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Article

Evaluating the Toxicity of Electronic Cigarette Aerosols for Firsthand and Secondhand Exposure Under Different Device Operating Conditions

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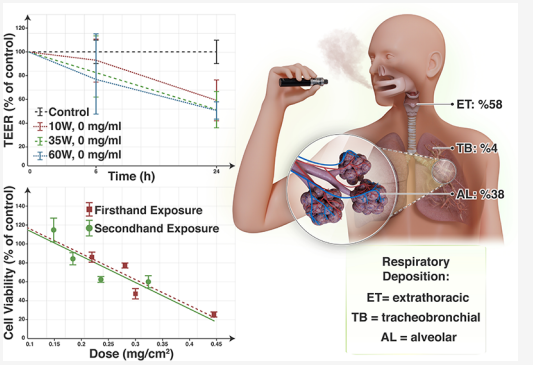
ABSTRACT: The rapid proliferation of electronic cigarettes (ECs) has raised significant concerns about their potential health effects on both users and bystanders. This study systematically investigates the impact of EC aerosol exposure on human alveolar epithelial cells (A549), considering variations in device parameters, nicotine concentration, and exposure type. Using a gravity-based air−liquid interface exposure system, we assessed cytotoxicity and epithelial barrier integrity by measuring cell viability and transepithelial electrical resistance (TEER). Our results indicate that EC aerosol exposure significantly reduces cell viability and disrupts monolayer integrity in a dose- and device-dependent manner. Notably, VUSE (pod-type) exposure led to a 16% decrease in viability and a 41% reduction in TEER, while VOOPOO (mod-type) exposure caused a 25% viability loss and a 61% reduction in TEER. Power settings played a critical role: at 60 W, cell viability dropped by 48% at 12 mg/mL nicotine concentration compared to 29% at 0 mg/mL. Moreover, under the same number of puffs (30 puffs), firsthand exposure resulted in a 73% viability decrease, whereas secondhand exposure showed a 47% reduction, indicating substantial bystander risks associated with EC usage. These findings underscore the importance of device specifications and exposure conditions in determining EC aerosol toxicity. The observed epithelial barrier disruption suggests increased vulnerability to respiratory diseases. Given the comparable toxicity of firsthand and secondhand aerosols, regulatory measures should extend beyond direct users to include bystander protection. This study highlights the urgent need for comprehensive toxicity assessments to inform public health policies on EC use.



1. INTRODUCTION

The number of electronic cigarette (EC) users has been exponentially increasing over the past decade, with approximately 68 million worldwide in 2023.[1,2] Users in the United States account for more than 10% of global users (~9 million), including 3.6 million middle and high school students.[1] ECs were originally developed mainly as a smoking cessation aid. However, increasingly, those who have never smoked have reported using them.[3] EC aerosols are produced by vaporizing the e-liquid, which is comprised of a buffer medium (e.g., propylene glycol (PG) and vegetable glycerin (VG)), nicotine, flavorings, and other additives,[4] and the compositions of different brands of e-liquid vary significantly. Chemical characterization of EC aerosols has revealed nicotine,[5−7] flavoring chemicals,[8,9] metals,[10,11] and derivatives generated from reactions (such as thermal degradation and hemiacetal formation),[12−15] all of which may lead to health complications in cases of excessive and extended use. Moreover, most EC aerosols contain submicron particles that can be inhaled deep into the lungs, causing respiratory diseases or aggravating existing diseases such as chronic obstructive pulmonary disease (COPD) and asthma.[16−18]

Because of the rapidly developing EC technology, with four generations that have already evolved within the first two decades of its existence, we urgently need to understand the physicochemical and toxicological properties of EC aerosols that affect users as well as bystanders who may passively inhale EC aerosols via secondhand aerosols.[19,20] Recent *in vitro* studies have helped us to understand the specific mechanisms of EC aerosol toxicity, including inflammation, DNA damage, and oxidative stress.[21−28] For instance, Martinez et al.[26] found that exposure to EC aerosol extracts for 24 h reduced the viability of cultured human vocal fold fibroblasts and induced

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DNA damage. Another group evaluated EC cytotoxicity and inflammatory responses by exposing the human bronchial epithelial cell line (BEAS-2B) and human monocyte-macrophage cell line (THP-1) to aerosolized e-liquid with varying nicotine contents and flavorings.[27] Their results indicated that EC aerosols only moderately induced cytotoxicity, and the damage to the bronchial epithelial cells was nicotine-content dependent. Several groups have also examined the toxic effects of nicotine and flavoring in e-liquids. Notable studies include Phillips et al.[29] who found that adding nicotine to PG/VG aerosols induces increased expression of xenobiotic enzymes (CYP1A1/FMO3) in the lungs and metabolic changes, such as lower serum lipid levels and changes in hepatic metabolic enzyme expression.

Although these studies offer certain essential aspects of EC usage on health effects of EC usage, a major challenge in EC aerosol toxicological studies is the accessibility of appropriate exposure systems that mimic the deposition of aerosols in the human respiratory system. This makes it difficult to perform studies to model and predict the health effects of EC aerosols. To address this problem, several research teams have used an air−liquid interface (ALI) setup to standardize aerosol exposure conditions. Noël et al.[30] exposed human bronchial epithelial cells (H292) to subohm EC aerosols using the ALI VitroCell system. The results indicated cytotoxicity, membrane disintegration, reactive oxygen species production, inflammation, and dysregulated gene expression related to biotransformation and oxidative stress. Khalil et al.[28] reported adverse effects of power setting, nicotine concentration, and e-liquid composition on cells. Their results revealed induced cell death, DNA damage, and apoptosis in A549 cells exposed to 30 puffs at the 40 W EC power setting. ALI has also allowed comparisons between popular e-liquid brands (with and without nicotine) for their effects on physiologically relevant human bronchial and alveolar lung mucosa models.[31] An extensive analysis of studies using ALI to assess EC toxicity has been reported in a comprehensive review.[32]

Importantly, EC aerosols may be inhaled by bystanders.[33,34] A survey conducted in 2020, reveals that one in four US middle and high school students had been exposed to secondhand EC aerosols within the past month.[35] Exposure to secondhand and thirdhand EC aerosols by living with an EC smoker has also been documented and is linked to worse mental health.[36,37] Several laboratory studies on EC secondhand aerosols inhaled by nonsmokers have primarily focused on utilizing and characterizing aerosols exhaled by volunteers.[34,38,39] Although these studies offer reasonably accurate measurements of secondhand aerosols exhaled by smokers of varying ages, physical conditions and smoking habits, carrying out such research necessitates complicated human subject compliance, which restricts the amount of repeatable data that can be obtained from these studies. Firsthand and secondhand EC aerosols may differ markedly in their physicochemical properties due to compositional changes during exhalation.[40] Volatile and water-soluble compounds are partially retained in the user's respiratory tract, resulting in reduced nicotine levels, altered particle size distributions, and modified chemical profiles in secondhand aerosols.[41] Despite these changes, harmful constituents such as fine particulates, nicotine, and VOCs remain at biologically relevant concentrations, underscoring the importance of evaluating bystander exposure risks.[40−42] To address this problem, we recently developed a simulated respiratory system comprising a series of filter materials that mimicked the deposition efficiency of the human respiratory system.[43] This system allowed us to compare the toxicological properties of firsthand and simulated secondhand aerosols.

