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RESEARCH ARTICLE

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Electronic cigarette liquid exposure induces flavor-dependent osteotoxicity and increases expression of a key bone marker, collagen type I

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Abstract
Electronic cigarettes (e-cigarettes) are nicotine delivery devices advertised as a healthier alternative to conventional tobacco products, but their rapid rise in popularity outpaces research on potential health consequences. As conventional tobacco use is a risk factor for osteoporosis, this study examines whether exposure to electronic liquid (e-liquid) used in e-cigarettes affects bone-forming osteoblasts. Human MG-63 and Saos-2 osteoblast-like cells were treated for 48 hours with 0.004%-4.0% dilutions of commercially available e-liquids of various flavors with or without nicotine. Changes in cell viability and key osteoblast markers, runt-related transcription factor 2 and Col1a1, were assessed. With all e-liquids tested, cell viability decreased in a dose-dependent manner, which was least pronounced in flavorless e-liquids, most pronounced in cinnamon-flavored e-liquids and occurred independently of nicotine. Col1a1, but not runt-related transcription factor 2, mRNA expression was upregulated in response to coffee-flavored and fruit-flavored e-liquids. Cells treated with a non-cytotoxic concentration of fruit-flavored Mango Blast e-liquid with or without nicotine showed significantly increased collagen type I protein expression compared to culture medium only. We conclude that the degree of osteotoxicity is flavor-dependent and occurs independently of nicotine and that flavored e-liquids reveal collagen type I as a potential target in osteoblasts. This study elucidates potential consequences of e-cigarette use in bone.

KEYWORDS
Col1a1, cytotoxicity, electronic cigarette liquid, MG-63 osteoblast-like cells, RUNX2, Saos-2 osteoblast-like cells

1 | INTRODUCTION

Electronic cigarettes (e-cigarettes) are nicotine-delivery devices that are rapidly gaining worldwide popularity as a combustion-free alternative to conventional cigarettes. Emerging on the Chinese market in 2004 and in the USA in 2007, e-cigarettes are now a multi-billion dollar industry (US Department of Health and Human Services et al., 2016). A standard e-cigarette features a battery, heating element and electronic liquid (e-liquid) chamber. The e-liquid, which typically contains a mixture of nicotine, propylene glycol (PG), vegetable glycerin (VG) and flavoring agents, is vaporized into an aerosol when the e-cigarette battery warms the heating element. The aerosol is then inhaled by the user in a process known as vaping.

While e-cigarettes are advertised as a healthier alternative to tobacco, their rapid rise in popularity outpaces research on potential health consequences associated with their use. In the USA alone, over two million middle and high school students report using e-cigarettes, making e-cigarettes more popular than tobacco products in this age group (CDC,, & Prevention, C. f. D. C. a., 2017). Furthermore, the appeal to teenagers fuels growing concerns over health risks associated with e-cigarette use. A recent systemic review of case reports of e-cigarette users summarizes adverse health effects ascribed to e-cigarette use, including respiratory, gastrointestinal and cardiovascular complications (Hua & Talbot, 2016). Another important, although under-investigated, area of e-cigarette research is their potential impact on the skeletal system. Childhood and adolescence are critical times for optimal bone growth and development. Approximately 90% of bone mass is accrued by early adulthood at about 18 years of age (Bachrach, 2001). Hence, it is possible that young e-cigarette users are impairing their bone development, which may increase their risk of developing osteoporosis later in life. Osteoporosis, characterized by reduced bone mineral density and deterioration of the bone microarchitecture, is the leading cause of bone fractures (NIH Consensus Development Panel on Osteoporosis Prevention, D., and Therapy, 2001). Furthermore, osteoporosis is the most common metabolic bone disease in humans; thus, understanding risk factors associated with this disease is germane.

The effects of e-cigarette use on bone health are unknown; however, conventional tobacco cigarette use is linked to the pathogenesis of osteoporosis. Epidemiological studies demonstrate smoking conventional tobacco leads to reduced bone mineral density and increased risk for osteoporotic fractures (Kanis et al., 2005; Yoon, Maalouf, & Sakhaee, 2012). There are two proposed mechanisms by which smoking tobacco leads to reduced bone mineral density. First, tobacco smoke exposure can indirectly alter bone function by increasing parathyroid hormone release, increasing cortisol production or reducing vitamin D metabolism (Abate, Vanni, Pantalone, & Salini, 2013; Yoon et al., 2012). Second, tobacco smoke can directly act on bone by targeting the proliferation, differentiation and matrix deposition of bone-forming cells called osteoblasts (Ko et al., 2015). In either case, disturbance in the normal pattern of bone remodeling can contribute to the development of osteoporosis.

