HHS Public Access

Author manuscript

Toxicol Lett. Author manuscript; available in PMC 2019 June 05.

Published in final edited form as:

Toxicol Lett. 2018 May 15; 288: 143–155. doi:10.1016/j.toxlet.2018.02.025.

Mechanisms of toxicity and biomarkers of flavoring and flavor enhancing chemicals in emerging tobacco and non-tobacco products

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Abstract

Tobacco products containing flavorings, such as electronic nicotine delivery devices (ENDS) or e-cigarettes, cigars/cigarillos, waterpipes, and heat-not-burn devices (iQOS) are continuously evolving. In addition to increasing the exposure of teenagers and adults to nicotine containing flavoring products and flavoring enhancers, chances of nicotine addiction through chronic use and abuse also increase. These flavorings are believed to be safe for ingestion, but little information is available about their effects on the lungs. In this review, we have discussed the *in vitro* and *in vivo* data on toxicity of flavoring chemicals in lung cells. We have further discussed the common flavoring agents, such as diacetyl and menthol, currently available detection methods, and the toxicological mechanisms associated with oxidative stress, inflammation, mucociliary clearance, and DNA damage in cells, mice, and humans. Finally, we present potential biomarkers that could be utilized for future risk assessment. This review provides crucial parameters important for evaluation of risk associated with flavoring agents and flavoring enhancers used in tobacco products and ENDS. Future studies can be designed to address the potential toxicity of inhaled flavorings and their biomarkers in users as well as in chronic exposure studies.

Keywords

E-cigarette; flavoring chemicals; inflammation; oxidative stress; DNA damage; biomarkers

1. Introduction

Tobacco smoking is a prevalent habit all over the world. Due to its addictive alkaloid, nicotine, tobacco smoking is known to cause dependence and craving. Over time, tobacco products have evolved from chewing tobacco to cigars, pipes, and cigarettes even though

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Author contributions: GK, TM and IR wrote and revised/edited the manuscript.

Competing Conflict of Interests Statement: The authors declare no conflict of interest.

tobacco kills more than 7 million people each year (>6 million due to direct tobacco use and ~890000 due to second-hand smoke in non-smokers (WHO, 2015). Tobacco products have further evolved to include flavoring agents, such as menthol. Many new non-tobacco products, for example electronic nicotine delivery systems/devices (ENDS), i.e. electronic (e)-cigarettes have been introduced in the market with the apparent intent to reduce tobacco smoking. The addition of flavoring agents and their enhancers in these non-tobacco products is reasoned to decrease tobacco-based product use, but leads to an increase in product publicity and first-hand exposure of nicotine including to young adults. While flavoring agents are permitted for ingestion as their use through the digestive tract is well documented, recent reports of lung toxicity caused by inhaled flavoring agents in tobacco and non-tobacco products have emerged. This review summarizes the flavoring agents and chemical enhancers in emerging tobacco and non-tobacco products, their mechanism of lung toxicity, as well as existing and prospective biomarkers that can be utilized to predict lung toxicity.

2. Flavored tobacco and non-tobacco products

A broad class of tobacco products (combustible and non-combustible products), such as ENDS/e-cigarettes, little flavored cigars/cigarillos, and waterpipe have recently emerged, arising in part from efforts to evade existing regulations on tobacco. The consumption of e-cigarettes has increased in youth worldwide, posing a public health concern (Czoli et al., 2015; Krishnan-Sarin et al., 2015; Palipudi et al., 2016; Regan et al., 2013; White et al., 2015). E-cigarettes are battery-powered devices that heat and aerosolize a liquid mixture (also called e-liquid or e-juice), typically containing a vehicle-humectant, such as propylene glycol (PG) and/or vegetable glycerin (VG) along with nicotine and flavoring agents (Lerner et al., 2015a). Recently, a rapid growth is seen in both marketing and consumption of e-cigarettes (Ayers et al., 2011; Regan et al., 2013). With each “puff,” the heating element aerosolizes a small amount of liquid. In this format, the e-cigarette user is inhaling aerosols as vapor. With the recent emergence and increasing popularity of multiple devices for the recreational inhalation of non-combustible nicotine (e.g. e-cigarettes) among youth as well as in adults, Better understanding of effects of flavoring in e-cigarette aerosol is much needed. Although carcinogens appear to be reduced or eliminated in e-cigarettes (Cahn and Siegel, 2011; Cobb and Abrams, 2011; Etter and Bullen, 2011), health concerns of e-cigarette aerosols with flavoring chemicals including their enhancers based on toxicological effects on the lungs are not well understood.

3. Existing flavoring agents in emerging tobacco products

Flavorings in tobacco products were introduced in ~1924 to escalate the global market and include non-smokers or unconventional users. Menthol containing cigarettes have been marketed under ‘throat comfort’ cigarettes (Cruz et al., 2010), and menthol has been used as an additive in approximately quarter of cigarettes manufactured in the United States alone (Ferris Wayne and Connolly, 2004; Giovino et al., 2004). It is known that adolescents especially ethnic minority groups are more likely to progress from experimentation of menthol cigarettes to regular smoking (Kreslake et al., 2008; Robinson et al., 2006). Other flavored tobacco products have entered the market after menthol became much more prevalent and acceptable to users. There has been an exponential increase in flavored

tobacco and non-tobacco products since 2002. Currently, these products include menthol cigarettes, flavored smokeless tobacco, little and large cigars, cigarillos, e-cigarettes, hookah, and nicotine dissolvables, which are sold in a range of flavors from fruit flavors to candy or confectionery flavors, to alcoholic beverages to herbs and spices. This progression is concomitant to important legislation changes prohibiting flavoring of cigarettes in the United States (Section 907(a)(1)(A) of 2009 Family Smoking and Tobacco Prevention Act in USA legislature). However, many countries have yet to successfully enact similar laws (TCLC, 2015). Currently, the most common flavoring chemicals in cigarettes and e-cigarettes in the market are menthol, diacetyl and 2,3-pentanedione (Table 1). This list is merely representative and the readers may want to access the listed articles for complete list of identified flavoring chemicals in various tobacco and non-tobacco products.

An example of emerging non-tobacco products is e-cigarettes which entered the market in ~2007 and have been largely unregulated until 2016. Currently, there are an enormous amount of flavored e-cigarette (i.e. e-liquid) brands in the market (Zhu et al., 2014). Emerging studies have shown the link between e-cigarettes and e-liquids to lung tissue damage due to inflammation and immunogenic effects (Higham et al., 2016; Kreiss, 2007; Martin et al., 2016). While the flavorings used are believed to be safe for consumption, but not inhalation, the effect of specific flavoring chemicals differentially on lung cells is not much known.

4. E-cigarette flavorings

E-liquids come in a variety of flavors at various nicotine concentrations ranging from 0 mg to 36 mg/mL (Davis et al., 2015). However, e-liquid constituents and their potential adverse effects on respiratory health hazard are not known. The e-liquid manufacturers market these liquids with alluring names, such as Churrios, Muffin, Cotton Candy, Milk and Honey, Citrus/Lemon, Apple Pie, Melon Mania/Watermelon, Cheese Cake, Cherry, Chocolate, Coconut, Licorice, Cappuccino, Crème Brule, Oatmeal Cookie, Cinnamon Roll, and Tutti Frutti that are more appealing especially to youths (Allen et al., 2016; Chen and Zeng, 2017; Kim et al., 2016). Vaping exposes lungs to these flavoring chemicals when the e-liquids are vaporized and inhaled.

