shaking.

Finally, SA-PE-labeled beads were resuspended into Assay Buffer (125 µL), and the fluorescence of the beads was measured using a MAGPIX system (Luminex, Austin, TX, USA). Each cytokine concentration was calculated using individual standard curves, and the average for each group was calculated.

2.11 | Comet-Chip Assay

After 3 days of FA fume exposure, ALI airway culture cells were collected and the Comet-Chip assay performed as described in the manufacturer's alkaline assay protocol (Trevigen, Gaithersburg, MD, USA). As in our previous study (Wang, Wu, et al. 2021), a single cell suspension was prepared in 1× Dulbecco's phosphate-buffered saline (DPBS; pH 7.4; Corning), and 100 µL of cell suspension per insert were transferred into

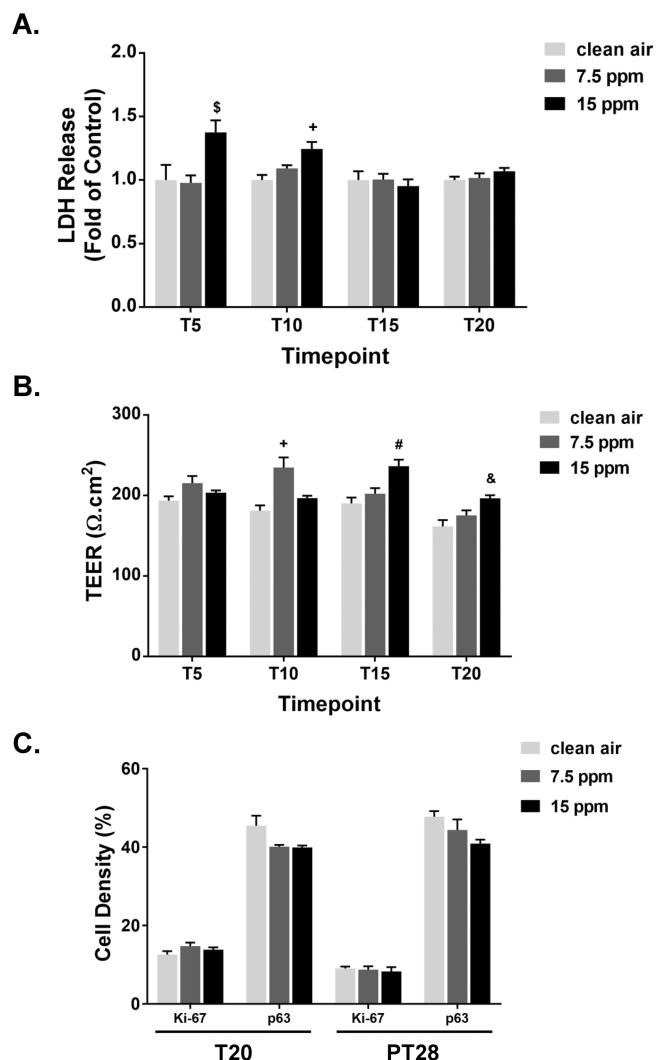


FIGURE 2 | Cytotoxicity assessment of FA fumes in airway ALI cultures. (A) Release of LDH into the basolateral media was assessed at T5, T10, T15, and T20. (B) Tissue integrity of the ALI cultures was measured at T5, T10, T15, and T20. (C) Cell proliferation and the percentage of basal cells were quantified in tissue sections immunohistochemically stained with Ki-67 and p63, respectively, at T20 and PT28. Data ($n=3$) are presented as means \pm SEM. +, #, & $p<0.05$ was considered statistically significant compared to the respective clean air-exposed control.

each of five wells of the 96-well Comet-Chip (Trevigen). The chips were incubated at room temperature for 30 min to allow the cells to settle into the microwells, the chip gently washed with 25 mL DPBS, and the wells sealed with low melting point agarose (Trevigen). To unwind DNA, the encased cells were treated with lysis solution (Trevigen) overnight at 4°C, followed by electrophoresis. The Comet-Chips then were neutralized using 0.4 M Tris-HCl buffer (pH 7.4; Sigma-Aldrich) and equilibrated using 0.02 M Tris-HCl (pH 7.4; Sigma-Aldrich). Finally, DNA was stained with 0.2× SYBR Gold (Invitrogen, Carlsbad, CA, USA) overnight at 4°C. Comet images were acquired using a Bio-Tek Cytation 5 Image Reader. Trevigen Comet Analysis Software was used to score the percentage of DNA in the comet tail (%DNA in Tail) for at least 300 cells per insert.

2.12 | Duplex sequencing (DS)

A positive control sample for DS analysis was prepared by treating ALI cultures basolaterally with 100 µg/mL ethyl methanesulfonate (EMS; Sigma-Aldrich) for 28 days as described previously (Wang, Mittelstaedt, et al. 2021).

Genomic DNA was extracted from control and experimental cultures using a QIAamp DNA Mini kit (QIAGEN, Valencia, CA, USA) and by following the manufacturer's instructions. DNA quality and concentration were measured using the Qubit dsDNA High Sensitivity Assay Kit (Invitrogen).

Duplex sequencing library preparation was performed using a TwinStrand Duplex Sequencing Human Mutagenesis kit (Rev 1.1; TwinStrand Biosciences, Seattle, WA, USA) as previously described (Wang, Mittelstaedt, et al. 2021). The human mutagenicity panel contains a balanced set of representative target sequences that are not likely to be significantly influenced by positive or negative selection. Briefly, 950 ng of DNA were fragmented using a Twist Library Preparation Enzymatic Fragmentation Kit 2.0 (Twist Bioscience), end-repaired, A-tailed, and ligated with Duplex Adapters. The DNA was then incubated with the kit-supplied library conditioning enzyme

cocktail. Each library was amplified by PCR and the target sequences captured through hybridization. The indexed libraries were pooled and sequenced on an Illumina NovaSeq 6000 (Illumina, San Diego, CA, USA) and analyzed as described previously (Wang, Mittelstaedt, et al. 2021).