Collectively, studies using factorial design to systematically assess the EC device parameters and their multifaceted effects related to various vaping conditions are relatively scarce. In addition, few studies have assessed the toxicity of secondhand EC aerosols owing to cumbersome ethical compliance. We hypothesized that both firsthand and secondhand EC aerosols would compromise bronchial epithelial barrier integrity in a manner dependent on device parameters (such as brands type, power, and nicotine levels) and vaping patterns (firsthand and secondhand exposures). At the molecular level, this compromise is primarily mediated by disruptions to tight junction proteins (TJPs) such as occludin, claudins, and ZO-1, which form the backbone of the epithelial barrier.[44,45] Emerging evidence indicates that EC aerosols induce oxidative stress, elevate pro-inflammatory cytokine production (e.g., IL-6, TNF-α), and trigger cytoskeletal disorganization—all of which are known to impair tight junction integrity.[46,47] For instance, Muthumalage et al.[22] demonstrated that flavored EC aerosols reduced ZO-1 expression and increased epithelial permeability through ROS generation and cytokine signaling. In another study, Khalil et al. reported late apoptosis and tight junction disruption in A549 cells exposed to EC aerosols under ALI conditions. These mechanisms suggest a biologically plausible pathway by which EC aerosols may weaken the epithelial barrier, increasing susceptibility to inflammation, infection, and chronic respiratory diseases such as asthma and COPD. When the epithelial barrier is compromised, subepithelial immune cells are directly exposed to environmental insults, potentially triggering excessive inflammatory responses and tissue remodeling.[48,49] This study used a simulated respiratory system and ALI system to examine cell viability and the monolayer influence of firsthand and simulated secondhand aerosols (simply referred to as secondhand aerosols). The wide range of EC device settings used in this study can also offer insights into the parameters affecting the toxicity of firsthand and secondhand EC aerosols.

2. METHODS

2.1. Cell Culture. A549 cells are human cell lines derived from respiratory epithelial cells. These cells produce surfactants, which are multipurpose lubricants that decrease surface tension and prevent alveolar collapse. In addition, A549 cells, generated from a type II pneumocyte lung tumor, express characteristic features of alveolar epithelial type II (ATII cells), including the synthesis of phospholipids, cytoplasmic lamellar bodies, and apical microvilli.[50] This cell line has been utilized as a model for ATII cells in the field of lung cell biology.[51] Herein, A549 cells (ATCC cat. no. CCL-185) were cultured in modified Ham's F-12 medium supplemented with 10% fetal calf serum and 1% penicillin/streptomycin at 37 °C with 5% CO_2. Cells were plated at a density of approximately 4×10^4 in 6-well Transwell plates (Nunc/Sigma) for TEER measurement and in 6-well plates without Transwell inserts for cell viability assessment and then grown to approximately 80% confluence. The use of Transwell inserts specifically for TEER measurements, but not for cell viability tests, was motivated by several factors. For instance, TEER measurements require cells to be grown on a permeable support to accurately assess epithelial barrier integrity, which is provided by the Transwell insert system. In contrast, cell viability could be reliably assessed using standard culture plates. Additionally, our cell viability assay relies on colorimetric or fluorometric readouts that could potentially be interfered with by the Transwell membrane, potentially compromising

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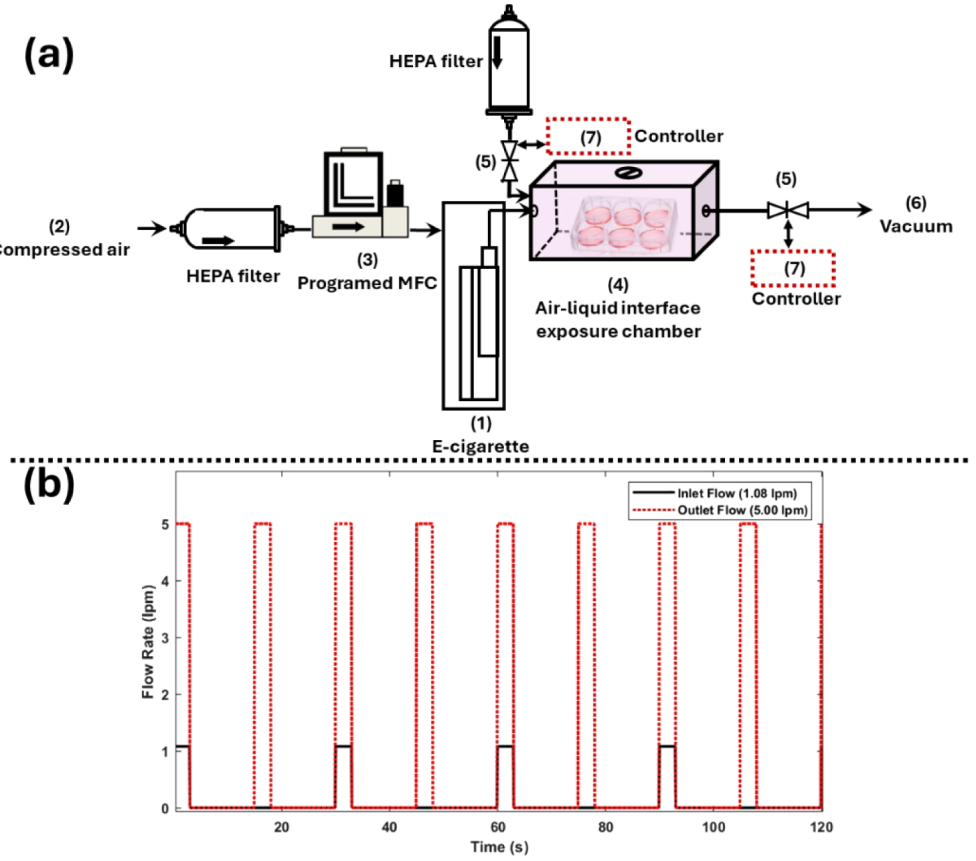

Figure 1. Experimental setup for gravity-based cell exposure to EC aerosols. (a) Schematic diagram of the exposure system: (1) EC device, (2) compressed air supply, (3) mass flow controller, (4) exposure chamber with 6-well plates, (5) on/off valves, (6) vacuum pump, (7) ELEGOO Uno R3 controller. (b) Flow rate profiles over time: The black continuous line represents the aerosol flow (1.08 lpm) controlled by the mass flow controller when activated. The red dotted line shows the intermittent operation of the on/off valve and clean air supply, with peaks indicating the 3-s periods of aerosol removal every 30 s.

the accuracy of the results. After the old medium was removed, the cells were washed with Phosphate-Buffered Saline, followed by addition of fresh medium. The cells were then exposed to the EC aerosols. For each treatment group, cells were exposed to EC aerosols or filtered air (control group) for 15 min and the aerosols were allowed to settle for 30 min. Following each experimental run, the exposed and control plates of cells were incubated at a 37 °C incubator operated with 5% CO_2 in a humidified environment. After 24 h, cells were collected to determine cell viability.