Various flavorings and flavoring enhancers are commonly included in e-liquids sold online and/or through vape shops. There are more than 250 e-cigarette brands and 8000 different flavorings in the USA market alone, and products have evolved rapidly in the past few years. By the time a product is considered unsafe or shown to have toxic properties, a new product emerges even by mixing flavoring chemicals with polyethylene glycerol/vegetable glycerin locally in a vape/smoke shop for desirable flavors to consumers. Numerous flavorings are sold without nicotine as non-tobacco products. Limited information is available on the adverse health effects of these emerging flavorings in tobacco products and non-tobacco products even if it differs from manufacturer to manufacturer within the same type of flavorings. The e-cigarette industry currently promotes these products with claims that they are less toxic or addictive. However, as noted by the Flavor Extracts Manufacturers Association (FEMA), flavors are safe for ingestion, and not for inhalation. Many tobacco flavoring ingredients labeled ‘Generally Recognized as Safe (GRAS)’ are intended for

foods, but have not been evaluated for inhalation toxicity. The composition of flavors differs as these are not FDA regulated. A significant concern exists regarding the purity of ingredients or chemicals employed, and the general lack of oversight in manufacturing or marketing/communication of flavorings (Hahn et al., 2014; Lisko et al., 2015). Several flavor groups can be classified according to the characteristics: e.g. berry/cherry, tropical fruit, classical tobacco, alcohol related drinks, chocolate/sweet flavor, vanilla, coffee/tea, and mint/menthol (Muthumalage et al., 2017). Certain flavorings include mixture of various flavorings in tobacco products (cigarillos and waterpipe). These flavoring chemicals may pose a major and potential hazard in ENDS users when they are aerosolized into ultrafine particles reaching distal and peripheral areas (smaller airways) of the lungs. Unfortunately, the exact mechanism of specific flavoring agent mediated respiratory system damage and toxicity is not known whilst the market is flooded with these products.

5. Common flavoring agents and their toxicities

5.1. Menthol

Menthol, a naturally occurring monocyclic terpene alcohol and a stimulant for cold receptors (Paschke et al., 2017), imparts mint flavor to traditional cigarettes and e-cigarettes. It was originally added to tobacco products to create an impression of reduced health risks. Its pharmacological actions reduce the harshness felt due to smoke and the irritation caused by nicotine, in turn increasing the likelihood of nicotine addiction in teenagers after experimental usage. It induces sensory effects, facilitating deeper inhalation and thus enhancing nicotine impact. It is interesting to note that menthol was the only permitted flavor in cigarettes in USA in Family Smoking Prevention and Tobacco Control Act 2009 (COTUS, 2009). Although eventually, the Tobacco Products Scientific Advisory Committee (TPSAC) concluded that ‘menthol cigarettes have an adverse impact on public health in the United States’ (TPSAC, 2011), despite this growing awareness, menthol containing traditional cigarettes and e-cigarettes have captured a major share of the market. While menthol is highly regulated in medical products, product standards are lacking in traditional cigarettes as well as e-cigarettes. As young adulthood appears to be a critical time for initiation of vaping and smoking, it is thought that presence of menthol increases the risk of dependence when compared to non-menthol cigarettes. Also, menthol is likely to be associated with altered physiological responses to tobacco smoke in cigarettes. Therefore, the FDA concluded that due to higher initiation rates with menthol cigarettes, they pose public health risk above that seen with non-menthol cigarettes (Food and Drug Administration, 2013).

5.2. Diacetyl and aldehydes

Diacetyl, also known as 2,3-butanedione, is a member of organic diketones. Diacetyl is found in e-cigarette aerosol with a concentration of 239 μg/e-cigarette (Allen et al., 2016). There are several reactive aldehydes including formaldehydes, that have been detected in e-cigarette aerosols (estimated formaldehyde level of 14.4±3.3 mg at high voltage) (Farsalinos et al., 2017a; Farsalinos et al., 2017b; Salamanca et al., 2017).

The generation is based on various power settings and usage methods adopted by users (Gillman et al., 2016; Talih et al., 2016). It provides a characteristic buttery flavor, and is both naturally found in foods and added as a synthetic flavoring agent in food products, e.g. butter, caramel, cocoa, coffee, dairy products, and alcoholic beverages (Hallagan, 2017; Mathews et al., 2010). In a recent study (Farsalinos et al., 2015b), 158 sweet-flavored e-cigarette liquids were evaluated for the presence of diacetyl and acetyl propionyl. It was shown that both flavoring chemicals were found in 74.2% of the samples with more samples containing diacetyl, which is approved safe for consumption through gastrointestinal tract. Diacetyl inhalation is manifested as decline in respiratory function and most commonly as development of bronchiolitis obliterans or popcorn lung, an irreversible respiratory disease (Kreiss et al., 2002; Rose, 2017; Wallace, 2017). The National Institute for Occupational Safety and Health (NIOSH), proposed upper limit for time weighted average exposure (TWA) at 5 ppb (18 μg/m^3) for 15 min and short-term exposure limit (STEL) at 25 ppb (88 μg/m^3) for 8 hr, while Scientific Committee on Occupational Exposure Limits (SCOEL) proposed upper limit for TWA is 20 ppb (70 μg/m^3) and STEL upper limit is 100 ppb (360 μg/m^3) (Farsalinos et al., 2015b).

The NIOSH, after investigating microwave popcorn and flavoring production facilities, has suggested that high diacetyl exposures may contribute to or cause severe respiratory disorders including *bronchiolitis obliterans* (Kreiss, 2007; Kreiss et al., 2002). Diacetyl has been shown to reduce lung capacity as measured by forced expiratory volume in one second (FEV_1) (Kreiss et al., 2002). Other flavoring chemicals, such as aldehyde cytotoxicity by cinnamon-flavored ENDS and other cytotoxic effects are correlated with the amount of cinnamaldehyde in the products (Behar et al., 2014). Benzaldehyde, a key ingredient in natural fruit-flavored products, has been shown to cause irritation of respiratory airways in cherry-flavored e-liquid (Goniewicz et al., 2014; Kosmider et al., 2016). Benzaldehyde concentration varies 15.7 to 10,300 ug/m^3 in flavored e-cigarette aerosol (Klager et al., 2017). High levels of furfural and 5-hydroxyfurfural present in sweet-flavored ENDS liquids (Soussy et al., 2016) are shown to cause irritation to the upper respiratory tract in humans. Furthermore, furfural compounds have tumorigenic activity in mice (Arts et al., 2004; HHS, 1990; Surh et al., 1994; Surh and Tannenbaum, 1994).

5.3. 2,3-Pentanedione

2,3-pentanedione or acetyl propionyl (a popular replacement for diacetyl) is also an α-diketone, chemically and structurally very similar to diacetyl (Day et al., 2011; Flake and Morgan, 2017). 2,3-pentanedione and acetoin (another popular diacetyl replacement) were detected in e-cigarette aerosol at concentrations up to 64 and 529 μg/e-cigarette (Allen et al., 2016). 2,3-pentanedione has been associated with airway fibrosis in rats (Morgan et al., 2012). The risks associated with 2,3-pentanedione inhalation are as high as diacetyl as shown in rats (Hubbs et al., 2012). The NIOSH proposed upper limit for TWA for 2,3-pentanedione is 9.3 ppb (38 μg/m^3) and STEL is 31 ppb (127 μg/m^3) (Farsalinos et al., 2015b).

5.4. Lesser reported flavoring agents

Another flavoring compound, 2,5-dimethylpyrazine, has been detected in recent studies in e-cigarette products from the Greek market using a multicomponent analysis utilizing gas chromatography (GC) and liquid chromatography-mass spectrometry (LC-MS) (Kavvalakis et al., 2015). The same study successfully evaluated many other flavoring agents (Table 1) including methyl-cyclopentenolone, ethyl maltol and 3,4-dimethoxybenzaldehyde in the 263 e-liquid samples tested (Kavvalakis et al., 2015). Recently, 2,5-dimethylpyarazine has been implicated in activation of apical ion flux in mouse tracheal epithelial cells and thus a suggested role in altering airway epithelial cell innate immunity (Sherwood and Boitano, 2016). Headspace solid phase microextraction technique can be employed for detection of flavorings (volatile compounds) in these products.