2.13 | Statistical Analysis

The data are presented as means ± standard error of the mean (SEM). Data analysis was carried out using GraphPad Prism (version 10.00, La Jolla, CA). One-way ANOVA, followed by Dunnett's test, was used for assessing treatment-related effects for each time point.

3 | Results

3.1 | Cytotoxicity and Cell Proliferation in Cultures Exposed to FA Fumes

The cytotoxicity of FA fumes was assessed by measuring LDH release into the basolateral compartment. The activity of released LDH was slightly, but significantly increased by approximately 40% and 20% following 5 (T5) and 10 (T10) repeated exposures, respectively, to 15 ppm of FA fumes (Figure 2A). However, 15 (T15) or 20 (T20) repeated exposures to 15 ppm FA did not appear to be cytotoxic. Repeated FA exposure also did not impair barrier integrity, as determined by TEER measurements. Rather than decreasing barrier function, significantly increased TEER measurements were observed after 10 days of exposure to 7.5 ppm FA (T10) and after 15 and 20 days of exposure to 15 ppm FA (T15 and T20), suggesting possible adaptive responses to repeated exposures (Figure 2B).

The effects of FA fumes on overall cell proliferation and on basal cell proliferation were investigated by IHC analysis using antibodies against Ki-67 and p63, respectively. The percentages of Ki-67- and p63-positive cells were not altered by 7.5 or 15 ppm of FA fumes at either timepoint investigated (T20 and PT28; Figure 2C).

TABLE 1 | Morphological evaluation of ALI cultures exposed to FA fumes.

Morphological findings	T20			PT28		
	Clean air	7.5 ppm	15 ppm	Clean Air	7.5 ppm	15 ppm
Apoptosis	3/3 (1.0) [100%]	3/3 (1.0) [100%]	3/3 (1.0) [100%]	3/3 (1.0) [100%]	3/3 (1.0) [100%]	3/3 (1.0) [100%]
Cyst, intraepithelial	2/3 (1.0) [67%]	2/3 (1.0) [67%]	2/3 (1.0) [67%]	0/3 (0.0) [0%]	2/3 (1.0) [67%]	1/3 (1.0) [33%]
Atrophy				0/3 (0.0) [0%]	0/3 (0.0) [0%]	1/3 (1.0) [33%]
Ciliation, decreased				0/3 N/A [0%]	0/3 N/A [0%]	1/3 N/A [33%]

3.2 | Effects of FA Fumes on General Tissue Morphology

Tissue morphological changes induced by FA fumes were assessed at T20 and PT28 by histopathology (Table 1). Apoptosis, cysts, and squamous differentiation were occasionally observed, but considered unrelated to FA exposure. Minimal apoptosis, as measured by cleaved caspase-3 antibody, was present at either T20 or PT28 in the FA exposed cultures. Intraepithelial cysts were not found at T20, whereas at PT28, cyst formation was detected in two of three cultures exposed to 7.5 ppm FA fumes and one of three cultures exposed to 15 ppm FA fumes. Cysts had a round to oval shape and were either empty or contained apoptotic epithelial cells, apoptotic bodies, amphophilic to basophilic amorphous materials and/or filamentous detritus. However, due to the small sample size (less than three) and the lack of a concentration-dependent response as regards both incidence and severity, this observation may better relate to cell culture variation, rather than FA exposure. There was no evidence for squamous cell differentiation related to FA exposure at either T20 or PT28. Note that squamous cell differentiation was noted in cultures treated for 5 days with 30 ppm FA in our previous study (Ren et al. 2022); no histopathology analysis was performed on any T5 cultures in this current study.

Atrophy and decreased ciliation was related to FA exposure at PT28, but not at T20 (Table 1). One of three cultures that was exposed to 15 ppm FA had areas with atrophy, which were characterized by a loss in the organization of the cell layers and a thinning of the ALI tissues, resulting from a reduction in cell numbers and cell height. In addition, one of three cultures in the 15 ppm FA exposure group also displayed decreased ciliation, consisting of an increased number of epithelial cells with no cilia, fewer cilia, and/or cilia localized in a focal area of atrophy. Again, the sample sizes ($n=1$) for these observations were too small to definitely be representative of histological abnormalities.

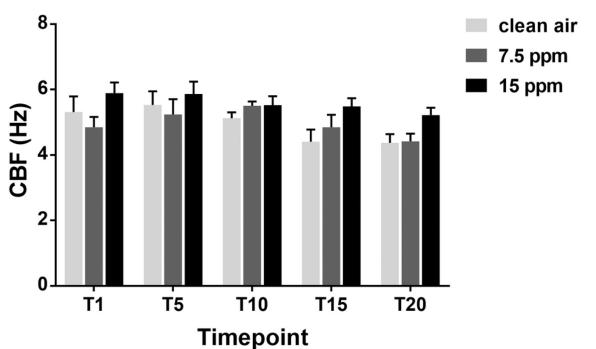
3.3 | Effects of FA Fumes on Ciliary Function and Structure

Repeated exposure of the ALI cultures to 7.5 and 15 ppm FA fumes for a total of 20 days elicited negligible effects on CBF (Figure 3A). Expression of select ciliary proteins, including acetylated α -tubulin, CDC20B, and DNAI1, also was measured at T20. There were nonsignificant decreases in the expression of acetylated α -tubulin and CDC20B in cultures exposed to 15 ppm FA fumes (Figure 3B); DNAI1 protein expression was unchanged in all FA-treated groups.

3.4 | Effects of FA Fumes on Mucin Homeostasis

Exposure to 7.5 and 15 ppm of FA fumes had no effect on the morphology (data not shown) and density of goblet cells at T20 and PT28 (Figure 4A). Secretion of three major airway mucin proteins, that is, MUC5AC, MUC5B, and CCSP, was measured in the apical washes collected at T20. There was only one significant, non-dose-related change, and considering that all fold-changes were less than 15% compared to the clean air-exposed controls (Figure 4B), the biological relevance of any alterations was considered low.

A.



B.

