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Toxicological Assessment of Aged Electronic Nicotine Delivery System Aerosols on Primary Human Small Airway Epithelial Cells

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ABSTRACT

The emerging popularity of electronic nicotine delivery systems (ENDS) has increased safety concerns. ENDS aerosols contain numerous hazardous components including acetaldehyde, formaldehyde, and heavy metals along with various other chemicals. Recent studies showed ENDS use can cause various health risks including gastrointestinal, cardiovascular, neurological, and immune system impairment. Moreover, the long-term respiratory effects of ENDS use have not been fully evaluated. In this study, we hypothesized during normal consumer use ENDS device ageing occurs, which may alter the toxicological properties of emitted aerosols. We propose that the altered toxicological properties of ENDS aerosols may elicit reactive oxygen species (ROS), oxidative stress, cell viability reduction and DNA damage in human primary small airway epithelial cells (SAEC). We utilized a custom built 4-channel puffing machine and a novel condensation trap to automatically generate and monitor aerosols from a mod type ENDS manufactured by VooPoo® using tobacco–flavored e-liquid. ENDS aerosols samples (1-25), (101-126) and (201-226) puffs were collected in the fluorinated ethylene propylene (FEP) tube trap and extracted to be assessed with the in-vitro toxicological assays. Our results revealed that the VooPoo® ENDS device produce different sizes of nanoparticles ranging from 10nm to ~1micron during the device aging process. Increasing particle concentrations were observed throughout the aging process where puffs 1-25 emitted 1 $\mu g/m^3$, puffs 101-126 emitted 4 $\mu g/m^3$, and puffs 201-226 emitted 7 $\mu g/m^3$. Along with the VooPoo® device aging process, the increasing levels of reactive oxygen species, glutathione level, and cellular viability reduction were observed. Moreover, a three-fold increase in DNA damage in SAEC was observed at puffs 201-226 aging stage compared to puffs 101-126 puff fraction. This work suggests ENDS aerosols become more hazardous during normal device usage, which may pose a threat to respiratory health.

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AUTHOR'S STATEMENT

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Introduction

According to the U.S. Food and Drug Administration (FDA, 2020), the electronic nicotine delivery system (ENDS) is defined as the non-combustible tobacco product that contains a heating element to create an aerosol by using an "e-liquid," which typically contains nicotine, flavorings, propylene glycol, vegetable glycerin, and other ingredients. ENDS aerosols are defined as the mist produced from the heating process, which contains liquid droplets in the sub-micrometer to the 200 μm size range (National Academies of Sciences et al., 2018). The popularity of ENDS as possible smoking cessation products has gradually increased over time. Still, people need to be aware that the long-term toxicological effect of ENDS use has not been fully evaluated. According to Goniewicz and his colleagues (2018), ENDS users had lower concentrations of biomarkers of exposures compared to combustible cigarette users. However, significant levels of biomarker concentrations were observed with the use of ENDS (Goniewicz et al., 2018). Therefore, even though ENDS are known to contain lower levels of harmful constituents than combustible cigarettes (Goniewicz et al., 2018; Lorkiewicz et al., 2019), it is still questionable if they are safe.

The number of ENDS users is increasing rapidly worldwide. According to Euromonitor International's global market research group, the number of ENDS users increased by 28 million from 2011 to 2016 within the 60 legal markets globally (Gordon, 2017). The top 10 global markets for ENDS products in 2016 were the United States, United Kingdom, Sweden, Italy, Algeria, Norway, India, Germany, and China. Among these 10 countries, the United States ranked the highest by exceeding 21,000 million dollars (Jones, 2018). In 2018, the global sales of ENDS products were $15.7 billion, and experts are estimating that ENDS sales will increase to

$40 billion in 2023 (Besaratinia & Tommasi, 2019). In the United States, the overall prevalence of ENDS users between 2017-2018 increased from 4.3% to 5.4% (Parekh & Desai, 2020).

The prevalence of adolescent and young adult ENDS uses in the United States also gradually increased from 2011 until 2018 (Cullen et al., 2018; Parekh & Desai, 2020; Wilson & Wang, 2017). More specifically, the young adult population showed a rapid increase in ENDS use compared to other age groups between 2017-2018 (Parekh & Desai, 2020). Young adult ENDS users are primarily males, Hispanics, college graduates, non-smokers, marijuana non-users, and heavy alcoholics (Parekh & Desai, 2020; Wilson & Wang, 2017). However, adolescent and young adult ENDS use prevalence is currently decreasing from 2019 to 2020 shown through several current studies (Gaiha, Lempert, & Halpern-Felsher, 2020; Stokes, 2020). According to the most current statistics from the Centers for Disease Control and Prevention (CDC), the prevalence of adolescent and young adult ENDS users in the United States decreased from 5.4 million (2019) to 3.6 million (2020) (CDC, 2019). Various factors have potentially contributed to this decrease, such as changes in federal and state regulations on ENDS, expanded public education/programs on ENDS use prevention, and lifestyle changes due to the COVID-19 pandemic (Gaiha et al., 2020; Stokes, 2020; Wang et al., 2021). Among these factors, the COVID-19 pandemic is a unique factor that affected ENDS use among the adolescent population. The COVID-19 pandemic has affected several industries globally and individual's lifestyles. However, the accessibility to the ENDS products was not the main contributor to the reducing ENDS products. The adolescent population could still obtain the ENDS products online so the accessibility remained similar prior to the stay-home order (Gaiha et al., 2020). The primary reason for this decrease in use was the advanced awareness about the potential adverse

health outcomes associated with COVID-19 when using the ENDS devices (Gaiha et al., 2020). A secondary reason was that the adolescents would not be able to use ENDS devices frequently after the pandemic since their parents are more likely to stay home (Gaiha et al., 2020). The long-term use of ENDS products can lead the youth population to addiction, nicotine poisoning, and other health injuries in the respiratory, gastrointestinal, cardiovascular, neurological, and immune systems (Hua & Talbot, 2016). Therefore, a comprehensive evaluation should be conducted to analyze and identify the adverse health effects of ENDS product use across all age groups starting from the cellular and extending to the public health level.

This study aims to address the following experimental questions: 1) What ENDS parameters influence each puff fractions? 2) What are the toxicological effects of ENDS aged aerosols on respiratory health? This study hypothesized that VooPoo® emitted aerosols may cause molecular and cellular adverse effects in human primary small airway epithelial cells (SAEC). In order to answer the research questions, dose-response cellular bioassays such as CellROX®, total glutathione (GSH), cell proliferation assay (MTS), and CometChip® were performed to measure reactive oxygen species (ROS) generation, oxidative stress, cellular viability, and DNA damage, respectively.

2. REVIEW OF THE LITERATURE

2.1 Main components of ENDS and their functions

ENDS have evolved with changes of some features and designs. However, all ENDS devices consist of four components: cartridge, atomizer, battery, and mouthpiece. The cartridge is a reservoir, an atomizer contains the heating element, the battery serves as a power source, and a mouthpiece is for inhalation. The ENDS devices function with the integration of all these main components. Puffing action through the mouthpiece activates the ENDS device. Then, when the device is heating up, it vaporizes the e-liquid in the cartridge. This heating process generates ENDS aerosol that contains nicotine, flavorings, and other chemical components (NIDA, 2018).

ENDS emissions contain various compounds such as volatile organic compounds (VOCs), nicotine, and particulate matter (National Academies of Sciences et al., 2018). Protano and his coworkers (2018) determined that all generations of ENDS emitted a higher level of Particulate Matter (PM) concentration than the limits recommended by the World Health Organization, which is a 24-h mean concentration for greater PM size fractions (25 μg m^{-3} for PM2.5 and 50 μg m^{-3} for PM10) (Protano et al., 2018; WHO, 2006). Also, his study recognizes the increasing PM emission over the generations of ENDS. This is due to the battery power adjustment feature, which has evolved over the first to fourth generations of ENDS. (Protano et al., 2018) The PM level emitted from ENDS aerosols was higher than the PM emitted from traditional cigarettes. (Protano, Cattaruzza, Osborn, & Vitali, 2014).