Importantly, although e-liquids do not contain all the known carcinogens found in tobacco smoke, many contain nicotine. In vivo and in vitro studies report alterations in bone metabolism in response to nicotine concentrations comparable to that found in saliva (0.6 μM-10 mM) or blood (0.03-0.5 μM) of tobacco consumers (Benowitz, 1988; Russell, Jarvis, Iyer, & Feyerabend, 1980). Several in vitro studies report a biphasic effect of nicotine exposure on normal or tumor-derived osteoblasts with low concentrations stimulating proliferation and gene upregulation, and high concentrations eliciting the opposite effect (Marinucci, Bodo, Balloni, Locci, & Baroni, 2014; Rothem, Rothem, Soudry, Dahan, & Eliakim, 2009). The mRNA expression of collagen type I (Col1a1), the main organic component of bone extracellular matrix, is upregulated in MG-63 osteoblast-like cells exposed for 24 hours to 0.1-100 μM nicotine but downregulated upon exposure to 10 mM nicotine (Rothem et al., 2009). More recently, Marinucci et al. (2014) reported that several other key osteoblast genes, including the critical mediator of the osteoblast phenotype runt-related transcription factor 2 (RUNX2), are upregulated or repressed in normal human osteoblasts cultured in the presence of 0.1-10 μM nicotine (Marinucci et al., 2014). RUNX2 is a transcription factor essential for the development, maturation and maintenance of osteoblasts (Ducy et al., 1999).

Besides nicotine, flavoring agents in e-liquids could negatively alter osteoblast proliferation, differentiation or matrix deposition. Several surveys indicate that the primary reason for increased e-cigarette use in youth is the wide variety of flavorings available (Dai & Hao, 2016; Patel et al., 2016; Villanti et al., 2017). There are over 8000 e-liquids flavors on the market ranging from “Peanut Butter and Jelly Sandwich” and “Mango Blast” to Tobacco flavors (Zhu et al., 2014). In 2016, the US Federal Drug Administration began regulating e-cigarettes under tobacco products. However, unlike conventional tobacco products where flavorings (except menthol) are no longer permitted, e-liquids may still contain flavoring agents. Furthermore, many of these flavoring agents are categorized as “generally recognized as safe” by the Flavor Extracts Manufacturers Association for ingestion; however, the classification does not pertain to inhalation. To this point, several in vitro studies using human, rat or mouse cells demonstrate that some flavoring agents in e-liquids are cytotoxic (Bahl et al., 2012; Behar et al., 2014; Behar, Wang, & Talbot, 2017; Farsalinos et al., 2013; Lerner et al., 2015; Otreba, Kosmider, Knysak, Warncke, & Sobczak, 2018; Rowell et al., 2017). Cinnamon-flavored e-liquids are particularly cytotoxic in rat cardiomyoblasts and human CALU3 airways cells (Farsalinos et al., 2013; Rowell et al., 2017). Another recent study using primary human oropharyngeal mucosal cultures found fruity-flavored e-liquids to be overly cytotoxic and more so than tobacco-flavored e-liquids (Welz et al., 2016). Notable is that the cytotoxic effect of flavored e-liquids can occur independently of the presence of nicotine, suggesting that flavoring agents alone can induce cellular damage (Bahl et al., 2012; Kaur, Muthumalage, & Rahman, 2018; Rowell et al., 2017). Taken together, these studies provide a compelling rationale for the current research, which investigates the impact of in vitro exposure to flavored e-liquids on osteoblasts.

Because smoking conventional cigarettes is a risk factor for osteoporosis, we hypothesize that vaping impairs bone by targeting bone-forming osteoblasts. We used human MG-63 and Saos-2 osteoblast-like cell lines in this study. Cells were exposed to a variety of flavored unvaped e-liquids, with or without nicotine, from four commercially available brands and assessed for changes in cell viability, RUNX2 and Col1a1 mRNA expression, and collagen type I protein expression. Several recent in vitro studies found comparable results between aerosolized and unvaped e-liquids on cell viability, justifying the use of unvaped e-liquid exposures in this study as a first-pass screening method to assess osteotoxicity (Behar et al., 2017; Rowell et al., 2017). This study aims to increase awareness of possible bone-related health risks associated with e-cigarette use.

