A REVIEW OF THE CYTOTOXICITY OF FREQUENTLY USED FLAVOR CHEMICALS IN REFILL FLUIDS

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Abstract

E-Cigarettes were introduced to the United States in the early 2000s and have gained popularity rapidly since. E-Cigarettes aerosolize a liquid, a “refill fluid” that the user then inhales. Although most E-Cigarettes contain nicotine, flavorings in refill fluids are what has drawn youth to use E-Cigarettes. Evidence is growing that flavor chemicals in the refill fluids can cause harm upon inhalation. Seven of the most popular flavor chemicals in refill fluids were chosen to study in this review. Ethyl vanillin, vanillin, cinnamaldehyde, maltol, ethyl maltol, benzyl alcohol, and menthol. Fifty-seven original articles (collected from PubMed, and Google Scholar) on the cytotoxicity of flavor chemicals were collected and critically analyzed. Many of these flavor chemicals are found frequently and are present in high concentrations. The cytotoxic effects of these flavor chemicals reducing mitochondrial reductase activity, decreasing cellular viability, increasing the production of reactive oxygen species, increasing the expression of proinflammatory molecules, disrupting epithelial barriers, and more. More research is needed on the specific effects of these flavor chemicals, as well as the cytotoxic effects of other flavor chemicals that can be found in refill fluids of E-Cigarettes.

Introduction

Electronic nicotine delivery systems (ENDS), also known as electronic cigarettes (ECs), e-cigarettes, vapes, E-cigs are battery powered devices that heat up a liquid solution to aerosolize its components, which are meant to be inhaled by the user in the form of a vapor. Originally meant to mimic the traditional smoking experience, ECs have rapidly evolved due to their popularity since their introduction in the United States marketplace in 2007 **(Schmidt 2020)**. All ECs have the same four basic components, (1) a cartridge to store the refill fluid (or e-liquid), (2) an atomizer, which is a heating coil that vaporizes the refill fluid, (3) a mouthpiece, and (4) a battery **(Breitbarth, et al. 2020)**. Currently there are "4 generations" of Electronic Cigarettes that have been released, which can be seen in Figure 1 below.

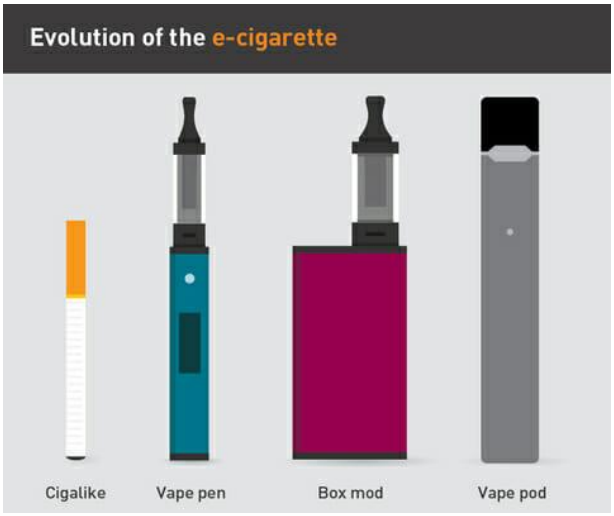


Figure 1: The evolution of electronic cigarettes, from 1st generation to 4th generation. Image freely available at https://truthinitiative.org/research-resources/emerging-tobacco-products/e-cigarettes-facts-stats-and-regulations

First generation ECs mimicked the appearance of the traditional filter cigarette and was sometimes referred to as a "cig-a-like." These ECs aimed to provide the user a smoking experience as close to the traditional cigarette as possible **(Williams, et al. 2019, Schmidt 2020)**. Pre-assembled and already containing e-liquid, these ECs were one time use, and were offered in

different nicotine concentrations for the user's convenience. The atomizer would vaporize the e-liquid and illuminate the tip (to resemble the burning of a traditional cigarette).

Second generation ECs were larger than the cig-a-likes and tended to have rechargeable batteries with a significantly more power to deliver more nicotine and thicker clouds of vapor. Most notably however was the presence of tanks that could be refilled with fluids as often as the user desired **(Williams, et al. 2019, Schmidt 2020)**. The appearance of 2nd generation ECs allowed for users to customize their vaping experience with new flavors of liquids, cheapened costs, and allowed the refill fluid industry to boom **(Breitbarth, et al. 2020)**. Some models only allowed for a specific length puff, while other models allowed the user to modulate how long and deep their puffs would be, offering a variety that the original ECs did not.

Third generation ECs tended to be even larger than their predecessors and included larger, longer lasting batteries with the option to customize their vape by changing the coil. Called "tanks" because of their bulky size, 3rd generation ECs would allow users to modify the coil temperature, which in turn let them control the properties of the aerosol, such as its concentration and thickness. This allowed users to have a customizable vaping experience, which helped to boost the popularity of ECs as users started to experiment to find their "fit" **(Wiliams, et al. 2019, Schmidt 2020)**.

Fourth generation ECs implemented a "pod" system. The pods were pre-filled cartridges that were one use, but allowed users to keep the same battery, to reduce costs. The main appeal of 4th generation ECs, however, was their sleekness and unique design. Brands such as JUUL™ resembled USB devices and were sleek, and easily concealable. This made them especially popular with middle schoolers and high schoolers, as they could easily hide their device or pretend it was just a USB if an adult were nearby. These ECs came in many different styles and

colors, and their flavors varied as companies, such as JUUL™ attempted to market them to youth and underage users. With smaller batteries and coils, the clouds produced would remain small, allowing for what came to be known as "stealth vaping."

ECs have rapidly gained popularity amongst youth and adults alike for the cheaper price, better taste/smell, variability in flavors, and misleading marketing by retailers. By 2020, 25% of EC users in the United States were under the age of 18 **(Wang, et al. 2020)**. In a survey, over 80% of users between the ages of 12-17 years stated that the variety of "cool flavors and nice smells" was a major reason for use **(Ambrose, et al. 2020)**. Another reason that ECs tend to be so popular is due to misleading claims made by manufacturers that ECs are "healthier", and "safer" ways to smoke. These claims also tend to target those who currently smoke to get them to switch over to using ECs instead. **(Chen, et al. 2020)**. Retailers tend to market their EC products to youth populations through catchy advertisements, and even going as far as giving a presentation at a high school **(Chen, et al. 2020)**. These sorts of claims and marketing strategies have helped EC retailers solidify themselves in the world of smoking and have greatly boosted the popularity and usage of their product.

