Mutagenicity of Formaldehyde Fumes in Organotypic Human Air–Liquid-Interface Airway Cultures

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The US Food and Drug Administration (FDA) has prioritized the adoption of New Approach Methodologies (NAMs) that reduce or replace animal testing while enhancing human relevance in regulatory toxicology. In line with this initiative, we have been exploring several advanced cell culture models, particularly those derived from primary human cells, as *in vitro* alternatives for toxicity testing. These models offer significant advantages, including improved physiological relevance, cost-effectiveness, higher throughput, and the potential for enhanced predictive capabilities compared to traditional animal models. Our laboratory is focusing on advancing the integration of genotoxicity assessments into toxicity testing with NAMs.

In particular, we have used Organotypic human air–liquid-interface (ALI) airway cultures, derived from primary human tracheobronchial epithelial cells, as an *in vitro* model for evaluating the toxicity of inhaled substances. Models can be constructed with cells from various parts of the human airway, but the model we have used in most studies recapitulates the structure and function of large airway epithelium. Because the cultures have an air interface, exposures can be conducted using particulates, gases, and various aerosols in a manner that mimics *in vivo* inhalation exposure in the human airway. Of importance to the FDA, exposures can be conducted with smoke and vapors from various tobacco products.

In previous studies with this model, we have developed a panel of structural and functional toxicity endpoints that provides a robust platform for evaluating the toxic effects of inhaled substances under exposure conditions that closely mimic *in vivo* inhalation. In a study published in *Environmental and Molecular Mutagenesis* (Wang et al. EMM 62:306-318), we demonstrated that mutagenesis, in addition to DNA damage endpoints, can be evaluated in ALI airway cultures using error-corrected next generation sequencing. We further showed that, as might be expected for an *in vivo*-like culture system, mutations increased in ALI airway cultures with repeat exposure to the model mutagen, ethyl methanesulfonate (EMS).

In this most recent study (Le et al EMM 66:6-21), we used EMS as a positive control and applied an integrated NAMs approach to investigate the effects of formaldehyde (FA), a known human carcinogen and potent inhalation toxicant, on human ALI airway cultures. Building on our previous short-term exposure study of FA toxicity, we exposed human ALI airway cultures to 7.5 and 15 ppm FA fumes at the air interface 4 hours per day, 5 days a week for 4 weeks, followed by a 28-day recovery period. Due to acute toxicity, cultures exposed to 30 ppm FA were limited to a 5-day treatment. Throughout the study, we monitored tissue responses including cytotoxicity, epithelial barrier integrity, inflammatory cytokine signaling, and DNA repair markers. Genotoxicity was assessed via the Comet-Chip assay after 3 days of exposure, and mutagenicity was evaluated using Duplex Sequencing following the recovery period.

The study found that while moderate concentrations of FA induced modest tissue stress and cytokine alterations, no increases in DNA damage or mutation burden were detected. This finding suggests that

FA fumes have limited genotoxic potential in this *in vitro* human airway model, even after repeated exposures. Although the negative results require further investigation and cautious extrapolation to human risk, this study demonstrates how NAMs can be used to evaluate the inhalation toxicity and mutagenic potential of airborne compounds. These NAM-based approaches provide a biologically relevant tool for assessing the genotoxic potential of inhaled toxicants and valuable information for extrapolation to human health risk.