2 | MATERIALS AND METHODS

2.1 | Cell culture

The human osteosarcoma cell lines Saos-2 and MG-63 were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) and maintained using established culture conditions (Arbon, Christensen, Harvey, & Heggland, 2012; Coonse, Coonts, Morrison, & Heggland, 2007; Ha, Burwell, Goodwin, Noeker, & Heggland, 2016; Smith et al., 2009). Briefly, Saos-2 cells were cultured in McCoy's 5A medium and MG-63 cells in minimal Eagle's medium (EMEM), each supplemented with 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA, USA), 2 mM L-glutamine, 100 IU/mL penicillin and 100 μg/mL streptomycin (Sigma-Aldrich, St. Louis, MO, USA). Cells were cultured at 37°C in air containing 5% CO_2. For routine maintenance, medium was changed every 3-4 days and cells were subcultured weekly.

2.2 | Sources of e-liquids

Twenty-three commercially available e-liquids from four different brands were purchased for this study. Vapor Emporium and Lotus brands were bought from retail shops in Nampa (ID, USA). Mister-E-liquid (https://www.mister-e-liquid.com/) and Vape Dudes (https://www.vapedudes.com) were purchased online. Each e-liquid arrived packaged in a sealed bottle that was labeled by the manufacturer as containing 0 mg/mL nicotine (used as a nicotine-free control) or 24 mg/mL nicotine. Refer to Table 1 for information on e-liquid flavors

TABLE 1 EC_{50} values for e-liquids from Vapor Emporium, Lotus, Mister-E-liquid and Vape Dudes brands

Flavor	Brand	Name	PG/VG	Stock concentration nicotine (mg/mL)	MG-63 EC_{50} (% volume)	Saos-2 EC_{50} (% volume)
Flavorless	Vapor Emporium	Flavorless	Not reported	0	3.10	3.10
				24	2.38	3.26
	Mister E-liquid	Clear	50/50	0	6.19	3.27
				24	4.71	2.38
	Vape Dudes	Flavorless	50/50	0	5.40	3.92
				24	7.30	3.14
Watermelon	Lotus	Sweet Melon	Not reported	0	2.68	2.12
				24	2.01	1.62
	Mister E-liquid	Watermelon	50/50	0	3.47	3.32
				24	2.93	3.21
	Vape Dudes	Watermelon Drip	50/50	0	2.71	2.63
				24	2.13	2.04
Mango	Lotus	Mango Blast	Not reported	0	2.41	2.10
				24	2.76	2.18
Mixed Fruits	Lotus	XXX Berry	Not reported	0	1.93	1.64
				24	2.07	1.63
	Mister E-liquid	Heartbreaker	50/50	0	2.57	2.08
				24	2.74	2.64
	Vape Dudes	Possum Sauce	50/50	0	2.73	2.60
				24	2.51	2.28
Coffee	Lotus	Irish Latte	Not reported	0	3.20	2.49
				24	2.30	1.98
	Mister E-liquid	G.T.F.O.	50/50	0	2.40	2.36
				24	1.65	1.50
	Vape Dudes	Irish Coffee	50/50	0	6.67	2.96
				24	5.92	2.93
Apple Pie	Mister E-liquid	Gran-E's Apple Pie	50/50	0	2.11	1.78
				24	1.77	1.01
	Vape Dudes	Apple Pie	50/50	0	3.02	2.62
				24	2.63	2.41
Menthol & Watermelon	Vape Dudes	Watermelon ICE	50/50	0	2.12	2.38
				24	2.00	1.68
Menthol	Lotus	Menthol	Not reported	0	1.75	1.99
				24	1.50	2.21
	Mister E-liquid	Mister E's Menthol	50/50	0	2.15	2.32
				24	1.62	1.43
	Vape Dudes	ICE ICE	50/50	0	1.86	2.12
				24	1.93	2.19
Hot Cinnamon	Lotus	Fireball	Not reported	0	1.23	<0.004
				24	0.97	<0.004
	Mister E-liquid	Napalm	50/50	0	1.21	<0.004
				24	0.54	<0.004
	Vape Dudes	Cinn Candy	50/50	0	1.76	<0.004
				24	1.56	<0.004
Menthol & Cinnamon	Vape Dudes	FIRE & ICE	50/50	0	1.66	2.02
				24	0.97	0.01

PG, propylene glycol; VG, vegetable glycerin.