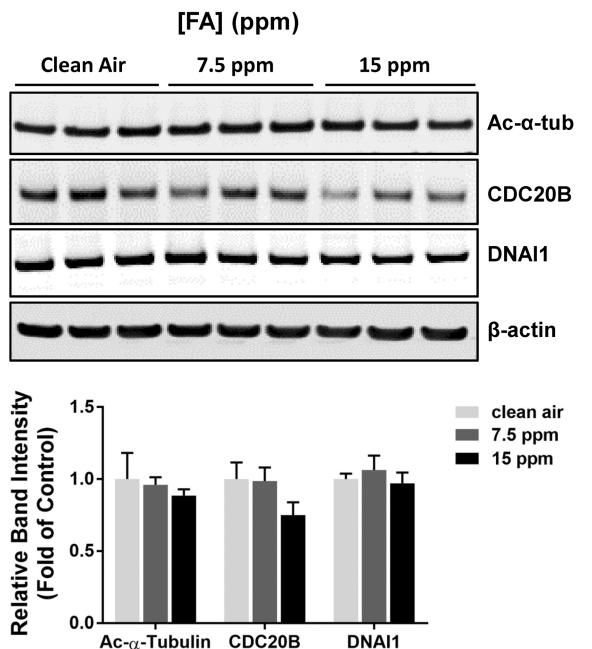


FIGURE 3 | Effects of FA fumes on ciliary function and structure of ALI airway cultures. (A) CBF was measured at T1, T5, T10, T15, T20, and TP28. (B) Protein expression of acetylated- α -tubulin, DNAI1, and CDC20B was measured at T20 by immunoblotting. Representative blots are presented in the upper panel. Densitometry data ($n=3$) are expressed as means \pm SEM.

3.5 | Effect of FA Fumes on Alcohol/Aldehyde Metabolic Enzymes

Expression of two alcohol/aldehyde metabolic enzymes, AKR1B10 and ADH1C, was measured at T20. The expression of ADH1C was not altered by exposure to either 7.5 or 15 ppm of FA fumes (Figure 5). Exposure of the ALI cultures to 15 ppm of FA fumes, however, moderately upregulated the expression of AKR1B10 by approximately 20%.

3.6 | Modulation of Inflammatory Proteins by FA Fumes

The secretion of a panel of cytokines and chemokines was measured in cultures exposed to 7.5 or 15 ppm FA fumes at T1, T5, T15, T20, and PT28. Among the inflammatory molecules screened, only the secretion of IL-1RA and IL-6 was modulated

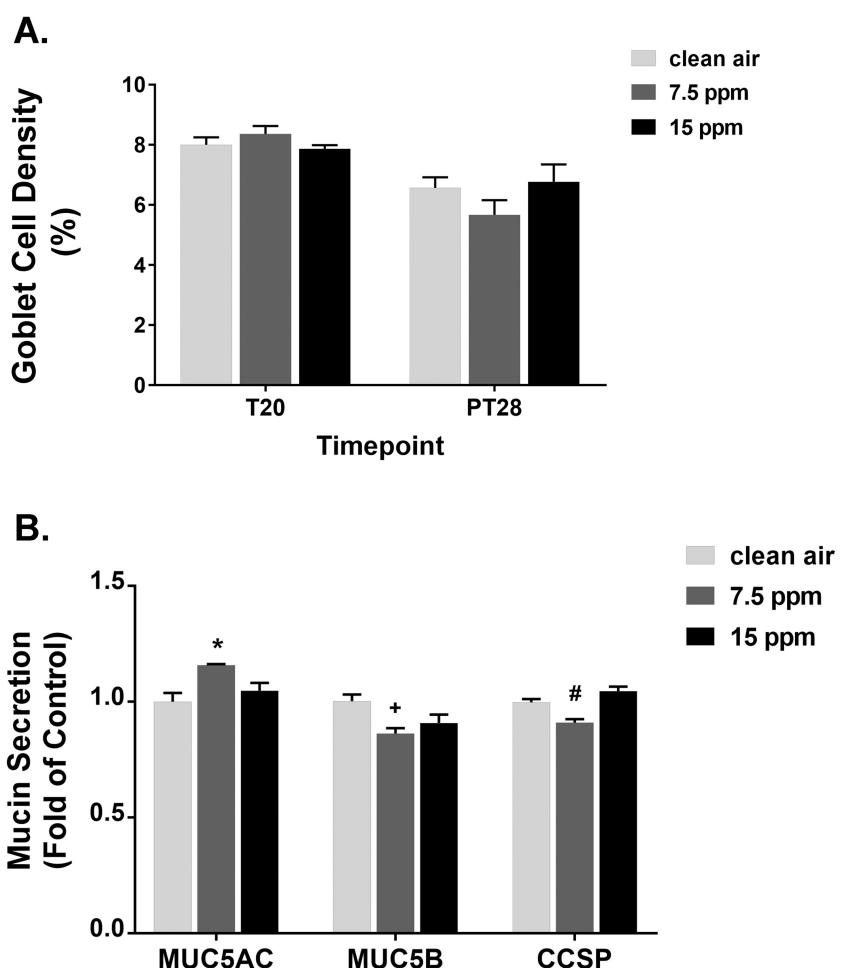


FIGURE 4 | Effects of FA fumes on goblet cell density and mucin homeostasis in ALI airway cultures. (A) Goblet cell density was quantified at T20 and PT28. (B) Secretion of MUC5AC, MUC5B, and CCSP was quantified at T20. Data ($n=3$) are presented as means \pm SEM. *, +, # $p < 0.05$ was considered statistically significant compared to the clean air-exposed control.