Caffeine has been introduced in e-liquids to give a taste of coffee, tea, chocolate or energy drink and is marketed as energy enhancer. One study (Lisko et al., 2017) quantified caffeine in 44 flavored e-liquids using GC-MS and determined that 42% of coffee-flavored products, 66% of tea-flavored products, 50% of chocolate-flavored e-liquids, 91% of energy enhancers contained caffeine in varying concentrations. It is not known whether inhalation of caffeine in e-liquid flavors will have any impact on respiratory health. Caffeine may impose allergic responses due to activation of eosinophils or mast cells. Flavor compounds, such as eucalyptol and pulegone have also been shown in the tested e-cigarette brands (Lisko et al., 2015). However, no inhalation toxicity studies are available on these flavors.

A study on e-cigarettes in Germany (Hutzler et al., 2014) reports the presence of flavoring agents in varying amounts upon analysis of 28 nicotine-free e-cigarette liquids. More hazardous ethylene glycol (replacement of glycerol and propylene glycol) in 5/28 and carryover of 0.1–15 μg/ml nicotine in 7/10 nicotine-free labeled samples is presented (Hutzler et al., 2014). Other agents include emerging CBD, cannabidiol (derivatives of cannabis derived oil from cannabis hemp i.e. phytocannabinoid oil) was reported. A study on adult smokers displayed presence of sucralose, an additive to enhance sweetness of e-cigarettes using LC/MS detection (Rosbrook et al., 2017). No information on toxicological effects of these flavors is yet available.

6. Flavoring agents mediated toxicity in e-cigarettes

There is a lack of information on toxic effects of many flavors in e-cigarettes especially via inhalation. Emerging evidence suggests that e-cigarettes cause oxidative stress, inflammatory response, and DNA damage in lung cells. It has also been shown that e-cigarette aerosol dampens the innate immune response, and affects mucociliary clearance (Figure 1).

6.1. Flavoring agents and oxidative stress

Recent studies have highlighted the need for research on flavoring chemicals present in e-cigarette aerosols, their potentially toxic degradation products, and the consequences of inhaling these flavor chemicals and byproducts. We have shown that human lung epithelial cells and fibroblasts (HFL-1) release reactive oxygen species (ROS) upon e-cigarette

flavoring agents exposure (Gerloff et al., 2017; Lerner et al., 2015a; Sundar et al., 2016). Furthermore, other flavoring chemicals which are found in e-cigarette aerosols/e-liquids based on the emission GC-MS data, such as acetoin, ortho-vanillin, and maltol, also trigger an inflammatory response by release of IL-8 inflammatory cytokine in these cells (Beas2B and HFL-1) (Gerloff et al., 2017). Certain additional chemicals were found in e-liquids using GC-MS, such as nicotyrine, benzaldehyde, furanone, propylpyridine, pyrol, and benzene derivatives, but characterizing their effects requires study in cell culture and mouse models (Gerloff et al., 2017). Another study using differentiated THP-1 macrophages demonstrated decreased phagocytosis as displayed by reduced phagocytic recognition molecules along with increased IL-8 secretion (Ween et al., 2017). Similarly, Muthumalage and co-workers have shown increased ROS and inflammatory response by human monoctyes (Monomac6 cells) (Muthumalage et al., 2017).

Other toxicants found in e-cigarette vapor include flavoring-related compounds like diacetyl and apple oil (3-methylbutyl-3-methylbutanoate). Flavoring chemicals exposure has also been shown to significantly and rapidly (within 20 minutes) decrease transepithelial resistance in human bronchial epithelial cells, suggesting epithelial barrier dysfunction and an impaired inflammatory response (Gerloff et al., 2017). The health effects of passive inhalation of e-cigarette flavorings in humans are not well characterized (Barrington-Trimis et al., 2014; McAuley et al., 2012; Schober et al., 2014; Schripp et al., 2013).

6.2. Flavoring agents and cytotoxic responses

Despite the emergence of flavored cigarettes and e-cigarettes, only few reports are available on respiratory exposure to flavoring chemicals especially in e-cigarettes. Flavoring agents mediated cytotoxicity in e-cigarettes is reported in few cellular models (Bahl et al., 2012; Behar et al., 2014; Cervellati et al., 2014; Hutzler et al., 2014; Kavvalakis et al., 2015; Lisko et al., 2015). ‘Cinnamon Ceylon’, an e-cigarette brand, was shown to be the most cytotoxic of 36 e-cigarette products tested in a cell culture based metabolic/toxicity MTT assay on human adult pulmonary fibroblasts and human embryonic stem cells (Behar et al., 2014). In the same study, while cinnamaldehyde, 2-methoxycinnamaldehyde, dipropylene glycol, and vanillin were identified in tested e-cigarettes using GC-MS and HPLC, cinnamaldehyde and 2-methoxycinnamaldehyde were shown to be highly toxic in MTT assay. A study (Welz et al., 2016) investigating the cytotoxic effects of different dilutions of e-liquids containing fruit flavor or tobacco flavor on human pharyngeal tissue cultures, and thus risk factors for head and neck squamous cell carcinoma, has shown a significant reduction in cell viability with fruit flavors as compared to tobacco flavored e- liquids. Another study, evaluating acute cytotoxicity (24 h treatment) of 15 e-liquid brands vapor extract on human bronchial epithelial, fibroblast and macrophage cell lines in vitro presented flavoring compounds with variable cytotoxic effects on the tested cell lines (Leslie et al., 2017). Cell viability was significantly decreased in both monoculture of human epithelial cells and a three dimensional co-culture of alveolar and lung microvascular endothelial cells after exposure to mint and cinnamon containing aerosols (Bengalli et al., 2017). Chronic inhalation of these flavoring chemicals cause airway epithelium injury, ultimately resulting in formation of pro-fibrotic lesions (Flake and Morgan, 2017; Morgan et al., 2012; Wallace, 2017). The

mechanisms for this may due to the release of pro-fibrotic mediators and myofibroblast differentiation.

6.3. Flavoring agents and immune-mediated responses

Toxicity by any compound usually starts with an immediate/early immunogenic response and includes progressive responses on different lines of immune defenses. Respiratory tract contains barriers to defend itself from any possible irritants. The first line of defense in the lung against inhaled particulates, nanoparticles, pathogens, allergens, noxious gases, and toxins in respiratory tract is provided by ciliated airway epithelium (Vareille et al., 2011). In addition to providing a physical barrier for protecting underlying tissue and maintaining salt and water movement to keep the lumen hydrated, it also coordinates particulate filtering through mucous and cilia, as well as the secretion of antimicrobial defense factors (Vareille et al., 2011). Ineffectiveness to sustain normal immune functions disposes an individual to infection and inflammation. Therefore, compromised airway epithelium is an acceptable model to study effects of tobacco and non-tobacco cigarettes. While tobacco cigarettes and more recently, e-cigarette products are described to suppress respiratory immunity by inducing oxidative stress and inflammatory response thus dampening the innate immune response. Not much is known about the individual effect of flavoring in non-tobacco cigarettes/ENDS on human risk assessment.

