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Article

Differential Toxicity of Electronic Cigarette Aerosols Generated from Different Generations of Devices *In Vitro* and *In Vivo*

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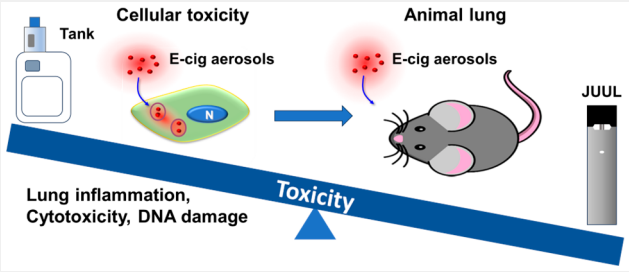
ABSTRACT: Electronic cigarettes (e-cigs) have become increasingly popular, especially among youth, raising concerns about their potential health risks. JUUL and Tank devices are two common types of e-cigs that deliver aerosols with varying nicotine levels and flavors. However, the differences in the aerosols generated from different devices and their corresponding cytotoxicity and pulmonary injury effects remain poorly understood. This study addresses these knowledge gaps by characterizing the aerosols of JUUL and Tank e-cig devices and testing their toxic effects on THP-1 and BEAS-2B human cell lines as well as the C57BL/6J mouse model. In our study, the lower-voltage device, the 3.7 V JUUL generates 2.72 mg/puff aerosols by using e-liquid containing 3% nicotine salt (i.e., nicotine benzoate), which is less than the 11.06 mg/puff aerosols generated by the 7.5 V Tank using e-liquid containing 2.4% freebase nicotine. Yet, the cytotoxicity results reveal that JUUL aerosols induced higher toxicity and increased production of pro-inflammation cytokines compared to Tank aerosols per puff. Additionally, we observed that JUUL induced more severe pulmonary inflammation and DNA damage compared to Tank after normalizing for cotinine, a nicotine metabolite, *in vivo*. Our findings suggest that the device design plays a more important role in e-cig aerosol-induced toxicity than the composition of the e-liquid or voltage. These results provide valuable insights into the health risks associated with various electronic-cig devices and offer an approach for evaluating them.



KEYWORDS: *electronic cigarette, aerosol, acute lung inflammation, oxidative stress, DNA damage*

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■ INTRODUCTION

The use of electronic cigarettes (e-cigs) or electronic nicotine delivery systems (ENDS) has gained huge popularity, especially among adolescents and young adults. E-cigs work by heating the e-liquid in cartridges to produce an aerosol or vapor that typically contains propylene glycol (PG), vegetable glycerol (VG), nicotine, flavors, and other chemicals, which are inhaled by the user. Although e-cig aerosols generally contain fewer particles and lower levels of toxicants than conventional tobacco cigarettes, they still contain potentially harmful substances, including fine and ultrafine particles, volatile organic compounds (e.g., formaldehyde, acetaldehyde), and heavy metals. Metals, such as Cr, Cu, Ni, and Fe, some in the nanoparticle format, have been found in e-cig aerosols at higher levels than in tobacco smoke.[1−3] Previous studies have shown that e-cig could lead to gas exchange abnormalities, reduce functional residual capacity in mice, cause mucociliary dysfunction, induce acute inflammation in the lungs, and increase susceptibility to respiratory infection by enhancing microbial capacity for cell invasion.[4−8] However, the continuous evolution of e-cig devices and e-liquid formulations poses a significant challenge in understanding the toxicity of e-cig aerosols and the health risks associated with using e-cigs. Despite the extensive information on e-cig toxicity provided by previous studies, there are very few studies that specifically compare the toxicity of e-cig aerosols from devices of different generations.

According to the Centers for Disease Control and Prevention, e-cigs are classified into four generations by device characteristics.[9] The Subohm Tank, the third generation e-cig, features low-resistance coils that produce a large cloud (aerosol) with a stronger delivery or hit of freebase nicotine. The latest e-cigs (aka., the fourth generation e-cigs), such as JUUL, typically utilize nicotine salt instead of freebase nicotine to deliver higher doses of nicotine.[10] Based on data from the National Youth Tobacco Survey in 2022, disposables were the most prevalent device type among youth who currently used e-cigarettes, accounting for 55.3% of usage. Prefilled/refillable pods or cartridges followed closely behind, representing

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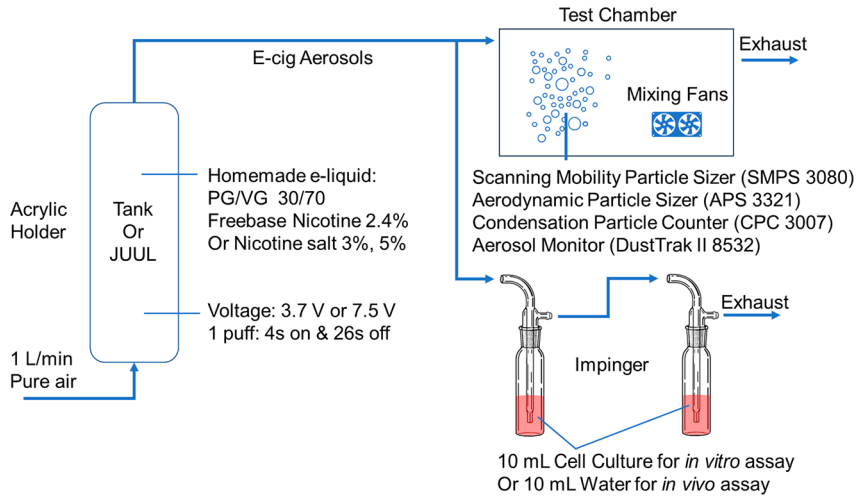
Scheme 1. Self-Built Puffing Machine Was Constructed in the Laboratory to Collect E-cig Aerosols[a]



[a]This machine enables precise control of voltage, air flow rate, and puff duration for e-cigarettes, ensuring reliable and accurate collection of e-cigarette aerosol samples. The aerosols were then introduced into the test chamber or impinger for further analysis.