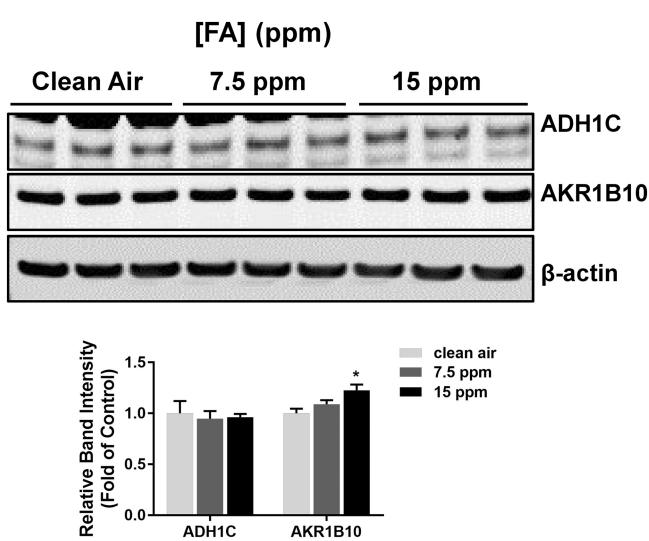


FIGURE 5 | Effect on metabolic enzymes in ALI airway cultures exposed to FA fumes. Protein expression of ADH1C and AKR1B10 was measured at T20 by immunoblotting. Representative blots are presented in the top panel. Densitometry data ($n=3$) are expressed as means \pm SEM. * $p < 0.05$ was considered statistically significant compared to the clean air-exposed control.

by FA exposure. Release of IL-1RA was increased by exposure to 15 ppm FA fumes at T1, T15, and T20 by approximately 1.3- to 1.6-fold (Figure 6A). This induction was sustained and slightly increased to approximately 2-fold relative to the clean air control at PT28.

Modulation of IL-6 secretion followed a similar temporal pattern to IL-1RA. However, instead of the stimulatory effect seen for IL-1RA secretion, FA fumes suppressed the release of IL-6. A single exposure (T1) to 15 ppm FA fumes decreased the level of IL-6 secretion by approximately 30% compared to the control air group (Figure 6B), followed by approximately 50% inhibition at T15 and T20. This inhibition was sustained following the recovery at PT28 and reached approximately 60%.

3.7 | Downregulation of Select DNA Repair Enzymes by FA Fumes

The effects of FA fumes on the expression of two DNA repair enzymes, O^6 -methylguanine (O^6 -meG)-DNA methyltransferase (MGMT) and Fanconi anemia group D2 (FANCD2), was evaluated at T20 (Fritz et al. 1991; Furukawa et al. 2005; Smogorzewska et al. 2007). MGMT is a suicide enzyme that

repairs the alkylated base, O^6 -meG (Cartularo et al. 2016). The Fanconi anemia (FA) and BRAC pathways cooperate to repair DNA damage, with mono-ubiquitination of FANCD2 leading to formation of nuclear foci for initiating the DNA repair response (DRR) pathway (Swarts et al. 2013; Nepal et al. 2017). We detected a significant, approximately 40% reduction in FANCD2 expression in the ALI cultures exposed to 15 ppm FA fumes; there also was a slight reduction in MGMT protein levels in cultures exposed to 15 ppm FA, but the reduction was nonsignificant (Figure 7).

3.8 | DNA Damage in ALI Cultures Exposed to FA Fumes

The Comet-Chip assay (Ge et al. 2014) was employed to evaluate whether or not DNA damage could be detected in ALI airway cultures exposed to up to 30 ppm FA fumes. After 3 days of FA exposure (T3), small reductions in % Tail DNA were detected in

cultures treated with 15 and 30 ppm FA, as would be expected if DPX were formed. However, the reductions were all nonsignificant (Figure 8).

3.9 | Mutagenicity of FA Fumes in Human ALI Airway Cultures

Human ALI airway cultures were exposed to 7.5 and 15 ppm FA fumes for a total of 20 days over a 28-day period, or to 30 ppm FA fumes for 5 consecutive days. Cultures were then allowed to recover and proliferate for another 28 days before conducting mutagenicity analysis by DS. Neither the low nor mid FA concentrations caused significant changes in mutation frequency (MF) compared to their clean-air-exposed vehicle controls (Figure 9A and Table 2), and the high concentration (30 ppm FA for 5 days of exposure) produced a small, but significant decrease in MF compared to the control air group. Consistent with our previous reports (Wang, Mittelstaedt, et al. 2021; Wang et al. 2024), the