For each e-liquid, its brand, name, PG/VG ratio and stock concentrations of nicotine are identified. MG-63 and Saos-2 cells were treated for 48 h with e-liquid containing a final nicotine concentration of 0.001, 0.01, 0.1, 0.5 or 1.0 mg/mL or an equivalent volume of e-liquid without nicotine at 0.004% 0.04%, 0.4%, 2.0% and 4.0%, respectively. Each EC_{50} value (expressed as a percentage volume of the e-liquid) was calculated using a linear model of the compiled cell viability data, with or without nicotine, for each cell line.

and PG/VG ratios for each e-liquid. PG and VG are humectants that keep flavorings and nicotine in suspension and facilitate vaporization when heated. Note the PG/VG ratio for the Lotus Brand and Vapor Emporium were not included on the label.

2.3 | Cell treatment

Cells were plated at different densities depending on the assay. The culture medium was changed after 24 hours and treatment was initiated in Opti-MEM medium, which is serum-free and phenol-red free (Invitrogen, Carlsbad, CA, USA). Sterile filtered e-liquid treatments were prepared by diluting unvaped e-liquid in Opti-MEM medium. Cells were treated for 48 hours with e-liquid containing a final nicotine concentration of 0.001, 0.01, 0.1, 0.5 or 1.0 mg/mL or an equivalent volume of e-liquid without nicotine at 0.004%, 0.04%, 0.4%, 2.0% and 4.0%, respectively. Volumes of diluted e-liquid were selected based on previously reported cell culture conditions as well as nicotine concentrations that were comparable to human blood and saliva of tobacco users (Bahl et al., 2012; Behar et al., 2017; Benowitz, 1988; Rowell et al., 2017; Russell et al., 1980). As scientific analyses of e-liquids report up to 10% inaccuracy in the actual nicotine concentrations compared to what is indicated on the manufacturer's label (Davis, Dang, Kim, & Talbot, 2015), cells were also treated with 0.001, 0.01, 0.1, 0.5 or 1.0 mg/mL nicotine purchased from Sigma-Aldrich (catalog no. 612596) for 48 hours. All experiments included an additional control whereby cells were treated with Opti-MEM serum-free medium only.

2.4 | Cell viability assay

Cells were plated at a density of 8 × 10^4 cells/well in a 96-well culture plate. After 48 hour treatment, cells were washed with phosphate-buffered saline (PBS) and incubated at 37°C with 10 μg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide (MTT; ATCC) for 4 hours. The conversion of tetrazolium salt MTT to a colored formazan by mitochondrial dehydrogenase was used to assess cell viability. After the supernatant was removed, 100 μL of dimethyl sulfoxide was added to each well and absorbance was read at 570 nm.

2.5 | Immunofluorescence detection of collagen type I protein

MG-63 cells were plated at 6 × 10^4 cells/well in poly-D-lysine/laminin eight-well culture chamber slides (BD BioSciences, Bedford, MA, USA). After treatment, cells were washed with EMEM serum-free medium, fixed with 3.7% formaldehyde, rinsed with PBS and permeabilized with methanol before being blocked for 1 hour with 2% bovine serum albumin + 0.1% Triton X in PBS. Cells were incubated with a primary antibody to collagen type I (AbCam, Cambridge, MA, USA) for 90 minutes, washed twice with 0.1% Triton X in PBS, and then followed by a 1 hour incubation with a secondary antibody conjugated to Alexa Fluor 488. Cells were washed three times with 0.1% Triton X in PBS. All incubations were done at 37°C. Collagen type I was visualized using a Nikon Epifluorescence microscope and digital images were captured using ImagePro software by media Cybergenetics (Silver Spring, MD, USA) using the same exposure time and filter setting for all images.

2.6 | RNA isolation and quantitative real-time polymerase chain reaction analysis

MG-63 cells were plated at a density of 6 × 10^5 cells/well in six-well culture plate. After treatment, cells were washed twice with PBS and total RNA was extracted using the EZNA® Total RNA Kit (Omega Bio-Tek, Norcross, GA, USA). RNA concentrations and purity were measured by ultraviolet absorbance, and quality was assessed on an agarose bleach gel. RNA was reverse-transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA, USA). Gene-specific primers (listed below) were used for quantitative reverse transcription-polymerase chain reaction, which was performed using Roche FastStart Essential DNA Green Master reaction mix on a LightCycler® 96 thermocycler (Roche, Indianapolis, IN, USA) (Table 2).