Refill fluids are hygroscopic substances (usually a mix of propylene glycol [PG], and vegetable glycerin [VG]), ethanol, water, nicotine, and flavor chemicals **(Stefaniak, et al. 2021)**. Refill fluids come in many forms, where users can customize the ratio of PG:VG depending on the type of vaping experience they would like and can alter nicotine concentrations by using nicotine salts. The components of interest are the flavor chemicals. Flavor chemicals are the chemicals that bind to olfactory and gustatory receptors to give the sensation of "flavor." Common flavor chemicals include cinnamaldehyde and vanillin that give rise to flavors such as cinnamon, and vanilla. There are thousands of flavors that are a combination of many fewer

flavor chemicals. Flavor chemicals have been found in alarming concentrations in ECs; however, bringing to light another concern about the safety of ECs **(Omaiye, et al. 2019, Hua, et al. 2019, Behar, et al. 2018)**. Although the Flavor and Extracts Manufacturer's Association (FEMA) are generally listed as safe to consume, this designation pertains to ingestion, not inhalation **(Hallagan 2014)**. More data regarding the potential dangers of flavor chemicals in refill fluids is necessary.

The purpose of this paper is to review literature dealing with some of the most popular flavor chemicals in refill fluids and to understand their cytotoxic effects by analyzing previously discovered data from published scientific literature.

Methods

Figure 2 summarizes the methods used to gather articles for this review. Peer-reviewed literature from Google Scholar and PubMed was gathered using the specific keywords: (EC, Electronic Cigarettes, vapes, E-Cigs, ENDS, E-cigarettes), and (Vanillin, Cinnamaldehyde, Ethyl Vanillin, Ethyl Maltol, Maltol, Benzyl Alcohol, Menthol), and (cytotoxicity, toxicity, oxidative stress, inflammatory responses, cellular effects/responses) by June 2021. The flavor chemicals looked at in this review are chemicals that tended to be found most often in high concentrations, and more frequently than other flavor chemicals based on previously collected data. The abstracts of all PubMed and Google Scholar articles were screened and any non-English articles or articles outside the scope of the project (experimental procedure, policy, only nicotine) were disregarded. We merged the articles from the two data bases and during a secondary screening eliminated any duplicates, or any other articles that were out of the scope of the paper. Tertiary review involved in-depth review of article content for consideration in the final review. Any

studies that did not involve toxicological effects of flavor chemicals, pure or as a part of refill fluids, *in vitro* or *in vivo* were eliminated. The review was updated and supplemental studies involving in depth information regarding specific cytotoxic effects caused by flavor chemicals were included. The articles in this review were primarily *in vitro*, and *in vivo* studies involving the effects of vaporized flavor chemicals present in refill fluids or as pure standards.

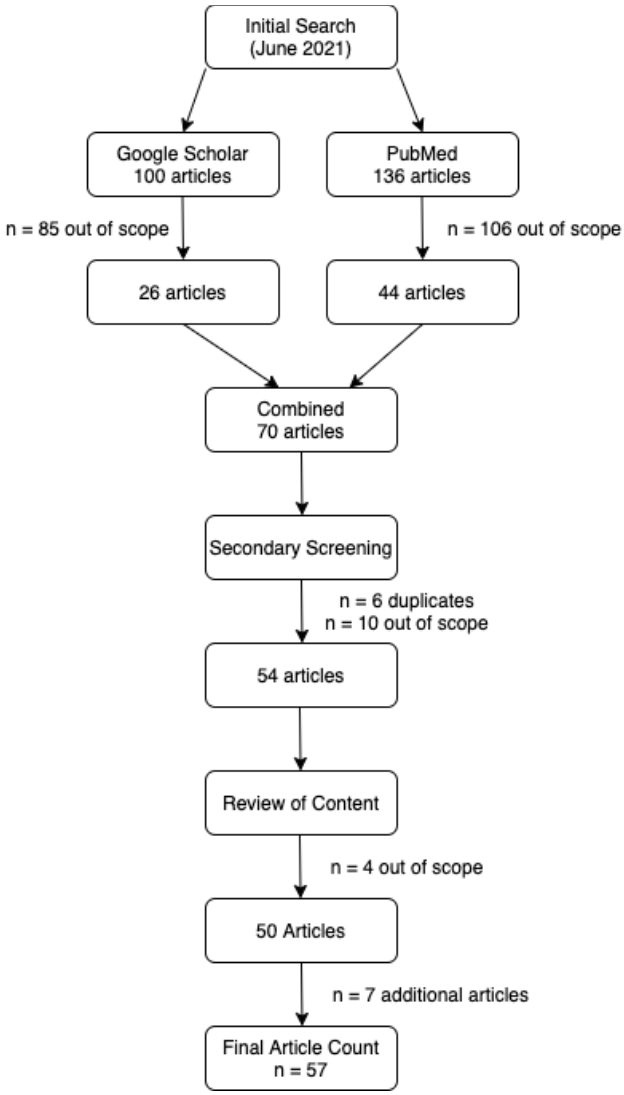


Figure 2: Flow diagram of literature search

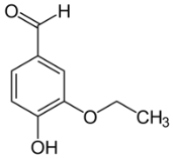
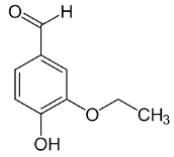
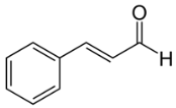
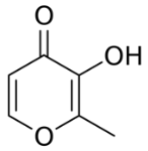
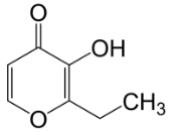
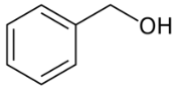
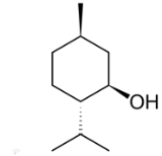
Name	Chemical Formula	Structure	Molecular Mass	Boiling Point	Flavor Profile	CAS Number
Ethyl Vanillin	$C_9H_{10}O_3$		166 g/mol	295.1 ℃	Aromatic, Caramel, Creamy, Smooth, Sweet, Vanilla	121-32-4
Vanillin	$C_8H_8O_3$		152 g/mol	285-286 ℃	Creamy, milky, phenolic, sweet, vanilla, vanilla bean	121-33-5
Cinnamaldehyde	C_9H_8O		132 g/mol	248 ℃	Cinnamon, spicy	104-55-2
Maltol	$C_6H_6O_3$		284.7 g/mol	289-290 ℃	Sweet, Berry, caramelly, cotton candy, fruity, jammy	118-71-8
Ethyl Maltol	$C_7H_8O_3$		140.14 g/mol	289-290 ℃	Sweet, Burnt Cotton, Caramel, Jammy, Strawberry, Sugar candy	4940-11-8
Benzyl Alcohol	C_7H_8O		108.14 g/mol	205 ℃	Almond, Balsamic, Bitter, Chemical, Cherry, Fruity, Weak	100-51-6
Menthol	$C_{10}H_{20}O$		156 g/mol	216 ℃	Cooling, mentholic, minty	2216-51-5

