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Impact of nicotine-free and nicotine-rich flavored electronic cigarette refill liquids on primary human melanocyte function

Shilpi Goenka [a,b,*]

[a] Department of Biochemistry and Cell Biology, Stony Brook University, Stony Brook, NY, United States
[b] Department of Biomedical Engineering, Stony Brook University, Stony Brook, NY 11794-5281, United States

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ABSTRACT

In this study, five popular EC liquid flavors–strawberry, banana, vanilla, tobacco, and menthol–were examined on human melanocyte functions. Each flavored e-liquid (in 80/20 PG/VG vehicle) was tested without or with 18 mg/mL nicotine. The effects of PG/VG and nicotine-containing vehicles were also evaluated. Results revealed that nicotine-free and nicotine-containing e-liquids had comparable cytotoxicity, with menthol> > banana> tobacco> vanilla> strawberry. This cytotoxicity was unrelated to either nicotine or the vehicle. PG/VG (1 and 2 %) increased melanin production without influencing cellular tyrosinase activity. The flavored e-liquids did not further affect melanin production, suggesting that the vehicle's effect, not the flavor, was responsible for the increased melanin production. Interestingly, nicotine at 2 % in the vehicle restored the stimulated melanin production to the control. Flavors suppressed cellular tyrosinase activity, with vanilla and banana flavors robustly inhibiting it. Vanilla and banana e-liquids also enhanced reactive oxygen species (ROS) production, which did not originate from the vehicle or nicotine-containing vehicle. Banana e-liquid with nicotine lowered ROS generation compared to nicotine-free banana e-liquid. Common flavors in e-liquids can cause cytotoxicity and influence melanogenesis even without nicotine, indicating that the use of ECs may not completely avoid the harmful effects of cigarette smoking. Further studies are warranted to investigate e-liquid aerosol effects on melanocytes.

1. Introduction

E-cigarettes (ECs) continue to gain popularity in their use as an alternative nicotine delivery system for tobacco smoking and comprise a battery-powered heating component featuring a cartridge that houses the e-liquid that comprises vehicle propylene glycol and vegetable glycerin (PG/VG), flavorings, and may or may not contain nicotine [1]. ECs were originally sold as a harm reduction device that could assist in smoking cessation [2]. However, EC has gained tremendous popularity, especially among the younger generation, who have initiated its use for recreational purposes. Young people are more likely to initiate EC use due to the appeal of the various flavorings offered in e-liquids [3]. Owing to the relatively shorter duration of airborne persistence exhibited by EC vapor in comparison to tobacco smoke, adolescents surreptitiously engage in EC usage within school and home environments, evading detection [4]. The representation of the flavor (by using bright flavor colors, sensory appeal, and images) shown on the packaging of EC products contributes to increasing the attractiveness of EC products among youth [5]. According to a recent survey [6], 89.4 % of students who use ECs prefer flavored products. Additionally, 25.2 % of students reported using ECs daily, with the most frequently mentioned EC brands among these students being JUUL, Esco Bar, Elf Bar, and Mr. Fog. Despite regulations imposed on the sale of certain flavors in ECs, many adolescents are still able to access ECs products from smoke shops, online retailers, or their friends, thus indicating the continued use by these susceptible populations. Erna et al. [7] analyzed 320 e-liquids and reported the average number of flavorings per e-liquid to be 6 ± 4, with the tobacco flavoring shown to have significantly fewer flavorings/e-liquid than the fruit, beverage, sweet, and dessert categories. Most flavorings used in ECs are also additives used in food and have been generally recognized as safe (GRAS) [8]. However, the GRAS status given by the Flavor Extracts Manufacturers Association (FEMA) is based on the nontoxicity of flavor when ingested and not inhaled or via dermal exposure. The proportion of flavoring substances is 1–4 % in several e-liquids [9]. Despite the Food & Drug Administration (FDA) enacting a flavor restriction in 2020 that prohibited the sale of all flavors

* Correspondence to: Department of Biochemistry & Cell Biology, Stony Brook University, Stony Brook, NY 11794-5281, United States.
E-mail address: shilp.goenka@gmail.com.

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except menthol and tobacco in cartridges [10], some vendors found an alternative route where they spiked fruity flavors in tobacco e-liquids. Using fruity flavors with sweet taste has retained their appeal for youngsters who, despite this flavor ban, found alternate ways to access the banned flavors owing to loopholes in the ban policies [11,12]. Interestingly, a study revealed that some e-liquids labeled as 'tobacco flavored' contained chemicals typically present in sweet flavors, thus raising the alarm [13].

Current data suggests that vaping can potentially cause detrimental effects on nearly every organ within the human body. For example, the use of ECs has been shown to adversely impact oral [14], pulmonary [15,16], and cardiovascular health [17]. In addition, studies have shown that the use of EC can result in neurotoxicity [18] and harm to the human ear [19]. Furthermore, studies have demonstrated that ECs can cause mutagenicity and genotoxicity [20]. Melanocytes are specialized cells present in the oral cavity, lungs, heart, brain, ears, eyes, and skin [21]. Melanocytes in vertebrate embryos originate from the dorsal region of the neural tube [22]. These cells have embryologic origins and signaling pathways that are comparable to neurons [23]. The synthesis of the pigment melanin occurs within melanosomes by specialized cells known as melanocytes. The multiple dendritic extensions of melanocytes aid in transferring melanosomes to adjacent keratinocytes. The enzyme tyrosinase plays a crucial role in regulating the rate of melanin pigment synthesis within melanosomes [24]. Melanin pigment of melanocytes confers a multitude of biological benefits encompassing free radical scavenging, UV photoprotection, chelation of harmful metal ions, UV photoprotection, antimicrobial effects, and immune regulation [25,26].

The skin, being the largest organ, functions as the primary barrier against environmental toxicants. There has been a rise in anti-pollution cosmetics designed to protect the skin from the harmful effects of cigarette smoke and particulate matter (also found in EC emissions) [27,28]. Thus, it suggests that modern society is more aware of skincare and aesthetics. Additionally, due to societal pressures and racial disparities, some persons with darker skin may desire lowered melanin production, while others with lighter skin may aim to enhance melanin production. However, both cosmetic skin lightening and skin tanning can have potential risks [29–31]. Dysregulation of melanin synthesis leads to pigmentation abnormalities categorized as hypopigmentation or hyperpigmentation dermatological disorders [32]. Hypopigmentation leads to leukoderma or vitiligo, characterized by white depigmented patches or spots on the skin, while hyperpigmentation occurs in periorbital melanosis (POM), post-inflammatory hyperpigmentation (PIH), melasma, and solar lentigines [33]. The increased activity of melanocytes and augmented melanin production disrupt collagen degradation, leading to collagen accumulation that induces fibrogenesis and the development of keloids, a prevalent form of pathological scar [34–37]. These disorders not only negatively affect personal aesthetics but also impose a psychological burden that impacts mental health and quality of life [38–40]. Elevated melanogenesis is a risk factor for melanoma formation and is more pronounced in Caucasians compared to other ethnic groups [41,42]. Environmental exposure to toxins or chemicals has been shown to induce melanocyte death or result in pigmentation alterations. The primary risk of melanocyte dysfunction and loss of growth regulation, specifically the development of melanoma, is typically linked to initial UV light exposure but may also be connected to systemic exposure to environmental toxins such as arsenic [43] and specific drugs, including hydrochlorothiazide [44,45] and various immunosuppressants [46]. Tobacco smoke, as well as its constituents such as nicotine and polycyclic aromatic hydrocarbon benzo(a)pyrene, have been shown to stimulate pigmentation [47–49]. While the adverse effect of cigarette exposure and nicotine on melanocyte functions has been reported previously, with a recent meta-analysis suggesting the paradoxical effect that exposure to cigarette smoke lowered the risk of skin melanoma [50], no studies have examined the potential effects of flavored EC exposure on melanocyte functions, including melanin synthesis.