The potential health outcomes from ENDS use can vary from the different features and characteristics of ENDS devices. Especially for the mod-type devices, there is more concern due

to its characteristics and customization-friendly features. The heating process of the ENDS e-liquid is the process of most concern during aerosol generation. The heating elements of the ENDS use various types of metals such as nickel, chromium, tungsten, iron, and aluminum (Laucks & Salzman, 2020). E-liquids contain propylene glycol and glycerol, and heating this liquid at a high temperature can generate formaldehyde and hemiacetals such as acetaldehyde (Laucks & Salzman, 2020). According to Laucks and Salzman (2020), there was an increase in the quantities of the byproducts in a higher voltage device and higher coil temperature. These byproducts, including hemiacetals, can cause nasal irritation, cardiovascular effects, lung mucosal impairment, and respiratory system damage (Laucks & Salzman, 2020).

2.2 Evolution of ENDS

The history of ENDS starts from the first-generation devices called cig-a-like products (Williams & Talbot, 2019). This nickname of the first-generation ENDS is derived from its shape, which has a similar size and shape compared with regular combustible cigarettes. Unique features of the first-generation ENDS are LED lights and fixed low voltage batteries (Williams & Talbot, 2019). LED lights are attached at the end of the device and monitor when the devices are in use. The first generation can be divided into 1-piece style, 2-piece style, and 3-piece style, depending on their unit compositions. The major pieces of the first generation, such as atomizing unit, fluid reservoir, and battery, were divided or combined in three different styles for the first generation ENDS products (Williams & Talbot, 2019).

The second-generation ENDS also has two nicknames: personal vaporizer and “clearomizers” (Protano et al., 2018; Williams & Talbot, 2019). Unlike the first-generation, this

second-generation ENDS comes with a larger size that allows longer battery life and a voltage adjustment feature (Protano et al., 2018). The second-generation ENDS products use pen-style batteries with a removable atomizing unit and a larger fluid reservoir (Williams & Talbot, 2019). Moreover, the clear and larger fluid reservoir size of the second-generation ENDS allowed users to refill the fluids (Williams & Talbot, 2019). The second generation's adjustable and stronger battery power with a larger fluid reservoir allowed users to customize the device with their preferences (King, Smith, McNamara, & Cao, 2018). Therefore, first-generation users were likely to convert to second generation ENDS to obtain more customized nicotine experiences (King et al., 2018).

Third generation ENDS come in different sizes and shapes. The common components of the different styles of the third-generation ENDS devices are an atomizer and a lithium battery (Noël et al., 2018). Third generation ENDS devices contains the "Mod", which is an energy source of ENDS (Protano et al., 2018). There are two types of mods: mechanical mods and regulated mods (Protano et al., 2018). One of these two types of mods, regulated mods, can adjust the voltage and the output in watts (Noël et al., 2018). The atomizing units of third-generation ENDS products come in three different types: various styled, replaceable dripping, and sub-ohm (Williams & Talbot, 2019). This different style of atomizing units allows the users to customize the device shapes and coil composition by their preference compared to other generations. The replaceable dripping atomizer style devices allows the users to build their own coils or filaments, while the sub-ohm style devices allow users to adjust the voltage and wattage with the low resistance coils (Williams & Talbot, 2019).

The fourth generation is the most recent product in the ENDS market. Its features are very similar to the previous generations, and it is often called as “pod-style” device (Protano et al., 2018; Williams & Talbot, 2019). Fourth generation ENDS products have fixed voltage similar to the first generation but with different battery styles, such as USB or teardrop shapes (Williams & Talbot, 2019). The fourth-generation ENDS devices can reach up to 300 Watts (W) and maintain the coil temperatures during the puffing process through their advanced electronic circuitry system (Dibaji, Guha, Arab, Murray, & Myers, 2018).

The components of all these generations of ENDS devices are similar but each of them has unique features that differentiate each from another. These unique features of each generation can affect the performance level of each device and produce different chemical byproducts, leading to different endpoints for each generation (Dibaji et al., 2018). This evolution of ENDS devices and their features also indicates the increasing popularity among the population, which leads people to desire customizable features based on their preferences. Increasing functionality over the generations may also increase exposure to aerosols. Increasing wattage can result in increased exposure to aerosols, volume of puffs, and volume of inhaled aerosols, which all enhance hazard risks. (Protano et al., 2018).

2.3 Toxicological Effects of ENDS Aerosols

Several studies have shown different toxicological effects of ENDS aerosol that result from their operation (Protano et al., 2018; Yingst et al., 2019). To operate ENDS, heating e-liquids to high temperatures is required (V. Yu et al., 2016). This heating process of e-liquids produces carcinogenic carbonyl compounds, such as formaldehyde, acetaldehyde, and acrolein

(V. Yu et al., 2016). Toxicants also exist in the emitted aerosols in the form of nicotine, nanomaterials, flavoring chemicals, volatile organic compounds, carcinogenic chemicals, and heavy metals (NIDA, 2018). After ENDS aerosols are directly or indirectly inhaled, the respiratory system can become a target where the ENDS emitted particles are deposited (Tsuda, Henry, & Butler, 2013).

Recent studies have shown that bronchial epithelial cells are sensitive respiratory cell types that show decreased cell viability and increased oxidative stress after ENDS exposure. (Aug et al., 2015). Moreover, ENDS use can also induce DNA damage on the micronuclei across epithelial cell lines (Tellez et al., 2021). These hazardous reactions in the epithelial cells of the respiratory system can result in adverse health effects such as reduced immune responses, acute lung injury, chronic obstructive pulmonary disease, and more (Gotts, Jordt, McConnell, & Tarran, 2019; Oriakhi, 2020). For example, the ENDS emitted particles with sizes less than 100 nm can cause DNA damage, which will induce pro-inflammatory cytokine expression and produce free oxygen radicals by affecting the immune system (National Academies of Sciences et al., 2018).

2.3.1 Oxidative Stress

Oxidative stress is defined as the imbalance of ROS and antioxidant defenses (Betteridge, 2000). Inhaling carcinogenic chemicals from ENDS aerosols can induce oxidative stress (Munakata et al., 2018; Wills, Soneji, Choi, Jaspers, & Tam, 2021). Toxicants from inhaled ENDS aerosols impair airway epithelial cells, which provide critical barrier protection and important defense mechanisms including mucus production (Wong, Magun, & Wood, 2016).

Cellular processes such as aerobic respiration are also disrupted during ENDS use and exposure (Anderson, Majeste, Hanus, & Wang, 2016; Pearce et al., 2020b). Inhalation of several hazardous components within ENDS aerosols can elicit antioxidants decrease leading to oxidative stress (Anderson et al., 2016; Betteridge, 2000; Pearce et al., 2020b).

The level of oxidative stress can be evaluated through in-vitro assays such as glutathione assay using GSH-Glo Glutathione assay kit (Promega), the ratio of glutathione to oxidized glutathione (GSH/GSSG), and antioxidant response element (ARE) reporter activity (Betteridge, 2000; Pearce et al., 2020b). According to a recent in-vitro study, there was a significant decrease in ARE reporter activity after the exposure to ENDS emitted aerosols with increased dose dependent result (Munakata et al., 2018). This shows that the increased concentration of emitted ENDS aerosol results in decreased level of antioxidant, which leads to oxidative stress (Munakata et al., 2018). Another recent in-vivo study also suggests the acute ENDS generated aerosol exposure can result in pulmonary oxidative stress (Kuntic et al., 2020). According to Kuntic and his colleagues (2020), toxic aldehyde acrolein in the ENDS aerosol was the factor that induced the oxidative stress. The presence of oxidative stress can induce several adverse health effects by causing chronic and degenerative diseases (Pizzino et al., 2017). Moreover, the oxidative stress can also increase the aging process rate leading to trauma and stroke (Pizzino et al., 2017).