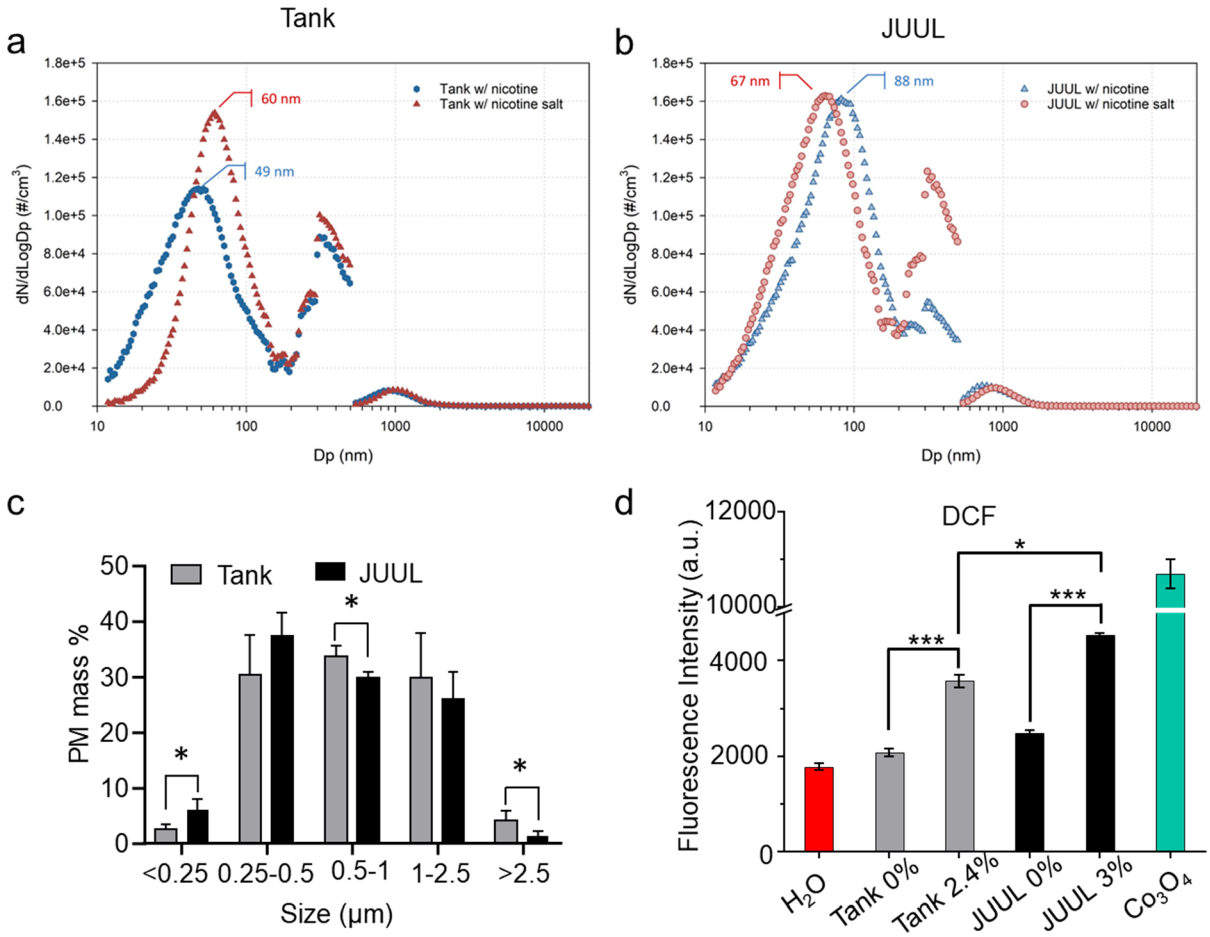


Figure 1. Particle size distributions of e-cig aerosols from (a) Tank and (b) JUUL devices with either freebase nicotine or nicotine salt in the e-liquid. (c) Total PM emission and size-resolved PM fraction of e-cig aerosols from the Tank system using freebase nicotine and JUUL using nicotine benzoate salt. (d) Intensity of DCF fluorescence at 525 nm after incubation with samples containing 120 puffs of e-cigarette aerosols from the Tank system using e-liquid without nicotine (referred to as Tank 0%) or containing 2.4% freebase nicotine (referred to as Tank 2.4%) and from JUUL using e-liquid without nicotine salt (referred to as JUUL 0%) or containing 3% nicotine benzoate salt (referred to as JUUL 3%). DI water and Co_3O_4 NPs (10 μg/mL) were used as the negative and positive control, respectively. For (c) and (d), * indicates $p < 0.05$, and *** indicates $p < 0.001$.

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Environ. Health 2023, 1, 315−323

25.2%.[11] Given the high popularity of the third and fourth generations of e-cigs, it is necessary to understand the toxicological differences between these product generations.