In our earlier study [51], we showed that PG/VG vehicle solutions combined in specific ratios can augment melanin production in HEMn-LP cells, while in our recent study [52], we reported that e-liquids of vanilla, banana, cinnamon, tobacco, and menthol of a panel of twenty e-liquids (ten e-liquids with nicotine and ten e-liquids without nicotine) induced moderate to high cytotoxicity that was independent of nicotine in melanocytes from lightly pigmented (Caucasian) and darkly pigmented (African American) newborn donors. In the current study, four of these e-liquid flavors (vanilla, banana, tobacco, and menthol) were selected based on our previous study, along with strawberry e-liquid, a popular flavor. A total of ten e-liquids (with five flavored e-liquids with no nicotine and the remaining five flavors containing 18 mg/mL nicotine) were examined for alterations in melanocyte functions, with a focus on melanin production, tyrosinase activity, and ROS generation. The objective of the study was to investigate whether the presence of nicotine or flavors with the PG/VG vehicle in e-liquids have any adverse effects on melanocytes (from a Caucasian donor), including their key impact on the activation of melanin production and related indicators of tyrosinase activity and ROS generation. Since ECs are touted as a less harmful alternative to cigarette smoking and a large number of youngsters are attracted to ECs due to the availability of multiple flavors with the option to omit or add nicotine, the investigation of the impact of these flavored e-liquids on melanocyte functions is significant. The primary impetus for subjecting skin melanocytes directly to pure e-liquids is the frequent occurrence of e-liquid contact with the skin in both occupational and consumer settings. EC factory workers mix e-liquids and fill cartridges on a daily basis. EC users, mostly younger, using cartridges that are prefilled and replaceable, as well as tank and mod systems that can be refilled and creating their own e-liquid blends, have an elevated risk of direct exposure to e-liquids [53,54]. Due to lower safety standards, younger people utilizing refillable tanks, modular systems, or prefilled cartridges were more likely to be exposed to e-liquids [55]. Mixing e-liquids, replenishing cartridges, and modifying EC devices increased exposure [56]. Refillable tank-based EC users reported 18.8 % spillage while refilling and 18.5 % leakage during operation [57]. One leak was reported by 50 % of users, 3–10 spills by 33 %, and $>$ 10 spills by 16.7 %. Additionally, 23.3 % of consumers indicated children could open e-liquid bottle tops [57]. Lack of gloves, childproof bottle closures, and warning labels leads to unintentional spills and leakage, causing direct skin exposure.

2. Experimental section

2.1. Materials

E-liquids in five flavors (vanilla, strawberry, banana, tobacco, and menthol), available in both nicotine-free and 18 mg/mL nicotine concentrations, were obtained from an online retailer (My Freedom Smokes, Charlotte, NC, USA). The procured e-liquids had market label names of French Vanilla, Ripe Strawberry, Banana Pudding, Captain's Choice Tobacco, and Fresh Menthol; their descriptions and flavor classifications have been documented in our prior report [58]. According to the vendor, these ten e-liquids were formulated with a vehicle base ratio of 80/20 % v/v PG/VG. PG and VG were sourced from the same supplier and blended in an 80 % PG to 20 % VG ratio to formulate the vehicle base to be used as vehicle control in all experiments. A nicotine base devoid of flavor, given as PG/VG in a 75:25 % v/v ratio, was provided by the same supplier as a nicotine control. The substrate L-3,4-dihydroxyphenylalanine (L-DOPA) was purchased from Sigma-Aldrich (St. Louis, MO, USA). The 2′,7′-dichlorodihydrofluorescein diacetate (H2DCFDA) reagent was procured from Molecular Probes Inc. (Eugene, OR, USA). The human melanocyte growth supplement (HMGS) and medium 254 were acquired from Thermo Fisher Scientific Inc. (Waltham, MA, USA). MTS reagent CellTiter 96® AQueous One Solution was acquired from Promega Corporation (Madison, WI, USA).

2.2. Cell culture

Human skin melanocytes isolated from the foreskin of a lightly pigmented neonatal male donor (HEMn-LP; Cat#: C0025C) were purchased from Thermo Fisher Scientific and cultured using medium 254 containing 1 % HMGS with 1 % penicillin-streptomycin antibiotics (Gibco™) at 37 °C in a 95 % air-5 % CO_2 incubator.

2.3. MTS cytotoxicity assay

A total of 2×10^4 HEMn-LP cells per well were grown in a 96-well plate for 48 hours. After that, the culture medium was replaced with a fresh medium containing 0.5–2 % e-liquids diluted from the neat e-liquid (100 %) and maintained for 48 hours. The rationale for the melanocyte counts used in cytotoxicity assay has been described in the supplementary material (Section I). Upon treatment completion, 100 μL of culture media with 20 μL of MTS reagent was replaced and incubated at 37 °C for 2 hours. The absorbances were recorded at 490 nm using a microplate reader (Versamax™, Molecular Devices, MN, USA) after aliquoting 100 microliters to a new 96-well plate. Cell viability was determined by normalizing the absorbance values of treatment groups to those of the untreated control and expressed as percentages.

2.4. Cellular melanin determination

HEMn-LP cells were seeded in 12-well plates at a density of 1.1×10^5 cells per well in 1 mL of complete medium. The cells were then incubated for 72 hours. Thereafter, the culture medium was replaced with a fresh medium that contained the e-liquids, and the cultures were incubated for 48 h. At the end of the 48-hour incubation, the cellular melanin levels were estimated using the method described in our previous study [51].

2.5. Cellular tyrosinase activity assay

A total of 50,000 HEMn-LP cells were placed in each well of a 24-well plate. After 72 h, a fresh culture medium containing different e-liquid concentrations was added, and cultures were maintained for 48 h. After incubation, cells were processed for tyrosinase activity analysis using the methodology described in our previous study [59]. A microplate reader was used to measure the absorbances of 25 μL of lysate and 75 μL of 3 mM L-DOPA solution at 475 nm in kinetic mode. The estimation of tyrosinase activity was conducted by normalizing the linear range of slopes to the total protein content.

2.6. Cellular ROS determination

HEMn-LP cells (5×10^4 cells/well) were grown in a 24-well plate for 96 h and then treated with e-liquid samples for 48 h. After the treatments, the intracellular ROS production was determined using the fluorescent probe H2DCFDA, using the method described in our prior report [60].

2.7. Statistical analysis

For the analysis of the groups, a one-way ANOVA followed by Tukey's or Dunnett's multiple comparisons test was run using GraphPad Prism version 9.5.0 for Windows and GraphPad Software (San Diego, CA, USA). The level of statistical significance was determined to be $p < 0.05$, and the results were given as the mean ± standard deviation.

3. Results

3.1. Effects of e-liquids on cell viability

The treatment of HEMn-LP cells with the PG/VG vehicle had no impact on cell viability at the concentration ranges of 0.5–2 % (Fig. 1A). The strawberry e-liquid was noncytotoxic across the entire concentration range (Fig. 1A). However, the banana e-liquid markedly lowered the cell viability to 7.11 % at the concentration of 2 %, while the vanilla e-liquid significantly lowered the viability to 70.03 % at 2 % (Fig. 1A). Tobacco e-liquid resulted in significant lowering of cell viability to 79.75 % and 66 % at 1 % and 2 % concentrations, respectively (Fig. 1A). In comparison, treatment with menthol e-liquid resulted in the more pronounced loss of cell viability. Specifically, the viability was dramatically lowered to 8.04 % and 6.72 % at 1 % and 2 % concentrations, respectively (Fig. 1A).

Next, the nicotine vehicle and the nicotine-containing strawberry e-liquid did not impact the cellular viability impact over the 0.5–2 % concentration range (Fig. 1B). Nicotine-containing banana e-liquid significantly lowered cell viability to 13.20 % at 2 % (Fig. 1B). Nicotine-containing tobacco e-liquid significantly lowered cellular viability to 84.06 %, 76.41 %, and 65.25 % at 0.5 %, 1 %, and 2 %, respectively (Fig. 1B). At the same time, nicotine-containing vanilla e-liquid significantly lowered viability to 85.04 % and 63.28 % but at 1 % and 2 %, respectively (Fig. 1B). The effects of nicotine-containing menthol e-liquid were most marked on cells; the viability was significantly lowered to 85.48 %, 8.47 %, and 7.83 % at 0.5 %, 1 %, and 2 %, respectively (Fig. 1B).

The IC_{50} values were determined for banana and menthol e-liquids. These were 1.53 ± 0.25 %, 1.72 ± 0.22 %, 0.66 ± 0.07 %, and 0.67 ± 0.03 % for nicotine-free banana, nicotine-containing banana, nicotine-free menthol, and nicotine-containing menthol e-liquids, respectively. The cytotoxicity profiles of both nicotine-free and nicotine-containing e-liquids were observed to be similar, with the order of cytotoxicity being menthol >> banana > tobacco > vanilla > strawberry. Based on these results, only non-cytotoxic e-liquid concentrations were utilized in subsequent experiments.

3.2. Effects of e-liquids on cellular melanin production

The visual inspection of the cell pellets after treatment with the PG/VG vehicle group (1 and 2 %) showed darker-colored pellets relative to the untreated control (Fig. 2A). In contrast, pellets of the nicotine-base group at 0.5 and 1 % appeared similar in coloration to that of the untreated control (Fig. 2A). However, the pellet for the nicotine-base group at 2 % appeared lighter than the PG/VG group at 2 % (Fig. 2A), suggesting that nicotine in the vehicle diminished vehicle-stimulated melanin production. The pellets for flavored e-liquid groups showed no marked change from the untreated control group in the case of banana and menthol flavors (Fig. 2B). However, the pellets were darker for vanilla (1 %) and strawberry (1 and 2 %) flavored e-liquids (Fig. 2B).

The spectrophotometric quantitation of relative intracellular melanin contents corroborated our results from visual inspection. Treatment of cells with PG/VG vehicle significantly increased melanin content by 24.46 % and 35.07 % at concentrations of 1 % and 2 %, respectively (Fig. 2C). Treatment with nicotine-base at 2 % significantly decreased the melanin content by 20.31 % relative to the PG/VG vehicle. Consequently, the cellular melanin contents by nicotine-base at 1 and 2 % were not different from the untreated control group (Fig. 2C).