Figure 3: Basic chemical information about the 7 flavor chemicals analyzed in this review

Ethyl Vanillin

Ethyl vanillin, a frequently found flavor chemical in refill fluids, has a negative effect on ATP production and oxygen consumption rates **(Hua, et al 2019, Bitzer 2018, Jabba, et al 2020)**. In a study of the 20 top-selling refill fluids purchased in Southern California, 10 dominant flavor chemicals were identified and evaluated in the MTT assay **(Hua, et al 2019).** The concentrations of ethyl vanillin met or exceeded the IC_{50} in the MTT assay **(Hua, et al 2019)**. Ethyl vanillin was the 4th most potent flavor chemical, and its cytotoxicity was strongly correlated with its concentration ($R^2 = 0.68$) **(Hua, et al 2019).** The MTT assay showed that many refill fluids that contained ethyl vanillin also produced a significant decrease in mitochondrial reductase activity **(Hua, et al 2019)**. Jabba, et al. 2020 then showed through live-cell metabolic assays and Seahorse XF mitochondrial stress tests that ethyl vanillin decreased basal Oxygen Consumption Rate (OCR) in a concentration dependent manner at 1,3,5 mM **(Jabba, et al 2020)**. Maximal respiration and Spare Respiratory Capacity (SRC) were reduced in both cells at concentrations of 3 mM and 5 mM. This led to an observed decrease in the ATP production of BEAS-2B cells at 1,3,5 mM and at 3,5 mM for A549 cells **(Jabba, et al 2020)**. SRC is frequently used as an indicator of cellular ability to respond to increased energy demand, which is seen in cell division, differentiation, stress response, and cell fate determination. A decrease in this metric is an indicator of mitochondrial dysfunction. This is an important finding because the decrease in ATP production can have significant effects on cellular function since production of ATP is critical for a wide range of cellular processes in lung epithelial cells, especially for ciliary beating, and surfactant production. This diminished ATP production in cells can then decrease defenses against reactive oxygen species causing cellular stress and increasing cytotoxicity.

Ethyl vanillin's effects on cells is also seen in its's ability to decrease oxygen consumption rates and decrease free radical production. In a pure ethyl vanillin study conducted by Hickman, et al. 2019, the authors showed that in neutrophils isolated from healthy human subjects, ethyl vanillin decreased Total Oxygen Consumption Rate (OCR) in a dose-dependent manner and the highest doses of Ethyl Vanillin tested (5 mM) significantly decreased time to max OCR **(Hickman, et al. 2019)**. This correlates with the research performed by Hua, et al. 2019 since a decrease in max time to OCR indicates a decrease in mitochondrial activity since it shows that oxygen is not being as efficiently used in processes such as oxidative phosphorylation, which correlates with a decrease in ATP production, as demonstrated by Jabba, et al 2020. Furthermore, this decrease could also be due to the key proteins of glycolysis or the pentose phosphate pathway undergoing thiol modifications or forming NADPH complexes. Hickman, et al. 2019 also discovered that ethyl vanillin had the potential to drastically reduce neutrophil phagocytosis at the highest doses tested by ~50% (**Hickman, et al. 2019)**. Since neutrophils are instrumental in disposing of bacterial pathogens, a decrease in function could lead to increased susceptibility of respiratory disease and infection. Furthermore, neutrophil activity leads to the creation of radicals and reactive oxygen species (ROS) , so a decrease in activity would also show a decrease in ROS. This is backed up Bitzer et al. 2018, who showed how ethyl vanillin had one of the highest tested negative correlation coefficients to free radical production, with a Pearson coefficient of -0.0943 **(Bitzer, et al. 2018)**. This decreased free radical production by 42% at an ethyl vanillin concentration of 0.8 mg/mL. At a baseline of 0.8 mg/mL lipid peroxidation was also reduced by 60% with malondialdehyde being used as a marker **(Bitzer, et al. 2018)**. This decrease in ROS is further backed up by a 45% decrease in 8-isoprostane, a molecule produced by the free radical-catalyzed peroxidation of arachidonic acid

(Bitzer, et al. 2018). This antioxidant capability may be because phenols can break chains by reacting with peroxyl radicals having weak O-H/N-H bonds and because of the formation of Schiff bases by the aldehyde on ethyl vanillin.

Vanillin is another very commonly found flavor chemical that is often present in high concentrations in refill fluids and was found to be the second most cytotoxic flavor chemical in a study of 65 flavor chemicals **(Behar, et al. 2018)**. It reduces mitochondrial activity, impairs NO production, increases proinflammatory mediators, increases oxidative stress, and causes cell death. Behar, et al. 2018 found that vanillin was found in 56% of analyzed fluids at concentrations of at least 1mg/mL, while the highest concentration found was 31 mg/mL **(Behar, et al. 2018)**. Using MTT assays, the researchers demonstrated that vanillin could have a significant effect on mitochondrial reductase activity and decreased activity lead to an overall decrease in mitochondria function **(Behar, et al. 2018)**. Using a DAF-2DA assay, Fetterman, et al 2018 discovered that vanillin impaired A23187 induced nitric oxide production (**Fetterman, et al. 2018)**. This is significant because nitric oxide is not only an important neurotransmitter, but also plays roles in maintaining homeostasis and is a regulatory molecule in cells. qPCR assays of IL-6 expression at most of the concentrations tested (4.6 E^-5 mg/mL – 0.00046 mg/mL) and showed increased ICAM-1 expression at 0.46 mg/mL (**Fetterman, et al. 2018)**. This is significant for epithelial cells because in Kreuger, et al. 1991 enhanced levels of IL-6 were seen in many solid tumors and hyperproliferative lesions of psoriasis. Furthermore, *in vitro*, IL-6 can increase proliferation of keratinocytes, and in breast carcinoma cell lines (ZR-75-1, and T-47D) can elicit a major change in cell phenotype which is characterized by a fibroblastic morphology, enhanced motility, increased cell-cell separation, and decreased adherens type junctions **(Kreuger, et al. 1999)**. This shows the importance of IL-6 as an inflammatory mediator as well