The JUUL system, positioned as a representative of the fourth generation of e-cigarettes, has been marketed as less harmful than Tank systems. This claim is attributed to its low-power design, operating at approximately 6.3 W with a voltage of 3.7 V, which is believed to result in the production of fewer harmful byproducts compared to higher-powered devices such as the Tank device, which typically operates at 20 W or even higher, with the rated voltage exceeding 5 V.[12,13] Notably, studies have indicated the presence of benzene, a known human carcinogen, in the e-cig aerosols produced by certain Tank systems, but not in the aerosols from the JUUL system.[14] However, the JUUL system may also have certain aspects that make it potentially more toxic compared to the Tank systems. By utilizing the nicotine benzoate formula, the JUUL pods have a much higher nicotine concentration in e-liquids (5%) than those used in Tank systems (2.4%).[15,16] In addition, the wick material in JUUL pods was subsequently modified after their first launch in Europe, to further increase the aerosol generation leading to an even higher delivery dose of nicotine per puff.[17] On the other hand, the addition of benzoic acid in e-liquids increases the concentrations of protonated nicotine, which was shown to activate/desensitize nAChRs on the inner surface of the respiratory tract while reducing the concentrations of unprotonated nicotine that potentially produce more toxic effects in the lungs, including lung cancer promotion.[18] Furthermore, pod e-cigs were shown to produce smaller particles than tank/box-mode e-cigs and traditional cigarette smoke, with a greater tendency to penetrate deeper into the lungs.[19] Overall, very limited studies have assessed and compared the toxicity of these two popular generations of e-cig devices as well as the differences between e-liquids containing nicotine salt and freebase nicotine within the same framework.

To address the aforementioned knowledge gaps, we specifically selected Tank and JUUL devices for analysis in the present study, which aimed to investigate and compare the physicochemical properties, cytotoxicity, inflammation, and oxidative stress induced by e-cig aerosols from these two devices with varying nicotine levels and different flavors. Two different cell lines, the human macrophage cell line THP-1 and the human bronchial epithelial BEAS-2B cell line, as well as wildtype C57BL/6J mice, were used to assess the toxic effects of e-cig aerosols on cells (*in vitro*) and mouse lungs (*in vivo*). The findings of this study provide comprehensive information on both *in vitro* and *in vivo* toxicity profiles of aerosols from two popular types of e-cig devices considering various device settings and puffing regimens, offer valuable insights into the various health risks associated with specific e-cig devices, and introduce a means to evaluate their toxic potential through cotinine-normalized measurements.

■ MATERIALS AND METHODS

Materials are provided in the Supporting Information (SI).

E-cig Aerosol Generation, Characterization, and Sampling

A Subohm Tank e-cig (VaporFi Volt Hybrid Tank, Vaporfi.com LLC., Miami Lakes, FL, USA) and a Pod mods e-cig (JUUL, JUUL Laboratories Inc., San Francisco, CA, USA) were used to generate e-cig aerosols by our self-built machine as shown in Scheme 1, using lab-made flavorless e-liquids containing a 30/70 (mass fraction) mixture of propylene glycol (PG)/vegetable glycerin (VG) and freebase nicotine or nicotine benzoate salt. While e-cig aerosols generated from two devices using both e-liquid formulations of freebase nicotine and nicotine benzoate salt were characterized in a test chamber, the e-cig aerosols from the Tank system using 2.4% freebase nicotine (24 mg/mL) formula and the JUUL device using nicotine benzoate salt at 3% (35 mg/mL) and 5% (59 mg/mL) formulas were also collected with an impinger and were assessed for their *in vitro* and *in vivo* toxicity in this study. These devices and formulations were chosen as representatives of common usage patterns in the real world. The methods for making the e-liquids are described in the SI.

The e-cig aerosols were generated using our developed self-built puffing machine as described in a previous work.[20] Briefly, the e-cig devices were subjected to continuous puffing cycles (i.e., 4 s/puff, every 30 s) carried out by filtered air at 1 L/min (66 mL puff volume), to mimic a typical puff topography of e-cig users.[21−23] JUUL devices were powered at 3.7 V and 2.5 A, while the Vapor-fi Volt Hybrid Tank (referred to as the "Tank device" below) was equipped with a 0.5-ohm heating coil and powered at 7.5 V and 2.5 A according to their user manuals. As shown in Figure 1, to characterize the generated e-cig aerosols from the two devices using e-liquids containing either 2.4% freebase nicotine or 3% nicotine benzoate salt, three puffs of e-cig aerosols were introduced into a 460 L stainless steel chamber.[20] A Scanning Mobility Particle Sizer (SMPS 3080, TSI Inc., Shoreview, MN, USA) and an Aerodynamic Particle Sizer (APS 3321, TSI Inc., Shoreview, MN, USA) were used to measure the size-resolved particle number concentrations in the ranges of 12−496 nm and 0.54−19.8 μm, respectively. The particle number concentration (PNC) and mass concentration of fine particles (particulate matter with diameters that are generally 2.5 μm and smaller, $PM_{2.5}$) in the e-cig aerosols were measured using the Condensation Particle Counter (CPC 3007, TSI Inc., Shoreview, MN, USA) and Aerosol Monitor (DustTrak II 8532, TSI Inc., Shoreview, MN, USA), respectively.

To obtain e-cig samples to be used in the following *in vitro* and *in vivo* toxicological assessments, e-cig aerosols of 5, 10, 20, 120, 240, and 360 puffs generated using the automatic puffing machine were directly collected into a two-stage impinger (SKC Inc., Eighty Four, PA, USA) in series filled with DI water or cell culture media of total 20 mL. The impinger collection efficiency of e-cig aerosols was determined to be ∼83% for Tank and ∼73% for JUUL. The aerosol emission rates were checked multiple times throughout continuous 480-puffing cycles by measuring the filter samples of 10-puff e-cig aerosols collected using a five-stage cascade impactor (Sioutas, SKC Inc., Eighty Four, PA, USA). The obtained e-cig samples were also analyzed for metal elements using Inductively Coupled Plasma Mass Spectrometry (ICP-MS, NexION 2000, PerkinElmer Inc., Waltham, MA, USA) and for reactive oxygen species (ROS) using the 2′, 7′-dichlorodihydrofluorescein diacetate fluorescence (DCF) assay (Invitrogen, Thermo Fisher Inc., Carlsbad, CA). The assays were conducted following the manufacturer's instructions, and a brief description is provided in the SI.