The melanin contents of cells treated with strawberry e-liquid without nicotine at 1 % and 2 % showed melanin contents that were significantly higher by 22.07 % and 26.64 %, respectively, relative to the untreated control; these increases were identical to the PG/VG vehicle-induced increases at similar concentrations (Fig. 2C). Cells treated with strawberry e-liquid containing nicotine significantly increased melanin contents by 22.33 %, 21.89 %, and 25.56 % at 0.5 %, 1 %, and 2 % concentrations, respectively, as compared to untreated control (Fig. 2C).

The melanin contents of cells after treatment with 0.5 % concentration of either vehicle or nicotine base or the vanilla e-liquid had no effect (Fig. 2D). However, at 1 %, the cellular melanin contents after

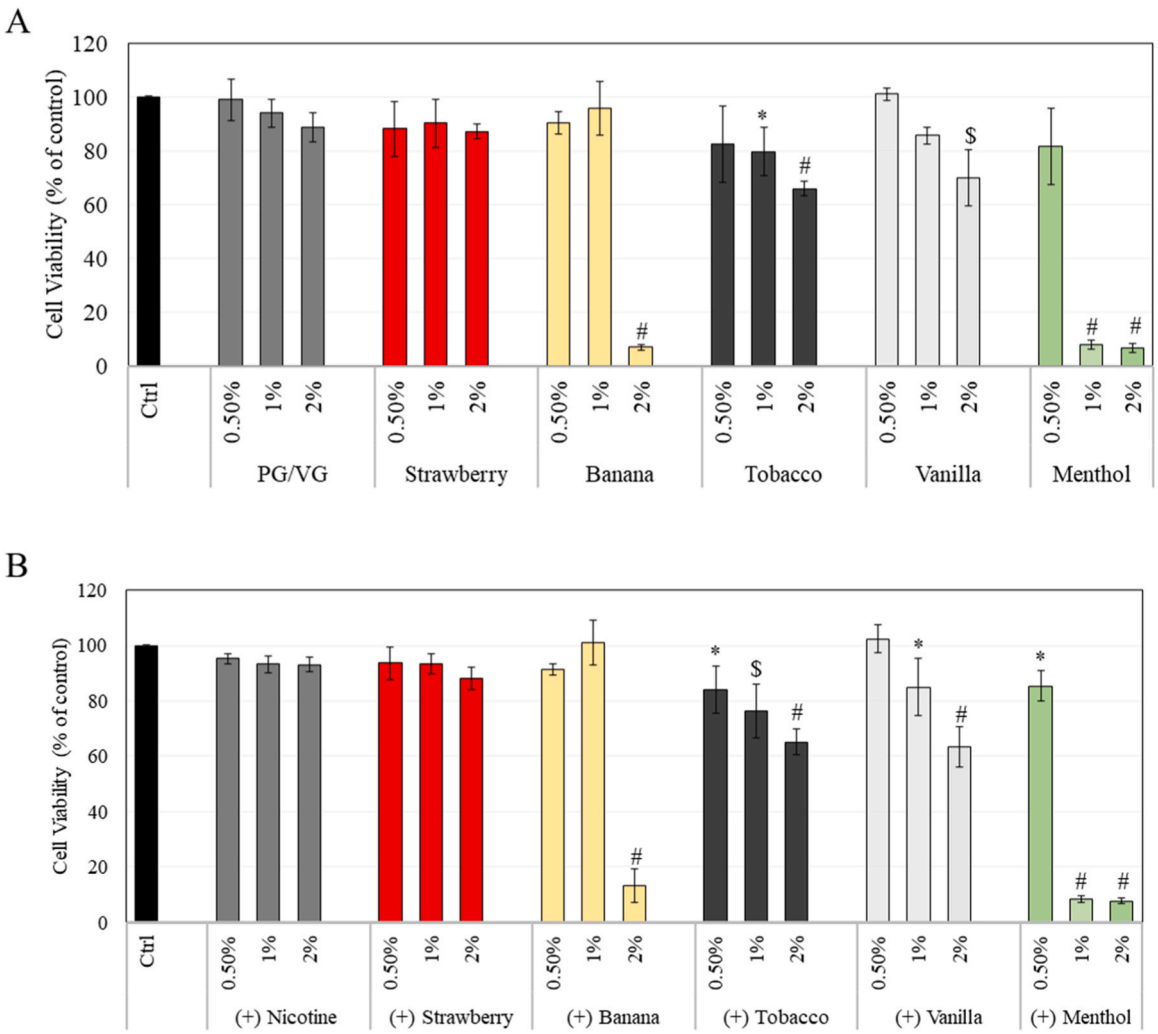

Fig. 1. Viability of melanocytes in the presence of (A) nicotine-free (−) and (B) nicotine-rich (+) e-liquids of strawberry, banana, tobacco, vanilla, and menthol flavors over 48 hours, as measured by MTS assay; PG/VG denotes the unflavored base-liquid (80/20 % v/v), and (+) Nicotine refers to unflavored base-liquid containing 18 mg/mL nicotine in PG/VG; one-way ANOVA with Dunnett's test; * $p < 0.05$; * * $p < 0.01$; $ $p < 0.001$ and # $p < 0.0001$ vs. untreated control (Ctrl). All data are mean ± SD of three independent experiments.

treatment with the vehicle e-liquid (increased by 27.83 %) or the vanilla e-liquid (increased by 29.80 %) were significantly increased in an identical manner (Fig. 2D). This indicates that vanilla flavor by itself did not exert any additional impact on melanin synthesis when present with the PG/VG vehicle.

The banana e-liquid exhibited no noticeable effect on the cellular melanin levels at a concentration of 0.5 % (Fig. 2E). At a concentration of 1 %, both the PG/VG vehicle and the nicotine-containing banana e-liquid significantly increased melanin levels compared to the untreated control, with enhancements of 24.41 % and 21.78 %, respectively (Fig. 2E). Interestingly, the melanin content of cells trated with banana e-liquid without nicotine exhibited a 12.95 % increase compared to the untreated control. However, this change did not achieve statistical significance. This indicates that the banana flavor exhibits a mild suppressive effect on the PG/VG-mediated increased melanin content at 1 %. However, this effect diminishes in the presence of nicotine, as the nicotine-containing banana e-liquid maintained the elevated melanin content observed with 1 % PG/VG alone.

The tobacco e-liquid led to a significant increase in cellular melanin levels by 18.83 % at a concentration of 0.5 %. This result was significantly greater than that of the untreated control. However, it did not reach significance when compared to the vehicle group, which had a mean value of 105.39 % (Fig. 2F). At the same time, the menthol e-liquid exhibited no effect at the concentration of 0.5 % (Fig. 2F).

Collectively, these findings indicate that at a concentration of 0.5 %, none of the flavors exert a distinct effect on melanin production when compared to the PG/VG vehicle. At a concentration of 1 %, the vehicle PG/VG significantly enhances melanin production, while the addition of vanilla, strawberry, or banana flavors yields no additional effects. At the highest concentration of 2 %, the vehicle PG/VG maintains a significant role in promoting melanin production, while the strawberry flavor shows no additional influence. However, when nicotine is present with the vehicle at 2 %, a significant reduction in melanin content is observed, which is restored to elevated levels when strawberry flavor is present with nicotine, suggesting an influence of strawberry flavor at this concentration.

3.3. *Morphology of cells after e-liquid treatment*

The morphology of melanocytes, particularly the dendrite structures, was also examined microscopically after treatment with the e-liquids. The results showed that cells exhibited a highly arborized multidendritic structure with many dendritic spines after treatment with the PG/VG vehicle at 1 % and 2 % concentrations (Fig. 3A); this was in contrast to the untreated control group, which showed primarily two or three dendrites (Fig. 3C). Cells treated with the nicotine-containing vehicle