as a regulator of epithelial growth and cell-cell association. As part of the immunoglobin superfamily, ICAM-1 plays important roles intercellular cell adhesion stabilizing cell-cell interactions. Increase in ICAM-1 levels due cytokine stimulation or increased levels of IL-6 can lead to improper or unnecessary immune system activation which can lead to cell damage and cell death.

Vanillin can lead to cell damage due to oxidative stress and cell death. In Fetterman's 2018 study using pure vanillin and human aortic endothelial cells (HAECs), she performed fluorescent DHE assays to test oxidative stress of cells when exposed to vanillin. Oxidative stress increased at the highest tested concentrations (0.46 mg/mL), and these concentrations were also enough to cause death of cells. TUNEL assays were performed to detect DNA breaks which occur in the last steps of apoptosis. Cell death occurred at the highest vanillin concentrations tested, 0.46 mg/mL – 4.6 mg/mL (**Fetterman, et al. 2018)**. Vanillin plays several roles in decreasing mitochondrial activity, impairing nitric oxide production, increasing proinflammatory mediators, increasing oxidative stress, and causing cell death.

Cinnamaldehyde

Like vanillin, cinnamaldehyde is found in high concentrations in many refill fluids and is frequently reported to be the most cytotoxic flavor chemical found in Electronic Cigarette refill fluids **(Behar, et al. 2018)**. Cinnamaldehyde is cytotoxic *in vitro* **(Omaiye, et al. 2020, Behar, et al. 2016)**, impairs immune functions of cells **(Clapp, et. al 2017, Gerloff, et al. 2017, Fetterman, et. al 2018)**, alters cellular morphology **(Behar, et al. 2016)**, and increases oxidative stress along with decreasing mitochondrial function in cells **(Gerloff, et al. 2017, Fetterman, et al. 2018, Clapp, et al. 2019, Muthumalage, et al. 2018)**. A 2016 refill fluid study using Human Embryonic Stem Cells (HESCs) and Human Pulmonary Fibroblasts (HPFs) using an MTT assay

found cinnamaldehyde to be highly cytotoxic. After only 2 hours of exposure at the IC_{50} concentration, HPFs were past a point of no return and could not recover. For hESCs, this point of no return occurred at 8 hours of exposure **(Behar, et al. 2016)**. Other studies calculate the IC_{50} to be in the LIQUA range with lethal concentrations being 0.053 mg/mL for HESCs, and 0.049 mg/mL in HPFs **(Omaiye, et al. 2020)**. A 2017 study by Clapp, et al., also confirmed the cytotoxicity of cinnamaldehyde as cells at 10 mM (1.32 mg/mL) cinnamaldehyde.

Cinnamaldehyde impairs immune functions of respiratory cells through multiple mechanisms. Macrophage phagocytosis was reduced at any concentration tested greater than 312 µM, or 0.00132 mg/mL **(Clapp, et al. 2017)**. A Natural Killer Cell Assay also showed that cinnamaldehyde prevented the killing of target cells, and also killed Natural Killer cells, preventing them from carrying out phagocytotic activity at any tested concentration greater than 625 µM, or 0.0825 mg/mL **(Clapp, et al. 2017)**. Further *in vitro* refill fluid studies found that cinnamaldehyde can rapidly impair epithelial cell barrier function in 16-HBE cells **(Gerloff, et al. 2017),** leaving cells defenseless against foreign pathogens. In pure cinnamaldehyde studies, cinnamaldehyde impaired A23187 induced NO production in Human Aortic Endothelial Cells (HAECs), which was correlated to endothelial disfunction and further suppression of the immune system **(Fetterman, et al. 2018)**.

Cinnamaldehyde affects cellular morphology and increases oxidative stress, while decreasing mitochondrial function in cells. In a 2016 refill fluid study, an immunocytochemistry assay showed that cinnamaldehyde depolymerizes microtubules in HPF cells decreasing cell growth, attachment, spreading, and effectively altering cellular morphology and motility. A Comet assay showed an increase in DNA strand breakage and an increase in cell death **(Behar, et al. 2016).** Clapp, et al. 2019 also found that membrane permeability of cells was reduced at

concentrations between 1-5 mM **(Clapp, et al. 2019)**. Cinnamaldehyde plays a role in the release of IL-8, inflammatory molecules, in cells such as Human Lung Epithelial Cells (BEAS-2B), Human Lung Fibroblasts (HFL-1), in Human Blood Monocytes (MM6), which led to proinflammatory responses and potential irreversible scarring **(Gerloff, et al. 2017, Muthumalage, et al. 2018).** Pure cinnamaldehyde may play a role in the release of the proinflammatory mediator, IL-6, at most concentrations (0.001-0.01 mmol/L) tested, which can be harmful to the cellular endothelium **(Fetterman, et al. 2018)**. Pure flavor chemical and refill fluid studies have found an increase in oxidative stress at higher concentrations of cinnamaldehyde (10 mmol/L) and concentration dependent ROS generation in cell free systems **(Fetterman, et al. 2018, Muthumalage, et al. 2018).** Cinnamaldehyde can also decrease the mitochondrial function of Human Bronchial Epidermal Cells (HBE), and BEAS-2B cells, which led to a drop in glycolysis at cinnamaldehyde concentrations up to 0.5 mM, and a drop in intracellular ATP at cinnamaldehyde concentrations up to 5 mM, which could be correlated with a drop in ciliary beating that was found in cells **(Clapp, et al. 2019)**. Cinnamaldehyde can have effects in cellular function and well-being by playing a role in cellular cytotoxicity, suppressing immune functions, altering cellular morphology, decreasing mitochondrial function, and increasing oxidative stress.