2.3.2 Inflammation

Inflammation can occur when toxicants from emitted ENDS aerosols injure tissues. When cells are damaged, they release chemicals that leak into the surrounding tissues causing

inflammation. In a study conducted by Glynos and his coworkers, emitted ENDS aerosols were determined as a possible trigger of inflammatory response, especially with flavorings. (Glynos et al., 2018). From this result, it was shown that the exposure to ENDS aerosols can cause adverse effects on the respiratory system. Another study conducted by Lerner and his coworkers also showed exposure to ENDS aerosols produced ROS and inflammatory cytokines in both human epithelial cells and mouse models, which indicates inflammation process in the system. (Lerner et al., 2015). These two studies suggest that ENDS aerosols adversely affect pro-inflammatory cytokines that can result in acute inflammation and lead to acute respiratory distress syndrome (ARDS), asthma, and chronic obstructive pulmonary disease (COPD) (Moldoveanu et al., 2009). Moreover, inflammation can also contribute to the development of various types of cancers (Moldoveanu et al., 2009). Chemicals in the ENDS aerosol can be changed into propylene oxide, acrolein, acetaldehyde and formaldehyde through the vaping process (Moldoveanu et al., 2009). These chemicals may lead to inflammation and can potentially cause genomic instability, neoplastic development, and tumor growth (Merecz-Sadowska et al., 2020).

2.3.3 Genotoxicity

Genotoxicity is the phenomenon that occurs when cells are exposed to toxic materials and result in a degradation of genetic material, such as DNA and RNA (Ren, Atyah, Chen, & Zhou, 2017). The difference between the genotoxicity and cytotoxicity is that the genotoxins can cause mutations since their genetic structures are deformed. According to an in-vivo study, Ganapathy and his co-workers used DNA damage detection assay (q-PADDA), RT-PCR, Western blot, and 8-oxo-dG ELISA assay to measure the DNA damage after the exposure to ENDS aerosols. (Ganapathy et al., 2017) This study showed that there was more oxidative DNA

damage in cells with ENDS aerosol exposures compared to smoke extracts with the presence of 8-oxo-dG. (Ganapathy et al., 2017; Pearce et al., 2020b) This study also indicated that the exposure to ENDS aerosol showed more oxidative genotoxicity and gene mutation than combustible tobacco, which suggests the possible emergence of chronic respiratory disease from vaping in the future (Ganapathy et al., 2017).

Another current study also suggests the ENDS emitted aerosol exposure induces genotoxicity even at lower dose (Pearce et al., 2020b). According to Wright and colleagues (2020), genotoxic potential was shown at a dose of 25 puffs after 24-hours exposure to ENDS emitted aerosol. Their study used the CometChip® assay to determine and quantify the single stranded DNA damage of human bronchial epithelial (NHBE) cells after the ENDS exposure with different puff concentrations (Pearce et al., 2020a, 2020b). Other evidence also suggests that the exposure to ENDS aerosol can damage DNA not only at the chromosomal level by breaking the strands in leukocytes, but also at the gene level causing mutation (Canistro et al., 2017). The mutation at the gene level can be critical on human health by inducing tumor proliferation and metastasis, which will make the cells resistant to chemotherapies and other therapeutic methods (Mishra et al., 2015). These studies suggest that the genotoxicity of ENDS aerosols can contribute to development of lung cancer, bladder cancer, and cardiovascular diseases (Canistro et al., 2017; Lee et al., 2018; Pearce et al., 2020b).

2.3.4 Cytotoxicity

Cytotoxicity occurs when the living cell goes through necrosis or apoptosis after exposure to toxicants (Elmore, 2007). In the recent study conducted by Behar and coworkers

(2018), they found among the 35 ENDS aerosol samples, 74 percent of the aerosols showed cytotoxic effects in the cells. Also, they classified the harmful e-liquid flavorings and the result showed that creamy/buttery flavors presented most harmful effect with its abundance of glycerin, diacetyl, and 2,3-pentanedione that cause lung disease in human cells and rats. (Behar, Wang, & Talbot, 2018). Among those toxicants in the ENDS emitted aerosols, glycerin is a popular ingredient because it contributes to thicker aerosol cloud formation. However, the aerosolized glycerin produces a higher concentration of carbonyl compounds that increase the cytotoxic effect in human cells. (Behar et al., 2018).

Another study also suggests that ENDS emitted aerosol contains cytotoxic mediators impacting on cell viability after exposure (Hua et al., 2019). According to Hua and his colleagues, cytotoxicity was observed from the cell proliferation (MTT) assay results after ENDS aerosol exposure with the e-liquid concentration greater than 1 mg/ml (Hua et al., 2019). The cytotoxicity of the ENDS emitted aerosol on epithelial cells after exposure can induce DNA strand breakage (Yu et al., 2016). All these results suggest that the toxicants present in e-liquids may lead to cytotoxicity effects in the cells, which can cause various adverse health outcomes after the exposure to ENDS emitted aerosols (Behar et al., 2018; Hua et al., 2019; Vicky Yu et al., 2016).

2.4 Federal Regulations of ENDS

The level of toxicity of ENDS aerosols can differ depending on the composition of the e-liquids, exposure time, frequency of use, presence of nicotine, and types of flavorings. However, it has been shown by several studies that long-term exposure to emitted ENDS aerosols can

cause significant adverse health effects (Glantz et al., 2018; Callahan-Lyon P, 2014; Drummond, M.B., & Upson, D., 2014). The population most vulnerable to those toxicological effects are adolescent who are still in the process of mental and physiologic development. Therefore, more policies that are specific are needed to protect adolescent and young adult populations with more substantial restrictions on selling e-cigarettes. Currently, there are some regulations and policies that exist to regulate ENDS use in the United States.

Starting in June 2009, the U.S. Food and Drug Administration (FDA) exerted authority to regulate ENDS products (Bhalerao et al., 2019). This led all the ENDS products to be under regulation by the FDA. However, more specific regulatory actions were needed to address the loopholes in the ENDS marketplaces that still existed. Therefore, the FDA published new guidelines under authority given under the Federal Food, Drug, and Cosmetic Act to place stronger restrictions for ENDS products (Behar et al., 2018; Dave, Feng, & Pesko, 2019; Gottlieb, 2019). More specific limitations were applied for the age restriction of ENDS sales, ENDS marketing, and the advertisement (Collins, Glasser, Abudayyeh, Pearson, & Villanti, 2019). Previously, the ENDS manufacturers were targeting the adolescent and young adult population by promoting fruit flavors that appeal to children. However, the new guidance under the Federal Food, Drug, and Cosmetic Act set limitations on these ENDS marketing tactics. Moreover, the FDA and Federal Trade Commission warned four ENDS companies about advertisements that targeted the adolescent population and the online distribution of the ENDS products (Collins et al., 2019). Another regulation, Tobacco-21, is also being implemented in many states by raising tobacco products' minimum legal sale age (Bhalerao et al., 2019). According to a recent study, the Tobacco-21 law that raises the minimum legal sale age of

tobacco products from 18 to 21 was effective showing a 39% reduction in smoking among 18- to 20-year-olds (Friedman et al., 2019). However, there are still significant gaps that need to be addressed to protect public health from ENDS use. Therefore, more regulations and interventions are required to reduce ENDS product use in the United States.

3. METHODS AND PROCEDURES

3.1 ENDS Aerosol Sample Collection

Aerosol sample collection was conducted in the 5 m^3 environmental exposure chamber, located at the Underwriters Laboratories facility in Marietta, GA. A custom-built 4-channel puff machine was used to generate aerosols from the mod generation electronic nicotine device (Figure 6). A VooPoo® mod device was selected for the aerosol sample collection device with American Patriot tobacco-flavored e-liquid at 3 mg/ml nicotine strength. The VooPoo® device was connected to the custom-built 4-channel puff machine, which was installed inside an environmental exposure chamber (Figure 5), to produce the ENDS aerosol. The ENDS device was automatically operated using the pneumatic actuator, which was set to produce 50 ml of each puff with 3.5 seconds duration following the Center for Scientific Research Relative to Tobacco (CORESTA) Recommended Method No.81. In order to simulate realistic puff topography, an inter-puff interval of at least 30 seconds was added into the puff generating process, also following the CORESTA No.81 method. The generated ENDS aerosol was directed into the buffer chamber, located underneath the devices, and diluted with clean air at a flow rate between 0-10 liters/minute. Then the mixed aerosol from the buffer chamber was emitted through the 7-port manifold and collected in the fluorinated ethylene propylene (FEP) condensation tube trap. The aerosol samples were collected at three different stages to compare the device and coil aging effect during the sample generating process. The initial sample was collected during puffs 1-25, the second sample during puffs 101-126, and the last sample during puffs 201-226.