2.7 | Statistical analysis

For all experiments, the mean ± SEM values represent at least three independent experiments. MTT data were analyzed using the Dunnett's test for comparisons between e-liquid treatments and the medium-only control. For multiple comparisons to assess the influence of the cell line or nicotine, we used a two-way ANOVA followed by a Tukey post-hoc test. Each EC_{50} value (expressed as percentage volume of e-liquid) was calculated using a linear model of the compiled cell viability data, with or without nicotine, for each cell line. Relative mRNA levels were estimated using the $\Delta\Delta C_q$ method normalized to GAPDH, and data were presented as a fold-change compared to culture medium only control. ΔC_q values were used for statistical testing using the Bonferroni post-hoc test. Collagen type I immunofluorescence staining was quantified using Image J software (National Institutes of Health, Bethesda, MD, USA), and the amount of staining was expressed as the percentage of the total area of the captured image. $P < 0.05$ was considered statistically significant. All statistical analyses were carried out using the software program SigmaPlot 13.0.

TABLE 2 PCR primers used in this study

	Primer sequence (5′-3′) for qRT-PCR	Temp (°C)
RUNX2	TAT GGC ACT TCG TCA GGA TCC	64°C
	AAT AGC GTG CTG CCA TTC G	
Col1a1	AAC ATG ACC AAA AAC CAA AAG TG	63°C
	CAT TGT TTC CTG TGT CTT CTG G	
GAPDH	CTC TGC TCC TCC TGT TCG AC	53°C
	TTA AAA GCA GCC CTG GTG AC	

qRT-PCR, quantitative reverse transcription-polymerase chain reaction.

3 | RESULTS

3.1 | Degree of osteotoxicity occurs independently of nicotine and is flavor-dependent

A variety of flavored and flavorless e-liquids from four different brands were selected for cytotoxicity screening. To assess the effect of nicotine, a nicotine-free matched control was used. Each experiment also included a culture medium only control. Table 1 summarizes the results of cytotoxicity screening of 23 nicotine-containing e-liquids and their matching nicotine-free e-liquids with EC_{50} values for both MG-63 and Saos-2 osteoblast-like cell lines.

Dose-response MTT experiments using MG-63 and Saos-2 cells exposed to selected flavorless, fruity, coffee, menthol and cinnamon flavored e-liquids are shown in Figures 1 and 2. An important finding from these experiments was that a dose-dependent decrease in viability was detected after 48 hours exposure to all e-liquids tested in both cell lines compared to culture medium only. Several of the e-liquids were highly cytotoxic at 2% volume or higher, contributing to data variability (Figures 1D, 1E, 1I, 1J and 2E and 2J). However, there were no consistent differences between e-liquid treatments with or without nicotine, suggesting that the changes in viability occurred independently of nicotine.

Another key finding from these cell viability experiments was that flavored e-liquids caused a more pronounced reduction in viability compared to e-liquids without flavorings (Figures 1 and 2). Consistent among the brands tested, the least cytotoxic e-liquids were flavorless (Figures 1A, 1F and 2A, 2F). Concerning the flavored e-liquids, the degree of osteotoxicity varied. The least cytotoxic flavored e-liquids were coffee (Figures 1C, 1H and 2C, 2H) and fruity (Figures 1B, 1G and 2B, 2G), followed by menthol (Figures 1D, 1I and 2D, 2I). The most cytotoxic e-liquids were cinnamon-flavored e-liquids Fireball (Figures 1E and 2E) and Napalm (Figures 1J and 2J). These results were confirmed by treating cells with known nicotine concentrations diluted to 0.001-1.0 mg/mL. Consistent with the e-liquid nicotine-containing treatments, there were no significant changes in viability in MG-63 or Saos-2 cells exposed to nicotine purchased from Sigma-Aldrich at all concentrations tested (data not shown).