It has been shown that e-cigarette users have increased impedance, peripheral airway flow resistance, and innate defense protein profile as well as oxidative stress as main pathological changes (Reidel et al., 2017; Vardavas et al., 2012; Wu et al., 2014). One such case of a woman diagnosed with exogenous lipoid pneumonia after 7 months of e-cigarette use has been clinically reported (McCauley et al., 2012). The patient showed recovery after discontinuing e-cigarette use. It was observed that the clinical symptoms (dyspnea, productive cough and subjective fevers) were caused by oil-based humectants in e-cigarette composition (McCauley et al., 2012). Numerous symptoms after e-cigarette use are also reported using an internet-based surveys (Hua et al., 2013). However, such a human-based study on flavors inhalation and chronic animal studies by e-cigarettes is lacking and requires immediate attention for proper risk assessment.

As discussed above, from the workers exposed in food manufacturing, diacetyl leads to subclinical alterations of lung function and airway obstruction and eventually life-threatening bronchiolitis obliterans (Kreiss, 2007; Kreiss et al., 2002). Symptoms, such as cough and shortness of breath are also observed by short-term respiratory exposure to diacetyl (Kreiss, 2007). A recent study on effect of 2,5-dimethylpyrazine on immortalized human bronchial epithelial and primary mouse tracheal epithelial cells has identified a role of 2,5-dimethylpyrazine on the regulation of chloride secretion by activating apical ion efflux via cystic fibrosis transmembrane conductance regulator and reducing the physiological response to signaling molecules important in airway epithelial cell innate immunity (Sherwood and Boitano, 2016). Effect of different flavoring agents- acetoin, diacetyl, 2,3-pentanedione, maltol, vanillin, coumarin and cinnamaldehyde, on human bronchial epithelial cells, human mucoepidermoid carcinoma epithelial cells, and human lung fibroblasts and monocytes (Gerloff et al., 2017) showed no significant change in cell

viability at tested concentrations, but induced proinflammatory cytokine, IL-8 release from lung epithelial cells lines, where acetoin and maltol were more potent inducers of IL-8 release than even TNFα (Gerloff et al., 2017). All flavoring chemicals tested showed impaired epithelial barrier function in human bronchial epithelial cells. These data suggest toxicity mechanism of flavors proceed through significant loss of epithelial barrier function culminating from proinflammatory response in lung cells (Gerloff et al., 2017).

Another study on primary human alveolar macrophages, neutrophils, and natural killer cells using their functional endpoint responses, such as phagocytic capacity and proinflammatory cytokine production to pathogenic stimuli after treatment with seven flavored, nicotine-free e-liquids reported dose-dependent, broad immunosuppressive effects, in particular using the three cinnamaldehyde-containing e-liquids (Clapp et al., 2017). Furthermore, it has been shown that menthol, potent natural agonist at cold receptor TRPM8, suppresses natural defense mechanisms (involuntary coughing against fumes through TRPM8) and thus, cigarettes or e-cigarettes at a menthol level exceeding 50μg must be declared as 'mentholated' (Paschke et al., 2017). Many studies have shown that e-cigarette exposure can dampen host immunity against bacteria, such as *streptococcus pneumonia, staphylococcus aureus* and viruses, such as influenza A in mice (Hwang et al., 2016; Sussan et al., 2015).

6.4. Flavoring agents and DNA damage

A few studies have indicated toxicity associated with the use of e-cigarette products on DNA damage. E-liquids were mainly cytotoxic to oropharyngeal tissue with some inducing significant DNA damage. This is in agreement with our recent findings that e-cigarettes cause oxidative stress, DNA damage, and inflammatory responses in human lung epithelial cells and mouse lungs (Lerner et al., 2015a; Lerner et al., 2015b).

Interestingly, e-cigarette liquids have shown higher cytotoxicity on human embryonic stem cells and mouse neural stem cells in comparison to human pulmonary fibroblasts (Bahl et al., 2012). Subsequent study determined cinnamaldehyde, 2-methoxy cinnamaldehyde, vanillin, dipropylene glycol as responsible agents in cytotoxicity assay on human embryonic stem cells and human pulmonary fibroblasts (Behar et al., 2014). The inhibition of the response of GABA receptors, main inhibitory neurotransmitter receptors, by xanthin derivatives, allantoin, chlorogenic acid, 3-hydroxy-2-methyl-4-pyrone (maltol), trigonelline hydrochloride, and 2,3,5 trimethylpyrazine, are also reported to partially contribute to CNS stimulation via DNA damage (Hossain et al., 2003). Hence, flavoring chemicals may have adverse effect on neurological system apart from nicotine. Menthol increases the sensation of airflow and hinders respiratory activity via DNA damage, masking any reflex actions (coughing), allowing increased lung exposure to cigarette constituents, such as tar in traditional cigarettes and nicotine in e-cigarettes. This, in turn, increases lung permeability and absorption of harmful cigarette constituents. As menthol also interacts with nicotine metabolism, higher levels of nicotine are maintained in the body. Cell death has been shown to be significantly enhanced by menthol derived smoke than non-menthol smoke indicating a synergistic effect (Noriyasu et al., 2013). Low levels of menthol suppressed respiratory irritation by smoke irritants, acrolein and cyclohexane in mice (Ha et al., 2015). Further studies are required to understand the effects of flavoring agents on airway epithelial and/or

3-dimensional cell culture models using an air-liquid interface system. Biomarkers of e-cigarette flavorings and flavoring agents/chemicals would be critical for proper human risk assessment. This would provide an understanding of pathological processes as well as strategy for targeted therapeutic approaches.

7. Flavoring chemicals and mucociliary clearance

Recent evidence suggests that flavoring chemicals induce mucin by goblet cells. For example, chocolate flavoring chemical, 2.5-dimerthylpyrazine is shown to alter cystic fibrosis transmembrane conductance regulator (CFTR) expression, which could have adverse effects in immune mechanisms, such as mucociliary clearance, dampening the epithelial defense against inhaled particulates and pathogens (Sherwood and Boitano, 2016). It has been shown that α7 nicotinic receptor regulates CFTR function in airway epithelium (Maouche et al., 2013), suggesting that loss of CFTR may have adverse outcome in mucociliary clearance in response to tobacco products especially in patients with cystic fibrosis. Mucus hypersecretion can hinder the respiratory pathogen clearance and exacerbate respiratory function in pulmonary diseases, such as COPD and asthma (Vareille et al., 2011). Martin *et al* observed down-regulation of CSF-1 and CCL26 inflammatory genes i.e. suppression of immune and inflammatory-response genes in nasal epithelial cells (Martin et al., 2016). Reidel *et al* found increased neutrophilic activation and mucin hypersecretion by e-cigarette in users (Reidel et al., 2017). However, the e-cigarette vaping is associated with dry mouth/throat in users. Nevertheless, there is a lack of information on e-cigarette flavoring mediated mucin secretion in users, and the reason for dry mouth/throat or affecting host-defense and mucociliary clearance is not known.