3.2 FEP Tube Condensation Trap Sample Extraction

The emitted ENDS aerosol samples were collected in the fluorinated ethylene propylene (FEP) condensation tube (Savillex, EdenPrairie, MN, USA). Before each ENDS aerosol sample collection process, all the FEP tubes were prepared as 15-inch lengths and cleansed using the hydrochloric acid and nitric acid cleaning solution. A total of 24 ml of cleaning solution was prepared with 1% hydrochloric acid, 2% nitric acid, and deionized (DI) water. The caps from both sides were removed from the FEP tube before inserting the cleaning solution. A total of 24 ml of cleaning solution was inserted into the FEP tube from one side using the 50-ml syringe. The FEP tube filled with the cleaning solution was sonicated for 5 minutes at 25°C to agitate any remaining residuals in the tube from the manufacturing process (Figure 8). Then, a total of 250 ml of DI water was inserted into the FEP tube to ensure the complete removal of the cleaning solution from the tube. Then, the FEP tube was connected to the DI water faucet and rinsed by running DI water for 10 minutes and sonicated for 5 minutes at 25°C. After the sonication process, the FEP tube was connected to the air blower for an hour to be dried completely and weighed.

All the FEP tubes were maintained below 4°C in the refrigerator after the sample collection and transferred to the Georgia State University campus to be weighed and extracted. The condensed aerosol inside the FEP tubing trap was extracted using the extraction solution (75% Methanol). Each FEP tube was capped and filled with 50 ml of extraction solution before the sonication process. The coiled FEP tubes were divided into two zones and marked with colored tape. Each zone, zone 1 and zone 2 were sonicated for 5 minutes at 25°C (Figure 8). After the sonication process, another 50 ml of extraction solution was added, and 50 ml of initial

volume was collected in the clean 50-ml conical tube. The collected 50-ml sample was vacufuged at 60°C with 1000 rpm for 12 hours until the volume was reduced to 10 ul in preparation for dilutions (Figure 9).

3.3 SAEC Cell Culture and Exposure

The SAEC, located at the key sites of airway obstruction and destruction in pulmonary diseases, were used for the toxicological assays. SAEC were cultured in the T-75 cell culture flask using PneumaCult™-Ex Plus Medium, which is an effective cell culture media, especially for the SAEC. Prior to cell culture, the stock SAEC cells were added to 6 ml of PneumaCult™-Ex Plus Medium and centrifuged for 8 minutes at 250xg. Then, the supernatant was removed, and only the cell pellet was suspended with the 1 ml of PneumaCult™-Ex Plus Media. Lastly, the cell pellet was transferred into the T-75 cell culture flask and incubated at 37°C until it expanded to 70-80% confluency.

For the VooPoo® ENDS aerosol exposure, the SAEC was subcultured into the trans-well inserts of the 24-trans-well cell culture plate at a density of 100,000 cells/ml. The sub-cultured cells were incubated at 37°C for approximately 10 days until the monolayer was formed. The sub-cells were maintained by changing the media every 2-3 days with PneumaCult™-Ex Plus Medium. Once the monolayer was formed, the cells were air-lifted by removing the media from the apical surface. During the air-lifted phase, the cells were maintained by changing the basolateral media (PneumaCult™-ALI media) every 2-3 days. After the air-lifted phase, diluted 100 ul of each exposure (NC, PC, puffs 1-25, puffs 101-126 and puffs 201-226) was added

directly into the cell's apical surface and incubated at 37°C for 24 hours and 7 days. During the 7-day exposure, all the exposures were changed every day and the basolateral media (PneumaCult™-ALI media) was changed every 2-3 days.

3.4 Toxicological Assays

3.4.1 Reactive Oxygen Species Measurement

CellROX® Orange was used to measure the level of ROS after the 24 hours and 7 days exposure to the VooPoo® American Patriot 3% ENDS aerosol. This assay is based on the mechanism of oxidation, which changes non-fluorescent dye into fluorescent with reactive oxidative species (ROS). The CellROX® assay is a fluorescent assay that indicates the presence of ROS through the generation of a detectable fluorescent signal. The CellROX® reagent was made with 5 µl of CellROX® Orange and 2.50 ml of PBS in the 15-ml conical tube and wrapped with aluminum foil. The exposures were removed from the wells and washed twice with 100 ul of PBS. A total of 50 ul of CellROX® Orange was added to each well and incubated at 37°C for 60 minutes. After the incubation, CellROX® reagents were removed from each well and washed with 200 ul of PBS three times. A total of 50 µl of 3% paraformaldehyde (fixative) (Invitrogen) was added to each well and incubated at 37°C for 15 minutes. Then, 3% paraformaldehyde (fixative) (Invitrogen) was removed from all the wells and washed with 200 µl of PBS twice. DAPI stain was prepared with 6 drops of DAPI and 2 ml of PBS in the 15-ml conical tube. For each well, a total of 100 µl DAPI stain was added and left for 30 minutes. Finally, the fluorescence signal was detected and imaged using the Cytation 1 spectrophotometer (Biotek).

3.4.2 Total Glutathione Level Measurement

Total glutathione level was measured to detect the oxidative stress of the SAEC after 24 hours and 7 days exposure to the VooPoo® American Patriot 3% ENDS aerosol. Oxidative stress is caused by an imbalance of antioxidants and ROS. Therefore, the oxidative stress can be determined by the glutathione level measured through the total glutathione (GSH) assay. Glutathione is a small peptide that consists of three amino acids that play a role as antioxidants in the cell. Therefore, the decreased total GSH indicates increased ROS in the cells. The GSH reagent was prepared with 2 ml of GSH Glo reaction buffer, 20 µl of GST, and 20 µl of Luciferin in the 15-ml conical tube. The exposures were removed from all the wells and washed with 100 µl of PBS twice. A total of 100 µl of GSH- Glo reagent was added to each well and incubated at 37°C for 60 minutes. After the incubation, the GSH-Glo reagent was removed, and 100 ul of Luciferin was added to each well. The plates were wrapped with aluminum foil and incubated at 37°C for 30 minutes. Finally, the luminescence result was observed by using a Cytation 1 spectrophotometer.

3.4.3 Cellular Viability Measurements

The MTS assay was used to measure the cellular viability of the SAEC after the 24 hours and 7 days exposure to the VooPoo® American Patriot 3% ENDS aerosol. The MTS assay is a colorimetric and metabolic assay that uses soluble yellow tetrazolium salts (Hillegass, Shukla et al. 2010). In this assay, a color shift occurs where the color changes from yellow, which indicates there are no cells available to metabolize the salt, to brown or reddish color indicating a higher level of cell viability in wells with viable cell population. The MTS reagent was prepared with

250 µl of MTS solution and 2.25 ml of Ex-Plus media in the 15-ml conical tube. The exposures were removed from all the wells and washed with 100 µl of PBS buffer once. A total of 100 µl of prepared MTS reagent (CellTiter 96â Aqueous One Solution Cell Proliferation Assay) (Promega) was added to each well and incubated at 37°C for 2 hours. After the incubation, all the samples were transferred to a black 96-well plate to measure the absorbance using the Cytation 1 spectrophotometer at 490 nm.

3.4.4 DNA damage detection

The CometChip® assay was used to detect the DNA damage of the SAEC after the exposure to VooPoo® American Patriot 3% ENDS aerosol. The CometChip® assay is an electrophoresis assay that quantifies the DNA damage level for each cell by measuring the length of the "comet tail." This assay is designed to denature the single-strand DNA fragments or damaged DNA and allow them to migrate out from the cell with the electrophoresis process, which creates a structure that resembles a comet. The length and intensity of the comet tail indicates the level of DNA damage the exposed cell incurred (Ge et al., 2014). After the electrophoresis process, cells are stained with SybrGold, which allows the image of the "comet head" from the healthy DNA and "comet tail" from the damaged DNA, which is measured under the fluorescent microscope. The CometChip® platform was prepared with the microwell array mold stamped on molten agarose until the gel solidifies. While the CometChip® platform is being prepared, the exposures were moved from the apical surface and treated with 150 µl of Trypsin-EDTA and incubated at 37°C for 1 hour. After the incubation, 150 µl of TNS was added to each well and mixed thoroughly. Then, all the cells were transferred into the CometChip®

platform and incubated at 37°C for 1 hour. The platform was stained overnight and imaged under the microscope and analyzed using the Image J (NIH) software and OpenComet (cometbio.org).