Table 3 depicts EC_{50} values for MG-63 and Saos-2 cells treated with e-liquids with or without nicotine and grouped as flavorless, coffee, fruity, menthol or cinnamon. The grouping of e-liquids further illustrates that flavorless e-liquids were the least cytotoxic and cinnamon-flavored e-liquids were the most cytotoxic. In addition, there were no significant differences between e-liquids with or without nicotine in any flavor category in either cell line. Thus, this confirms the cytotoxicity occurs independently of nicotine. Both cell lines responded similarly to the e-liquids with the only differences being that Saos-2 cells were more sensitive to the flavorless and cinnamon flavors compared to MG-63 cells. Because both osteoblast-like cell lines responded similarly to the e-liquid treatments, MG-63 cells were used in subsequent experiments. Based on EC_{50} values, solutions of 0.4% coffee- and fruity-flavored e-liquids either with or without 0.1 mg/mL nicotine were chosen for use in subsequent experiments to avoid excessive cell death. Cinnamon-flavored e-liquids were not used due to their high cytotoxicity.

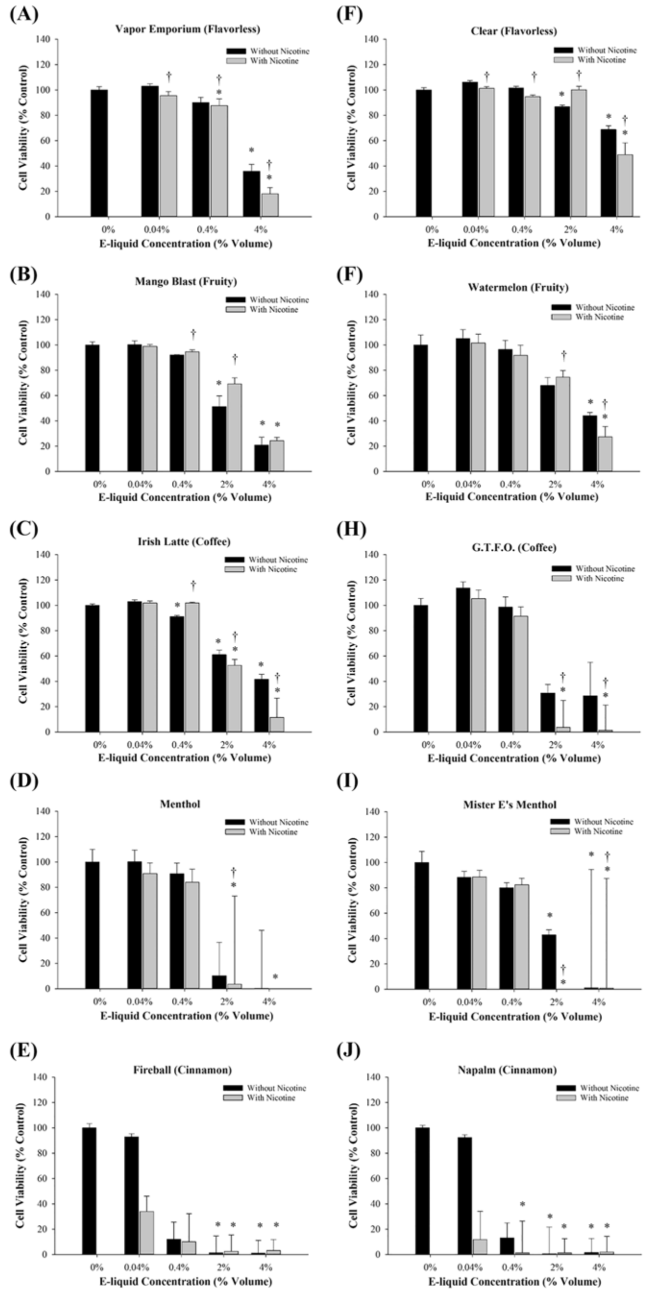


FIGURE 1 Effect of e-liquids on cell viability. MG-63 cells were treated for 48 h with e-liquid containing a final nicotine concentration of 0.01, 0.1, 0.5 or 1.0 mg/mL or an equivalent volume of e-liquid without nicotine at 0.04%, 0.4%, 2.0% and 4.0%, respectively. Cell viability was determined using the MTT assay. Results are expressed as percentage cell viability. Each bar represents the mean ± SEM of at least three independent experiments. *Significant difference from culture medium only control ($P < 0.05$). †Significant difference from the matched control without nicotine ($P < 0.05$). A-J, E-liquids tested. A, Vapor Emporium brand. B-E, Lotus brand. F-J, Mister-E-Liquid brand

3.2 | Col1a1 mRNA expression increases in response to flavored e-liquid exposure

We were interested in whether flavored e-liquids with or without nicotine altered mRNA expression of the key osteoblast genes RUNX2 and Col1a1 in MG-63 cells. Based on the results described above, we specifically chose fruity and coffee flavors, which were not overly