2.2. Experimental Setup. Two popular commercial EC brands, VUSE (Vuse Alto Disposable Pen, Reynolds Vapor Company, USA) and VOOPOO (Drag X, Shenzhen Woody Vapes Technology Co., China), were purchased and used in this study. The VUSE Alto pod (closed) system (fourth-generation EC system) contained prefilled pods with a 70/30 PG/VG ratio and 5.5% nicotine by weight (approximately 55 mg/mL), delivered as nicotine benzoate (salt). The VOOPOO, a mod (open) system (third-generation EC system), used PnP coils (0.15 Ω) with a commercial e-liquid containing 70/30 PG/VG ratio and varying nicotine concentrations (0, 3, 6, and 12 mg/mL) as specified in the experimental design. Both products used unflavored e-liquids to eliminate potential confounding effects from flavoring chemicals. All e-liquids were purchased directly from manufacturers to ensure consistency. A gravity-based cell exposure apparatus was used to simulate realistic human exposure to aerosols. The system consisted of a sealable exposure chamber (2 L) equipped with inlet and outlet ports designed to hold a 6-well plate (n = 6). Aerosols were generated using an EC device connected to a chamber inlet. The EC was activated by compressed air, regulated by an automated mass flow controller (Pneucleus Technologies LLC, Hollis, NH) to ensure a consistent flow rate of 1.08 L per minute (lpm). The outlet was connected to an on/off valve controlled by a microservo motor (SG90) and ELEGOO Uno R3 controllers. This valve was linked to a vacuum pump that removed aerosols from the chamber at a flow rate of 5 lpm controlled by an orifice. To mimic a typical breathing pattern, the pump was operated for 3 s every 30 s, starting 15 s after aerosol introduction, simulating the average breath-hold time in humans.[52] Additionally, a microfan inside the chamber ensured that the aerosols were well mixed. Figure 1 shows the schematic of the experimental setup. The system design, incorporating automated controls for aerosol generation and removal, allowed precise and reproducible exposure conditions. This setup enables the study of both acute and prolonged exposure effects, as the exposure duration and intensity can be easily adjusted to mimic realistic human exposure scenarios.

For firsthand aerosol exposure characterization, the experiments were conducted following a previously described protocol.[10] Briefly, a computer-programmed mass flow controller generates EC aerosols through the push mechanism, following the ISO 20768 standard puffing protocol: 55 mL puff volume, 3 s puff duration, and 30 s puff interval.[10,53] The valve connected to the vacuum was switched on during the puff period, and 30 puffs were used for each run. Another plate, housed in an exact duplicate of the exposure chamber, was exposed to filtered air as the control. Secondhand aerosols were generated using a method we recently developed, with the experimental setup illustrated in the Figure S1.[43] To validate our approach for studying secondhand smoke exposure, we developed and characterized a simulated respiratory system using filter-based media that replicates particle deposition patterns observed in human respiratory tracts (Figure S2). The system employs a combination of fabric materials, including Thai silk and polyester, configured to

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Table 1. Study Design: Task 1: Brand Comparison, Task 2: Effect of Nicotine Concentration and Power on Firsthand Aerosols, Task 3: Comparison Between Firsthand and Secondhand Aerosols

Task	EC Brand	Exposure	Nicotine (mg/mL)	Power (W)	Dose (mg/cm^2)
1	VUSE	Firsthand aerosols	55[a]	Default	0.15 ± 0.03
	VOOPOO		0	10	0.22 ± 0.04
2	VOOPOO	Firsthand aerosols	0	10	0.22 ± 0.04
				35	0.34 ± 0.09
				60	0.39 ± 0.07
			3	10	0.21 ± 0.03
				35	0.28 ± 0.09
				60	0.39 ± 0.09
			6	10	0.19 ± 0.06
				35	0.43 ± 0.05
				60	0.44 ± 0.08
			12	10	0.20± 0.06
				35	0.42 ± 0.04
				60	0.43 ± 0.10
3	VOOPOO	Firsthand aerosols	0	10	0.22 ± 0.04
		Secondhand aerosols		10	0.18 ± 0.06
		Firsthand aerosols	0	60	0.39 ± 0.07
		Secondhand aerosols		60	0.32 ± 0.05
		Firsthand aerosols	6	10	0.19 ± 0.06
		Secondhand aerosols		10	0.20 ± 0.06
		Firsthand aerosols	6	60	0.44 ± 0.08
		Secondhand aerosols		60	0.28 ± 0.04
	VUSE	Firsthand aerosols	55[a]	Default	0.15 ± 0.03
		Secondhand aerosols			0.11 ± 0.04

[a]The calculated concentration is based on the manufacturer-provided percentage and propylene glycol (PG)/vegetable glycerin (VG) ratio of 70/30. The dose reported is the total mass of particles delivered in each well (9.6 cm^2), collected on a preweighed filter, and measured using an accurate analytical balance (US Solid 0.1 mg Analytical Balance) after 30 puffs.

match the filtration efficiencies of the extrathoracic, tracheobronchial, and alveolar regions across the submicron size range (30−500 nm) relevant to secondhand smoke particles (Figure S3). Comparative analysis of aerosol size distributions measured upstream and downstream of the simulated system demonstrated its effectiveness in mimicking human respiratory processing of primary smoke from both tobacco cigarettes and ECs, showing characteristic alterations in particle concentration and size distribution that occur during respiratory deposition (Figure S4). This validated system provides a controlled, repeatable platform for generating representative secondhand smoke without human subject involvement, enabling detailed characterization of secondhand aerosol properties from various smoking devices.

A complete set of experimental parameters, including the EC brand, power setting, nicotine concentration, and mass of particles deposited in each well after 30 puffs, is presented in Table 1.

To establish the dose−response relationship between EC aerosols and cell viability for firsthand and secondhand aerosols, we introduced different puff numbers (15−90 puffs) to achieve a wide range of doses. For this test, we used a VOOPOO brand at 60 W and 6 mg/mL nicotine concentration. To ensure consistency and standardization, all exposures were normalized by puff count, which serves as a practical surrogate for user behavior and device output in EC aerosol studies. The use of deposited mass per unit surface area (mg/cm^2) is aligned with standard *in vitro* toxicology practice and allows for direct comparability across conditions. Moreover, deposition patterns were verified for spatial uniformity across the exposure chamber to further ensure dose consistency. The deposition dose in each well was quantified using the following procedure: Preweighed glass fiber filters (Whatman, 47 mm diameter) were placed in each well before aerosol exposure. After exposure, filters were carefully removed and postweighed using an analytical balance with 0.1 mg precision (US Solid USS-DBS8, readability 0.1 mg, repeatability ± 0.1 mg). Three consecutive measurements were averaged to determine the deposited mass. The deposited dose was calculated as

$$\text{Dose}\left(\frac{\text{mg}}{\text{cm}^2}\right) = \frac{m_{\text{post}} - m_{\text{pre}}}{A}$$

where m_{post} is the postexposure filter mass (mg), m_{pre} is the preexposure filter mass (mg), and A is the exposed surface area (9.6 cm^2 for each well). This approach ensured consistent quantification across all experimental conditions. Blank controls with filtered air exposure were processed identically to account for potential ambient particle deposition or filter mass changes due to humidity.