Determination of E-cig Samples Cytotoxicity *In Vitro*

The cell cultures of the human monocyte cell line (THP-1) and the human epithelial cell line (BEAS-2B) were prepared according to the manufacturer's instruction (American Type Culture Collection, Manassas, VA, USA). Before being exposed to e-cig aerosol samples by coculture, cells were cultured with fresh medium overnight at 37 °C in the 5% CO_2 incubator, and THP-1 cells were additionally pretreated with 1 μg/mL phorbol 12-myristate acetate (PMA). Aliquots of 3×10^4 primed cells were cultured in 0.1 mL of medium with e-cig samples of different puffs in 96-well plates (Costar, Corning, NY, USA) at 37 °C for 24 h. For the IL-1β release, coculturing THP-1 cells with 10 ng/mL LPS was necessary to initiate the transcription of pro-IL-1β. After exposure, the supernatants of THP-1 cell culture were collected for the measurement of IL-1β and TNF-α, while the supernatants of BEAS-2B cell culture were collected for IL-8 and TGF-β1 measurement, using enzyme-linked immunosorbent assay (ELISA) (R&D Systems Inc., Minneapolis, MN, USA) following the manufacturer's instructions. The cells were kept for cell viability assessment using the CellTiter 96 Aqueous One Solution

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Environ. Health 2023, 1, 315−323

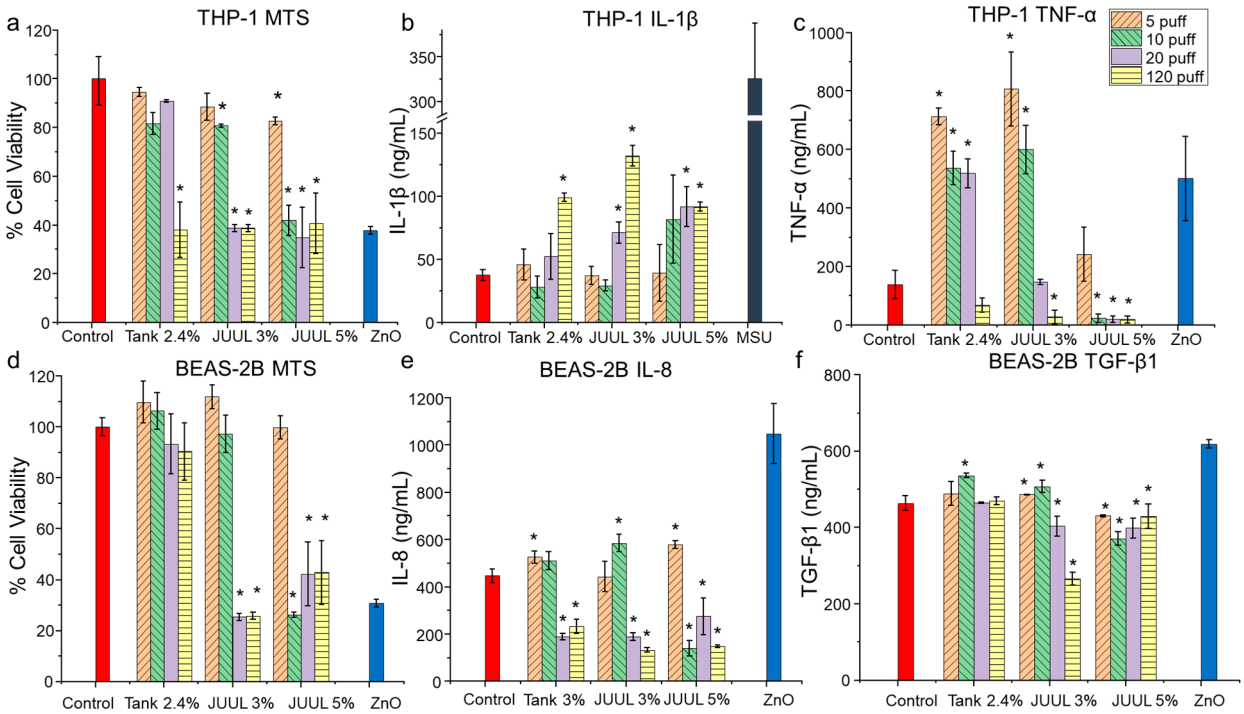

Figure 2. (a) Cell proliferation of THP-1 cells was determined by MTS after incubating with the e-cig samples for 24 h. Production of (b) IL-1β and (c) TNF-α in THP-1 cells. (d) Cell viability of BEAS-2B cells after incubating with the e-cig samples for 24 h. Production of (e) IL-8 and (f) TGF-β1 in BEAS-2B cells. All bar plots share the same legend. Cell culture medium was used as the control for all the above assessments (the red bar). Monosodium urate (MSU) of 100 μg/mL was used as the positive control in the IL-1β assessment. ZnO NPs of 10 μg/mL were used as the positive control in the assessment of cell viability, TNF-α, IL-8, and TGF-β1. *$p < 0.05$, compared to the control.

Cell Proliferation Assay (MTS) (Promega Inc., Madison, WI) and the Adenosine triphosphate (ATP) assay (ATPlite firstep Luminescence Assay, PerkinElmer Inc., Waltham, MA, USA). The assays were conducted following the manufacturer's instructions, and a brief description is provided in the SI.