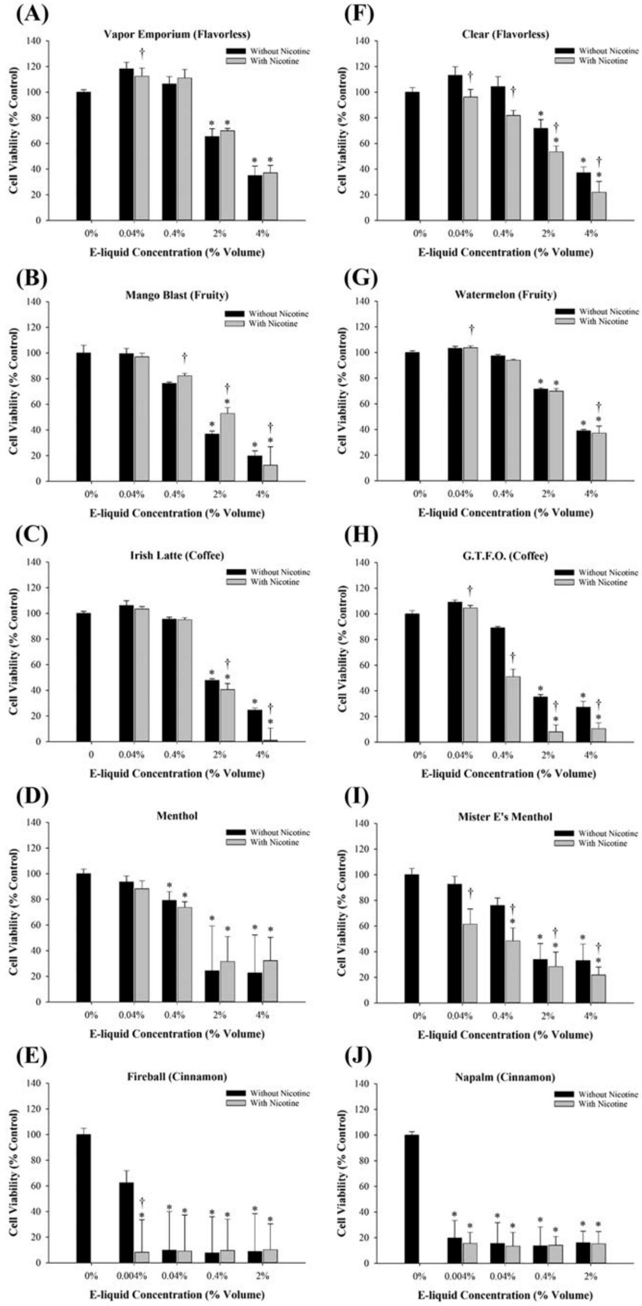


FIGURE 2 Effect of e-liquids on cell viability. Saos-2 cells were treated for 48 h with e-liquid containing a final nicotine concentration of 0.001, 0.01, 0.1, 0.5 or 1.0 mg/mL or an equivalent volume of e-liquid without nicotine at 0.004% 0.04%, 0.4%, 2.0% or 4.0%, respectively. Cell viability was determined using the MTT assay. Results are expressed as percentage cell viability. Each bar represents the mean ± SEM of at least three independent experiments. *Significant difference from culture medium only control ($P < 0.05$). †Significant difference from the matched control without nicotine ($P < 0.05$). A-J, E-liquids tested. A, Vapor Emporium brand. B-E, Lotus brand. F-J, Mister-E-Liquid brand

cytotoxic. Cells treated with 0.4% flavored Lotus e-liquids, with or without 0.1 mg/mL nicotine, showed no detectable changes in RUNX2 expression, while there was an increase in Col1a1 expression compared to culture medium control (Figure 3A-D). Mango Blast and Irish Latte flavors induced an approximate fivefold increase in Col1a1 expression (Figure 3B and 3C), whereas Sweet Melon elicited a 10-15-fold increase (Figure 3D). In contrast, treatment with flavorless e-liquid had no impact on Col1a1 expression (Figure 3A). There were no consistent differences between the nicotine-free and nicotine-containing treatments. These results suggest that the flavorings in e-liquids alone may specifically target Col1a1 in MG-63 cells although at the mRNA level these trends were not statistically significant.