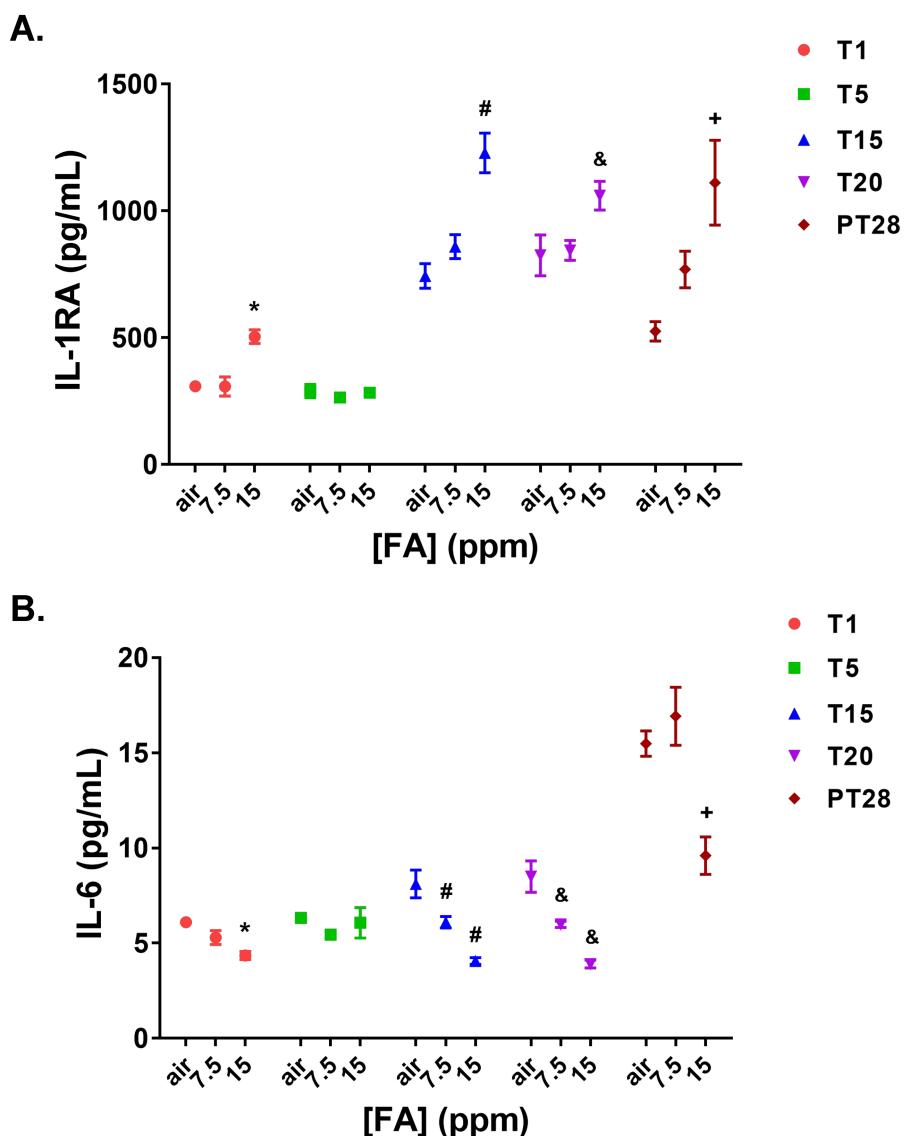


FIGURE 6 | Effects on inflammatory cytokines secreted by ALI airway cultures exposed to FA fumes. Expression of IL-1RA (A) and IL-6 (B) was measured using the Bio-Rad Bio-plex Pro Human cytokine 27-plex assay. Data ($n=3$) are expressed as means \pm SEM. *, #, & $p < 0.05$ was considered statistically significant compared to the clean air-exposed control at the respective timepoints.

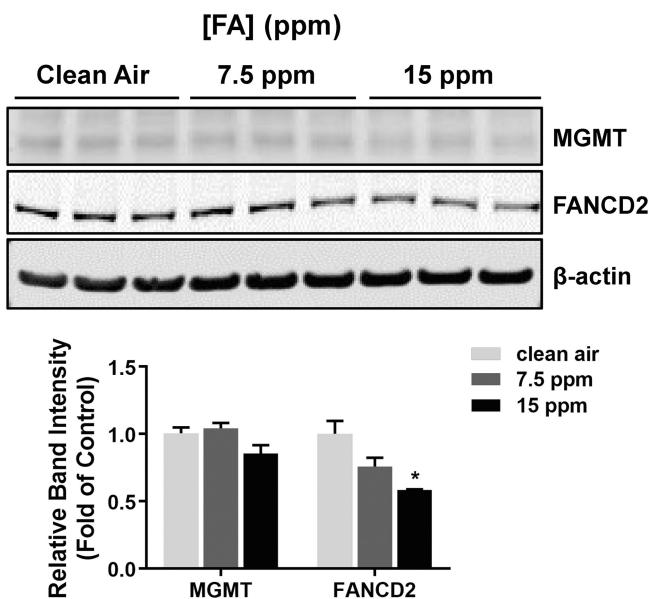


FIGURE 7 | Modulation of DNA repair enzymes by FA fumes. Protein expression of MGMT and FANCD2 was measured at T20 by immunoblotting. Representative blots are presented in the top panel. Densitometry data ($n=3$) are expressed as means \pm SEM. * $p < 0.05$ was considered significant compared to the clean air-exposed control.

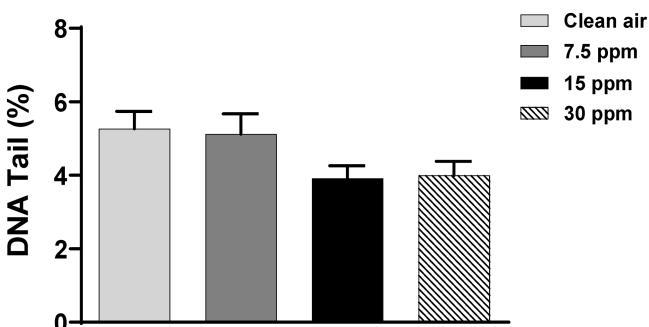


FIGURE 8 | Genotoxicity of ALI cultures exposed to FA fumes. DNA damage (DNA strand breaks and alkali-labile sites measured as %DNA in Tail) was detected using the Comet-Chip assay. Densitometry data ($n=4$) are expressed as means \pm SEM.

positive control, 100 μ g/mL EMS, significantly increased MF by approximately 10-fold.

Simple base-substitution spectra as well as their trinucleotide context were further examined to confirm the lack of FA mutagenicity in the ALI model under the current exposure regimens. As shown in Figure 9B and Table 3, the proportions of all six sub-types of simple base-substitutions were not altered by either the FA exposure schedule or exposure concentration; whereas, consistent with our previous observations (Wang Mittelstaedt, et al. 2021), EMS caused a significant increase in the proportion of pyrimidine-normalized C \rightarrow T transitions. The trinucleotide contexts for each simple base-substitution were categorized to investigate any possible changes in context-specific base substitution caused by FA fumes. The low and mid concentration FA groups exhibited similar patterns to their vehicle-exposed control (Figure 9C). In the high-concentration/short-exposure group, an increasing trend was observed for C \rightarrow A transversion

in the CCG and GCA trinucleotide context; however, the changes did not reach statistical significance.

4 | Discussion

We previously described an in vitro respiratory test platform consisting of a spiking system linked to a system for exposing human ALI airway cultures to airborne toxicants and we used this platform to evaluate respiratory responses to FA fumes (Ren et al. 2022). A 5-day exposure to the low and mid concentrations of FA fumes (7.5 and 15 ppm) elicited marginal to moderate responses. As a chronic rodent study demonstrated that exposure to lower FA concentrations for longer durations is unlikely to increase the damage caused by shorter exposures (Kerns et al. 1983), we investigated the effects of exposure duration on the nature and magnitude of the toxicity responses using the ALI exposure system. In this follow-up study, we extended the treatment duration for the low and mid concentration groups from 5 days to a total of 20 days of exposure over a 4-week period. The high concentration of FA fumes (30 ppm) caused severe damage by the end of the 5-day exposure and thus the exposures were terminated at this point to avoid measuring the effects of overt toxicity.

To facilitate a direct comparison of toxicity responses produced by the 5- and 20-day exposure regimens, we employed the same panel of toxicological endpoints for this 20-day exposure study as used for our previous 5-day exposure study (Ren et al. 2022). The noninvasive measurements made at T5 in the previous 5-day exposure study were repeated in this current study to confirm the consistency of the findings between these two studies. Overall, as was observed in chronic rodent study of Kerns et al. (1983), extending the FA exposure duration only marginally impacted both the nature and magnitude of the responses.