Assessment of Toxicological Responses in Mouse Lung *In Vivo*

Eight week old male C57BL/6J mice purchased from Charles River Laboratories International Inc. (San Francisco, CA, USA) were housed at the UCLA Center for Health Sciences barrier facility. Animal exposures to e-cig samples were carried out by oropharyngeal aspiration as described at NIOSH.[24] Briefly, with the anesthetized animals (intraperitoneal (i.p.) injection of ketamine (100 mg/kg)/xylazine (10 mg/kg) in a total volume of 100 μL) held in a vertical position, 50 μL suspensions containing e-cig samples from JUUL and Tank devices at different puff numbers in water were instilled at the back of the tongue to allow aspiration into the lungs (six mice for each group). Control animals received the same volume of PBS. The positive control using ZnO nanoparticles (NPs) at 2 mg/kg. The mice were sacrificed at 24 h postexposure, and bronchoalveolar lavage fluid (BALF), serum, and lung tissues were collected as previously described.[25] Briefly, the trachea was cannulated, and the lungs were gently lavaged three times with 1 mL of sterile PBS to obtain BALF. BALF was used to perform total and differential cell counts and to measure the levels of IL-1β. The serum was used to measure the level of 8-hydroxy-2′-deoxyguanosine (8-OHdG) and cotinine.

Statistical Analysis

Mean and standard deviations (SD) were calculated for each parameter. Results were expressed as the mean ± SD based on multiple determinations. Group comparisons were evaluated using one-way ANOVA followed by pairwise *t* test with Bonferroni. All analyses were performed using R Statistical Software (v4.2.3; R Core Team 2023). A statistically significant difference was assumed when the *p*-value was <0.05.

RESULTS AND DISCUSSION

Physicochemical Characterization of E-cig Aerosol

The particle size distributions of e-cig aerosols from the two devices using e-liquid of either freebase nicotine or nicotine salt formula are shown in Figures 1a and b. The observed e-cig aerosol size distributions are trimodal, with peaks in the ultrafine (<100 nm), submicron (~300 nm), and micron (~1000 nm) regimes, which are consistent with a previous study.[26] When using free-base nicotine in the e-liquid, JUUL generated more ultrafine but fewer submicrometer particles than the Tank system. Besides, using nicotine salt in the e-liquid enhanced the emission of ultrafine particles for the Tank system and submicron particles for JUUL. This is possibly due to the increased particle partitioning of the nicotine salt than the freebase nicotine according to Pankow theory.[27,28] As a result, higher levels of PNC and $PM_{2.5}$ mass concentrations were also observed for both devices when using nicotine benzoate salt than freebase nicotine (Table S1). Previous studies have presented evidence of the deposition of small particles in the lower (smaller) airways, which suggested that JUUL (using nicotine salt) may deliver more aerosol particles into the deep lung than the Tank (using freebase nicotine).[29]

Additionally, the total and size-resolved mass concentrations of e-cig aerosols from the Tank system using freebase nicotine and JUUL using nicotine benzoate salt were determined using a five-stage cascade impactor. As shown in Figure 1c, the PM emission of JUUL is significantly lower compared to the Tank system. However, the mass fraction that small particles constitute, i.e., particles smaller than 0.5 μm, is higher for JUUL than for the Tank system. The estimated PM mass collected in the samples were 11.06 mg/puff for Tank and 2.72 mg/puff for JUUL. The abiotic ROS generation in e-cig

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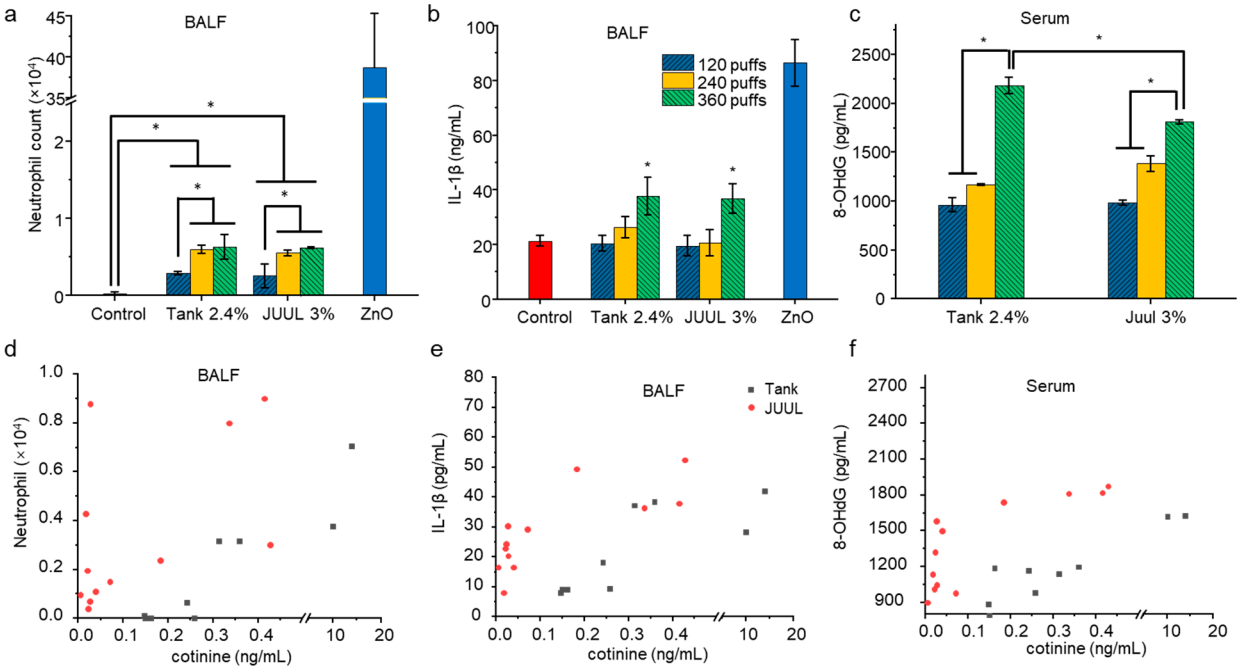