8. Biomarkers for detection of flavoring agents

Lung toxicity biomarkers for tobacco products involving oxidative stress, DNA damage, and inflammatory mediators are well studied. For example, differential systemic thiol status has been shown to altered by cigarette smoke (Rossi et al., 2009). Plasma nicotine level has long been used to assess nicotine intake and pharmacologic effects immediately after exposure (half-life: 2h). Blood, salivary, or urinary cotinine levels, a metabolite of nicotine, are useful as sustainable biomarkers due to longer elimination time and half-life of 16–18 hours (Hukkanen et al., 2005). Increase in cotinine has been associated with lung cancer risks (Yuan et al., 2011). TNE or total nicotine equivalent gives the sum of urinary nicotine, cotinine and several metabolites in the nicotine metabolite profile. This is considered a good biomarker for daily nicotine intake and takes into account all environmental factors influencing nicotine metabolism (McKinney et al., 2014). Assessment of TNE levels can be used for e-cigarettes as biomarker of nicotine intake (Goney et al., 2016). Even if e-cigarettes deliver less nicotine per puff than tobacco cigarettes, vapors (e-cigarette users) take longer inhalations with increased puffing topography than smokers (Lee et al., 2015). Other biomarkers, like carbon monoxide (Scherer, 2006), aromatic and heterocyclic amines (Riedel et al., 2006; Turesky and Le Marchand, 2011) and tobacco-specific nitrosamines (TSNAs) (Kavvadias et al., 2009; Stepanov and Hecht, 2005, 2008) are irrelevant for e-cigarettes as carbon monoxide is produced only after tobacco combustion, absent in e-cigarettes and TSNAs are only detected in e-cigarettes if the nicotine is not of

pharmaceutical grade (Farsalinos et al., 2015a). Similarly, only low levels of polycyclic aromatic hydrocarbons (PAHs) are detected in e-cigarettes (Cheng, 2014) as compared to traditional cigarettes (Li et al., 2008; Suwan-ampai et al., 2009). Volatile organic compounds (VOCs) are found in traditional cigarette as a result of incomplete combustion of tobacco (Yuan et al., 2011), as well as e-cigarette smoke by chemical transformations due to vaporization (Marco and Grimalt, 2015), and diacetyl and alpha-diketones mediated biotransformation induced respiratory toxicity (Anders, 2017). Some metals, such as lead and cadmium have long been associated with tobacco exposure due to adsorption of these metals in tobacco plants from the soil (Bernhard et al., 2005; Saffari et al., 2014). Recently these metals have been found in e-cigarettes (Hess et al., 2017; Saffari et al., 2014).

In light of lower toxicity by e-cigarettes flavorings (O'Connell et al., 2016), e-cigarette flavoring mixture evaluation should not be undermined especially because vapors take longer puffs than smokers (D'Ruiz et al., 2016). Recently, the early detection of airway complications by diacetyl exposure has been discussed (Rose, 2017) with inhaled dosimetry, puffing profile/topography, and regional toxicity (Cichocki and Morris, 2017).

There are studies on e-cigarette aerosol exposures suggesting that aerosol constituents could be harmful to human tissues for triggering inflammatory responses (Ji et al., 2016; Lerner et al., 2015b; Rubenstein et al., 2015). However, the validity of these studies is dependent on the aerosol extracts as representative of what e-cigarette users are exposed to and cannot be directly translated to disease. Four biomarkers of oxidative stress: sNox2-dp, 8-isoPGF2α, NO bioavailability, vitamin E, relevant in vaping associated cardiovascular disease and respiratory disease have been identified (Carnevale et al., 2016; Martin et al., 2016). Exhaled breath condensate (EBC) and excreted metabolites (exhaled breath or urine) are more likely to predict actual levels in vapors than studies using exposure of cell models to e-liquids or aerosols (Carnevale et al., 2016).

Currently, the biomarkers for detection of flavoring agents are almost non-existent. There has been a recent report that toxic aldehyde production due to flavoring agent could be a potential biomarker in e-cigarettes (Khlystov and Samburova, 2016). The research group measured higher levels of toxic aldehydes (formaldehyde, acetaldehyde, benzaldehyde, acrolein and propionaldehyde) upon vaping with popular e-liquid brands. The effects were found to be exponentially proportional to flavor concentration and generated after thermal decomposition, producing aldehyde levels higher than occupational safety standards. Normally, aldehydes are shown to be produced by thermal decomposition of propylene glycol and glycerol. These findings strengthen the need for detailed investigation on contribution of flavoring agents in e-cigarette induced lung toxicity especially after thermal decomposition. Additionally, previous studies have been performed on e-liquids rather than vapor produced after heating of e-liquids. Interestingly, increased aldehyde levels are reported in smoke from menthol cigarettes (Baker et al., 2004a; Baker et al., 2004b), however these aldehydes are derived as a product of sugar combustion. Benzene formation has been detected in benzaldehyde containing e-cigarette (for imparting cherry flavor) using high power settings in refill tank systems (Pankow et al., 2017). We have compiled various detection methods for flavoring agents in Table 1 that can be used to monitor levels of flavoring compounds during manufacturing as well as regular inspection in marketed brands.

These flavors may have differential toxicity patterns as reflected by two aerosol capture methods for qualitative analyses of e-liquid flavors (Eddingsaas et al., 2018). No genetic biomarkers predisposing to flavoring chemicals upon inhalation are currently known.

9. Flavor enhancing chemical agents in ENDS

Flavor enhancing chemicals are additives added to enhance the given flavor, taste, and freshness of the products such as flavoring additives in an e-liquid. These are based on basic key tastes, e.g. salt, sweet, bitter, sour and savory with aromas. Originally, monosodium glutamate, known as MSG, was included in soups, snacks and sauces. Glutamate can be reacted with sodium, potassium, and calcium providing different characteristics of additives. Various amino acids and derivatives (hydrolyzed proteins) are also used as additives including inositol, leucine, and glutamic acid. Some of the enhancers are ethyl pyrazine (toasty/crispy taste, bakery products cookies), caffeine, capsicum, ethylguaiacol (smoky/ashy flavor), ethyl maltol (sweetner e.g. in candy), γ-octalactone (creamy taste), isobutavan (thickening agent for cloud vaping), malic or citric acid (sour- salt and vinegar), sucralose (sweetner), syringol (smoky), triacetin (buttery), vanillin/maltol, and sucralose (sweetness in candy flavors juices), tartarazine (imparting colors in candy, dessert, ice crème, alcoholic beverages), and sulfites-preservatives (for throat hit, usually trigger allergic responses, headache, sneeze and cough) which are now available in e-liquid for vaping.

Some of these chemicals including MSG can cause allergic responses. The toxicological aspects of these flavoring additives are not known, however, it is perceived that some of the agents can act as oxidative and inflammatory agents, and lead to pro-fibrotic, proatherogenic and pro-adipogenic and pro-diabetic responses (insulin metabolism, resistance, and secretion), neurological, developmental and reproductive disorders. As e-liquid/juices are popular in teenagers as well as adults, it will affect behavior and trigger allergies, hyperresponsiveness and asthma responses. It is not yet known whether these additive chemicals are possible carcinogens especially when charged at high voltage settings with flavors during inhalation.

10. Flavoring chemicals risk assessment based on toxicology

The current inadequate *in vitro* and *in vivo* data suggests that many constituents including flavoring and its enhancer chemicals may lead to respiratory pathology, allergic and immune-inflammatory responses after inhalation and may induce toxicity (Costigan and Lopez-Belmonte, 2017; Costigan and Meredith, 2015). As discussed before, thermal decomposition of flavoring chemicals e.g. diacetyl, aldehydes can lead to tissue damage. This tissue injury may be studied using three dimensional cell culture models in the future. In addition, explicit cell viability effects of newer flavorants on specific lung cell types in airway epithelia (e.g. mucus producing goblet cells and ciliated cells) will add to proper risk assessment. Therefore, we propose that a combination of assessment of ingredient purity, screening for hazard exclusion on respiratory sensitizers, allergic and sensitivity responses, vaping topography and determination of thermal breakdown products would be useful for regulatory toxicity and risk assessment.

11. Heat-not-burn tobacco smoke with flavoring chemicals and their toxicity

The tobacco industry claims it has created a healthier alternative to smoking combustible cigarettes. iQOS, heat-not-burn devices, deliver nicotine without reaching high temperatures compared to conventional cigarettes (Kogel et al., 2016; Oviedo et al., 2016; Sewer et al., 2016; Smith et al., 2016; Wong et al., 2016). Traditional cigarettes produce smoke from the smoldering end along with ash. The rationale for the use of heat-not-burn devices is the harm reduction attempt due to lack of combustion in these cigarettes and the resulting health effects.