3.5 Statistical Analysis

All the assay exposures were completed in triplicate throughout the experiment, and each trial was duplicated for the toxicological analysis. All the data were analyzed using the one-way ANOVA parametric test with GraphPad Prism software (Prism version 9.0.0). The exposure samples of puffs 1-25, puffs 101-126, puffs 201-226, negative control, and positive control were analyzed by one-way ANOVA and GraphPad Prism software with the significance level less than or equal to 0.05.

4. RESULTS

4.1. ENDS Aerosol Generation Parameter Analysis

Prior to every experiment, each labeled FEP tube was weighed when clean and completely dried to minimize the potential error for the mass calculation. The pre-experiment weight of the e-liquid filled VooPoo® tank was also measured to determine the total sample mass collection percentage. After the FEP tubes and VooPoo® tanks were reweighed following the sample collection process, the mass was calculated by comparing the post-experiment weight to the pre-experiment weights. For example, vaporized e-liquid mass during the experiment was calculated by subtracting post-weight from the pre-weight and resulted in 1558 mg from the January 25th experiment (Table 1). From the same day experiment, the mass collected in the tube was calculated by subtracting pre-weight from the post-weight and resulted in 1065 mg (Table 1). Then, the percent collection was calculated by dividing the sample mass collected in the tube (1065 mg) into the mass of vaporized e-liquid (1558 mg) and multiplied by 100 (Table 1). As shown in several lines of Table 1, the mass collected was sometimes higher than the mass vaporized. This is due to condensation of water vapor present in the exposure chamber air supply. In addition to the sample mass collected, Table 1 also shows the coil resistance (Ohm) and the power (W) used for each experiment. Regarding the resistance and the power, the result shows that the low resistance coil vaporizes more e-liquids than the high resistance coils. This is because the low resistance coil will deliver more power to the coil and lead to the coil's temperature increase. Moreover, the increasing wattage also observed as the coil ages resulting in a higher coil temperature and more vapor concentration at later device ageing stages.

4.2 Reactive Oxygen Species Generation in ENDS Exposed SAEC

The ROS were detected by the CellROX fluorescence assay, which uses cell-permeant CellROX dye that exhibits bright orange fluorescence when oxidized and shows the presence of ROS. The nuclei of the SAEC were counterstained with blue color to be distinguished. Figure 1 shows the qualitative data for the ROS in SAEC after the exposure to VooPoo® American Patriot 3% ENDS aerosol for 24 hours and 7 days at different device aging stages. After 24-hours exposure, significant ROS levels were detected in all exposures compared to the negative control (Figure 1A). The result also showed the linear dose-response that correlated with dose signal comparing with negative control after the 24 hours exposure (Figure 1C, 1D, 1E). ROS levels were significantly higher after the repeated exposure for 7 days in the puffs 0-25 and puffs 101-126 compared to the 24-hours exposure (Figure 1H, 1I). After the 7-days exposure, the larger orange fluorescence area was observed at puffs 0-25 (Figure 1H) and puffs 101-126 (Figure 1I). At the puffs 201-226 device aging state after 7-days exposure (Figure 1J), less bright orange fluorescence and blue counterstain were observed, which may be due to a technical error where insufficient volume of CellROX stain was used.

4.3 ENDS Aerosols Elicit Oxidative Stress

The oxidative stress of the SAEC was detected by using the GSH-Glo Glutathione assay, a luminescent assay that detects and quantifies the level of glutathione in the cells. The glutathione is the antioxidant in the human cell, often used as the biomarker of oxidative stress. After 24-hours exposure to the VooPoo® American Patriot 3% ENDS aerosol, the total GSH levels were decreased in all exposures compared to the negative control (Figure 2). The result

showed that the initial aging stage of the device (puffs 0-25) led to a higher level of oxidative stress compared to the later aging stage of the device (puffs 201-226) after 24-hours exposure (Figure2). After the 24 hours exposure, the middle stage (puffs 101-126) showed the significant reduction of the total GSH level. After the 7 days repeated exposure, the middle aging stage of the device (puffs 101-126) showed the significantly highest reduction of the glutathione level (Figure 2). Both glutathione level results after 24 hours and 7 days exposure indicate oxidative stress and ROS compared to the negative controls (Figure 2).

4.4 Impact of ENDS aerosol on SAEC viability

The level of cellular viability of the SAEC was measured through the MTS assay, which examines the metabolic capacity of the cells using the tetrazolium salt. It is a colorimetric assay that shows the color changes from yellow to brownish red with the healthy cells. The Figure 3 shows that there was a significant decrease of cell viability in the later device age stage. In the 24 hours exposure result, puffs 101-126 and puffs 201-226 showed the most decrease of the cell viability but only puffs 101-126 was found to be statistically significant (Figure 3). In the 7 days exposure, all the treated groups showed the decrease of cell viability, but the reduction was not significant (Figure 3). This can be due to the repeated exposure process, which could potentially wash off the dead cells. The cells in the puffs 201-226 might be still viable but there could be more molecular changes observed after the further analysis. Moreover, it could be due to the nature of the ALI cell culture system, which provides more resistant environment for the toxicant exposures through the multilayer formation.

4.5 Induction of DNA damage by ENDS Aerosols Exposures in SAEC

The increasing level of DNA damage at higher concentrations of VooPoo® American Patriot 3% ENDS aerosol was observed through the CometChip® assay result (Figure 4). The percent tail DNA damage was compared across the different device aging phases of puffs 0-25, puffs 101-126, and puffs 201-226, along with the positive and negative controls. Throughout the CometChip® assay, 0.1% hydrogen peroxide (H_2O_2) was used as a positive control to ensure that cells responded correctly. The negative control was also tested to verify the potential error within the experiment and set up the baseline data to compare with the ENDS aerosol exposed groups. As shown from the percent Tail DNA result (Figure 4), a significant level of DNA damage was observed from the puffs 101-126 device aging level after 24 hours of exposure. All the ENDS exposed groups showed a higher percentage of DNA damage, even at the lowest concentration group at puffs 0-25, compared to the negative control after 24 hours of exposure (Figure 4). Like the 24-hours exposure, all the exposed groups showed higher DNA damage levels than the negative control after repeated exposure for 7 days (Figure 4). However, we observed a three-fold increase in DNA damage caused by puffs 201-226 versus puffs 101-126 after the 7 days exposure (Figure 4). These findings are consistent with our hypothesis that the toxicity of ENDS aerosol increases as the heating element ages, and this may have adverse implication for human health.

5. DISCUSSION AND CONCLUSION

Acute and chronic exposure to ENDS aerosols may cause several adverse health outcomes such as COPD, lung diseases, and cardiovascular diseases (Behar et al., 2018; Canistro et al., 2017; Ganapathy et al., 2017; Gotts et al., 2019; Lee et al., 2018; Merecz-Sadowska et al., 2020; Pizzino et al., 2017). This study examined the effect of VooPoo® ENDS device lifecycle and toxicological profiles of VooPoo® aerosols by assessing ROS generation, oxidative stress, cellular viability, and DNA damage. There is still limited knowledge about the safety of ENDS and its potential health outcomes from acute and short-term exposures. However, recent studies suggest hazardous compounds from the ENDS aerosol increase along with the device aging process. This study was designed to demonstrate the potential toxicological effect of ENDS device lifecycle on the ENDS emitted aerosols exposure.

This study accounted for several factors to effectively examine the hazardous effects of the ENDS emitted aerosols in realistic conditions. First, current ENDS market research was carried out for the device and e-liquid selection before the sample collection process to increase the representation of the experimental result. The most used 3rd generation ENDS device with the e-liquid tank and e-liquid flavor was selected and purchased from the local vaping store in Marietta, GA. Despite several other top-selling e-liquid flavors, the neutral flavor e-liquid with a mid-range nicotine level was selected. Second, the ENDS aerosol samples were collected at different time points throughout the device's lifecycle to examine the effect of the ENDS device aging process on the SAEC. It was divided into three different phases, which were at puffs 1-25, puffs 101-126, and puffs 201-226. The ENDS device was constantly generating aerosols between each sample collection time point inside the environmental exposure chamber with 3.5-seconds

duration and 30-seconds interval period. Third, the puffing duration and time interval were operated using a custom-built puffing machine to mimic actual ENDS operation based on the CORESTA 81 standards. Fourth, the environmental exposure chamber located at the Underwriters Laboratories (UL) allowed this study to control independent variables, such as humidity, air input, air flows, and temperatures throughout the sample collection process. Lastly, the air-liquid-interface (ALI) system, a highly effective model to deliver aerosolized toxicants to the human airway epithelial cells because it provides realistic ALI conditions (Lenz et al., 2014), was used for the in-vitro portion of this study. The air-liquid-interface (ALI) system was used to effectively measure the potential toxicological effect by accounting for the confounding factors that the respiratory system may encounter during the exposure and toxicant metabolic process.