Figure 3. Results of different toxicity biomarkers from *in vivo* assessments. (a) Neutrophil cell count, (b) IL-1β level in BALF samples, (c) 8-OHdG level in serum samples. Both BALF samples and serum samples were collected from mice (C57BL/6J, $n = 6$) that were exposed to aerosol samples in water from Tank and JUUL devices for 24 h. Dot plots of (d) neutrophils in BALF, (e) IL-1β in BALF, and (f) 8-OHdG in serum versus cotinine concentration in mice serum after exposure to samples from Tank and JUUL devices for 24 h were generated based on (a to c).

samples containing 120 puffs from the Tank system using e-liquid containing no nicotine or 2.4% freebase nicotine and JUUL using e-liquid containing no nicotine or 3% nicotine benzoate salt were determined by the DCF assay. As shown in Figure 1d, despite lower PM emission from the JUUL device, higher ROS generation in e-cig samples of JUUL than that of Tank are observed. Besides, either freebase nicotine (Tank 2.4%) or nicotine benzoate salt (JUUL 3%) significantly enhanced the intensity of DCF fluorescence, suggesting increased total ROS generation, which is consistent with our previous study.[7] We further measured the total aldehyde content in the e-cig samples, and results showed that the aldehyde level in samples containing various puffs of e-cig aerosols from JUUL is significantly higher than that from Tank at the same puff number (Figure S1). The levels of metal elements in the e-cig samples are shown in Table S2. Higher levels of Mg, Ni, and Zn were found in JUUL than in Tank samples. Previous studies have reported high levels of metals such as Al, Cd, Cr, Cu, Pb, and Fe in e-cig samples, which were not found at such high levels in our results.[30,31]

Toxicity Assessment of Tank and JUUL E-cig Aerosols Produced *In Vitro*

We customized the e-liquids to be tested in toxicological assessments, recovering those available on the market despite the flavor. E-cig samples to be exposed to THP-1 and BEAS-2B cells were obtained by collecting a certain number of puffs (i.e., 5, 10, 20, and 120 puffs) of e-cig aerosols into the culture medium from the Tank system using e-liquid containing 2.4% freebase nicotine (presented by Tank 2.4%) and from JUUL using e-liquid containing nicotine benzoate salt at 3% (presented by JUUL 3%) and 5% (presented by JUUL 5%). As shown in Figure 2a, the THP-1 cell viability results suggest that the device type and nicotine levels are associated with cell toxicity induced by e-cig aerosols. Generally, for both devices, samples containing more puffs of e-cig aerosols caused more cytotoxicity in THP-1 cells. No significant impact on cell viability was observed for the samples containing 20 puffs of e-cig aerosols collected from the Tank system, while the samples collected from JUUL significantly reduced the cell viability. In addition, higher nicotine levels (5%) showed higher toxicity than lower ones (3%) for the JUUL device, as indicated by different impacts on cell viability caused by 10-puff samples. Comparable impacts on cell viability were observed on samples from the Tank system and JUUL device using 3% and 5% nicotine benzoate salt that contain 120, 20, and 10 puffs of e-cig aerosols, respectively. Similar trends were also observed in BEAS-2B cells, as shown in Figure 2d. In addition to cytotoxicity, exposure to e-cig aerosol samples also showed proinflammatory effects, as demonstrated by the production of IL-1β and TNF-α in THP-1 cells. Figure 2b shows the device-type- and puff-number-dependent IL-1β production. For example, samples containing 20 puffs of e-cig aerosols from the JUUL device caused significantly higher IL-1β levels in THP-1 cells than those from Tank. Also, the exposure dose as indicated by the number of e-cig aerosols puffs contained in the samples was shown associated with the IL-1β production for both devices. In contrast, opposite trends regarding the effects of exposure doses and nicotine levels were observed on the production of TNF-α in THP-1 cells (Figure 2c) and IL-8 and TGF-β1 in BEAS-2B cells (Figure 2e and f). Generally, low-dose exposure to e-cig aerosols (e.g., exposure to samples containing 5 or 10 puffs of e-cig aerosols) showed increased pro-inflammatory effects, while high-dose exposure (e.g., exposure to samples containing 20 or 120 puffs of e-cig aerosols) inhibited the production of pro-inflammatory biomarkers.

Since being introduced in the United States in 2015, JUUL has gained huge popularity, especially among youth and young adults. As a representative of the fourth generation e-cigs,

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Environ. Health 2023, 1, 315−323

JUUL features operation at low power and uses a nicotine salt formula, which is unique from the previous generations of e-cigs. The huge popularity of JUUL among youth has attracted intensive attention about its potential toxicity. However, to date, there is insufficient evidence to assess the potential toxicological risks of using JUUL products, which also leads to the FDA's marketing denial orders to market JUUL products issued in 2022.[32] Our study showed that e-cig aerosols from JUUL caused stronger cytotoxicity and inflammation *in vitro* and resulted in more severe oxidative stress and inflammation *in vivo* under comparable exposure doses. Our study revealed that, despite the lower PM emission, JUUL exhibited higher toxicity potential compared to the Tank system. Previous studies have found that higher battery output voltages of e-cig can increase the levels of carbonyl compounds in e-cig aerosols, including carcinogens such as formaldehyde and acetaldehyde, and aerosols generated at higher voltages are generally more toxic than those generated at regular voltage from the same device.[12,13] However, in the present study, the aerosols generated from JUUL were observed to exhibit higher ROS generation than those from the Tank system, indicating a greater potential to induce oxidative stress and pro-inflammatory responses.