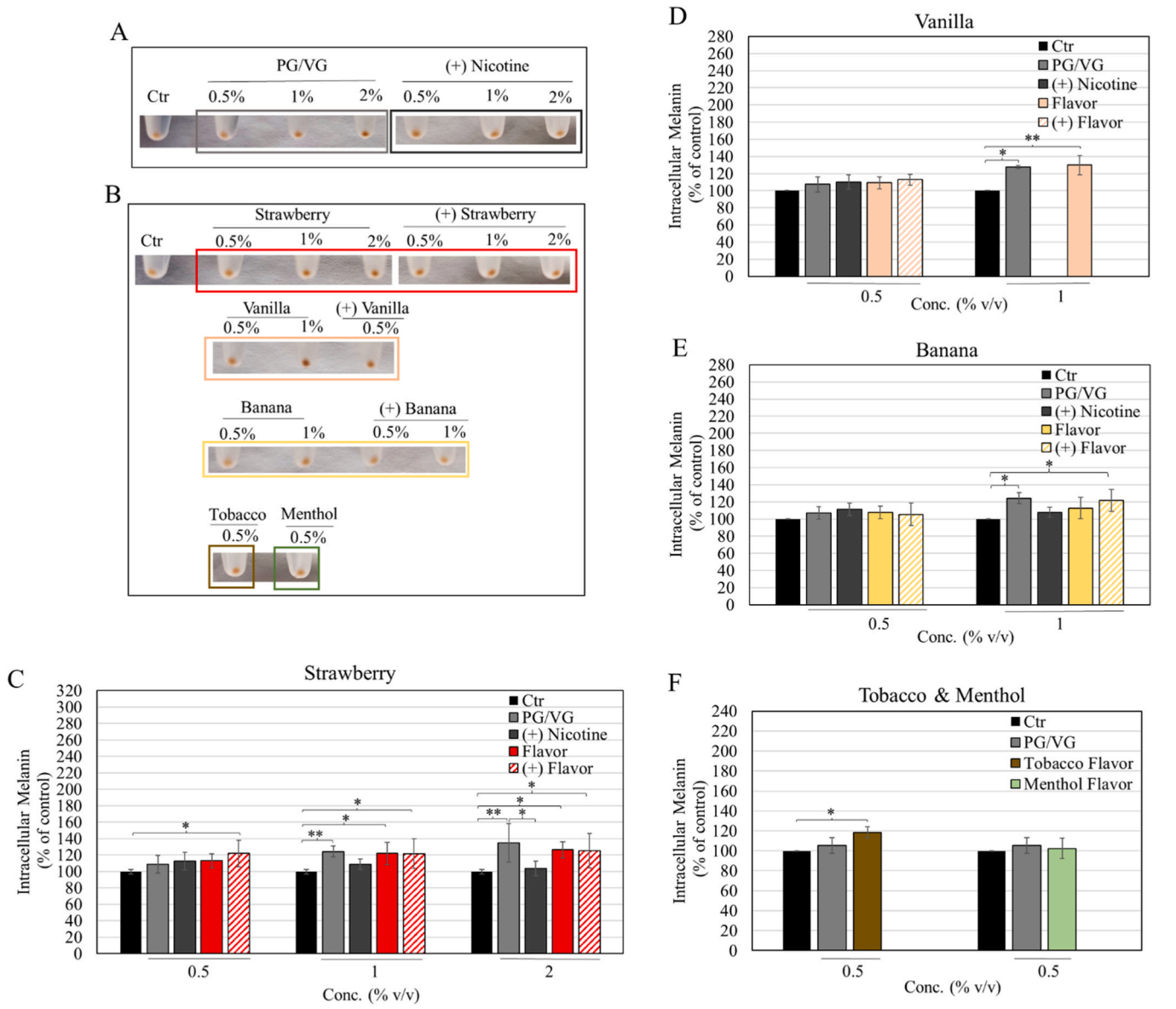

Fig. 2. Photos of melanocyte pellets denoting the experimental groups: (A) untreated control (Ctr), PG/VG vehicle, and nicotine-containing PG/VG vehicle [shown as (+) Nicotine in plots]; and (B) untreated control, flavored e-liquids (strawberry, vanilla, banana, tobacco, and menthol) without nicotine and with nicotine [shown as (+) Flavor in plots], shown from two different experiments; Melanin contents quantified in the pellets spectrophotometrically for the five flavored e-liquids of (C) strawberry; (D) vanilla; (E) banana and; (F) tobacco and menthol; (*$p < 0.05$ and **$p < 0.01$; one-way ANOVA with Tukey's test). Data for C) is the average of values from four independent experiments; Data for D), E), and F) are mean ± SD of values from three, four, and three independent experiments, respectively.

group at 2 % showed fewer dendritic cells as compared to the 2 % PG/VG vehicle base (Fig. 3B).

The cells treated with strawberry-flavored e-liquid at concentrations of 1 % and 2 % showed longer dendrites as compared to the untreated control group; however, the dendritic network was less branched as compared to the PG/VG group at 1 % and 2 % concentrations, respectively (Fig. 3A). Similarly, comparison of cells treated with 1 % PG/VG with 1 % vanilla e-liquid showed a somewhat diminished dendrite number for the vanilla flavor (Fig. 3A). Interestingly, cells treated with vanilla e-liquid with nicotine at 0.5 % consisted of several dendrites as compared to the nicotine base at 0.5 % and the untreated control (Fig. 3B). Treatment of cells with banana-flavored nicotine-containing e-liquid resulted in elongated dendrites, which was apparent at 0.5 and 1 % concentrations. The treatment of cells with 0.5 % tobacco-flavored e-liquid showed visibly elongated dendrites compared to the untreated control group and the 0.5 % PG/VG group (Fig. 3A).

Overall, these results indicate that the PG/VG vehicle markedly enhances dendrite number and branches at 1 % and 2 %, while the vanilla and strawberry flavors seemed to lower the dendritic numbers compared to the vehicle. Moreover, nicotine also appears to suppress the dendritic arborization of PG/VG vehicle-treated cells.

3.4. *Effects of e-liquids on cellular tyrosinase activity*

Both the PG/VG vehicle and the nicotine-containing vehicle exhibited no impact on the tyrosinase activity of melanocytes across the concentration ranges of 0.5–2 % (Fig. 4A). Strawberry-flavored e-liquid significantly suppressed cellular tyrosinase activity to 75.56 % and 47.80 % at 1 % and 2 % concentrations, respectively (Fig. 4A). These values were significantly lower than those of the untreated control and the vehicle-treated group, indicative of the effect of the flavor on the biological effects. Vanilla e-liquid led to a concentration-dependent marked inhibition of tyrosinase activity of melanocytes; the activity was inhibited to 42.04 % and 22.67 % at concentrations of 0.5 and 1 %,

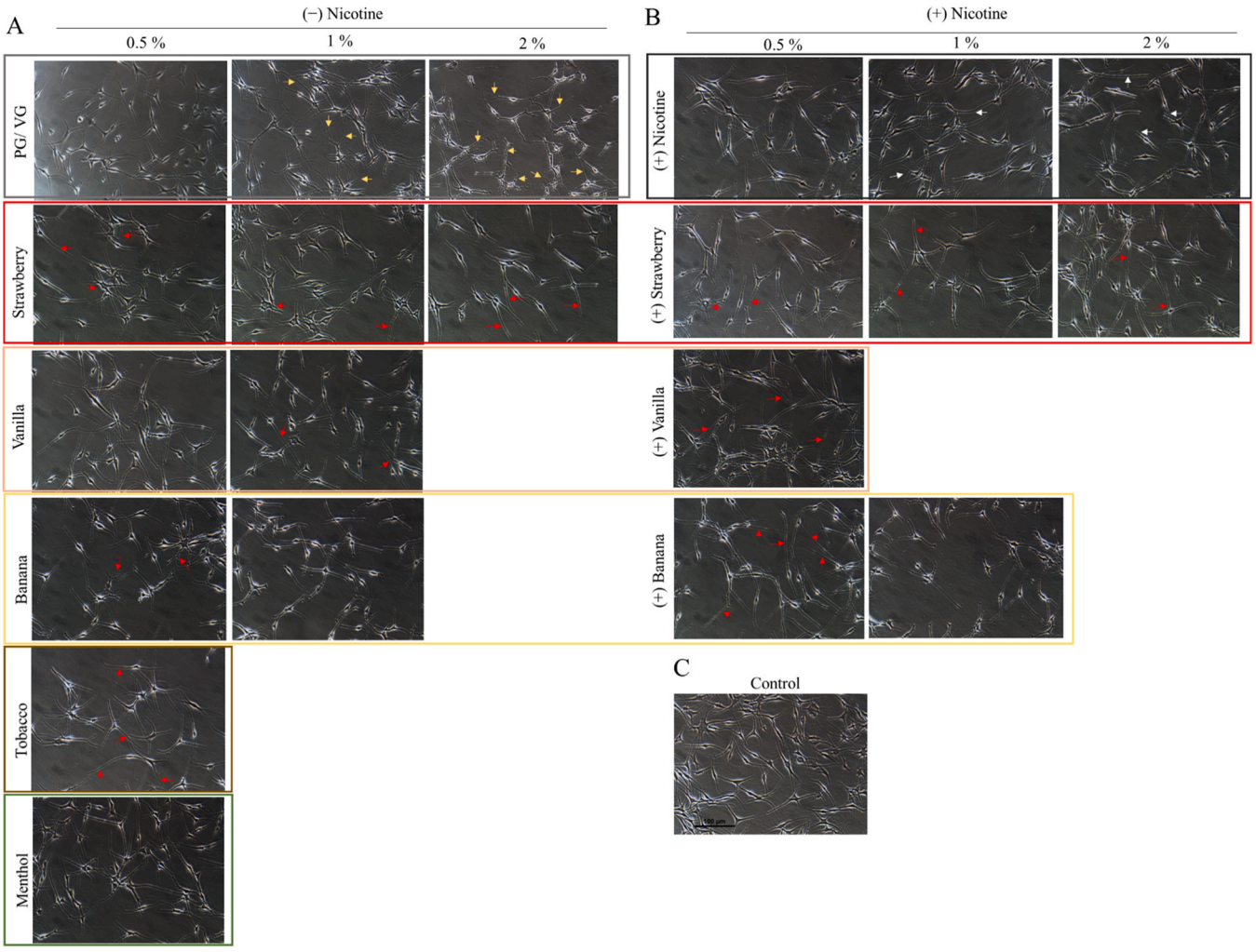

Fig. 3. Phase-contrast images of melanocyte cultures after a 48-hour treatment with various noncytotoxic concentrations of e-liquids (A) without (–) nicotine; (B) with (+) nicotine; and (C) untreated control; photos are shown from a representative experiment; yellow arrows denote the multidendritic cells of PG/VG groups, white arrows denote the bidendritic cells of PG/VG with nicotine group, and red arrows in images indicate the extended dendrites of cells in various other groups.