2.3. Cell Viability and TEER Measurement Methods. Cell viability and TEER were selected as primary biological end points based on their direct relevance to respiratory epithelial health and function.[54] Cell viability assessment provides a quantitative measure of cytotoxicity, which is a fundamental indicator of cellular damage caused by EC aerosols.[55] TEER measurements specifically evaluate epithelial barrier integrity, which is critical for maintaining proper respiratory function and preventing exposure of subepithelial tissues to environmental toxicants.[56] Disruption of epithelial barriers represents a key pathophysiological mechanism in respiratory diseases including asthma, COPD, and increased susceptibility to respiratory infections. Together, these complementary end points provide insight into both general cytotoxicity and specific functional impairment of the epithelial barrier. Cell viability was evaluated using sulforhodamine B (SRB) staining assay. 24 h after exposure to EC aerosols, the cell medium was removed, and cells were fixed with 10% trichloroacetic acid (TCA) for 1 h at 4 °C. After washing three times with deionized water, cells were stained with 0.2%w/v SRB (in 1% acetic acid) for 30 min on a rocker platform. Unbound SRB were removed by washing with 1% acetic acid. Bound SRB was resolved using a 10 mM Tris base. SRB staining was quantified photometrically (λ = 565 nm) with a FLUOStar Omega plate reader (BMG LabTech). Cell viability was expressed as the percentage of viable cells relative to the control.

The ALI culture was used to test the cellular monolayer integrity of A549 cells after exposure to EC aerosols. A549 cells were seeded in

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Transwell inserts (4.67 cm^2 polyester, 0.4-μm pore size; Corning Costar, Tewksbury, MA, USA) at 5 × 10^4 cells/well with 1 mL of culture medium in the apical chamber and 2 mL of culture medium in the basal chamber. After 24 h, the apical medium was removed and the basal medium was changed daily. The cells were grown under ALI conditions for 4 days to form complete monolayers and differentiate into inserts. On day four, 2 mL of medium was added to the apical chamber, and the cells were exposed to EC aerosols or filtered air. The cells were left in the exposure chamber for 30 min and then placed in an incubator at 37 °C with 5% CO_2 for 24 h. A549 cell monolayer integrity was evaluated by the TEER measurement using an Epithelial Voltohmmeter (EVOM) with an STX2 electrode (World Precision Instruments Inc., Sarasota, FL) at specified time points. The electrode was soaked in 70% ethanol and rinsed with the culture medium before use. The TEER was calculated using eq 1.[56]

$$\text{TEER}\ (\Omega \times \text{cm}^2) = (R_{\text{sample}} - R_{\text{blank}}) \times \text{effective membrane area}\ (\text{cm}^2) \quad (1)$$

The effective membrane area was defined as the area of the Transwell insert base. TEER values were expressed as the percentage of the TEER value of the exposed samples relative to the TEER value of the control measured simultaneously after EC aerosol exposure.

2.4. Statistical Methods. Statistical analyses were performed using MATLAB (R2024b) software. Analysis of variance (ANOVA) followed by Tukey's honest significant difference (HSD) test was used to compare TEER values and cell viability between the control and treatment groups at 24 h postexposure, with significance set at $p < 0.05$. The analysis examined the differences between the control and exposed groups, as well as the potential effects of EC power settings, nicotine concentrations, and exposure type on both cellular end points. All experiments were performed in six independent runs ($N = 6$), and data are presented as mean ± standard error of the mean (SEM).

3. RESULTS

3.1. Effect of EC Brands on Cell Viability and TEER. Figure 2 shows the cell viability and TEER values of A549 cells 24 h after exposure to EC aerosols of VUSE and VOOPOO ECs. In comparison to the control, the cell viability decreased to 84 ± 5% ($p < 0.05$, $N = 6$) and 75 ± 4% ($p < 0.01$, $N = 6$) for the VUSE (default conditions) and VOOPOO (10 W and 6 mg/mL nicotine), respectively (Figure 2a). The TEER values, representing cellular monolayer integrity, observed 24 h after exposure, reduced to 59 ± 18% and 39 ± 26% relative to the control for VUSE ($p < 0.05$, $N = 3$) and VOOPOO ($p < 0.05$, $N = 3$), respectively (Figure 2b). TEER values appear to decrease with time after exposure to EC aerosols.

This time-dependent effect suggests that extended exposure periods (36 or 48 h) may reveal significant differences and warrants investigation in future studies. Collectively, exposure to EC aerosols of both brands adversely affected cell viability and cell monolayer integrity. In addition, the results illustrate that the usage of EC exhibits toxic effects regardless of the generation of devices, which was also observed for the deposited dose of aerosols (Table 1). The difference in toxicity observed between mod-type (VOOPOO) and pod-type (VUSE) ECs is consistent with previous studies, suggesting that third-generation devices emit higher amounts of toxic compounds than fourth-generation do. This difference may be attributed to the higher customizability and power output of mod-type devices, potentially leading to increased aerosol production and subsequent cellular damage.

3.2. Effect of EC Power Setting and Nicotine Concentration. Figure 3 shows the viability of A549 cells 24 h after exposure to EC aerosols under varying power

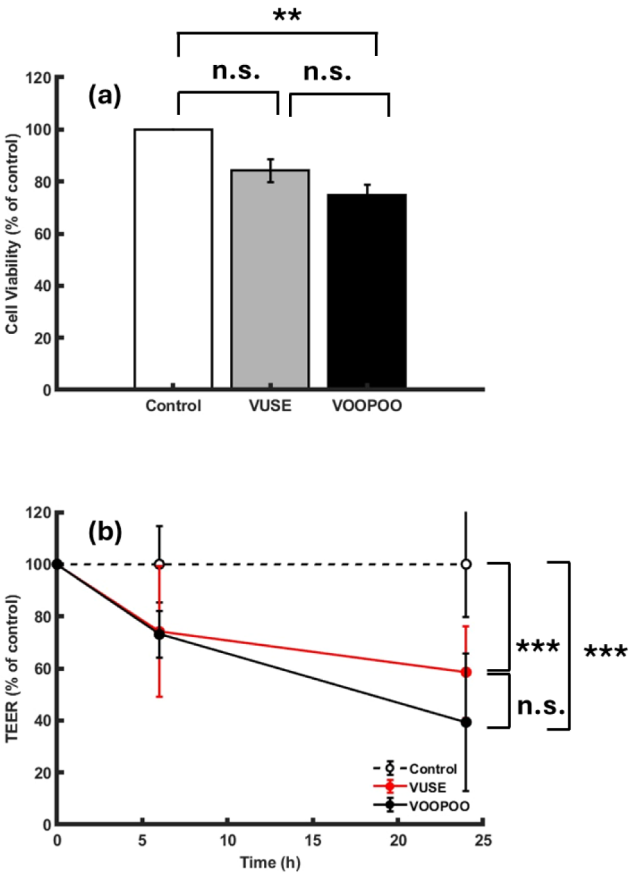

Figure 2. (a) Cell viability and (b) TEER for VUSE (default power, 55 mg/mL nicotine) and VOOPOO (10 W and 6 mg/mL nicotine) brands, 24 h after exposure to firsthand EC aerosols. Data are averages of six independent runs ($N = 6$) with triplicates of each run; error bars represent the standard error of the mean. Statistical analysis was performed using one-way ANOVA followed by Tukey's HSD posthoc test. *: $p < 0.05$ ($p = 0.038$ for VUSE vs control); **: $p < 0.01$ ($p = 0.009$ for VOOPOO vs control); ***: $p < 0.001$; n.s.: difference not significant ($p > 0.05$).

settings on VOOPOO EC (10, 35, and 60 W) and nicotine concentrations in the e-liquid (0, 3, 6, and 12 mg/mL). Compared to the control, cell viability decreased in all groups exposed to EC aerosols. At 10 W, we found that the cells exposed to aerosols with 0, 3, 6, and 12 mg/mL nicotine had cell viabilities of 108 ± 3%, 96 ± 1%, 95 ± 4%, and 93 ± 3% (vs control), respectively.