The increased ROS level observed from the CellROX® assay after the 24-hours and 7-days exposure to VooPoo® emitted aerosols compliments the decreased total GSH level result from the GSH assay. After the 24-hours exposure, the most significant changes in ROS level and GSH level were observed in the later ENDS aging process, which are puffs 201-226 (Figure 1.1) and puffs 101-126 (Figure 2), respectively. These two results show the presence of oxidative stress in the cells due to VooPoo® emitted aerosol. Oxidative stress occurs when an imbalance exists between antioxidants and ROS (Birben, Sahiner, Sackesen, Erzurum, & Kalayci, 2012). When the ROS level increases, the antioxidant level decreases and results in oxidative stress with reduced cell viability (Birben et al., 2012). Therefore, ROS and GSH level results suggest that when the SAEC is exposed to ENDS emitted aerosols, ROS production increases and occurs oxidative stress, resulting in adverse health outcomes (Chatterjee et al., 2019). Observed percent tail DNA determined by using the CometChip® assay showed increases of DNA damage in both

24-hours and 7-days exposure across all the aging points compared to the negative control (Figure 4). The significant DNA damage was observed at puffs 101-126 after 24-hours exposure and puffs 201-226 after 7-days exposure (Figure 4). Although the levels of DNA damage were not at the critical stage, the presence of genotoxicity cannot be excluded since the disease could develop at later stages. This result shows the acute ENDS aerosol exposure can induce genotoxicity by increasing the risk of developing diseases, such as cancer (Khalil et al., 2021).

Since this was a preliminary study, there were some limitations encountered through the experiment due to the COVID-19 pandemic, equipment utilization, and methodology. Due to the COVID-19 pandemic, the equipment and materials that were needed for the experiment were backordered and delayed the experiment schedule. Also, the sample collection process was delayed for five months since it had to be done inside the environmental exposure chamber located at UL, and the facility limited visitors. Therefore, the complete analysis from the experimental data, including physiochemical data, are not yet evaluated. Another limitation was the utilization of the custom-built 4-channel puffing machine. The orientation of the puffing machine required numerous attempts to generate the ENDS aerosol without damaging the coils until puffs 201-226. However, it was addressed by placing the puffing machine horizontally and refilling more e-liquids in the tank after each sample collection point.

In conclusion, this study suggests that the age of ENDS device and the duration of usage may pose a threat to respiratory health by causing oxidative stress, DNA damage, and reduced cellular viability and glutathione levels. The DNA damage might not affect the cell viability level immediately, but it may transmit the deformed DNA to the next generations. Even though

the physicochemical data has not been evaluated in this study due to pandemic mediated delays, several studies show the biotransformation and metabolism process after the ENDS aerosol exposure can lead to various adverse health outcomes (Cirillo et al., 2019; Clapp & Jaspers, 2017; Noël, Hossain, Perveen, Zaman, & Penn, 2020). Moreover, the recent in-vivo study suggests the exposure to ENDS aerosol affects biotransformation genes, such as CYP1A1 and CYP2E, and changes hematological profile after the exposure (Noël et al., 2020). Therefore, it is recommended to continue addressing our knowledge gaps to fully understand the potential toxicological effects of acute and chronic ENDS aerosol exposures.

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TABLES

Table 1. ENDS Aerosol Generation Parameter Analysis

Table 1. Mass of e-liquid and vaporized during the sample collection					
Start Date	**Device, Coil, and Wattage**	**Device Age**	**Mass vaporized (mg)**	**Mass collected (mg)**	**Percent Collected**
1/25/21	Voopoo® 0.2Ω, 40 W	1-25	1558	1065	68%
		101-125	1647	1170	71%
		201-225	1550	636	41%
2/3/21	Voopoo® 0.6Ω, 20 W	1-25	1302	997	77%
		101-125	706	1094	155%
		201-225	756	713	94%
2/10/21	Voopoo® 0.2Ω, 60 W	1-25	2648	225	8.5%
		101-125	2716	633	23%
		201-225	2562	554	22%
2/17/21	Voopoo® 0.6Ω, 28 W	1-25	1230	265	22%
		101-125	1240	930	75%
		201-225	1254	820	65%

FIGURES

Figure 1.1 Reactive Oxygen Species Qualitative Results after 24hrs and 7 days Exposures.

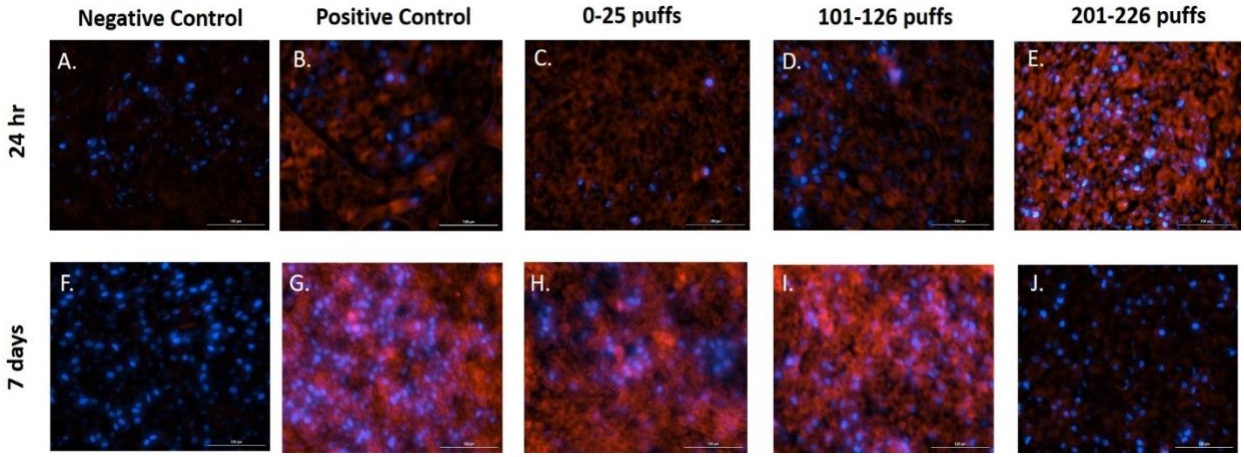

Figure 1.2 Reactive Oxygen Species Quantitative Result after 24hrs and 7 days Exposures