In addition, we also measured DNA damage by the Comet-Chip assay and mutagenesis by DS. A study by Wang et al. (2024) showed that mutation induction accumulates with repeated treatment of ALI airway cultures. We therefore hypothesized that, whereas extended treatments may not impact the extent of other toxicities and functions, the 20-day treatment duration may facilitate the detection of FA-induced mutations. In this case, cultures exposed 20 times to 15 ppm FA received twice the cumulative dose of FA as cultures treated 5 times with 30 ppm FA. None of the three concentrations of FA that we tested had a measurable effect on the induction of DNA damage, as measured by the alkaline comet assay. Also, neither 20 treatments with 7.5 or 15 ppm FA nor 5 treatments with 30 ppm FA increased MFs. We observed a small reduction in mutagenesis in cultures with the 5-day treatment, 30 ppm FA exposure. This could have been due to a bottleneck created by the highly toxic treatment, perhaps related to the formation of DPX adducts, or the effect of squamous differentiation followed by recovery (Ren et al. 2022).

Since we did not conduct histological analysis on cultures treated with 30 ppm FA for 5 days in this current study, our previous FA study was used as the reference. Even though the total amount of FA deposited on the apical surface of the ALI cultures by the 5-day exposures to 7.5 and 15 ppm FA increased by a factor of four for the 20-day exposures, this extension of the

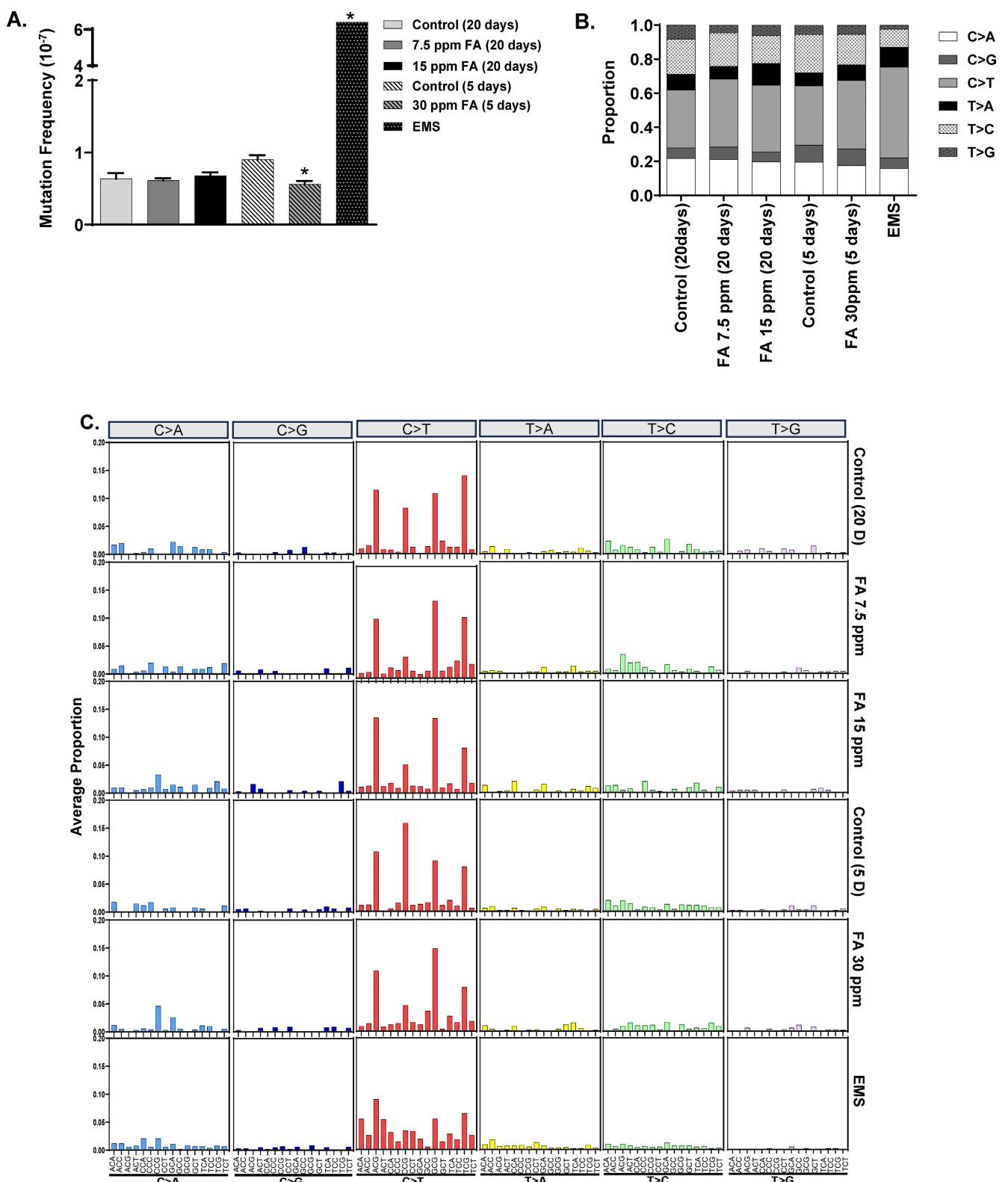


FIGURE 9 | Mutation analysis of ALI airway cultures exposed to FA fumes. (A) Mutation frequency in each treatment group was calculated; EMS was included as a positive control. Proportions of simple base-substitution (B) and trinucleotide spectrum (C) were analyzed by mutation spectra analysis. Data ($n=3$) are presented as means \pm SEM. * $p<0.05$ was considered statistically significant compared to the respective clean air-exposed control.

exposure duration did not impact most tissue responses to FA fumes. This suggests that the tissues may have adapted to the exposures, perhaps through some form of repair. Although there was some evidence of increases in TEER with repeated dosing

of 15 ppm FA, and increased TEER can be associated with squamous differentiation, we observed neither evidence of squamous differentiation nor increased expression of the squamous differentiation marker, AKR1B10 (Fukumoto et al. 2005). These

TABLE 2 | Quantitative data describing duplex sequencing mutation analysis of FA-treated human ALI airway tissue models.