Toxicity Assessment of Tank and JUUL E-cig Aerosols Produced *In Vivo*

To further verify the above-observed results of toxicological effects of e-cig aerosols, we conducted an *in vivo* assessment of e-cig aerosols using wild-type C57BL/6J mice (n = 6). After the mice were exposed to e-cig samples collected from Tank and JUUL devices using e-liquid containing 2.4% freebase nicotine and 3% nicotine benzoate salt, respectively, the inflammation in mouse lungs and systemic oxidative stress were assessed by counting the inflammatory cells (Figure S2) in BALF, measuring IL-1β in BALF, and measuring 8-OHdG in serum. As shown in Figure 3a and b, exposure to e-cig aerosols caused acute inflammation in the lungs of mice, suggested by elevated neutrophil counts and IL-1β levels. In addition, results showed a clear dose-dependent acute inflammatory effects in mice lungs and oxidative stress in circulation, with exposure to e-cig samples of more puffs of e-cig aerosols inducing more neutrophil infiltration (Figure 3a) and IL-1β production in lungs (Figure 3b), and 8-OHdG in serum (Figure 3c). This effect was also confirmed by different levels of focal inflammation in the lung, as demonstrated by hematoxylin and eosin (H&E) staining (Figure S3). However, despite the higher level of 8-OHdG caused by exposure to e-cig samples containing 120 puffs of e-cig aerosols from the Tank system than that from the JUUL device, comparable levels of neutrophil cells and IL-1β were observed between the Tank and JUUL groups when exposed to e-cig samples containing the same number of puffs of e-cig aerosols. Furthermore, the levels of cotinine in serum (Figure S4), the primary stable metabolite of nicotine,[33] were measured as a biomarker of nicotine exposure. The relationships between the levels of cotinine and different biomarkers of effects are shown in Figure 3d−f. Each point in the figure represents an actual mouse, with the position of the x-axis determined by the cotinine level in its serum, and the position on the y-axis determined by the neutrophil cell counts and IL-1β in BALF, and levels of 8-OHdG in serum, respectively. The representation of JUUL by the red dot is predominantly located above the representation of Tank by the black squares, indicating that, under comparable nicotine exposure, JUUL results in higher levels of 8-OHdG in serum, as well as more neutrophil infiltration and IL-1β production, suggesting a greater extent of deoxyribonucleic acid (DNA) damage and greater severity of lung injury.

Our results revealed that both devices induced comparable levels of lung inflammation including neutrophil infiltrations, IL-1β production, and 8-OHdG production when considering the same number of puffs. However, the Tank induced higher serum nicotine levels than did the JUUL at the same puff number, as indicated by cotinine, a nicotine metabolite. On one hand, Tank generates a higher mass of aerosol particles per puff than JUUL. On the other hand, the benzoic acid in the JUUL e-liquid not only changes the taste and throat hit but also affects the behavior of e-cig aerosols *in vivo*.

In the lungs, the unprotonated free-base form of nicotine is lipophilic and thus readily diffuses across the membranes of the respiratory tract into the blood, whereas the protonated form of nicotine is hydrophilic and does not diffuse as readily across the membranes.[16] This implies the Tank's greater efficiency in delivering nicotine to the blood. To account for the influence of nicotine levels on the number of puffs taken by users for satisfaction, we normalized the toxicity biomarkers to cotinine levels. This normalization demonstrated JUUL's significantly higher toxicity than that of the Tank system, which was consistent with our *in vitro* results. The number of puffs is widely used because it provides a standard and easily understandable measure of the amount of nicotine and other substances inhaled, which can mimic the real-life conditions of e-cig use. However, given the differences in the nicotine content and status in the e-liquid, as well as in the aerosol quality per puff between Tank and JUUL, the number of puffs may not fully capture the differences between the two devices. This underlines the complexities in comparing these devices despite the utility of the number of puffs as a commonly used measure of inhaled substances in e-cig studies. As e-cigs were designed as an alternative nicotine delivery system to satisfy smokers' cravings without the harmful effects associated with tobacco combustion, the nicotine content should ideally reflect user exposure levels. Cotinine, the primary stable metabolite of nicotine, established as a reliable biomarker of nicotine exposure, has been found in the blood or urine of both mouse models and e-cig users.[34,35] Nicotine replacement therapy, such as nicotine patches, gum, and lozenges, works by replacing some of the nicotine obtained from cigarettes. Each of these medications comes in different nicotine strengths, highlighting nicotine's critical role in exposure. Cotinine levels offer a direct measurement of the nicotine absorbed by the body and can be detected in blood, saliva, and urine, providing flexibility in sampling methods.[34−36] Further, cotinine has a longer half-life than nicotine, approximately 16 h, meaning it remains in the body longer and thus provides a more accurate measure of nicotine exposure over time.[37,38] In some studies, traditional cigarette exposure was cigs/day or pack-year, while e-cig exposure was quantified in terms of puffs.[39−41] However, some other studies have noted that, due to recall bias or social factors, self-reports often do not align with true smoking prevalence.[42] Consequently, cotinine has emerged as the biomarker of choice for optimizing the evaluation of cigarette exposure. Therefore, using cotinine levels as an alternative method for defining exposure doses could lead to more accurate assessments of nicotine exposure.

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Environ. Health 2023, 1, 315−323