Maltol

Maltol is another chemical commonly found in refill fluids, which was found 18% of refill fluids analyzed **(Behar, et al. 2018)**. Maltol often is found in concentrations > 1mg/mL and in a study of 65 flavor chemicals, maltol was measured in concentrations up to 4.9 mg/mL **(Behar, et al. 2018)**. Maltol has been implicated in reducing the barrier function of cells **(Gerloff, et al. 2017**), increasing cellular proinflammatory responses **(Gerloff, et al. 2017,**

Muthumalage, et al. 2018), reducing mitochondrial activity **(Behar, et al. 2018)**, and increasing ROS generation in cells **(Muthumalage, et al. 2018)**. Using a Transepithelial/Trans endothelial Electrical Resistance (TEER) assay, which measures the blockage of electric signals through cell layers as AC currents are passed from one electrode to another showed a significant loss in barrier function in 16-HBE cells **(Gerloff, et al. 2017**). Loss of barrier function could lead to barrier dysfunction, a loss of molecular regulation, and potential inflammation of cells.

Maltol increased proinflammatory responses in cells. As a pure chemical and refill fluid studies, maltol increased in IL-8 secretion in Human Pleura/Lymphocytes (U937), BEAS-2B, and HFL-1 cells using ELISA assays **(Gerloff, et al. 2017, Muthumalage, et al. 2018)**. An increase in IL-8 secretion could lead to unneeded activation of the immune system.

Finally, maltol can play a role in mitochondrial activity and increasing ROS concentrations in cells. A 2018 refill fluid study utilized the MTT assay to show that maltol has a negative effect on mitochondrial reductase activity, reducing ATP production in HPFs **(Behar, et al. 2018)**. This effect was more pronounced as the voltage of the EC increased from 3V and 5V and transfer efficiency increased to 58% **(Behar, et al. 2018)**. A pure maltol study showed that dose dependent ROS generation occurred in cell free systems when H_2O_2 concentrations were measured **(Muthumalage, et al. 2018)**. Additionally, maltol had the highest H_2O_2 concentrations of any flavor chemical tested at each concentration **(Muthumalage, et al. 2018)**. Interestingly, maltol did not play a significant role in cell viability as an AO/PI assay, which measures viability of nucleated cells in culture, demonstrated that viability of cells when exposed to maltol did not drop under 70% **(Gerloff, et al. 2017, Muthumalage, et al. 2018)**. Since ROS production can play a vital role in oxidative stress responses, this is an important concept to explore further.

Ethyl Maltol

Ethyl maltol is similar to maltol but has an ethyl group at the 2nd carbon. Like maltol, ethyl maltol is commonly found and was in 80% of 31 refill fluids analyzed in a study, with the highest concentration being 4.2 mg/mL **(Behar, et al. 2018)**. In another study, ethyl maltol was higher in concentrations than any other flavor chemical **(Hua, et al. 2019)**. Ethyl maltol has cytotoxic effects on cells in the MTT assay **(Behar, et al. 2018, Hua, et al. 2019, Omaiye, et al. 2019)**, can cause physiological alterations in cells **(Sherwood, et al. 2016)**, can have potent effects on teeth **(Kim, et al. 2018)**, and can increase Ca^{2+} concentrations cytosolically **(Rowell, et al. 2020)**. MTT assays using a pure flavor chemical and refill fluid studies have shown that ethyl maltol is cytotoxic. Behar, et al. 2018 showed that the 3V and the 5V transfer efficiencies for ethyl maltol were similar as both had similar levels of cytotoxicity **(Behar, et al. 2018)**. An MTT assay showed a strong positive correlation (R^2 = +0.93) between cytotoxicity and the concentration of ethyl maltol. Furthermore, ethyl maltol was cytotoxic in every refill fluid in which it was found **(Hua, et al. 2019)**. A refill fluid study using the JUUL ECs and BEAS-2B cells showed a moderate to high correlation between the concentration of ethyl maltol and cytotoxicity, which was quantified using a MTT assay and a Neutral Red Uptake (NRU) Assay, which measures the cell's ability to endocytose and bind dye to lysosomes. Ethyl maltol was the only flavor chemical tested that was correlated with cytotoxicity **(Omaiye, et al. 2019)**. Ethyl maltol also reduces mitochondrial activity in cells. The MTT assay, which measures the activity of mitochondrial reductase showed that ethyl maltol significantly affects cellular respiration and ATP production, reducing mitochondrial efficiency and activity in BEAS-2B and mouse neural stem cells (mNSCs) **(Hua, et al. 2019)**. Behar, et al. 2018 also found that ethyl maltol could cause physiological alterations in Human Pulmonary Fibroblasts (HPFs), resulting in a loss of ciliary beating and reduced respiration **(Sherwood, et al. 2016)**.

Ethyl maltol may play a significant role in oral health. A refill fluid study by Kim, et al. 2018 looked at the carcinogenic potentials of ethyl maltol in refill fluids. ethyl maltol can play a role in the demineralization and loss of enamel hardness **(Kim, et al. 2018)**.This was assessed by indentations on teeth 100 μm from an initial indent after a 6-hour incubation period and resulted in a 7.8 ± 2.0% loss in enamel **(Kim, et al. 2018)**. Using *S. Mutans* UA 157, a bacterium commonly found in the mouth, the O'Toole-Kolter Method was used to quantify biofilm formation on human teeth. The researchers noted a significant decrease in biofilm formations when compared to the control indicating that ethyl maltol has the positive potential to act as a potent antimicrobial **(Kim, et al. 2018)**.

Ethyl maltol also increases cytosolic concentrations of Ca^{2+}. A 2020 refill fluid study, Human Bronchial Epithelial Cells (HBEC), Human Lung Cancer Cells (CALU-3), and transfected Human Embryonic Kidney Cells (HEK-293T) cells all showed an increase in cytosolic Ca^{2+} concentrations upon exposure to ethyl maltol **(Rowell, et al. 2020)**. The intracellular increase in Ca^{2+} concentrations was quantified by the Invitrogen™ Fluo-4 Direct™ Calcium Assay Kit, and the Fura-2 Calcium Flux Assay Kit ab176766, which measure intracellular concentrations of calcium. The increase in Ca^{2+} was speculated to be from the endoplasmic reticulum, which stores Ca^{2+}, due to a lack of extracellular calcium during the experimental procedure. Ethyl maltol was one of the three compounds tested that was associated with Ca^{2+} concentrations **(Rowell, et al. 2020)**. Ethyl maltol is a flavor chemical of interest because it is often used at high correlations, it is present in many refill fluids, it is cytotoxic, can physiologically alter the cell, can reduce mitochondrial activity, can play a significant role in oral demineralization, biofilm formation, and can increase Ca^{2+} intracellular concentrations to unhealthy levels.