respectively, which was significantly lower as compared to the PG/VG group and untreated control groups (Fig. 4B). The presence of nicotine in vanilla e-liquid did not alter the inhibited tyrosinase activity at 0.5 %, as the activity remained inhibited to 50.55 %, which was not significantly different from the flavored nicotine-free e-liquid (Fig. 4B). Next, the results of cells after treatment with banana e-liquid showed a similar trend as that of vanilla e-liquid, as the tyrosinase activity was inhibited to 64.44 % and 44.35 % at 0.5 % and 1 % concentrations, respectively, that were significantly lower relative to untreated and PG/VG vehicle groups (Fig. 4C). In the presence of nicotine-containing banana e-liquid, the residual tyrosinase activity of cells was 65.54 % at 0.5 % concentration, which was significantly lower than the corresponding nicotine-base group and the untreated control group (Fig. 4C). However, at the higher concentration of 1 %, the tyrosinase activity of the cells treated with nicotine-containing e-liquid was 69.29 %, which was significantly higher than that of the flavored e-liquid without nicotine, although it was significantly lower than the nicotine-vehicle and untreated control groups (Fig. 4C). Treatment of cells with tobacco e-liquid and menthol e-liquid at concentrations of 0.5 % inhibited the tyrosinase activity to 73.08 % and 68.98 %, respectively, which was significantly lower than untreated control and PG/VG groups (Fig. 4D).

Collectively, these findings demonstrate that e-liquids with vanilla and banana flavors exhibit a concentration-dependent, more pronounced inhibitory effect on the tyrosinase activity of melanocytes, with the vanilla flavor displaying a higher effect. Furthermore, the incorporation of nicotine into e-liquid with the banana flavor can effectively enhance the suppressed tyrosinase activity by substantial levels. Based on a comparison at a concentration of 0.5 %, the order of enzyme inhibitory activity is as follows: vanilla exhibits the most potency, followed by banana, with tobacco and menthol showing similar potency levels, and strawberry exhibits the lowest potency.

3.5. *Effects of e-liquids on cellular ROS production*

No impact on ROS production in melanocytes was observed after treatment with PG/VG vehicle or nicotine-containing vehicle over the concentration ranges of 0.5–2 % (Fig. 5A). In addition, both strawberry-flavored e-liquid with and without nicotine also did not affect ROS levels at any concentration tested (Fig. 5A). However, treatment with 0.5 % vanilla flavored e-liquid elicited marked production of ROS in cells that was significantly elevated to 120.77 % as compared to the untreated control and the PG/VG vehicle group (Fig. 5B). The ROS levels continued to remain significantly elevated (mean value of 120.65 %) at the higher concentration of 1 % of the vanilla-flavored e-liquid (Fig. 5B). Treatment with banana flavored e-liquid did not alter ROS levels at 0.5 %, but at the higher concentration of 1 %, the ROS levels were significantly elevated by 20.62 % as compared to the PG/VG vehicle group (Fig. 5C). In the case of nicotine-containing banana flavored e-

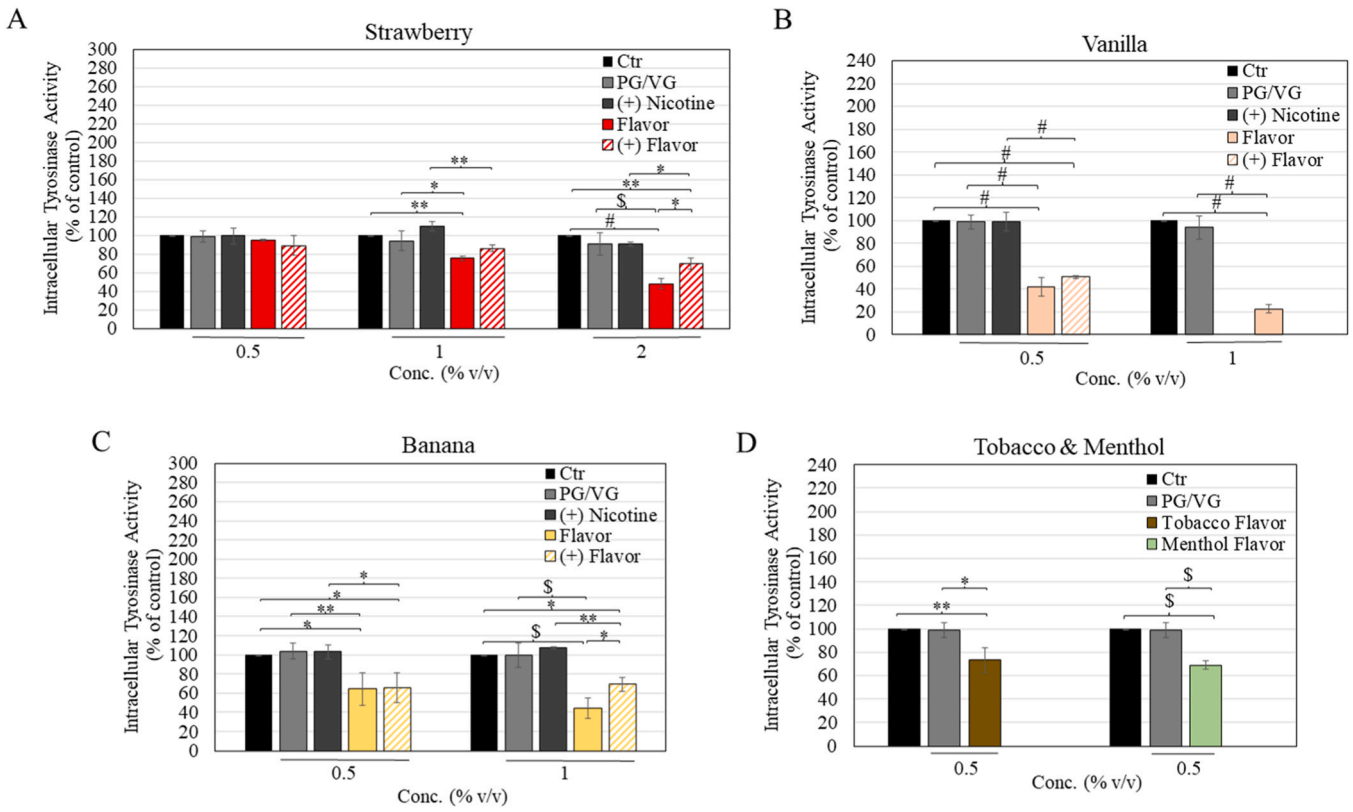

Fig. 4. Tyrosinase activity of melanocytes after 48-hour treatment with the five flavored e-liquid groups of (A) strawberry; (B) vanilla; (C) banana and; (D) tobacco and menthol. (*$p < 0.05$; **$p < 0.01$; \$$p < 0.001$, and #$p < 0.0001$; one-way ANOVA with Tukey's test). All data are mean ± SD of at least three independent experiments.

liquid, however, intriguingly, the ROS levels at 1 % concentration were significantly decreased by 28.55 % relative to the nicotine-free banana flavored e-liquid; this decrease was also significantly lower relative to the nicotine-containing vehicle (Fig. 5C). These results suggest that the inclusion of nicotine in banana e-liquid at a concentration of 1 % effectively mitigates the elevated levels of ROS. No effect of tobacco- or menthol-flavored e-liquid at 0.5 % on cellular ROS levels was observed (Fig. 5D).

These results show that the PG/VG or nicotine-containing vehicle does not impact ROS production in melanocytes over the noncytotoxic concentration range between 0.5 % and 2 %. Moreover, the three flavored e-liquids of strawberry (0.5–2 %), tobacco (0.5 %), and menthol (0.5 %) also had no impact on ROS levels. However, two specific flavors, namely, vanilla (0.5–1 %) and banana (1 %), elicit significant ROS production attributed to the flavors. In addition, nicotine in 1 % banana-flavored e-liquid can rescue the elevated ROS levels to baseline, indicating an interactive effect of nicotine with banana flavor.