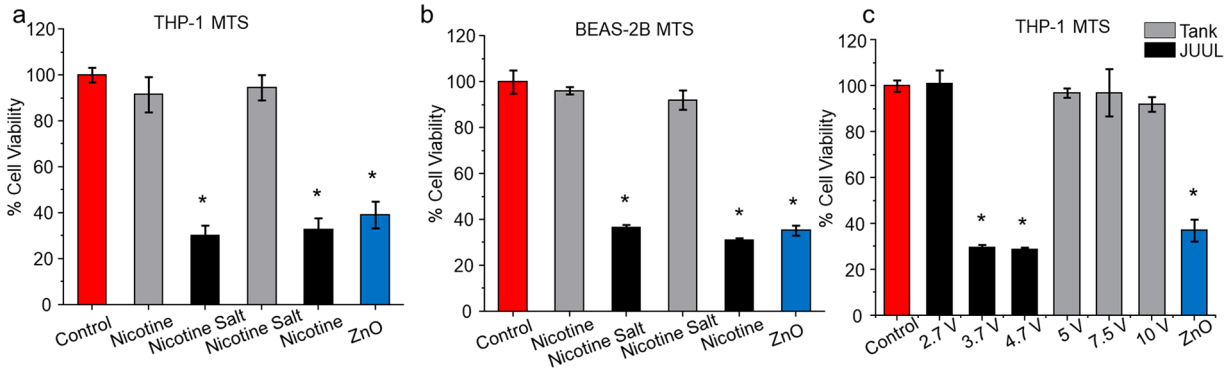

Figure 4. Determination of (a) THP-1 cell and (b) BEAS-2B cell proliferation by MTS after being treated with aerosols collected from Tank and JUUL devices with e-liquids containing either 2.4% freebase nicotine or 3% nicotine salt for 24 h. (c) Determination of THP-1 cell proliferation by MTS after treatment with aerosols (20 puffs) collected from Tank and JUUL devices at different voltages. ZnO NPs were used as the positive control with a concentration of 10 μg/mL. Cell culture medium was used as the negative control. $^*p < 0.05$, compared to the (negative) control.

The Impact of Flavor, Device Type, Nicotine Formula, and Voltage on Toxicity

To further determine the factors affecting the toxicity of e-cig aerosols, additional toxicological assessments were conducted with variable parameters, including flavors, nicotine formula, device type, and power voltage. JUUL pods with flavors including Classic tobacco, Virginia tobacco, and Menthol and 3% or 5% nicotine levels were used to obtain e-cig aerosol samples. As shown in Figure S5, aerosols with all flavors collected from higher numbers of puffs significantly inhibited THP-1 and BEAS-2B cell viability and exhibited heterogeneous effects on various pro-inflammatory factors. However, the flavored e-cig did not show higher toxicity than flavorless e-cig, and there were no significant differences observed among these flavors. In addition, the results are numerically and trend-wise similar to those in Figure 2, suggesting that flavor does not play a key role in affecting cell viability and inflammatory cytokines. The *in vivo* results were consistent with this conclusion.

To differentiate the effects of the device and e-liquid, Tank and JUUL were filled with e-liquid containing 2.4% nicotine or 3% nicotine salt. This arrangement and combination of two device types and two e-liquid types resulted in four scenarios, and the aerosols generated by them were incubated with THP-1 and BEAS-2B cells. As shown in Figure 4a and b, the 20-puff aerosols produced by JUUL were found to exhibit cytotoxicity, while Tank filled with nicotine salt did not significantly reduce cell viability. Taking 20-puff aerosols as an example, unlike Tank, which showed similar cytotoxicity across varying voltages of 5, 7.5, and 10 V, JUUL working at higher voltages of 3.7 and 4.7 V significantly induced greater cytotoxicity and reduced cell viability (Figure 4c). In conclusion, compared to Tank, the JUUL device design plays a key role in cytotoxicity regardless of the nicotine salt in the e-liquid or the lower voltage.

■ CONCLUSION

In this study, we assessed and compared the physicochemical properties, cytotoxicity, inflammation, and oxidative stress induced by e-cig aerosols from Tank and JUUL devices as well as the differences between e-liquids containing nicotine salt and freebase nicotine, within the same framework. The cell lines THP-1 and BEAS-2B, as well as wild-type C57BL/6J mice, were used to assess the toxic effects of e-cig aerosol. The present study found that all e-cig aerosols, including those from JUUL and Tank devices, induced cytotoxicity *in vitro* and acute inflammation *in vivo* at high puff numbers. However, the JUUL device exhibited a greater toxicity than the Tank device. JUUL pod systems generated more ultrafine particles and less $PM_{2.5}$ mass, as well as contained higher levels of ROS and aldehydes compared to Tank e-cigs. Aerosols from JUUL induced lower cell viability and higher pro-inflammatory cytokines than those from Tank in both THP-1 and BEAS-2B cell lines. In addition, the study also demonstrated that JUUL devices caused more oxidative stress, pulmonary inflammation, and DNA damage, as indicated by increased levels of the biomarker 8-OHdG in the mouse model. Furthermore, the differences in cytotoxicity and inflammation effects were found to be influenced by the device used, the number of puffs, and the nicotine concentration in e-liquids. Interestingly, the study suggests that the observed differences in toxicity between JUUL and Tank devices may be primarily attributed to the device design rather than to the nicotine salt, flavors, or voltage. The *in vivo* study suggested that using cotinine levels instead of puff numbers as an alternative method for defining the exposure dose of e-cig could lead to more accurate assessments of nicotine exposure. These findings highlight the importance of considering device design in assessing the potential health risks associated with e-cigs. This study has certain limitations that should be acknowledged. Our focus on *in vitro* and *in vivo* experimental models cannot fully replicate the complexities of human physiological responses to JUUL and Tank use in real-world settings. Furthermore, this study centered on acute exposures and responses, providing limited insights into the long-term effects of JUUL and Tank use. Further research is needed to better understand the underlying mechanisms and long-term health implications of e-cig use as well as to inform regulations and public health policies regarding e-cig devices.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/envhealth.3c00099.

Materials; preparation of lab-made flavorless e-liquids; determination of ROS level; cell culture; animal housing; particle number concentration and $PM_{2.5}$ mass concentration of aerosols; element analysis in aerosols (ICP-MS); aldehyde levels in aerosols; cell count in BALF after mice exposed to aerosols; histological staining of

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Environ. Health 2023, 1, 315−323

lung tissue after mice exposed to aerosols; cotinine levels in serum after mice exposed to e-cig aerosols; determination of cells proliferation and cytokines after cells exposed to aerosols and the cytokines and neutrophil count in BALF after mice exposed to aerosols (PDF)

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Notes

The authors declare no competing financial interest.

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