IQOS and other heat-not-burn devices use sheets of tobacco that reach lower temperatures to deliver nicotine to the user (Kogel et al., 2016; Oviedo et al., 2016; Smith et al., 2016; Wong et al., 2016). Heating tobacco cast-leaves form the tobacco constituents that are comparable to that of conventional cigarettes without producing sidestream smoke (Auer et al., 2017; Smith et al., 2016). Heat-not-burn devices reach temperatures between 300°C-350°C as opposed to the combustion zone temperature 700°C-900°C of a conventional cigarette (Smith et al., 2016).

These devices may not produce sidestream smoke due to lack of complete combustion. However, they do reach temperatures high enough for pyrolytic reactions to occur (Bekki et al., 2017; Li et al., 2018; Schaller et al., 2016a; Smith et al., 2016). The constituents of mainstream smoke from tobacco heating systems are less compared to that of conventional cigarettes. However, the deleterious compounds to human health are still present in smoke produced by IQOS (Kogel et al., 2016; Oviedo et al., 2016; Sewer et al., 2016; Wong et al., 2016). Ubiquitous constituents of conventional cigarette smoke such as tar, nicotine, carbonyl compounds (i.e., formaldehyde, acrolein, acetaldehyde), and nitrosamines are still present in mainstream smoke from tobacco heating systems. Tobacco heating systems may lower these toxic constituents of conventional mainstream smoke, but they are still present in varying concentrations or may have some additional volatiles, posing a significant health risk to the users (Bekki et al., 2017; Li et al., 2018; Schaller et al., 2016a; Schaller et al., 2016b). Studies have also shown that flavored iQOS smoke such as menthol, a flavoring agent, contains even higher concentrations of these harmful constituents (Bekki et al., 2017; Kogel et al., 2016; Oviedo et al., 2016). Mitova et al. showed that the exhaled portion of the iQOS smoke contained nicotine, acetaldehyde, and other volatile organic compounds (Mitova et al., 2016). This increases the risk of contaminating indoor environments, such as restaurants and workplaces, thus exposing the nonsmoking population to secondhand smoke contaminants.

Emission of the hazardous compounds by thermogenic degradation of tobacco causes adverse health effects, such as impaired endothelial function which can lead to cardiovascular diseases (Glantz, 2017). Presence of tar and VOCs increases the exposure to free radicals that can result in oxidative stress and inflammation, ultimately posing the same health risks induced by exposure to conventional cigarette smoke such as asthma, chronic obstructive pulmonary disease (COPD), and stroke. Thus, heat-not-burn systems (iQOS) do not eliminate the risk of exposure to hazardous constituents of conventional smoke. It can be

hypothesized that these novel tobacco heating systems will become the healthier/low-risk alternative to regular cigarettes in the next five years. Therefore, it is crucial to conduct more independent research studies on characterization and toxicity of the smoke, in particular as and when these iQOS will have flavoring chemicals and flavors on respiratory health.

12. Respiratory health effects of ENDS flavorings: shortcomings on lack of chronic respiratory health effect studies

Research on the characterization of e-cigarette aerosols and related health effects on humans have been emerging within the last five years as e-cigarettes are becoming vastly popular, especially among young Americans and the worldwide population (Chun et al., 2017; Huang et al., 2017). It is crucial to understand toxicological and chronic health effects of e-cigarettes as it has been introduced and marketed as a healthier alternative to conventional combustible cigarettes.

Particle characterization studies have shown that 95% of the e-cig aerosols are in the nanometer aerodynamic diameter range, mostly around 600 nm (Alderman et al., 2014). These small respirable particles can reach the alveolar and airways region. However, the deposition may be more complex due to the presence of humectants, such as propylene glycol and vegetable glycerin. Larger hygroscopic growth rate can increase the lung deposition of these particles. Exposure to e-cig particles can lead to pulmonary toxicity due to oxidative stress-mediated inflammatory responses. Moreover, nicotine in e-liquids can downregulate a7nAChR activity and impair the CFTR regulator, which in turn can result in decreased mucociliary clearance in chronic lung diseases (Maouche et al., 2013)

Acute exposures to e-cigarettes have shown decreased lung function. Vardavas et al., showed increased airway resistance and decreased FeNO in exhaled breath condensate after 5 minutes of exposure to e-liquid with nicotine (Vardavas et al., 2012). Previous studies have shown that decreased FeNO may suggest impaired CFTR function (Vardavas et al., 2012) (Korten et al., 2018). Kumral et al., observed sinonasal and mucociliary clearance symptoms in chronic e-cig users (Kumral et al., 2016). Other studies have demonstrated depressed cough reflex due to acute exposure to nicotine-containing e-cig (Dicpinigaitis, 2017; Dicpinigaitis et al., 2016a, b), and suppression of immune and inflammatory responses in nasal epithelium (Martin et al., 2016). These studies suggest that e-cig use can dampen protective mechanisms against foreign material entering human lungs.

On average 16% of high school children have smoked e-cigarettes with flavorings. Therefore, it is essential to understand the effects of exposure to e-cigs flavorings with and without nicotine in adolescents. Observational and survey-based studies on children have shown chronic bronchitis among e-cig users and increased asthma symptoms, even with sub-acute exposures (McConnell et al., 2017). Increased school absences have been noticed in children with exacerbated asthma symptoms due to e-cigarette use (Wang et al., 2016), suggesting the immune-inflammatory responses of e-cigs in vulnerable populations. A randomized study was performed to evaluate the safety profile of ENDS vapor products over 12 weeks where general protective effects were seen (Cravo et al., 2016), and harm

reduction in COPD smokers who switched to e-cigarettes as well as in a long-term cessation study (Campagna et al., 2016; Polosa et al., 2016).

Adverse respiratory effects observed in humans are consistent with *in vitro* and animal studies. These include inflammation, oxidative stress, decreased lung function, and suppressed protective mechanisms (cough reflex, mucociliary clearance) (Aufderheide and Emura, 2017; Aug et al., 2015; Larcombe et al., 2017) (Barber et al., 2017). These symptoms also suggest increased susceptibility to pathogens among e-cig users. It is also believed that flavorings can suppress the immune responses and augment the susceptibility to viral and bacterial infections in vulnerable and susceptible populations. Further, the flavoring chemicals used in e-cigarettes and other tobacco products may increase the risk of exacerbations in susceptible populations and in preexisting conditions associated with pulmonary diseases by generating extremely harmful chemicals upon aerosolization. Clearly there is a shortcoming on current state of scientific understanding due to lack of human exposure respiratory health effect data. Hence, further studies are required to study the respiratory health effects by chronic exposures to flavorings in ENDS and other tobacco products. The outcome of such studies will provide toxicological information on the regulation of e-cigarettes and flavorings for regulatory agencies.