Treatment	Replicate	Total duplex bases ($\times 10^8$)	Total mutant duplex bases	Mutant frequency ($\times 10^{-7}$)
Control (20 days)	1	12.6	100	0.79
	2	12.6	75	0.59
	3	11.4	70	0.53
Mean		12.2	82	0.64
FA 7.5 ppm (20 days)	1	13.3	75	0.56
	2	11.5	72	0.62
	3	10.5	70	0.66
Mean		11.8	72	0.61
FA 15 ppm (20 days)	1	12.7	95	0.75
	2	12.0	72	0.60
	3	5.9	41	0.69
Mean		10.2	69	0.68
Control (5 days)	1	15.4	145	0.94
	2	12.7	100	0.79
	3	11.0	108	0.98
Mean		13.0	118	0.90
FA 30 ppm (5 days)	1	11.6	70	0.60
	2	13.7	84	0.61
	3	17.1	82	0.48
Mean		14.1	79	0.56
Positive control	1	11.8	781	6.60

findings were in accord with the chronic rat study reported by Kerns et al. (1983), indicating that host defense mechanisms were sufficient to protect tissues from prolonged exposures to low doses of FA (Kerns et al. 1983). Additionally, DPX were detected in HNECs only at high FA concentrations (100–200 μM), but not at lower concentrations (20 and 50 μM) or with repeated 50 μM FA treatments (Neuss et al. 2010), suggesting a threshold to the toxic effects of FA exposure.

We and others have reported that FA reacts with glutathione, causing a redox imbalance, which potentially can result in an inflammatory response in rodent lungs, contributing to tumor development (Matsuoka et al. 2010; Aydin et al. 2015; Lima et al. 2015; Umansky et al. 2022; Bernardini et al. 2020; Ren et al. 2022). Unlike 5 days of exposure to 30 ppm FA, which elicits secretion of many pro-inflammatory and anti-inflammatory cytokines (Ren et al. 2022), the present study found that repeated dosing with 15 ppm FA produced only a few alterations in cytokine and chemokine secretion: increases of IL-1RA and decreases of IL-6 at T15 and T20. We hypothesize that the release

of IL-1RA, an anti-inflammatory cytokine, may have suppressed the secretion of pro-inflammatory cytokines, specifically IL-6, by binding to the IL-1 receptor (Garat and Arend 2003; Arend 2002; Lee et al. 2017; Dinarello 2018). In addition to these in vitro findings, long-term exposure to low levels (0.08 mg/m³) of FA in mice caused pathological changes in the lung, but did not affect the immune response as implied by a constant percentage of CDT4⁺ and CDT8⁺ cells in the blood (Cheng et al. 2016). A human study in nonatopic asthmatics further indicated that the inflammatory response had overwhelmed the protective function of anti-inflammatory cytokines, along with an increased IL-1RA/IL-1 β ratio (Mao et al. 2000). Similar to what appears to occur with cytotoxicity, host adaptive mechanisms may be able to counteract the inflammatory response at relatively low concentrations of FA, whereas high concentrations of FA (30 ppm) result in pro-inflammatory cytokine release and consequently induce tissue damage.

There is evidence that FA inhibits DNA repair. Grafstrom and colleagues found that co-treatment of cultured human bronchial fibroblasts with FA and X-rays reduced the level of DNA repair elicited by X-rays alone, possibly by alkylating chromatin or other proteins involved in DNA repair (Grafstrom et al. 1983). In the current study, we observed that exposure to 15 ppm FA significantly reduced FANCD2 expression at T20; in our previous study we observed that exposure to 30 ppm FA produced a similar reduction in FANCD2 expression at T5 (Ren et al. 2022). Pontel and colleagues reported that FANCD2 is mainly responsible for FA-induced DNA adduct repair in mice (Pontel et al. 2015). Other investigators found that the inhibition of FANCD2 gene expression by cigarette condensate was sufficient to cause genetic instability in normal airway epithelial cells, potentially leading to bronchogenic carcinogenesis through mutation and loss programmed cell death (Hays et al. 2008; Swarts et al. 2013). Although we found a modest decrease in FANCD2 expression following FA exposure, our Comet-Chip assay data produced no evidence of FA-induced DNA damage and there was no evidence that FA exposure increased MFs. These findings suggest that the residual DNA repair remaining in the human ALI cultures was sufficient to mitigate any FA-induced genotoxicity.

Several possible reasons may explain why FA was not genotoxic or mutagenic in human ALI airway cultures. Compared with EMS, FA is a relatively weak genotoxin in bacteria, *Saccharomyces cerevisiae*, human lymphoblastoid cells, and cultured fibroblasts, especially with regard to the induction of point mutations, the type of mutations detected most efficiently by DS (Chanet et al. 1975; Goldmacher and Thilly 1983; Kreiger and Garry 1983; Wehner and Brendel 1993; Kawanishi et al. 2014). In contrast, FA is a relatively potent cytotoxicant; in our study, exposures to 30 ppm FA for 5 days may have reduced the number of cells capable of fixing and manifesting mutation to a point at which DS was incapable of detecting a moderately increased mutagenic response. In addition, the toxicity produced by five daily exposures to 30 ppm FA made extending the treatment period, as we were able to do with 15 ppm FA, impractical. As pointed out by Kawanishi et al. (2014), the detection of FA mutagenicity is challenging because of its strong cytotoxicity coupled with its relatively weak mutagenicity. This may have contributed to why the combinations of dose and treatment duration

TABLE 3 | Proportions of simple base substitutions in FA-treated human ALI airway tissue models.