These results suggested that nicotine did not significantly affect cell viability under low-EC conditions. Cell viabilities at 60 W for 0, 3, 6, and 12 mg/mL nicotine were 71 ± 5%, 85 ± 4%, 52 ± 1%, and 56 ± 2% (vs control), respectively. At an intermediate power setting of 35 W, we observed varying impacts on cell viability depending on the nicotine concentration. Without nicotine, cell viability was 81 ± 6%, whereas at 12 mg/mL nicotine, it dropped to 57 ± 4%.

Importantly, under 60 W (35W), the cell viability with 12 mg/mL nicotine is significantly lower than without nicotine (52 ± 2% (57%) with nicotine vs 71 ± 6% (91%) without nicotine, $N = 6$), suggesting a combinatory effect of power and nicotine on cell viability, likely due to thermal transformation of nicotine at higher temperature created by the heating of the EC coil.[6−58] The dose difference, 0.39 ± 0.09 mg/cm^2 without nicotine and 0.43 ± 0.10 mg/cm^2 for 6 mg/mL nicotine

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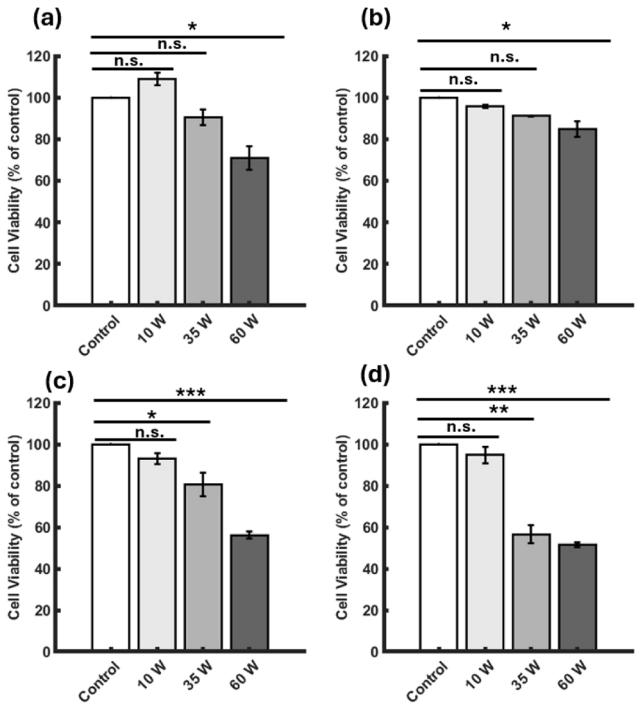

Figure 3. A549 cell viability postexposure to firsthand aerosols generated using a VOOPOO device at power settings of 10, 35, and 60 W, with (a) no nicotine, (b) 3 mg/mL nicotine, (c) 6 mg/mL nicotine, and (d) 12 mg/mL nicotine. Data represents the means of six independent runs, each with triplicates; error bars denote the standard error of the mean. Statistical significance was determined using one-way ANOVA followed by Tukey's HSD posthoc test. *p < 0.05, **p < 0.01, ***p < 0.001 indicate significant differences compared to the control group, n.s.: difference not significant.

(Table 1) is not significant for these two conditions, suggesting that the different cell viability is caused by potential chemical transformation of the aerosols. Previous studies concurred with our observation that higher nicotine concentrations lead to reduced cell viability.[59−61] Fuoco et al.[62] found that the number concentration of aerosols produced by nicotine-containing e-liquids was approximately double that of nicotine-free e-liquids. Furthermore, it has been reported that the EC power setting affects nicotine emissions, and the addition of PG enhances nicotine yield at low power. However, at higher power settings, there is an increase in nicotine yield regardless of the e-liquid composition.[63]

The TEER values in the groups with the power settings of 10, 35, and 60 W without nicotine were reduced to 58 ± 17%, 51 ± 16%, and 49 ± 6% of the control, respectively, 24 h after exposure (Figure 4a). The TEER values in the groups with the power settings of 10 W, 35, and 60 W and 6 mg/mL nicotine were reduced to 61 ± 32%, 61 ± 24%, and 62 ± 17% of the control, respectively (Figure 4b). These results suggest that EC power does not significantly affect cell monolayer integrity, nor does it show a combinatory power-nicotine effect, as observed in cell viability tests. Although the power level has no effect on the TEER value, it can be emphasized that even at low doses (10 W), it will cause damage to the integrity of the epidermis.[22,64] The measurement of TEER values at 6 and 24 h also showed that the effect of EC aerosol exposure on A549 cell monolayer integrity was time-dependent. Overall, the results showed that TEER values were reduced under the tested power setting and nicotine conditions.

TEER values are often used to indicate the tightness of epithelial cell layers. Therefore, a reduction in TEER values can indicate loss of cell monolayer integrity and increased permeability. These results suggest that EC aerosol exposure negatively impacts the integrity of A549 cell monolayers. These findings provide insight into the potential adverse effects of EC aerosol exposure on cell monolayer integrity. However, further research is required to fully understand its mechanisms and implications for human health. Although the TEER values were generally reduced following EC aerosol exposure, the extent of this reduction varied considerably depending on the exposure conditions.[65,66] The observed discrepancy between cell viability (Figure 3) and TEER measurements (Figure 4) may be attributed to the distinct aspects of cellular health assessed by each method. TEER primarily evaluates the integrity of tight junctions within epithelial monolayers, reflecting barrier function, whereas cell viability assays measure the overall cell survival. Studies have shown that TEER values can remain stable despite significant reductions in cell viability, particularly in the early stages of cellular damage.[67] This suggests that