13. Conclusions

The most common flavoring agent, menthol inhibits oxidation of nicotine to cotinine and thus increases the overall nicotine exposure (MacDougall et al., 2003). Menthol, in presence of ethanol, is reported to decrease excretion and thus increase tissue accumulation of nicotine-derived nitrosamine ketone (NNK) in porcine esophageal mucosa (Azzi et al., 2006). Similar pathways are yet to be established in other flavoring agents. Furthermore, as vaporizers have adjustable features (wattage/voltages, air flow), customization by users by altering their topography may add to the risk. There are effects that are yet not fully understood in terms of flavoring-induced toxicity, such as mitochondrial toxicity (lesions and increased permeability of inner mitochondrial membrane) of menthol at concentrations greater than 0.1 mM (Bernson and Pettersson, 1983). This involves impaired mitophagy in various cellular dysfunctions (apoptosis, necrosis/necroptosis, and cellular senescence). These pathways may also exist in other flavoring compounds mediated toxicity. Additionally, it is possible to extrapolate the data on biomarker and toxicity studies from animal models to humans (Schick et al., 2017). However, the studies on animal exposure by e-liquids and aerosols should be carefully interpreted due to the anatomical and physiological differences, as well as different susceptibilities in animals and humans (Rahman et al., 2017). As discussed above, recent studies on oxidative, inflammatory, and immune systems effects by e-cigarettes have been reported *in vitro* and *in vivo* mouse models, but studies on flavorings on lung toxicity are not available. Similarly, it is possible that dampening of innate-immune responses by flavorings lead to increased host-pathogen interactions in e-cigarette flavoring users. Flavoring may directly interact with host and modify host-pathogen interactions.

It is quite clear that flavors in e-cigarettes have been marketed to attract young adults (Ambrose et al., 2015; Harrell et al., 2017) with no apparent health benefits and product

switching from tobacco cigarettes to e-cigarettes may decrease health risks for other reasons than added flavoring agents or chemicals. This include various emerging ENDS include JUUL which uses salts, such as benzoic acid, lactic acid- nicotine salts (nicotine benzoate or lactate) with different flavorings and various vaping devices, such as nutrovape (energy vape, sleep/relax aid vape, aromatherapy happy and diet aid, vitamin B12 vape) are launched without regulatory testing.

14. Future directions

TPSAC (TPSAC, 2011) has already outlined the required steps to correctly assess the role of flavoring agents in human risk due to inhalation by smoker and aerosols/vapors. Many questions are still to be answered. It would help to establish a mechanism for tracking the favoring agents that are being used so that potential toxicities can be examined. Attempts have been already made as shown by detection methods in Table 1.

While many *in vitro* assays have been developed to predict the potential toxicities of flavorings, there is a need to use aerosol/vapor than e-liquids to correctly assess the exposure. Except menthol, no other flavor is implicated in addiction liability and nicotine dependence until now. Any additional addiction and neurological impact liabilities specific to introduction of flavor to e-cigarettes have not been pursued. There is a lack of chronic cohort studies, such studies would help understand the progression of pathophysiology related to flavor containing vapors. This demands a consolidated database approach to collect data from exposure to flavored products in cases such as bronchiolitis obliterans (popcorn lungs). This would also provide ways to evaluate pattern of use especially among adolescents. Furthermore, there is a lack of information if flavoring chemicals would metabolize in the body to more harmful compounds, affect host-pathogen interactions, and lead to inflammatory or immunogenic responses. Cohort studies with follow up to accurately measure human risk from e-cigarette use in active and passive e-cigarettes users are required. Finally, it is important that proper human risk assessment of flavoring compounds and additives or enhancers (e.g. JUUL containing nicotine benzoate or lactate and nutrovape as mentioned above in emerging tobacco and non-tobacco products is performed to pave way for strict regulatory laws and reduce usage in young adults and minimize respiratory toxicological health effects.

Acknowledgements and Funding

This study was supported by the NIH 1R01HL135613, NIH 1R01HL085613 and NIH 1R01HL085613-S1 and NIH-FDA-CTP 1R01DA042470 (to I.R). Research reported in this publication was supported by NIDA/NIH and FDA Center for Tobacco Products (CTP). The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH or the Food and Drug Administration.” The first author GK appreciates the unconditional support and guidance provided by Prof. Dr. Daniel Dietrich during GK’s ongoing post-doctoral fellowship.

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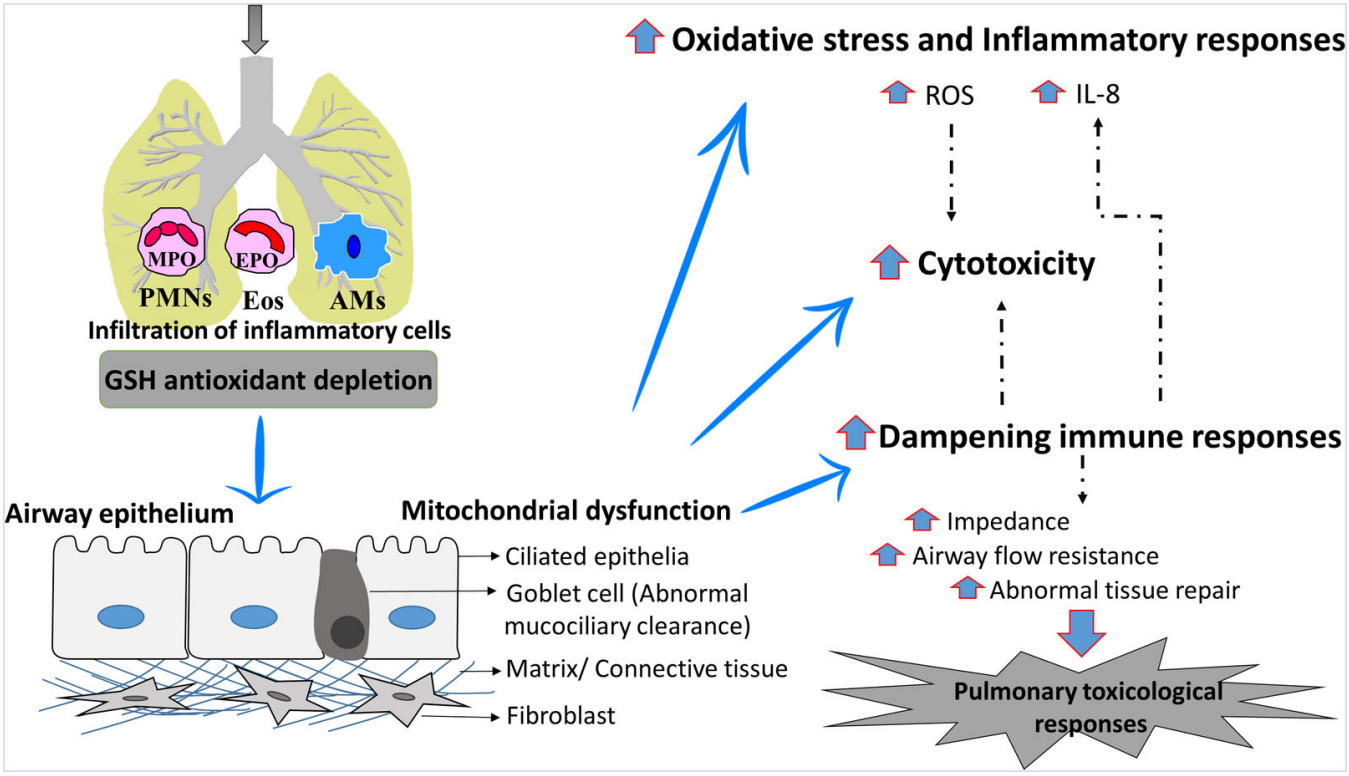

Figure 1. Currently known toxicity mechanisms in affected respiratory cells upon flavorant exposure:

The figure represents the pulmonary toxicity after inhalation of a flavoring chemical such as diacetyl. Once inhaled, the flavoring agent causes insult to the first line of defense (e.g. depletion of antioxidant thiols and barrier dysfunction) against particulates and antigens, the airway epithelium. Responsible for appropriate conduction of airflow, the airway epithelium usually consists of ciliated epithelium cells interspersed with mucus secreting goblet cells. Hence, the contact with flavoring chemicals (flavorant) results in induction of oxidative stress (as visible from increased ROS production and IL-8 secretion) as well as immune responses such as decreased lubrication, increased impedance and flow resistance (marked by coughing). While both mechanisms are capable of separately leading to cell toxicity (as observed in bronchiolitis obliterans in case of diacetyl inhalation), cytotoxicity may also proceed by yet not known mechanisms. Fibroblasts, present in the adjacent connective tissue, beneath the airway epithelium, are also affected. Upon oxidative stress and DNA damage, various cellular signaling cascades are activated, thereby leading to inflammatory responses.