Benzyl Alcohol

Benzyl alcohol is an aromatic and an alcohol and is frequently used to provide a fruity flavoring. Behar, et al. 2018 found the presence of benzyl alcohol in 18% of the products analyzed in a study of over 60 refill fluids **(Behar, et al. 2018)**. Benzyl alcohol has been found in concentrations up to 39 mg/mL, and thus is a chemical of interest **(Behar, et al. 2018)**. Benzyl alcohol reduces mitochondrial activity **(Behar, et al. 2018, Leigh, et al. 2016, Hua, et al. 2019, Omaiye, et al. 2020)**, reduces cellular viability **(Leigh, et al. 2016)**, breaks apart into new molecules upon aerosolization **(Behar, et al. 2018)**, and does not evoke an inflammatory response **(Leigh, et al. 2016)**. A 2018 study found, using an MTT assay, that the mitochondrial activity of HPFs and A549s was with HPFs being more sensitive to benzyl alcohol than the A549s **(Behar, et al. 2018)**. These results supported other research, which found benzyl alcohol in more than half of analyzed products, but in concentrations greater than 1 mg/mL with a relatively high positive correlation to cytotoxicity ($R^2 = 0.729$) in BEAS-2B cells. An NRU assay showed up to a 50% decrease in metabolic activity in H292 cells upon benzyl alcohol exposure. Furthermore, benzyl alcohol was present in cytotoxic concentrations in every refill fluid that was analyzed **(Hua, et al. 2019, Leigh, et al. 2016, Omaiye, et al. 2020)**.

Benzyl alcohol reduces cellular viability, decomposes into two molecules in aerosols, but did not evoke inflammatory responses. A Trypan Blue Assay, which selectively stains dead cells blue, showed that cellular viability was reduced to 60% of the control by benzyl alcohol exposure **(Leigh, et al. 2016)**. When aerosolized using a 5 V battery, two new compounds were present in the benzyl alcohol aerosols, hydroxyacetone and 2,3-butanedione, 3000 µg/mL, and 96 µg/mL respectively **(Behar, et al. 2018)**. Leigh's 2016 study utilized an ELISA assay to observe the inflammatory response in H292 cells upon exposure to benzyl alcohol treatment and

found that there was no significant release of proinflammatory molecules, such as IL-6, CXCL-1, and CXCL-2 **(Leigh, et al. 2016)**. Benzyl alcohol reduces metabolic activity in cells, reduces cellular viability, may decompose into new molecules upon aerosolization, and may inhibit inflammatory responses in cells, making it a molecule of interest as it tends to be found in high concentrations.

Menthol

Menthol is a popular and commonly found flavoring, offering a "minty" taste, and can have a local analgesic effect orally. Menthol is commonly found and is present in high concentrations making it an important flavor chemical to evaluate. In a convenience sample of refill fluids, menthol was found in concentrations up 84 mg/mL and was present in 44% of the refill fluids analyzed **(Behar, et al. 2018)**. Menthol reduces cellular viability **(Leigh, et al. 2016, Fetterman, et al. 2018)**, and mitochondrial activity **(Singh, et al. 2016, Behar, et al. 2018)**, while provoking an inflammatory response **(Sundar, et al. 2016, Fetterman, et al. 2018, Muthumalage, et al. 2018)** and impairing NO production **(Fetterman, et al. 2018)**. Using a trypan blue assay, Leigh, et al. showed that cellular viability was decreased to 70% of that of the control, indicating 30% of cells had died upon exposure to refill fluids containing menthol **(Leigh, et al. 2016)**. This impact on cellular viability was supported by TUNEL assays which showed that pure menthol would cause cell death at the highest concentrations of menthol tested (--just give the concentrations that caused death)**(Fetterman, et al. 2018)**. Menthol also reduces mitochondrial activity and increases oxidative stress. An MTT assay showed that menthol had some of the highest IC_{50}'s at 24, 48, and 72 hours (68.9 ± 0.1 mM, 32.9 ± 0.9 mM, and 19.3 ± 0.8 mM respectively). Menthol also became more toxic to cells the longer they were exposed **(Singh, et al. 2016)**. This was supported by other studies that used MTT assays and showed that

mitochondrial activity was reduced upon exposure to menthol **(Behar, et al. 2018)**. A fluorescent DHE assay showed that oxidative stress increased in HAEC cells at 0.46 mg/mL and tended to cause cell death at these concentrations **(Fetterman, et al. 2018)**. This was supported by a study that measured concentration dependent ROS generation in cell free systems by measuring H_2O_2 production and found that concentrations of H_2O_2 increased significantly when exposed to menthol **(Muthumalage, et al. 2018)**.

Menthol evokes inflammatory responses in exposed cells. IL-8 secretion was increased in U937 cells by the addition of 1000 μM menthol **(Muthumalage, et al. 2018)**. qPCR showed an increase in expression of the inflammatory mediator IL-6 at most concentrations that were tested (10-100 mmol/L), and ICAM-1 expression increased at 0.46 mg/mL **(Fetterman, et al. 2018)**. An ELISA showed a 150 pg/mL increase in IL-8 expression and a 1000 pg/mL increase in PGE2 expression compared to the control in fibroblasts (HPdLF) when treated with menthol. A BCA protein kit assay, which uses colorimetric detection and quantitation of total protein concentrations, found an increase in COX-2, S100A8, RAGE, phosphorylated γH2.X (by a factor of: x5, x5, x3, x2), decrease in HDAC2 (by a factor of: x0.6) in HPdLF cells. It also measured an increase in COX-2, RAGE, phosphorylated γH2.X (x3, x3, x4) in HGEPp cells, and an increase in COX-2, RAGE, phosphorylated γH2.X (x2, x2, x2) in EpiGin **(Sundar, et al. 2016)**.