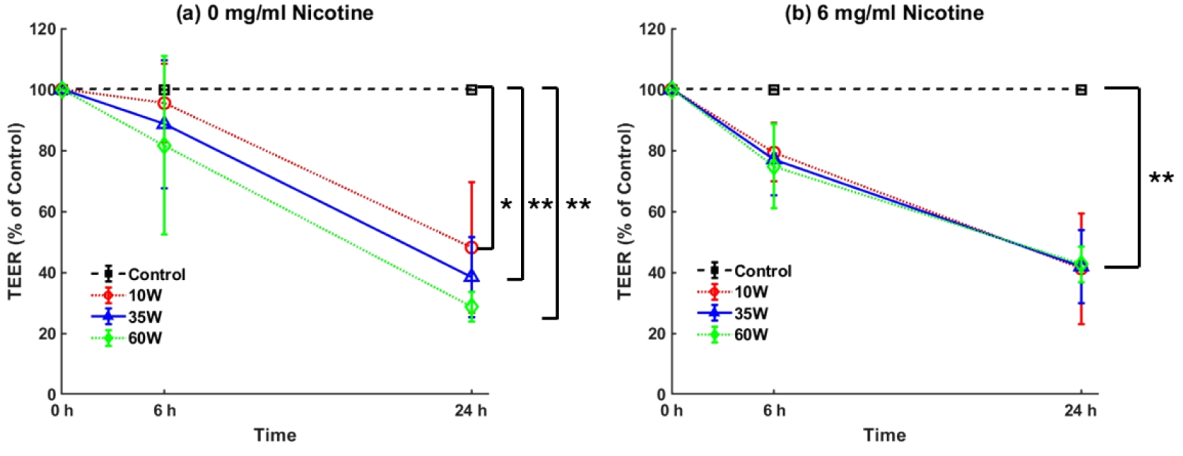

Figure 4. TEER response to firsthand aerosol exposure (VOOPOO EC) with varying nicotine concentrations (a) 0 mg/mL and (b) 6 mg/mL. Data are averages of triplicates (N = 3); error bars represent standard errors of the mean. Statistical significance was determined using one-way ANOVA followed by Tukey's Honest Significant Difference (HSD) posthoc test. *p < 0.05, **p < 0.01, ***p < 0.001 indicate significant differences compared to the control group, n.s.: difference not significant.

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TEER measurements may not immediately reflect cell death, as tight junction integrity can be maintained even when cell viability decreases. The relationship between cell viability and TEER values also appears to be complex, indicating that these two measures of cellular health are governed by different mechanisms.

3.3. Firsthand and Secondhand Aerosol Exposure.

Figure 5a shows the dose-dependent cell viability for both

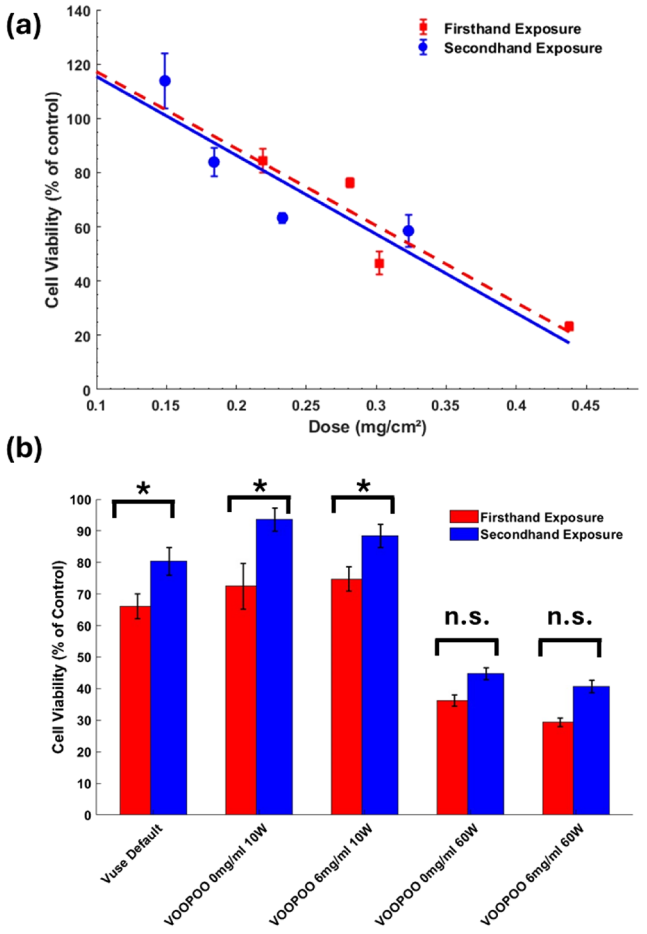

Figure 5. Viability for A549 cells exposed to firsthand and secondhand aerosols: (a) dose-dependency cell viability (VOOPOO, 10 W and 6 mg/mL nicotine), lines of best fit are added. (b) cell viability under firsthand or secondhand exposure at various power settings (10 and 60 W) and nicotine concentrations (0 and 6 mg/mL). Data are averages of 6 independent runs ($N = 6$) with triplicates for each run; error bars represent standard errors of the mean of replicates. Statistical significance was determined using three-way ANOVA followed by Tukey's HSD posthoc test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ indicate significant differences compared to the control group, n.s.: difference not significant.

firsthand and secondhand exposures. The data revealed an inverse relationship between dose and cell viability for both exposure types. For the firsthand aerosol exposure, we observed a dose-dependent decrease in cell viability. At the lowest dose of 0.22 mg/cm^2 (15 puffs), cell viability was 84 ± 4%, whereas the highest dose of 0.44 mg/cm^2 (90 puffs) resulted in 23 ± 4% cell viability. Secondhand aerosol exposure also resulted in a dose-dependent decrease in cell viability. The initial cell viability at the lowest dose (0.15 mg/cm^2) was higher than 100% (114 ± 10%), suggesting a potential stimulatory effect at low doses. Comparing the two exposure types, we observed that secondhand exposure to EC aerosols resulted in similar cell viability as firsthand exposure at comparable doses. This observation suggests that secondhand EC aerosols may be only slightly as toxic as firsthand aerosols are. Linear regression analysis of the dose−response relationship yielded strong negative correlations for both firsthand ($R^2 = 0.94$, $p < 0.001$) and secondhand ($R^2 = 0.92$, $p < 0.001$) exposures. The regression equations (viability firsthand = 149.3−286.4 × dose; viability secondhand = 153.8−293.1 × dose) indicate similar slopes for both exposure types, suggesting comparable dose-dependent cytotoxicity. No significant threshold effect was observed within the tested dose range, with toxicity increasing linearly from the lowest tested dose through the highest dose.

Figure 5b presents the cell viability data for firsthand and secondhand aerosol exposure under the same number of puffs but various EC operating conditions, including different EC brands, nicotine concentrations, and power settings. For the VUSE brand, firsthand exposure resulted in a cell viability of 66 ± 4%, whereas secondhand exposure showed higher viability of 80 ± 4%. The VOOPOO brand demonstrated varying results under the different conditions. At 0 mg/mL nicotine and 10 W power, firsthand and secondhand exposures resulted in cell viabilities of 73 ± 7% and 94 ± 4%, respectively. Interestingly, without nicotine and 60 W power, both firsthand and secondhand exposures showed relatively low cell viabilities (36 ± 2% and 44 ± 2%, respectively). As the data indicate, a significant difference between firsthand and secondhand exposure was observed for the VOOPOO at lower power settings, whereas no significant difference was observed at higher power levels. This substantial difference highlights the potential increased risk associated with direct EC use compared to secondhand exposure under certain conditions. This difference in viability was likely due to differences in the doses (Table 1).