4. Discussion

The first tier of the three-layer framework proposed for evaluating EC toxicity typically involves exposing cultured cells directly to the e-liquid [61,62]. Hence, in our current study, the melanocytes were directly exposed to unvaped neat e-liquids that were diluted in culture medium at 1:50, 1:100, and 1:200 (resulting in 2 %, 1 %, and 0.5 % v/v, respectively). Numerous studies have documented the use of unvaped e-liquids to evaluate biological effects across various cell types, including skin keratinocytes [63], lung cells [64–70], macrophages [71], neural stem cells [72], bone cells [73], embryonic stem cells [74], ear epithelial cells [19,75], as well as oral epithelial and gingival cells [76,77]. Moreover, we have also employed unvaped e-liquids to examine biological effects in retinal pigment epithelial cells [58,78] and primary skin melanocytes [52]. While this form of exposure does not simulate vaping, where users are exposed to aerosols produced by the heating of e-liquid inside ECs, our model is relevant under occupational and consumer exposure settings. The exposure of skin to e-liquids is pronounced in occupational settings where workers engage in the EC manufacturing process, combining nicotine with flavoring components and filling cartridges from e-liquid tanks on a daily basis, and in situations where EC users prepare their own e-liquids or formulate custom blends [53,54]. This has increased with EC users stocking e-liquid components due to regulatory bans on e-liquid sales. Personal protective equipment, including gloves, is necessary in the workplace, yet many facilities violate requirements and risk permeation. Teens and younger adults may make their own e-liquid mixtures and refill cartridges without gloves. Skin contact can also occur from direct contact with surfaces contaminated with exhaled aerosol residues. Nicotine was shown to permeate the skin from the clothing worn on which cigarette smoke was deposited [79]. Contact with EC refill liquids or pure nicotine on the skin resulted in considerable absorption of nicotine [80,81]. PG has been shown to permeate through the human skin [82,83]. Flavoring chemicals can also penetrate through the skin. Vanillin has high permeation and retention in human skin [84,85]. Linalool, another e-liquid flavoring [86], has been shown to penetrate through the skin [87]. Flavoring chemicals menthol and limonene increase dermal penetration [88]. An impaired skin barrier or injury can exacerbate exposure by facilitating direct interaction with the melanocytes. Many EC users previously smoked cigarettes, which impairs the skin barrier [89]. Consequently, the likelihood of increased exposure to melanocytes is feasible. The access of e-liquids to melanocytes (both in the skin and other body locations) can also occur indirectly via systemic pathways via the absorption of EC chemicals into the bloodstream by inhalation.

The rationale for the direct contact of e-liquids with the skin in various circumstances (spill/leakage/immersion), together with the

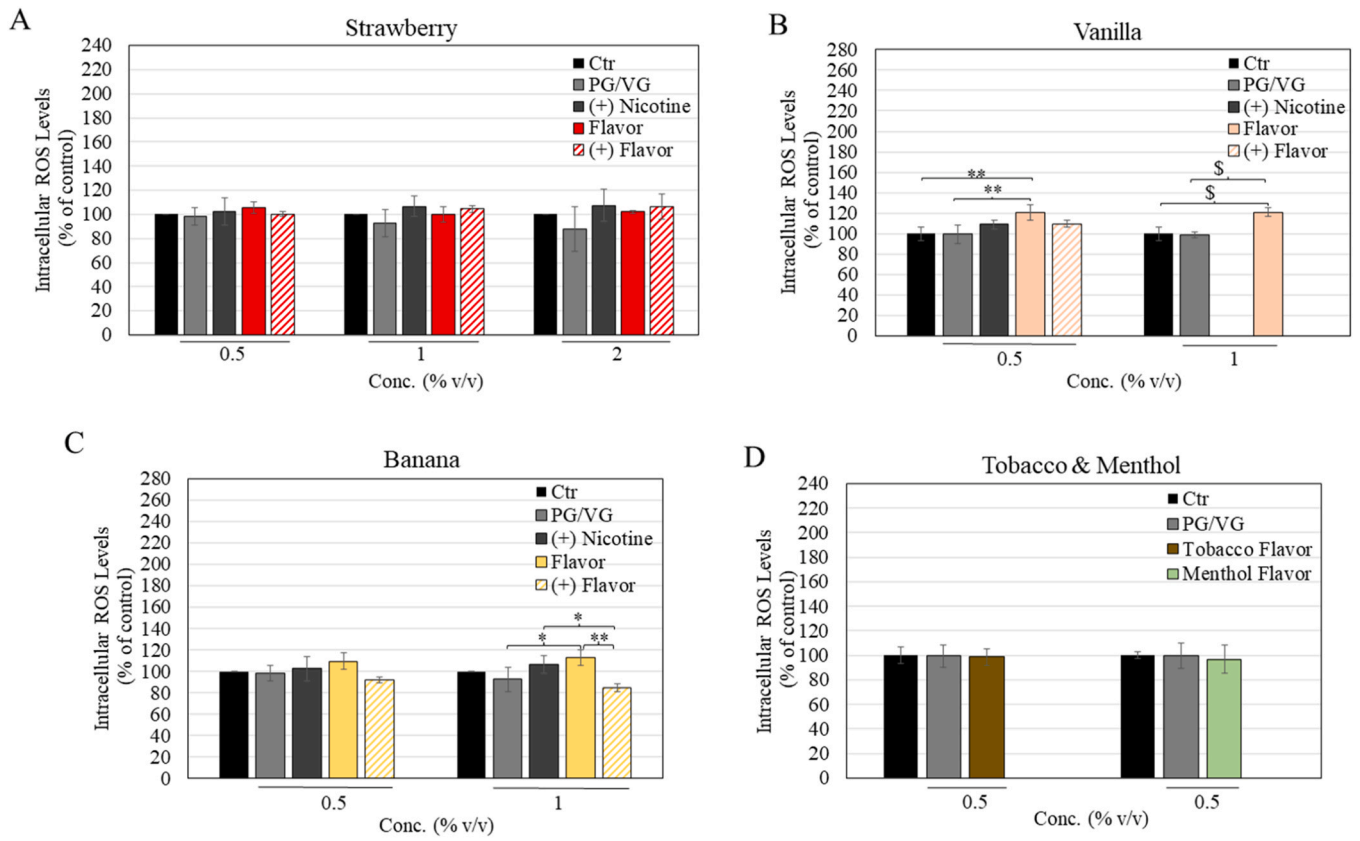

Fig. 5. ROS generation in HEMn-LP cells after a 48 h exposure to e-liquids containing (A) strawberry; (B) vanilla; (C) banana; (D) tobacco and menthol; (One-way ANOVA with Tukey's post hoc test; * $p < 0.05$, ** $p < 0.01$, and $ $p < 0.001$ vs. untreated control). Data for (A) and (C) are mean ± SD of at least three independent experiments. Data for (B) and (D) are mean ± SD of values combined from two independent experiments.

calculations indicating the concentrations of e-liquids attained on skin surfaces, is detailed in the supplementary material (Section II). The e-liquid concentrations in the first scenario of hand exposure (Fig. S1A, Table S1) are approximately 0.5 %, 2 %, and 7 % v/v, whereas in the second scenario of exposure of the thumb and index finger (Fig. S1B, Table S1), they are 1 %, 5 %, and 23 % v/v. Our data from the current study show that these concentrations are within the biological effects (0.5–2 %) range or exceed cytotoxicity thresholds. It should be noted that these calculations excluded the presence of stratum corneum and keratinocytes in the layer above the melanocyte suprabasal layer and employed a 48-hour continuous cell culture exposure. To simulate the pharmacokinetics of e-liquid spills or leaks, a 3-D skin tissue model, such as Melanoderm™ [90], which comprises stratum corneum, keratinocytes, and melanocytes, will be necessary. A previous study [63] demonstrated that a 4-hour exposure of neat PG/VG e-liquid onto Epiderm™ (a 3D organotypic tissue of human skin keratinocytes devoid of melanocytes) increased inflammatory mediators. Therefore, this may serve as a valuable reference for subsequent investigations. Direct exposure to e-liquids may not match the "48-h continuous duration" used in our experiments or reflect a spill/leakage, which might be short. In the context of finite dose studies, it was observed that nicotine-containing e-liquid, when spilled on human skin, formed a thin film, and its passage through the skin was monitored at different time points, including 24 and 48 h [80]. Comparable exposure durations of 48 h have been employed in previous studies for subjecting various cell types in the body to neat e-liquids [67,76,91]. A prior study [63] involved a 24-hour exposure of neat e-liquids on skin keratinocytes; however, we chose a 48-hour duration, as 24 h may not adequately reveal changes in melanin production in primary melanocytes. This decision is further supported by our earlier findings, which indicated increased melanin production following a 48-hour treatment with PG/VG e-liquids [51]. The potential spill and its components might only briefly expose melanocytes and be disseminated through the blood circulation systemically. Nonetheless, melanocytes may sequester chemicals and metal ions via melanin binding [92,93], leading to the potential accumulation of these substances inside the cells over time.