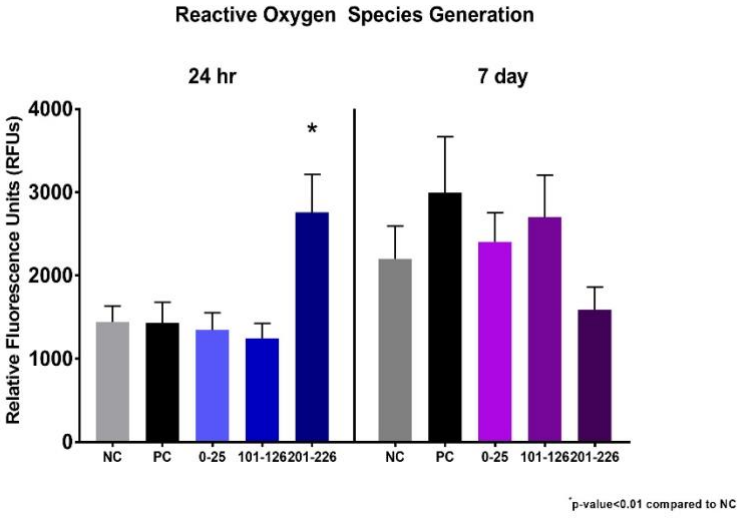

Figure 2. Total Glutathione Level after 24hr and 7 days Exposures

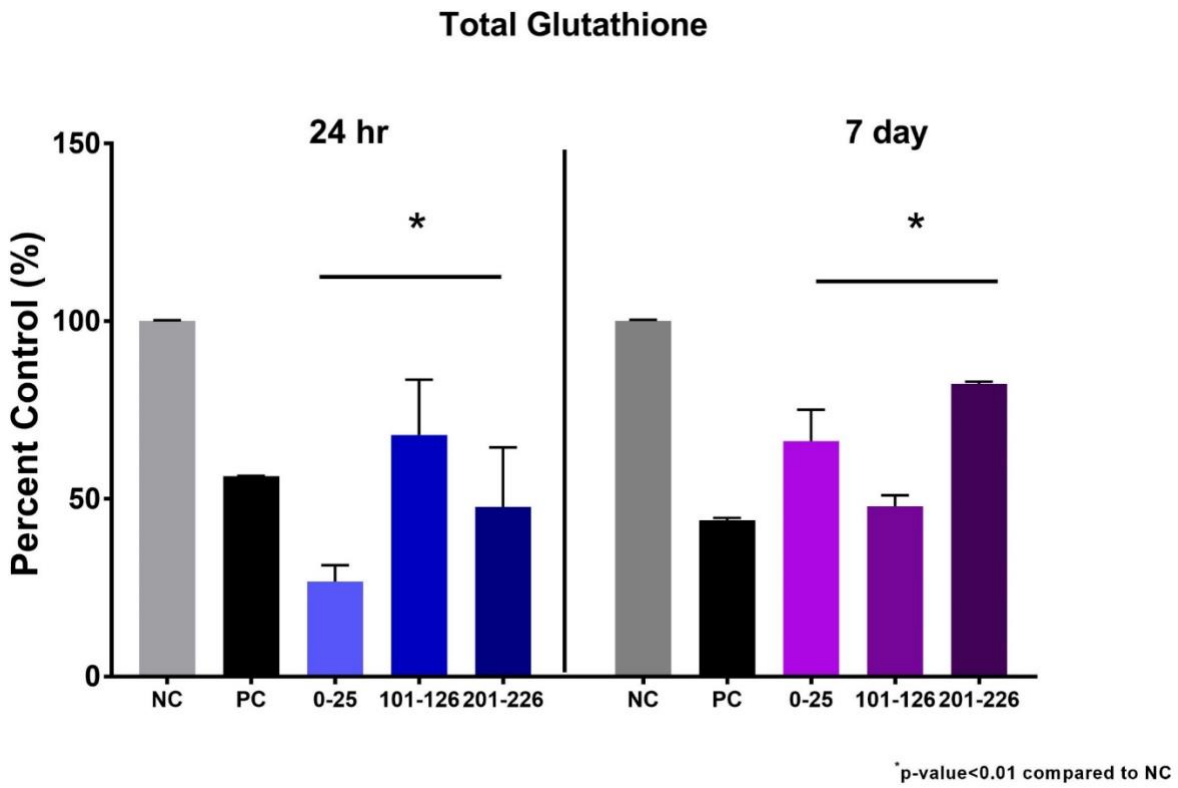

Figure 3. Cellular Viability Level after 24hr and 7 days Exposures

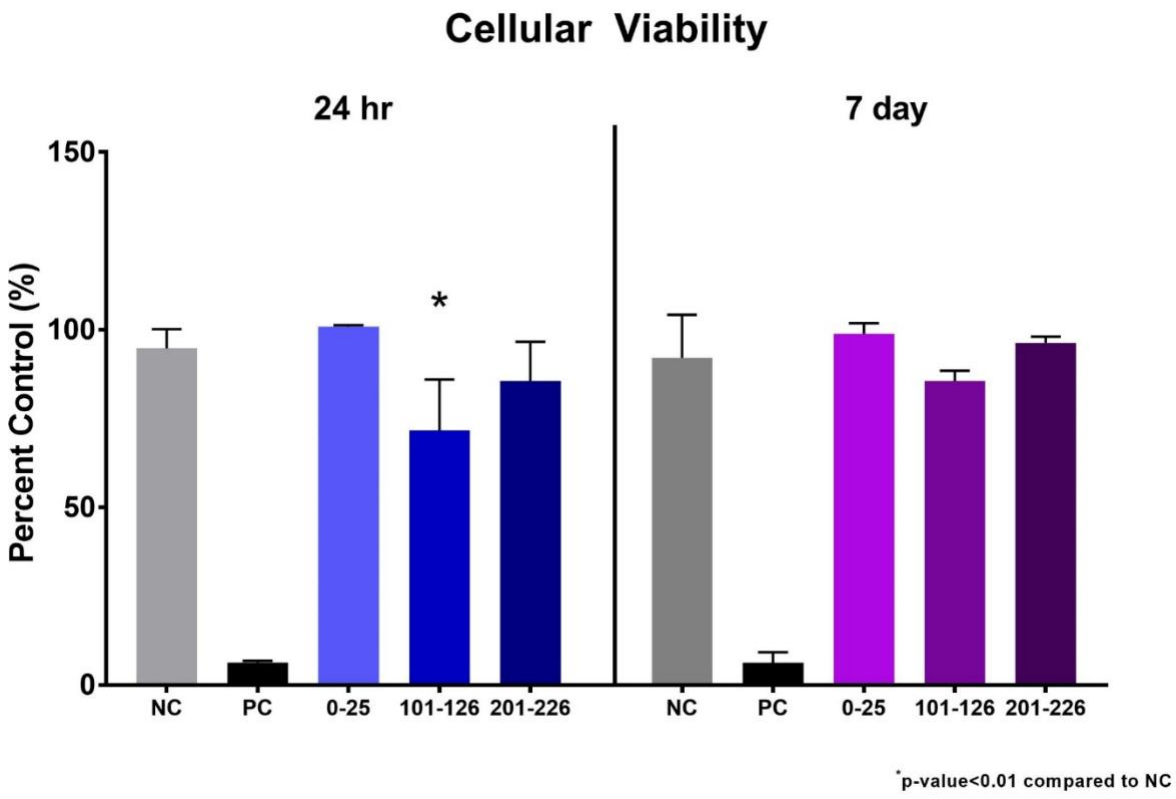

Figure 4. Percent Tail DNA Damage Level after 24hr and 7 days Exposures

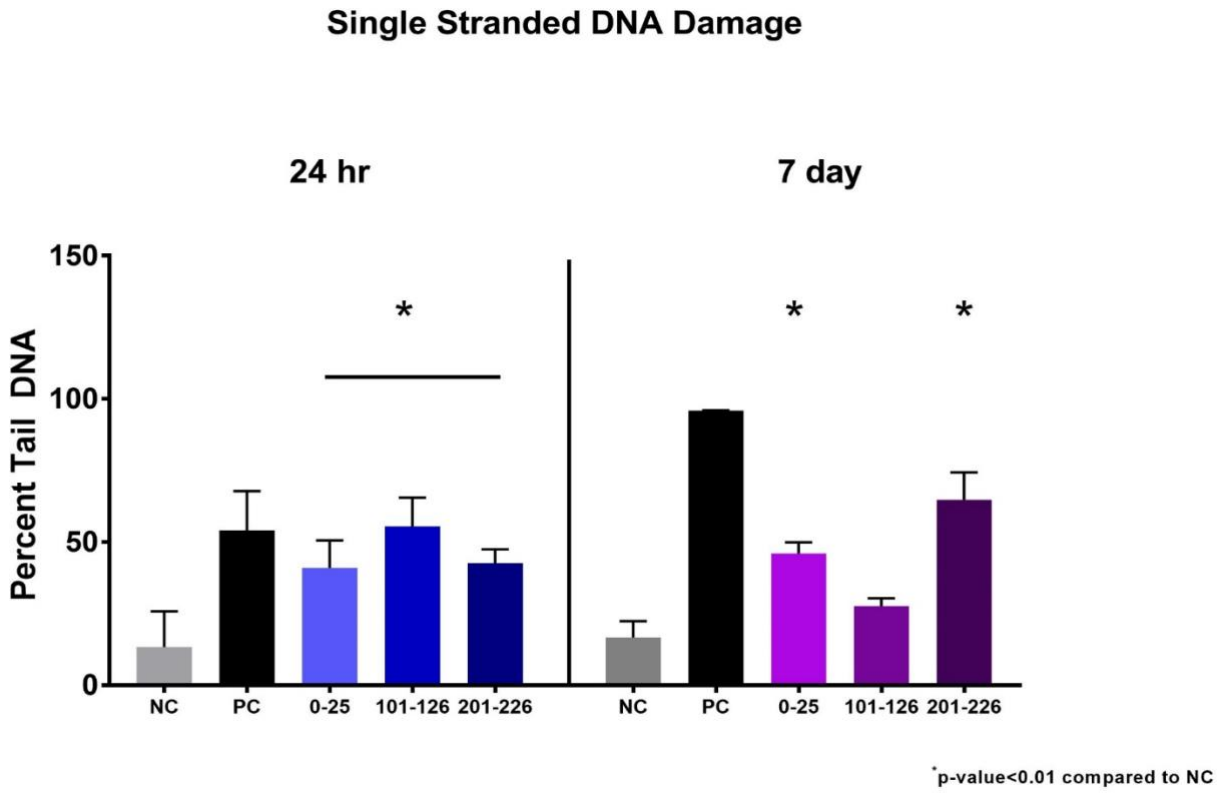