The comparison of TEER values between exposure groups (Figure 6) demonstrated that firsthand aerosol exposure

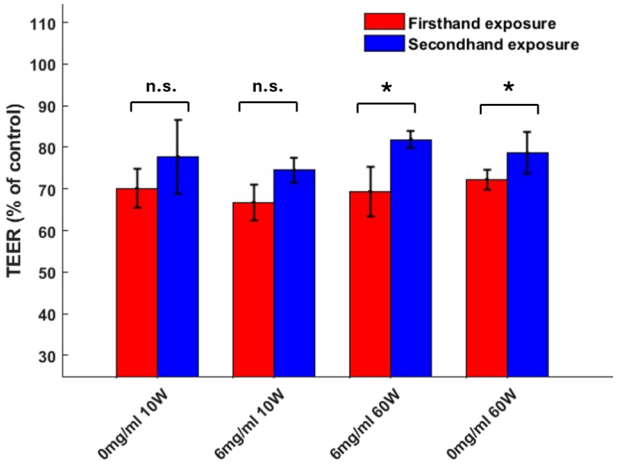

Figure 6. Impact of EC aerosol exposure on epithelial barrier integrity. TEER values of A549 cells exposed to firsthand and secondhand aerosols from VOOPOO EC at different power settings (10 W, 60 W) and nicotine concentrations (0, 6 mg/mL), measured 24-h postexposure. Data represents SEM from 6 independent experiments ($N = 6$). *: $p < 0.05$ represents a significant difference between the groups indicated.

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resulted in lower measurements than secondhand exposure. The data further revealed interesting patterns across the different EC settings and exposure types. For the VOOPOO device operated at 10 W with 0 mg/mL nicotine, TEER values decreased to 70 ± 8% and 78 ± 15% of the control for firsthand and secondhand aerosol exposure, respectively. When nicotine concentration increased to 6 mg/mL at the same power setting, TEER values were slightly lower, reaching 67 ± 7% for firsthand exposure and 75 ± 5% for secondhand exposure. At 60 W, with 0 mg/mL nicotine on the one hand, TEER values were 72 ± 4% for firsthand exposure and 78 ± 9% for secondhand exposure. On the other hand, with 6 mg/mL nicotine, TEER values were 69 ± 10% for firsthand exposure and 82 ± 3% for secondhand exposure. While there appeared to be a trend toward lower TEER values with firsthand exposure than with secondhand exposure, these differences were not statistically significant at 10 W whereas they were at 60 W.

4. DISCUSSION

This study provides insights into the cytotoxic effects of EC aerosol exposure on alveolar basal epithelial cells under various operating conditions. Our results demonstrated significant reductions in the cell survival and monolayer integrity of A549 cells upon exposure to EC aerosols, with variations observed based on device type, power settings, and nicotine concentration. 24 h after exposure to 30 puffs of EC aerosols, we observed cell viabilities of 75 ± 4% and 84 ± 5% compared to the control and TEER reduction to 39 ± 26% and 59 ± 18% of the control for the mod-type EC (VOOPOO, 10 W and 6 mg/mL nicotine) and pod-type EC (VUSE), respectively. Moreover, Tukey's HSD posthoc test showed a significant difference in cell viability between the tested brands. This difference in toxicity among the brands can be mainly explained by the fact that mod-type EC brands (third generation) emit higher amounts of toxic compounds than pod-type EC brands (fourth generation).[68,69] This observation confirms the dose-dependent toxicity observed in brand variety with the VUSE EC yielding 0.15 ± 0.03 mg/cm^2 of particles deposited onto cells, lower than the VOOPOO's doses at any operating condition.

We also observed a dependence of cell viability on the EC power setting, with 93 ± 3%, 81 ± 6%, and 56 ± 2% for the VOOPOO (without nicotine) device operated at 10, 35, and 60 W, respectively. A similar trend was also noted for all the nicotine concentrations tested. Our findings are consistent with earlier studies where various cell types exposed to EC aerosols resulted in an enhanced decrease in cell viability under various EC working conditions.[28,61,70] The power effects can be attributed to the increased aerosol production due to a higher wattage as confirmed by the mass of particles deposited on cells with 0.22 ± 0.04, 0.34 ± 0.09, and 0.39 ± 0.07 mg/cm^2 for 10 W, 35 and 60 W, respectively (without nicotine, Table 1). This observation aligns with established physicochemical principles of nicotine vaporization. Higher power settings increase coil temperature, which enhances the vapor pressure and transfer efficiency of nicotine from liquid to aerosol phase. At increased temperatures (typically above 200 °C), nicotine also undergoes chemical transformations including deprotonation, which changes the ratio of free-base to protonated nicotine forms and affects both delivery efficiency and respiratory tract patterns. Additionally, thermal degradation can produce nicotine decomposition products with potentially enhanced toxicity profiles. These temperature-dependent mechanisms explain the observed power-dependent increases in both nicotine emissions and cytotoxicity, consistent with our previous findings on metal emissions at different power settings.[10]

Our study also examined the combined effects of power settings and nicotine concentration in e-liquids. When the EC device was operated at 10 W, the nicotine concentration in the e-liquid had little effect on the cell viability ($p < 0.05$). In contrast, nicotine content significantly affected cell viability at higher EC power settings (35 and 60 W), suggesting a combinatory effect between power and nicotine. To illustrate, at an EC power of 60 W, cell viabilities of 85 ± 4%, 71 ± 6%, 56 ± 2%, and 52 ± 1% for 0, 3, 6, and 12 mg/mL nicotine, respectively, were observed. Tukey's HDS posthoc test confirmed these results, which revealed no significant difference between samples with and without nicotine at 10 W. At 35 W ($p < 0.05$) and 60 W ($p < 0.01$), there was a significant difference between samples with and without nicotine. Considering that nicotine concentration did not significantly affect the aerosol dose (0.39 ± 0.07, 0.39 ± 0.09, 0.44 ± 0.08, and 0.43 ± 0.1 mg/cm^2 at 0, 3, 6, and 12 mg/mL nicotine at 60 W, respectively, Table 1), this observation is likely due to the change in the thermal decomposition of emissions, particularly nicotine, at higher temperatures, owing to a power increase.[71,72] It should be noted that while power settings were precisely controlled in this study, direct coil temperature measurements were not performed. Previous studies have established that higher power settings directly correlate with increased coil temperatures, with approximately 10−15 °C temperature increase per 10 W power increment depending on device design, while coil wetness conditions can result in temperature differences exceeding 200 °C between dry and saturated conditions.[73−76] Moreover, coil degradation over repeated heating cycles can alter thermal properties and metal composition, potentially introducing additional experimental variability.[74] These temperature variations may contribute to the observed differences in cytotoxicity and could partially explain the variability in our biological end points across identical power settings.[76] Future studies should incorporate real-time temperature monitoring to better isolate the contributions of thermal effects from other device parameters and to enhance the reproducibility of exposure conditions.