Given the considerable diversity in the commercially available flavors of e-liquids, our research focused on five widely preferred flavors. These flavors along with additional flavors, have been evaluated for cytotoxicity to HEMn-LP cells and human retinal pigment epithelial cells (ARPE-19) in our previous studies [52,58]. A concentration range of 0.5 −2 % for a 48-hour duration was selected to evaluate e-liquid's effect on melanocytes, as similar concentrations and durations were used in our previous studies [52,58]. Moreover, another study [64] also used similar ranges for a 48-hour duration. Our use of all e-liquids with identical vehicle composition minimized any biological differences that might occur due to the changes in the PG/VG ratio of the vehicle itself since the PG/VG ratio can induce distinct cellular effects [63,94] including our recent study [51] where the PG/VG 80/20 ratio displayed the greatest biological effect on melanocytes.

Our results agree with our recent study [52] and others that showed flavor-dependent cytotoxicity regardless of the presence of nicotine in other cells, such as endothelial cells [95] and bone cells [96]. Moreover, our results of cytotoxicity profiles of nicotine-free and nicotine-containing strawberry and banana e-liquids are similar to our recent study [52]. However, our findings of cytotoxicity of menthol, tobacco, and vanilla e-liquids contrast with the cytotoxicity results of our previous study, where the mean IC_{50} values of menthol e-liquid with and without nicotine were 0.04 % and 0.34 %, respectively, banana e-liquid with and without nicotine was 1.38 % and 1.18 %, respectively, and tobacco e-liquid with and without nicotine were 1.19 % and 1.27 %, respectively [52]. This discrepancy may be attributed to batch-specific

effects as e-liquids of a new batch were used in the current study. Moreover, tobacco e-liquids may have been prepared using different materials in different batches by the vendor. Variation between batches of a flavored e-liquid by the same vendor has been noted before [97]. The use of cured tobacco leaves instead of industrial-grade tobacco flavors by some EC vendors introduces variability as cured tobacco can contain additional toxins [98]. It is plausible that the tobacco e-liquid of our previous study [52] may have been sourced differently and from cured tobacco, causing greater cytotoxicity than in the current study. However, because the vendor does not disclose the tobacco source in the product, it is difficult to ascertain the differences. Also, the metal ion concentrations in different e-liquids can differ, which can contribute to different cytotoxicity. For example, aerosols generated from e-liquid of different batches from the same vendor differed in metal concentrations, thus indicating the batch-to-batch variations of e-liquid formulations [99,100]. Hence, based on these reports, our MTS assays' differences are unsurprising. Intriguingly, all these three e-liquids in our current study seemed to be considerably less toxic to HEMn-LP cells compared to the e-liquids used in our prior study [52], where similar experimental conditions were utilized in MTS assays, with the only difference being the new batch of e-liquid purchased at different times. One potential issue is that it is not possible to know how long the e-liquid purchased from the manufacturer sits on its shelf after mixing or before mixing and dispatching. Because with time, e-liquids undergo chemical reactions [101], vanilla, tobacco, or menthol e-liquid may not remain that cytotoxic to melanocytes.

Vanillin has been recognized as the primary flavoring compound in vanilla e-liquids [102,103]. Our results of elevated ROS production by treatment with vanilla e-liquid agree with our recent study [78] and other reports that have also reported elevated ROS production by vanilla e-liquids in other cells [95,104]. Interestingly, in the previous study [95], the authors reported a decrease in ROS production after exposure to vanilla e-liquid containing a higher amount of nicotine (12 mg/mL), which is similar to our findings of 1 % banana e-liquid containing nicotine (18 mg/mL) that attenuated the ROS production of banana e-liquid without nicotine. Our results of the absence of any ROS production in cells after treatment with PG/VG (80/20) vehicle are similar to a prior study [104] that also showed no change in ROS production in human macrophages and lung cells after treatment with PG/VG (50/50).

Our findings of increased melanin production following treatment with the vehicle PG/VG 80/20, without any alteration in tyrosinase activity, align with our previous study [51]. Additionally, these effects occurred without cytotoxicity, which agrees with our prior studies [51, 52]. Our findings of decreased melanin production in HEMn-LP cells after treatment with a nicotine vehicle base at 2 % (2 mM nicotine) agree with a previous report [105] that showed that nicotine suppressed melanin production in these cells, although tyrosinase activity was also inhibited that contrasts with our results. This difference might be because we examined nicotine-containing PG/VG vehicles, while pure nicotine was examined in the previous study [105]. In other in vivo studies conducted on zebrafish embryos, nicotine at 50 μM was shown to lower melanocyte number due to toxicity and diminish melanin pigmentation in zebrafish [106]. However, other studies have demonstrated conflicting effects of nicotine on melanogenesis. Specifically, nicotine at 50 μM stimulated melanin content and tyrosinase activity in cells from a darkly pigmented donor (HEMn-DP cells) [107], whereas it decreased melanin content and tyrosinase activity in HEMn-LP cells at the same concentration [105]. However, at a higher concentration of 1 mM, nicotine diminished both parameters in HEMn-LP and HEMn-DP cells [105,107], indicating the pigmentation-dependent and concentration-dependent differential effects on melanogenesis. As the current study was conducted using HEMn-LP cells, it may be speculated that nicotine-containing PG/VG might further stimulate and not suppress melanin production in HEMn-DP cells, although expanding the results using HEMn-DP cells in future investigations is a necessary next step. Previous studies have reported some EC flavoring compounds' promoting or inhibiting effects on melanogenesis. For example, vanillin was shown to stimulate the activity of the enzyme tyrosinase in a cell-free mushroom tyrosinase assay [108] and increase skin darkening in human subjects when applied topically in polymeric form [109]. Conversely, the flavoring compound maltol induced pigment aggregation in a Xenopus melanophore model [110], indicative of its potential to induce skin lightening, as the aggregation of melanophores is associated with a diminished manifestation of pigmentation. Cinnamaldehyde is a known melanocytotoxic chemical that can result in depigmentation [111,112]. Our findings showed increased melanin production by vanilla and banana e-liquids that did not correlate to tyrosinase activity since it was suppressed. The findings are unsurprising, given that other compounds have previously shown inconsistent outcomes in enhanced melanin synthesis and diminished tyrosinase activity [51,113]. In addition to tyrosinase, tyrosinase-related protein 1 (TRP-1) and TRP-2 are also implicated in the melanogenesis pathway [114,115]. A previous study [116] demonstrated that methylanthranilate, a flavoring agent found in grape-flavored e-liquids [9], inhibited melanogenesis by reducing the protein and mRNA levels of TRP-2 and TRP-1, without a discernible impact on tyrosinase. The components PG/VG and flavoring agents in e-liquids may affect melanogenesis via their action on the tyrosinase protein or related proteins (TRP-1, TRP-2). The investigation of protein or gene expression of tyrosinase, TRP-1, and TRP-2 after treatment with e-liquids was not conducted due to a few limitations necessitating further research to explore the role of these proteins.

By including the vehicle control group of PG/VG (80/20) in the experiments, we could validate that the enhanced melanogenesis was unrelated to flavoring. Moreover, we previously [51] demonstrated that only pure PG or high PG vehicles increased melanin production in melanocytes, while pure VG and PG/VG (20/80) did not have any effect. Therefore, this suggests that if the vanilla or strawberry e-liquids were marketed with a vehicle base consisting of pure VG or high VG, we would not have observed any augmentation in melanin synthesis. Therefore, if the necessary vehicle control was not tested simultaneously, it is possible to mistakenly infer that the flavoring increased melanin production, even if that was not true. However, to verify this, acquiring the same flavored e-liquid prepared in various ratios of PG/VG vehicles from the same vendor and assessing them to validate this line of reasoning will be essential. This is especially necessary since vanillin, present in vanilla e-liquids, can react with PG and VG to produce chemical adducts such as acetals, whose biological properties may differ [101,117] and warrant further investigation.

In our earlier studies [118,119], we noted that specific compounds could alter dendricity without affecting cellular melanin levels. In the current study, e-liquids of banana (containing nicotine) and vanilla (containing nicotine) showed a qualitative enhancement in dendricity, while the cellular melanin content remained unchanged at a concentration of 0.5 %. Hence, it is plausible that these e-liquids might affect pigmentation by enhancing the export of melanin. Furthermore, cells exposed to 2 % strawberry e-liquid exhibited a comparable increase in melanin content to that of the 2 % PG/VG vehicle base, indicating that the flavor had no additional impact on melanin production. Nonetheless, a visual decrease in dendricity was evident for the 2 % strawberry group compared to the robust multidendritic arbors of the 2 % PG/VG vehicle base group. We did not explore the export of melanin through dendrites to keratinocytes in relation to flavored e-liquids because our study solely focused on the quantitative assessment of intracellular melanin levels and a qualitative analysis of dendritic morphology. To corroborate the impact of e-liquids on melanosome export, additional research employing melanocyte-keratinocyte cocultures [120] is necessary.