Table 1

Common flavoring agents in tobacco and non-tobacco products and their biological effects

Flavoring chemicals	Common flavors with chemical agents	Toxic effects	Detection methods in e-cigarettes	Study summary	References
Menthol	Mint	Oxidative, inflammatory and barrier dysfunction	GC-MS	Concentrations of menthol and other 9 flavors in 36 e-cigarette products were determined. Menthol concentrations ranged between 3700–12000 μg/g and was also found at low concentrations in 40% of the tobacco-flavored non-menthol products tested in this study.	(Lisko et al., 2015)
				30 e-cigarette fluids were analyzed for percentage of flavoring chemicals by weight using GC-MS. Menthol concentrations ranged between 5700–21600 μg/ml.	(Tierney et al., 2016)
				Flavoring agents including menthol were identified in 4 e-liquids and their emissions. Tested flavorings showed pro-inflammatory response and changes in barrier function when applied to different lung cells *in vitro*.	(Gerloff et al., 2017)
Diacetyl	Most common, used to simulate dairy products, chocolate, coffee, fruit etc.	Known to cause bronchiolitis obliterans and severe respiratory pathology.	GC	Diacetyl (up to 239 μg per cigarette) was identified using GC in 39 out of 51 tested e-cigarette samples. Other flavoring agents detected were 2,3-pentanedione and acetoin (see below).	(Allen et al., 2016)
				Diacetyl was detected in 62% of 24 e-cigarette vapors using GC. The study also identified presence of at least one aldehyde (propionaldehyde, acetaldehyde or formaldehyde) and acetoin in the e-cigarette vapors (see below).	(Klager et al., 2017)
			HPLC	More than 74.2% of 159 e-cigarette liquid and aerosol contained diacetyl using HPLC detection. Median diacetyl daily exposure levels were 56 μg/day (IQR: 26–278 μg/day). Also, 47.3% diacetyl containing samples	(Farsalinos et al., 2015b)

Flavoring chemicals	Common flavors with chemical agents	Toxic effects	Detection methods in e-cigarettes	Study summary	References
				exposed consumers to levels higher than the safety limits. 2,3-pentanedione levels were also detected (see below)	
2,3-pentanedione	Similar profile to diacetyl	Not studied	GC	2,3-pentanedione (64 μg per e-cigarette) was identified using GC in 23 out of 51 tested e-cigarette samples.	(Allen et al., 2016)
			HPLC	2,3-pentanedione was detected in 74.2% of 159 e-cigarette liquid as well as aerosol samples using HPLC. Median daily exposure levels were 91 μg/day (IQR: 20–432 μg/day). Also, 41.5% 2,3-pentanedione containing samples exposed consumers to levels higher than the safety limits.	(Farsalinos et al., 2015b)
Acetoin	Similar profile to diacetyl	Not studied	GC	Acetoin (up to 529 μg per cigarette) was identified using GC in 46 out of 51 tested e-cigarette samples.	(Allen et al., 2016)
				65% of 24 e-cigarette vapors had detectable levels of acetoin.	(Klager et al., 2017)
2,5-dimethypyrazine	Chocolate	Not studied	GC-MS	Levels of 2,5-dimethylpyrazine were detected in 5 out of 7 brands (total 263 e-liquid samples) with high accuracy. Other flavors such as 3,4-methoxybenzaldehyde (see below), humectants and polycyclic aromatic hydrocarbons were also detected.	(Kavvalakis et al., 2015)
3,4-Dimethoxybenzaldehyde	Cherry	Not studied	GC-MS	3,4-dimethoxybenzaldehyde was detected in 5 out of 7 brands (total 263 e-liquid samples with frequency of detection at 5.3%).	(Kavvalakis et al., 2015)
Vanillin, ethyl vanillin	Vanilla	Respiratory irritatant, inflammatory	GC-MS	141 volatile flavors including vanillin (in 22 out of 28) and ethyl vanillin (14 out of 28) were detected in 28 e-cig liquid aerosol samples. Other flavors detected include cinnamaldehyde and 3-methyl-1,2-cyclopentanedione (see below). Aldehydes, propylene glycol and	(Hutzler et al., 2014)

Flavoring chemicals	Common flavors with chemical agents	Toxic effects	Detection methods in e-cigarettes	Study summary	References
				glycerol were also detected.	
				14/30 e-cigarette liquids showed presence of vanillin and 10/30 samples showed ethyl vanillin using GC-MS. Concentrations up to 3300 μg/ml for vanillin was detected.	(Tierney et al., 2016)
				4 e-liquid samples were analyzed with GC-MS and various flavors including vanillin and ethyl vanillin were identified. In vitro cultures of lung cells (human bronchial epithelial cells, human lung fibroblasts) were treated with each flavoring chemical and analyzed for pro-inflammatory cytokines, IL-8. Rise in IL-8 and impairment in epithelial barrier function was noted.	(Gerloff et al., 2017)
Cinnamaldehyde, 2-methoxycinnamaldehyde	Cinnamon	Oxidative, inflammatory and barrier dysfunction	GC-MS	GC-MS analysis detected cinnamaldehyde in 2 out of 28 samples.	(Hutzler et al., 2014)
				Cinnamaldehyde was also identified using GC-MS in the 4 e-liquid samples but did not show a rise in IL-8 in this study.	(Gerloff et al., 2017)
			GC-MS, HPLC	Flavors were detected in Cinnamon Ceylon and other 8 cinnamon refill fluids. MTT assay screening in human embryonic stem cells and human adult pulmonary fibroblasts of above e-liquids showed cytotoxicity to varying degree with flavors, cinnamaldehyde and 2-methoxycinnamaldehyde being the most cytotoxic components.	(Behar et al., 2014)
Maltol, ethyl maltol	Caramel, Vanilla	Oxidative, inflammatory and barrier dysfunction	GC-MS	Out of 30 e-liquid samples, 8 contained maltol and 9 contained ethyl maltol at concentrations ≥0.5 mg/ml.	(Tierney et al., 2016)
				Maltol and ethyl maltol were detected using GC-MS in the 4 e-liquid preparations and showed	(Gerloff et al., 2017)

Flavoring chemicals	Common flavors with chemical agents	Toxic effects	Detection methods in e-cigarettes	Study summary	References
				a significant increase in IL-8 release.	
Other lesser known flavoring agents: Damascenone (α or β), 3-methyl-1,2-cyclopentanedione, acetamide, linalool, terpineol, citral, corylon, anisaldehyde, trimethylpyrazine, eugenol, benzaldehyde, limonene	Miscellaneous	Oxidative, inflammatory and barrier dysfunction	GC-MS	All the flavors listed were detected to varying degrees in tested 28 e-liquid samples using GC-MS analysis.	(Hutzler et al., 2014)
				Eugenol and other flavors were identified in 1/4 e-liquid vapors	(Gerloff et al., 2017)
				Eugenol was detected in 1/30 samples with concentration of 1.9mg/ml while benzaldehyde in 3/30 e-liquid samples with concentrations between 0.6–21.2 mg/ml and limonene in 2/30 samples with ≤ 2.7 mg/ml.	(Tierney et al., 2016)

Abbreviations: GC: gas chromatography, GC-MS: gas chromatography-mass spectrometry, HPLC: high performance liquid chromatography.