Furthermore, our results showed that secondhand EC aerosols induced cell death and membrane disintegration, as the secondhand EC aerosol dose was not significantly lower than that of the first and EC aerosols (Table 1). This is because of the relatively low respiratory deposition efficiency of submicron aerosols (10−800 nm),[43,77] where most EC aerosol particle sizes are concentrated in ref [18, 78], and 79. Specifically, compared to the control, firsthand VUSE EC aerosol exposure resulted in 66 ± 4% cell viability, lower than that of the secondhand aerosol exposure group, which was 80 ± 4%. The VOOPOO EC (10 W, 0 mg/mL nicotine) resulted in 73 ± 7% cell viability for firsthand aerosol exposure and 94 ± 4% for secondhand aerosol exposure. However, at higher power settings (60 W, 0 mg/mL nicotine), we found a significant difference in cell viability between firsthand and secondhand exposure, with cell viabilities of 36 ± 2% and 45 ± 2%, respectively. Meanwhile, no significant difference between the firsthand and secondhand aerosols was detected in TEER values. This is likely due to the change in aerosol physicochemical properties under high-power settings, which

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necessitates further study. The observed variability in deposited mass across experimental conditions (Table 1) introduces a potential confounder in attributing biological effects solely to device characteristics or nicotine concentration. To address this, we performed dose−response analyses (e.g., deposited mass vs cell viability, Figure 5a to identify patterns suggestive of composition-driven toxicity beyond mass alone. While dose-matching was achieved where feasible, we acknowledge the importance of implementing standardized exposure protocols with real-time deposition control to isolate the contributions of aerosol chemistry. Future work will incorporate such strategies to enhance causal inference across device types and exposure parameters. The differential responses observed between cell viability and TEER measurements highlight the complexity of the cellular responses to EC aerosol exposure. Although cell death may occur relatively quickly, the degradation of tight junctions and the subsequent loss of barrier function appear to follow a more gradual timeline. This temporal disconnection between immediate cytotoxicity and progressive barrier dysfunction suggests that even sublethal exposure to EC aerosols may lead to compromised epithelial integrity over time, potentially creating a vulnerability to secondary insults or infections.

In this study, we used an ALI setup to conduct EC aerosol exposure, allowing simulation of the surface contact between aerosols and cells. The masses deposited in our study were of the same order of magnitude as those reported in the literature. In comparison, Zhang et al. (2022) determined aerosol mass and nicotine delivered by an EC using two commercially available ALI Vitrocell systems.[80] Employing gravimetric analysis of mass recovered from filter pads, they found that the mass delivered, ranging from 0.16 to 1.3 mg/cm^2 increased linearly. Their findings offered valuable information on ALI exposure device functioning and aerosol delivery, although *in vitro* evaluations of EC aerosols have not been conducted. Noël et al. (2020) examined EC aerosols by quantifying the chemical composition.[30] Results revealed that aerosols generated from butter-flavored EC vaporized under "subohm" settings have elevated quantities of carbonyls, such as formaldehyde, acetaldehyde, and acrolein, in the range 210−450 μg whereas the EC operated under common conditions (resistance >1 Ω and voltage >4.5 V) yielded lower carbonyl levels in the vicinity of 60 μg for 30 puffs. These findings further indicate the mass contribution of pollutants to the EC aerosols. Exposure of human bronchial epithelial cells (H292) to subohm EC aerosols using a Vitrocell system ALI showed membrane disintegration, displaying significant reduction (∼50%) in TEER values between exposed and control groups, as in this study.

The complex relationships between power settings, nicotine concentration, and cytotoxic effects revealed by our study highlight the challenges in developing comprehensive regulations for EC devices. Our findings suggest that simply regulating nicotine content or power settings alone may not be sufficient to mitigate the potential health risks. Instead, a more nuanced approach that considers interactions between these factors may be necessary. Furthermore, the differences observed between the firsthand and secondhand exposure effects underscore the need for regulations that consider both direct users and bystanders. The rapid evolution of EC usage and evidence of secondhand aerosol exposure by bystanders, combined with the observations of this study, raise concerns about the health and safety of individuals exposed to secondhand EC aerosols. Secondhand aerosols have been proven to be sources of nicotine, VOCs, and $PM_{2.5}$, resulting from exhaling individuals.[81] Moreover, our previous study showed that secondhand aerosols contained approximately 80% of metals compared to firsthand EC aerosols. Exposure to secondhand EC aerosols is particularly worrisome, as in 2022, 5 million US middle and high school students reported exposure to secondhand EC aerosols in indoor or outdoor public places.[82−84] These numbers are concerning because secondhand exposure for more than 6 h can significantly change the concentrations of biomarkers for nicotine exposure and acrolein.[85]

While our study concentrated on cell viability and barrier integrity as primary end points, future investigations should incorporate additional mechanistic markers to more comprehensively characterize the cellular responses to EC aerosol exposure. Assessing oxidative stress indicators (e.g., 8-isoprostane, 8-OHdG, GSH/GSSG ratio), inflammatory mediators (IL-6, IL-8, TNF-α), apoptotic markers (caspase activation, Annexin V staining), and DNA damage parameters (comet assay, γ-H2AX staining) would provide critical insights into the molecular pathways involved in the observed cytotoxicity and barrier disruption. Such a mechanistic approach would enhance our understanding of how device parameters and exposure conditions influence specific toxicity pathways. In addition, our experimental design focused on cellular responses at 24 h postexposure, which captures many acute effects but does not address temporal dynamics or potential recovery processes. Future studies should include multiple assessment time points (immediate, 6h, 24h, 48h, and 72h) to characterize the progression of cellular damage, potential recovery or adaptation, and delayed cytotoxic effects. Additionally, studies incorporating recovery periods after exposure would provide valuable information about the reversibility of EC aerosol-induced damage and the potential for cellular repair after repeated or chronic exposures. Lastly, future investigations should incorporate real-time or batchwise chemical analyses of aerosol constituents, including carbonyls, VOCs, nicotine, and transition metals to better elucidate the mechanistic basis of observed toxicological effects. These studies will aim to correlate specific chemical signatures with cytotoxicity and epithelial dysfunction using advanced analytical techniques (e.g., GC-MS, LC-MS) in tandem with exposure systems.

5. CONCLUSION

This study revealed the interplay between factors influencing the toxicity of EC aerosols on human lung epithelial cells. Our findings demonstrate that device type significantly affects cellular responses, with mod-type devices generally producing more harmful effects than pod-type systems. Power settings have emerged as critical factors, with higher power levels consistently leading to decreased cell viability and compromised monolayer integrity. Notably, we observed a combinatory effect between the power settings and nicotine concentration, particularly at higher wattages, suggesting that the thermal decomposition of e-liquid components may play a crucial role in EC aerosol toxicity. Moreover, our results indicated that secondhand aerosols can be as harmful as firsthand aerosols, thereby challenging the notion that bystander exposure is significantly less risky. This observation challenges current public health messaging, which often emphasizes firsthand exposure risks while potentially under-

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standing the hazards of secondhand smoke exposure. The similar toxicity profiles between firsthand and secondhand aerosols, particularly at higher-power settings, suggest that bystander protection measures may need to be as stringent as those for direct users. While this study provides valuable insights using cell viability and barrier integrity end points in A549 cells, future investigations should incorporate additional mechanistic markers (oxidative stress, inflammatory mediators, DNA damage parameters) and multiple cell lines to more comprehensively characterize EC aerosol toxicity pathways and enhance the generalizability of findings to diverse respiratory cell populations. These findings underscore the need for comprehensive regulations addressing device parameters, e-liquid composition, and secondhand exposure to mitigate potential health risks associated with EC use.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.chemrestox.5c00064.

Simulated respiratory system design; filtration efficiency characterization; comparative aerosol size distribution analysis for electronic cigarette and tobacco cigarette smoke (PDF)

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Notes

The authors declare no competing financial interest.

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