Menthol also decreased NO production in cells. A DAF-2DA assay showed impaired NO production at 0.01 mmol/L exposure of menthol in HAECs. Flow mediated vasodilation was observed in patients and it was found that menthol smokers had lower flow mediated vasodilation and presented with an increased SYS/DIA and a decreased heart rate **(Fetterman, et al. 2018)**. Menthol has multiple effects on cells, including the ability to reduce mitochondrial

efficiency, increase ROS production, reduce cellular viability, evoke inflammatory responses, and even impair NO production in aortic cells.

Discussion

The flavor chemicals analyzed in this paper play a role in the generation of reactive oxygen species (ROS), oxidative stress responses, and cytotoxicity. The activation of transcription factors such as NF-κB, STAT3, AP-1, and Nrf2 can influence the propagation of other cellular and inflammatory responses such as secretion of inflammatory cytokines and regulating the antioxidant defense systems **(Kreiss, et al. 2002, Reuter, et al. 2010, Morgan, et al. 2011)**. MTT assays that measure mitochondrial reductase activity show that flavor chemicals tend to significantly reduce cellular respiration and lower ATP production. Lower ATP production could reduce cellular functions as ATP supply cannot keep up with the energy demanding cellular machinery and organelles. The eventual buildup of ROS can lead to cellular death or manifest itself through different diseases and pulmonary conditions **(Auten, et al 2009)**. Reactive oxygen species in refill fluids could cause injuries related to oxidative stress, such as bronchiolitis obliterans, asthma, pulmonary fibrosis, and COPD **(Park, et al. 2009)**. Human studies have shown that the use of ECs increases oxidative stress biomarkers, such as IL-8, in the blood as compared to non-smokers **(Carnevale, et al. 2016)**.

Inflammatory markers such as cytokine interleukin 8 (IL-8) and cytokine interleukin 6 (IL-6) play a role as biomarkers for stress mediated tissue damage and inflammation **(Lerner, et al. 2015)**. These markers tend to be activated by ROS, and IL-8 plays an important role in neutrophil recruitment, chronic inflammation, cancer detection and can dampen immunity against pathogenic invaders **(Mukaida 2003)**. IL-6 is secreted by macrophages and is an

important mediator of fever and the acute phase response., IL-6 plays a vital role in the stimulation of acute protein synthesis, the production of neutrophils, and growth of B-cells **(Wernstedt, et al. 2006)**. Both IL-6, and IL-8 play important roles in the immune response pathway, and overstimulation of either of these chemicals can lead to overexcitation of inflammatory responses, which could cause the immune system to start attacking tissues of the EC user and cause damage to the respiratory system, leading to difficulties in breathing and oxygenation **(Gerloff, et al. 2017, Muthumalage, et al. 2018)**. ICAM-1 is a member of the immunoglobin superfamily and is a transmembrane protein. It serves as an intracellular adhesion molecule and is present in the membranes of immune cells such as leukocytes, where it plays an important role in stabilizing cell-cell interactions **(Rothlein, et al. 1986)**. An increase in ICAM-1 expression has been implicated in increased infection by rhinovirus (cause of the common cold), and even a subarachnoid hemorrhage **(Bella, et al. 1998, Frijns, et al. 2002)**. PGE2 is induced by COX-2 and is an important lipid mediator in the inflammatory response during infections and regulates the activation, migration, and secretion of immune cells, such as macrophages, neutrophils, NK cells, and dendritic cells **(Nagamatsu, et al. 2010)**. Menthol acts on the transient receptor potential ankyrin 1 (TRPA 1) channel to activate the PGE2-COX-2 coupled response **(Willis, et al. 2011)**. An improper increase can lead to unhealthy levels of immune cells in the body without an infection. COX-2 plays an important role in inflammatory and analgesic responses by converting arachidonic acid to prostaglandin H2, which is expressed in inflammation. An improper expression of COX-2 can cause unnecessary inflammation in the body and bring forth an unnecessary immune response **(O'Banion, et al. 1999)**. RAGE is a pattern-recognition receptor that binds to S100A8 implicated in immune and inflammatory diseases and causes cellular senescence via oxidant stress **(Liu, et al. 2014)**. γH2.X is a known

marker of DNA damage as a result of oxidative stress and inflammation, and an increase in γH2.X indicates DNA damage, which could lead to cancer, and cell death **(Mah, et al. 2010)**. The uptick in production of interleukins, such as IL-6 and IL-8, and other inflammatory molecules can also alter the function of the airway epithelial barrier, rendering it less useful.

The airway epithelium acts as a regulated barrier against foreign chemicals. Barrier function can be altered by chemicals such as ethyl maltol allowing for increased infection and access by pollutants and pathogens. Barrier function plays a vital function in the integrity of tight junctions (TJs), intracellular contacts, and molecular regulation. Previous studies showed that dose dependent loss of endothelial barrier functions is associated with an increase in oxidative stress and inflammatory responses **(Schweitzer, et al. 2015)**. The exact mechanism for how flavor chemicals induce the disassembly of TJs is unknown. However, flavor chemicals and inflammatory mediators can cause a decrease in barrier function leading to impairment of the mucosal immune barrier and increase the possibility of infection **(Gerloff, et al. 2017)**. Cinnamaldehyde is also known to impair the immune functions of respiratory cells. Macrophages, neutrophils, and natural killer cells. The mechanism by which it is thought to do so is through thiolation of alveolar macrophages as a thiol reducing agent (DTT) blocked the cinnamaldehyde based effects **(Clapp, et al. 2017)**. Macrophages help eliminate inhaled pathogens and damaged host cells to prevent further injury and infection **(Horvath, et al. 2011)**. Neutrophils play a vital role in protecting the airway from bacterial infection by inhaled pathogens such as *Legionella P, Streptococcus P,* and others through phagocytosis. Cinnamaldehyde seems to impair neutrophil phagocytosis in a fashion like other α, β-unsaturated aldehyde flavorings by causing inflammation and an increase in bacterial populations **(Clapp, et al. 2017)**. NK cells recognize and kill virus infected cells and tumor cells by lysing target cells

through the presentation of antigens via exocytosis **(Topham, et al. 2009)**. Although the exact mechanism of cinnamaldehyde mediated inhibition is unknown, blocking NK cells would allow viruses and tumors to propagate more quickly. Toxicants such as cinnamaldehyde that impair the functions of macrophages, neutrophils, and NK cells would disrupt the delicate homeostasis present in the immune system and increase susceptibility to external infection.