Treatment	Replicate	C>A	C>G	C>T	T>A	T>C	T>G
Control (20 days)	1	0.25	0.01	0.39	0.07	0.24	0.04
	2	0.26	0.06	0.32	0.14	0.21	0.03
	3	0.14	0.12	0.31	0.07	0.18	0.18
Mean		0.22	0.06	0.34	0.09	0.21	0.08
FA 7.5 ppm (20 days)	1	0.27	0.09	0.33	0.07	0.20	0.04
	2	0.15	0.03	0.47	0.06	0.24	0.04
	3	0.21	0.09	0.40	0.10	0.15	0.05
Mean		0.21	0.07	0.40	0.08	0.20	0.04
FA 15 ppm (20 days)	1	0.28	0.07	0.38	0.07	0.16	0.04
	2	0.24	0.07	0.34	0.14	0.17	0.04
	3	0.07	0.03	0.46	0.17	0.17	0.1
Mean		0.20	0.06	0.39	0.13	0.17	0.06
Control (5 days)	1	0.22	0.09	0.33	0.06	0.24	0.06
	2	0.19	0.12	0.38	0.06	0.22	0.04
	3	0.18	0.09	0.34	0.11	0.22	0.06
Mean		0.20	0.10	0.35	0.08	0.23	0.05
FA 30 ppm (5 days)	1	0.13	0.08	0.40	0.10	0.21	0.07
	2	0.19	0.16	0.32	0.13	0.18	0.01
	3	0.20	0.05	0.49	0.04	0.14	0.08
Mean		0.17	0.10	0.40	0.09	0.18	0.05
Positive control	1	0.16	0.06	0.53	0.12	0.11	0.02

used in this study failed to detect measurable increases in point mutations.

In essence, we tried two approaches to maximizing our chances of detecting an increased frequency of point mutations in the human ALI airway model: treating the cultures with a relatively high dose of FA (30 ppm) for a short period of time (5 days) that produces relatively high levels of cytotoxicity, and treating with lower, better tolerated levels of FA (7.5 and 15 ppm) over a longer period of time (20 treatments over 28 days). Neither of these approaches succeeded in producing measurable levels of DNA damage or mutagenesis in the cultures.

It remains the case, however, that FA treatment of human TK6 cells and L5178Y mouse lymphoma cells not only produces gene mutations, such as mutations detected in the *Hprt* gene mutation assay, but also elicits positive responses for “large” chromosome events, including MN and CA (Goldmacher and Thilly 1983; Crosby et al. 1988; Ma and Harris 1988; Liber et al. 1989; Wehner and Brendel 1993; Merk and Speit 1998). In addition, TK6 and L5178Y cells are used for identifying genetic hazard because of their sensitivity for detecting chemical-induced genotoxicity and mutagenicity (OECD 2016a, 2016b). Contributing to this sensitivity is their rapid proliferation rates and absence of important DNA repair functions. For instance, L5178Y cells are deficient in

p53-mediated DNA damage repair functions, and have doubling times of approximately 10 h (Beer et al. 1983). In contrast, both *in vivo* human studies and *in vitro* human nasal and bronchial epithelium models likely possess functional pathways relevant to the repair of FA-induced DNA damage (Neuss et al. 2010; Costa et al. 2008, 2015; Cao et al. 2021). Furthermore, ALI airway cultures replicate very slowly: only 5% of total cells in human ALI airway cultures are actively proliferating (Wang, Mittelstaedt, et al. 2021). While these characteristics of human ALI cultures may make them unsuitable for hazard identification, these characteristics may be ideal for human risk characterization.

A limitation of the current study in terms of detecting FA genotoxicity is that only small alterations to DNA, mainly base pair substitutions, can be detected by DS, and FA is known to induce larger chromosomal alterations, like MN and CA and mitotic recombination (Kawanishi et al. 2014). This has been a weakness of the ALI airway culture system in generating a full accounting of genotoxicity. That is, it is conceivable that FA is generating a genotoxic response in the cultures, but the assay we used was incapable of detecting it. Until now, only one published study has evaluated MN frequency in human ALI airway cultures (Munakata et al. 2023). We are in the process of developing and validating this assay, which uses a combination of human epithelium growth factor to stimulate cell proliferation and the

cytokinesis-block assay to assay for MN only in cells that have undergone division. However, as the Comet-Chip assay failed to detect any DNA damage in FA-treated ALI airway cultures, it is unlikely that FA induces MN under the conditions of the current study.

Lastly, FA also can be generated endogenously, contributing to the formation of several DNA reactive metabolites (Hartwig et al. 2020). A previous animal study found that the levels of exogenous FA-induced DNA adducts were lower than those formed endogenously, indicating that the risk of inhaled FA may be overestimated (Yu et al. 2015). According to the German MAK commission, FA is classified as group four carcinogen, referring to a test substance that can potentially cause cancer, but mainly through nongenotoxic effects. In other words, other MoAs may also contribute to FA-induced carcinogenicity, aside from mutagenesis and chromosome damage. It has been reported that FA also exerts nongenotoxic effects, such as alternations in gene expression and cell proliferation, contributing to tumor development (Hartwig et al. 2020). Because of this, Hartwig et al. (2020) conclude that a significant cancer risk is not expected at or below the maximum FA workplace concentration. Again, the limited cell proliferation in human ALI airway cultures may make it difficult to investigate the significance of these potential nongenotoxic pathways.

In summary, the present study demonstrates that extending the exposure duration of human bronchial epithelium ALI airway cultures to relatively low concentrations of FA fumes had minimal effects on the tissue responses of the ALI cultures. As negative findings like this are difficult to prove conclusively, we cannot exclude the possibility that a combination of dose and treatment duration could be found that shows accumulation of some toxic responses in these cultures, specifically mutagenesis. However, as similar findings have been reported in human case studies, the *in vivo*-like ALI culture with a validated exposure system may have value in modeling the respiratory toxicity of airborne chemicals, like FA, in humans.

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Disclaimer

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.

Supplementary Table 1

Monitor FA fume concentration using FTIR:

Concentration	Formaldehyde (ppm)						AVG	SD	CV (%)
	1	2	3	4	5	6			
Control	0	0	0	0	0	0	0	0	0
FA-7.5	9.80	9.43	9.07	8.74	8.42	8.12	8.93	0.63	7.04
FA-15	16.97	16.28	15.69	15.10	14.58	14.13	15.46	1.07	6.89
FA-30	35.41	35.49	35.59	35.02	34.35	34.42	35.05	0.55	1.56