Figure 5. Environmental Exposure Chamber for ENDS Aerosol Sample Collection

Figure 6. Custom-built-4 channel Puff Machine

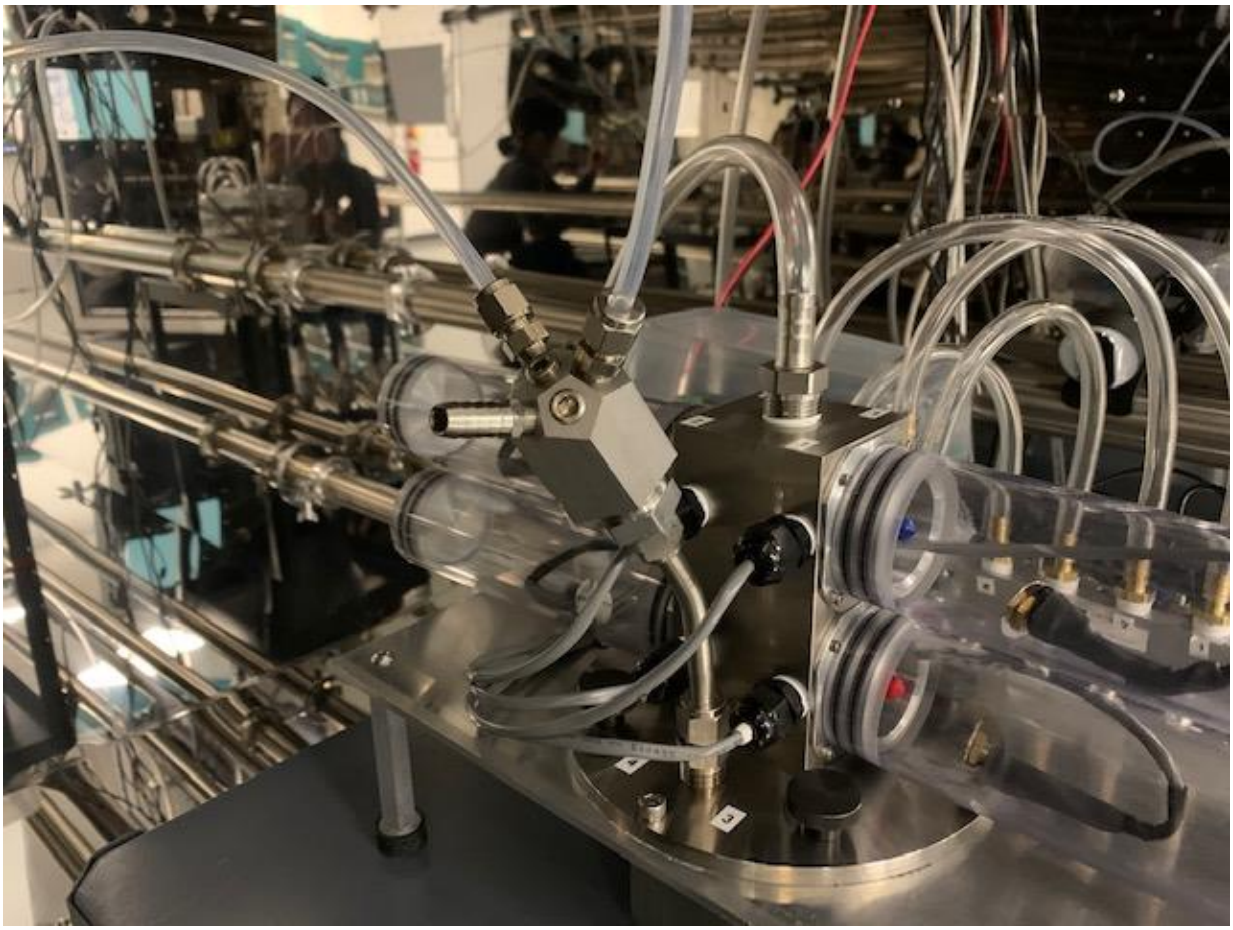

Figure 7. VooPoo® E-liquid Mass Measurement Setup

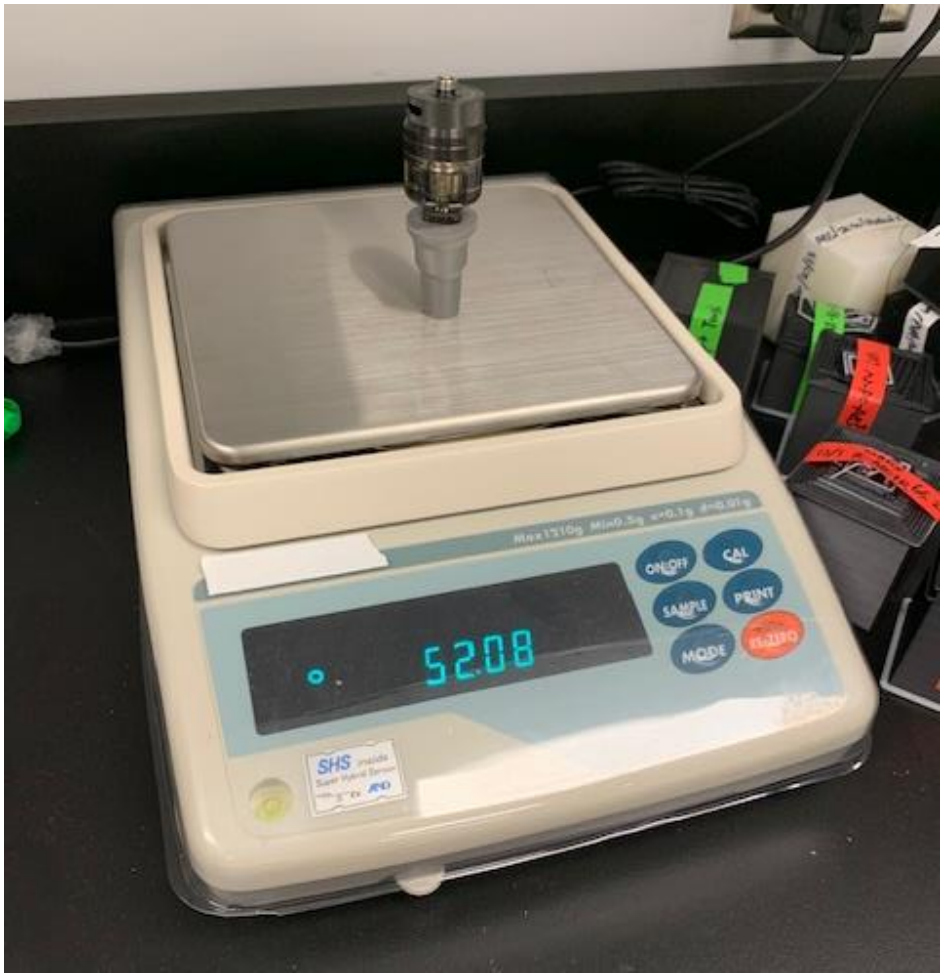

Figure 8. Sample Extraction Setup with Ultrasonic Bath Sonicator with FEP Tube

Figure 9. Vacufuge Process Setup After the FEP Tube Sample Extraction