After heating e-liquid in an EC device, PG and VG may undergo oxidation and decomposition to generate harmful compounds, including acrolein, formaldehyde, methylglyoxal, and dihydroxyacetone [121, 122]. Dihydroxyacetone is a melanogenesis stimulator with genotoxic

effects [123]. Acrolein and carbonyls increased melanogenesis, leading to photoaging-related skin yellowing [124,125]. Moreover, EC vapors induce higher ROS levels, resulting in heightened oxidative stress [126]. Higher ROS levels enhance melanogenesis and melanoma risk [127]. The effects of e-liquids after heating were not explored, as it was not the focus of our study. Despite this limitation, our findings are relevant in occupational and consumer exposure to ECs directly from the skin. Moreover, the cytotoxicity of e-liquids after aerosolization correlates to the cytotoxicity of unheated e-liquids by 74 %, validating that e-liquids are a representative model for toxicological screening [128]. Additionally, the flavor chemicals in e-liquids were also detected in aerosols, confirming effective transfer [128], and the PG/VG ratio in e-liquids was retained in aerosol, rather enriched in PG [129]. Vanillin flavoring continued to be cytotoxic to endothelial cells after aerosolization [130]. Other reports have also shown comparable cytotoxicity profiles of e-liquid and aerosol [131–133]. Elsewhere [63], neat e-liquids or EC exhaled aerosol condensates (from vape shops) were exposed to EpiDerm™. The authors reported upregulation of inflammatory mediators, including IL-1α cytokine, and validated that PG, rather than the flavor chemicals, was the key to driving the pro-inflammatory cytotoxic effects, which bears some similarity to our results. Interestingly, IL-1α enhances melanogenesis [134] and is also released from keratinocytes upon skin exposure to UV rays [135]. The absence of melanocytes in the 3D skin tissue used in the earlier study [63] precluded the assessment of e-liquids' effects on melanogenesis. Nonetheless, MelanoDerm™ would be a fitting model to expand our studies in the future to establish translational relevance. Based on these rationales, we hypothesize that post-aerosolization, e-liquid constituents may enhance cytotoxicity, oxidative stress, and melanogenesis, although further study is warranted. E-liquid vapor condensates generated by condensing vaped e-liquid aerosols have also been employed [136–138]. The variety of EC devices on the market, with adjustable power and delivery and various puffing topographies of EC users [139], and the absence of standardization make aerosol testing difficult. Seven methods have been reported for collecting e-liquid vapor condensates, bubbling being the most common method, yet it does not capture all flavor compounds, making the condensate unrepresentative of native EC vapor [140]. Due to aerosol components saturating heterogeneously in the test matrix solution, matrix saturation is inconsistent [141]. Despite e-liquid vapor condensates being a better model than e-liquids, they are less physiological than whole aerosol exposure, which directly exposes the skin to the aerosol. However, the impacts of e-liquid vapor condensate on human melanocyte function and underlying pathways warrant second-tier research in expanding toxicological effects [61,62].

Previous studies have reported the chemical analysis of various flavoring chemicals in commercial e-liquids using the gas chromatography-mass spectrometry (GC-MS) technique [7,102]. Due to some limitations, it was not possible to conduct a GC-MS analysis of the e-liquids in the current study. Nevertheless, based on prior studies, banana-flavored e-liquids contain the flavoring isoamyl acetate [130], while French vanilla e-liquid was shown to primarily consist of vanillin, ethyl vanillin, and maltol [9,142]. One important consideration pertains to the inherent variability observed in e-liquids sourced from different vendors, which poses a significant challenge in achieving results standardization across various studies. It is important to highlight that the HEMn-LP cells utilized in this study are derived from a male Caucasian newborn. Multiple studies have demonstrated a higher likelihood of males engaging in the use of EC products compared to females [143–145]. Furthermore, Caucasian students had a greater likelihood of having used ECs in high schools compared to African American students [146]. Hence, the cells employed in this study are representative of the broader impact of ECs. One of the key limitations of this study is the use of melanocytes from a neonatal foreskin instead of adult tissue, which fails to replicate the e-liquid spill/leakage that is more pronounced in Caucasian adults compared to newborns. However, neonatal human melanocytes were used because they exhibit superior proliferation rates and prolonged in vitro lifespan relative to adult human melanocytes [147] and have been used to evaluate nicotine's effects on melanocytes [105,148]. Furthermore, numerous 3D skin tissue models often incorporate melanocytes or keratinocytes derived from neonates [149,150]. Due to their environmental exposure to UV light irradiation and ambient air irritants, melanocytes derived from an adult have a higher dendricity than neonatal melanocytes [151] and are anticipated to exhibit greater susceptibility to EC exposure. However, another study [152] showed no difference in the dendritic morphology of melanocytes from adults and neonates when they were both isolated from matched donor sites, indicating the role of the donor site in dictating adult/neonatal melanocyte responses. Future studies using melanocytes from adult hand skin that replicate the e-liquid spill will be necessary. Furthermore, it is essential to assess the cytotoxicity of e-liquid on melanocytes derived from individuals who smoke cigarettes to facilitate future research endeavors aimed at augmenting existing data. We did not assess the specific mode of cell death induced by the menthol and banana e-liquids. Melanocyte apoptosis is a hallmark of vitiligo disorders, although other forms of melanocyte death, such as necrosis and ferroptosis, have also been observed [153]. Therefore, further investigations must be conducted to elucidate the underlying processes involved.

ECs have also been employed as a means of vaping cannabis, wherein certain e-liquids consist of mixtures of flavorings and cannabis extracts [154,155]. Flavored e-liquids containing cannabidiol (CBD) increased cytotoxicity, whereas no such effect was observed with flavored e-liquids containing nicotine [156] suggesting that CBD-containing flavors may pose a greater risk than nicotine. In our previous study [157], we elucidated the impacts of cannabis constituents, namely CBD and Δ9-tetrahydrocannabinol (THC), on HEMn-DP and HEMn-LP cells and showed that LP cells were more vulnerable to the detrimental effects induced by these cannabinoids, particularly THC. Therefore, future studies to investigate whether the combination of THC/CBD with flavorings and nicotine elicits a similar effect or potentially even greater cytotoxicity are warranted. UV radiation is a pro-melanogenic stimulus that increases melanogenesis in cells by p53 expression [158]. Nicotine was shown to be more cytotoxic in melanocytes with simultaneous UVA exposure [148]. The impact of EC flavors on melanocyte cytotoxicity and functional endpoints in the presence of UVA radiation will also be of interest for future investigations.

5. Conclusions

In summary, our findings validate that flavored e-liquids induce melanocytotoxicity irrespective of nicotine or the PG/VG vehicle. Menthol-flavored e-liquid demonstrated greatest cytotoxicity, whereas the strawberry flavor showed the lowest cytotoxicity. At noncytotoxic concentrations, strawberry, vanilla, and banana e-liquids enhanced melanin production, which was attributed to the PG/VG vehicle rather than the flavor or nicotine. Nevertheless, all the flavored e-liquids inhibited the tyrosinase enzyme activity, with vanilla and banana demonstrating the most significant effects; the flavors themselves, not the PG/VG or nicotine components, were responsible for the suppressed tyrosinase activity. Furthermore, vanilla and banana-flavored e-liquids augmented ROS production, which was attributed to the flavoring agents rather than the vehicle or nicotine base. However, the banana e-liquid that included nicotine resulted in a decrease in ROS production. Common flavors in e-liquids can impact melanogenesis and induce oxidative stress, suggesting that the use of ECs may not entirely mitigate the detrimental consequences of cigarette smoking. While our findings cannot be directly applied to real-life exposure in humans, they highlight potential concerns regarding the impact of these flavored e-liquids on melanocyte cytotoxicity and functions. Additional studies are necessary to examine the functions of melanocytes using e-liquid aerosols to replicate physiological EC usage. Moreover, it will be essential to assess the production of inflammatory cytokines by melanocytes to confirm that increased melanin production as noted in our study, with

increased ROS generation that indicates oxidative stress, extends beyond only cosmetic concerns of skin darkening. We contend that if future study using 3D skin tissues can substantiate the skin darkening potential of ECs, accompanied by elevated oxidative stress and inflammatory cytokine release, it might help in informing regulatory agencies and formulate guidelines. For a comprehensive risk assessment, the potential genotoxicity, mutagenicity, and carcinogenicity of EC exposure on melanocytes also need to be investigated.

List of abbreviations

BCA: bicinchoninic acid;
DCF: dichlorofluorescein;
DP: darkly pigmented;
EC: electronic cigarette;
HBSS: Hank's buffered salt saline;
HEMn: human epidermal melanocytes neonatal;
HMGS: human melanocyte growth supplement;
L-DOPA: L-3,4-dihydroxyphenylalanine;
LP: lightly pigmented;
PG: propylene glycol;
ROS: reactive oxygen species;
VG: vegetable glycerin

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Author statement

I, the corresponding author and the sole author take the full credit and responsibility of the work and confirm that there are no other persons who satisfied the criteria for authorship but are not listed.

CRediT authorship contribution statement

Shilpi Goenka: Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization.

Declaration of Competing Interest

The author declares that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.toxrep.2025.101924.

Data availability

Data will be made available on request.

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