Flavor chemicals such as cinnamaldehyde, and menthol impair the production of nitric oxide. This might be a result of ROS consuming nitric oxide, but nitric oxide plays a vital role in cardiological signaling by inhibiting vascular inflammation, thrombosis, helps regulate vascular tone and vasodilation **(Vita, et al. 2002)**. The flavor chemical impairment of nitric oxide production would lead to the eventual inflammation and degradation of the vascular network resulting in a plethora of cardiological conditions.

Some flavor chemicals, such as vanillin, cinnamaldehyde, and ethyl maltol can also alter cellular morphology, and function. Vanillin showed alterations indicative of cellular signaling where airway epithelial cells were unable to respond to signaling agonists imperative in salt and water balance. Further measurements showed changes in ion conductance and a loss in resistance in the trans epithelium. The researchers attributed this to the activation of a cystic fibrosis transmembrane conductance regulator (CFTR) ion channel **(Sherwood, et al. 2016)**. Vanillin and ethyl maltol reduce ciliary beating in epithelial cells, which can then lead to infection and disease due to decreased elimination and filtering of pathogens **(Sherwood, et al. 2016)**. Membrane permeability was greatly reduced upon exposure to cinnamaldehyde, which would reduce the ability of the cell to block bacterial/viral entry and increase rates of infection **(Clapp, et al. 2019)**. In addition, cinnamaldehyde depolymerized microtubules in pulmonary fibroblasts. Microtubules play many roles in cells, and their loss would limit cell growth, cellular attachment

to the basement membrane, and would alter their mobility **(Wade 2009)**. Altering cell mobility can prevent certain cells from spreading out and would force them to clump up in layers, which could eventually lead to cellular death in layers that are unable to get the proper nutrients or even the possibility of generating a tumor **(Wade 2009)**.

Ethyl maltol also plays a potentially significant role in oral health. Ethyl maltol demonstrated potent antimicrobial properties (Kim, et al. 2018). Biofilm is a dental microbial infection, composed of layers and colonies of micro-organisms [bacterium] distributed in sessile, mushroom shaped matrices **(Saini, et al. 2011)**. Biofilm is harmful to dental health and can cause halitosis, teeth yellowing, cavities, and in severe cases tooth, and gum disease. Ethyl maltol showed promise when it was able to significantly decrease the formation of biofilm on human teeth compared to the control **(Kim, et al. 2018)**. A limitation of this study is that this was tested on only one type of oral bacterium in the mouth but may work on other genetically similar bacteria . The mechanism by which ethyl maltol reduced the bacterial population is unknown, but ethyl maltol does destabilize the outer membrane of *E. Coli* by chelating Mg^{2+} and Ca^{2+} in a concentration dependent manner **(Schved, et al. 1996)**. However, ethyl maltol also demineralized enamel hardness, weakening the outer layer of human teeth. Again the exact mechanism is unknown, **(Kim, et al. 2018)**.

Ethyl maltol increased cytosolic Ca^{2+} concentrations in CALU-3, HBEC, and HEK-293T cells. Thapsigargin is an endoplasmic reticulum Ca^{2+}-ATPase pump inhibitor **(Lytton, et al. 1991)**. Ethyl maltol inhibits thapsigargin, allowing for the uncontrolled release of Ca^{2+} from ER stores, leading to a drastic increase of Ca^{2+} intracellularly **(Lytton, et al. 1991.** Ion influx can also lead to chronic inflammation and a decreased ability to fight off pathogens and infection, ER stress and dysfunction, and even abnormal cell growth **(Lerner, et al. 2015)**. Continuous/large

increases in the cytosolic concentrations of Ca^{2+} cause an influx of ions into the mitochondria to dysregulate metabolism, and influx into the nucleus can modulate and trigger the transcription of nucleases controlling apoptosis **(Pinton, et al. 2008)**. The improper increases of cytosolic Ca^{2+} can have significant impacts on cell function and survival.

Benzyl alcohol decomposed into hydroxyacetone and 2,3-butanedione upon aerosolization using a 5 V battery **(Behar, et al. 2018)**. Hydroxyacetone was not toxic for hPFs or A549 cells, but 2,3-butanedione was found to be toxic. Carbonyl compounds, such as 2,3-butanedione tend to form when the solvent is exposed to higher voltages **(Jensen, et al. 2015, Sleiman, et al. 2016)**. This happening only to Benzyl Alcohol may correlate especially when Benzyl Alcohol has a lower boiling point than any other flavor chemical analyzed in this paper. However, more studies need to be done to confirm this effect as carbonyls have been found in PG/VG solutions. 2,3-Butanedione is a toxic molecule that can cause obliterative bronchitis, can greatly affect epithelial barrier function, making respiration significantly harder, and can even lead to death **(Gerloff, et al. 2017, Harber, et al. 2006)**. A 2015 paper defined an "acceptable" daily inhalation limit of benzyl alcohol to be 65 µg, but it was estimated that the average user would be inhaling 248 µg of benzyl alcohol day based on smoking habits **(Farsalinos, et al. 2015)**. This is clearly higher than the average and would bring up serious concerns regarding the health of smokers and the toxicity of benzyl alcohol.

Conclusion

This study was limited by the literature that was available to analyze. Future studies should focus on exploring some of the cytotoxic effects of the flavor chemicals in more detail by using pure standards of the flavor chemicals rather than the refill fluids themselves. Refill fluids

have other chemicals present in solution that may play a role in addition to the flavor chemical of interest. This makes it imperative that there be more studies done with pure flavor chemical standards. Furthermore, a lack of standardization in the methods of exposing cells *in vitro/ in vivo* studies limits analysis as well as cross referencing studies. A study with a more standardized methodology would make it easier to cross reference it with other studies. This review aimed to explore and better understand the biological effects that flavor chemicals can have on cells in the high concentrations that they are found in ECs. This review described that it was shown that heating conditions can play a role in the chemistry of the flavor chemical, and this is something that is worth looking into further. The analyzed scientific literature indicates that flavor chemicals are found in high concentrations and can all have cytotoxic effects that vary based on concentration and effect but can influence different organ systems in addition to the respiratory system. More studies can be done focusing on other flavor chemicals that are found in ECs in order to better understand the threat that flavor chemicals may pose in the human body which can then be used to push for more governmental regulation.

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