3.3 | Collagen type I protein expression increases upon exposure to Mango Blast

Next, we explored whether the trend in mRNA expression would be reflected in collagen type I protein expression. Using the same concentration of e-liquid as for the mRNA experiments, MG-63 cells were treated for 48 hours with Mango Blast or Flavorless e-liquid and analyzed for collagen type I protein expression using immunofluorescence. Consistent with Figure 1B, treatment with 0.4% Mango Blast with or without nicotine resulted in no observable change in cell number (Figure 4A). The Mango Blast e-liquid treatments, with or without nicotine, significantly increased collagen type I protein expression compared to cells treated with culture medium only (Figure 4B). Consistent with the mRNA results, there were no significant changes in collagen type I expression in MG-63 cells exposed to flavorless e-liquids with or without nicotine.

4 | DISCUSSION

Nicotine delivery devices known as electronic cigarettes (e-cigarettes) are rapidly increasing in worldwide popularity among both adults and teenagers. Although advertised as a safer alternative to combustible tobacco, potential adverse health effects related to e-cigarette use remain underinvestigated. Extensive research, both in vitro and In vivo, demonstrates the detrimental impact of conventional cigarette smoke on the skeletomuscular system, in part by disrupting bone formation by osteoblasts (Ajiro, Tokuhashi, Matsuzaki, Nakajima, & Ogawa, 2010; El-Zawawy, Gill, Wright, & Sandell, 2006; Giorgetti et al., 2010; Liu et al., 2003; Marinucci et al., 2014; Vo et al., 2011). The end result can lead to decreased bone mineral density, which increases the risk for the development of osteoporosis (Abate et al., 2013). As conventional tobacco products are reported to impair normal bone formation, an understanding of how e-cigarettes may affect bone is essential to characterizing the overall health consequences of e-cigarette use. This study focuses on the impact of flavorings and nicotine found in e-liquids on human tumor-derived osteoblast-like cells and evaluates osteotoxicity and alterations of key osteoblast markers, collagen type I and RUNX2. The two cell lines used in this study, MG-63 and Saos-2, are well characterized and exhibit similar, though not identical, phenotypes to normal human osteoblasts (Czekanska, Stoddart, Richards, & Hayes, 2012; Pautke et al., 2004). It is important to note that recent publications indicate that unvaped e-liquid treatments accurately predict toxicity of corresponding aerosols, justifying the use of unvaped e-liquid treatments as a first screening model for e-cigarette in vitro studies (Behar et al., 2017; Rowell et al., 2017).

In this study, we use nicotine concentrations comparable to those found in blood and saliva of tobacco users that can range from

TABLE 3 Compiled EC_{50} values (percentage volume of e-liquid) by flavor categories

Cell line	Nicotine	Flavorless	Fruity	Coffee	Menthol	Cinnamon
MG-63	–	4.90 ± 0.13	2.64 ± 0.05*	4.09 ± 0.22	1.92 ± 0.04*	1.40 ± 0.09*
	+	4.80 ± 0.21	2.45 ± 0.06*	3.29 ± 0.29*	1.68 ± 0.05*	1.02 ± 0.20*
Saos-2	–	†3.08 ± 0.06	2.36 ± 0.06	2.60 ± 0.05	2.14 ± 0.03*	<0.004*,†
	+	†2.99 ± 0.06	2.23 ± 0.10	2.13 ± 0.14*	1.94 ± 0.09*	<0.004*,†

"Flavorless" e-liquids include Flavorless (Vapor Emporium), Clear (Mister-E-Liquid) and Flavorless (Vape Dudes). "Coffee" e-liquids include Irish Latte (Lotus), G.T.F.O. (Mister-E-Liquid) and Irish Coffee (Vape Dudes). "Fruity" e-liquids include Sweet Melon (Lotus), Watermelon (Mister-E-Liquid), Watermelon Drip (Vape Dudes), Mango Blast (Lotus), XXX Berry (Lotus), Heartbreaker (Mister-E-Liquid) and Possum Sauce (Vape Dudes). "Menthol" e-liquids include Menthol (Lotus), Mister E's Menthol (Mister-E-Liquid) and ICE ICE (Vape Dudes). "Cinnamon" e-liquids includes Fireball (Lotus), Napalm (Mister-E-Liquid) and Cinn Candy (Vape Dudes).

*Significant difference when compared to the flavorless category within a cell line ($P < 0.05$).

†Significant difference when compared to same e-liquid treatment in other cell line ($P < 0.05$).

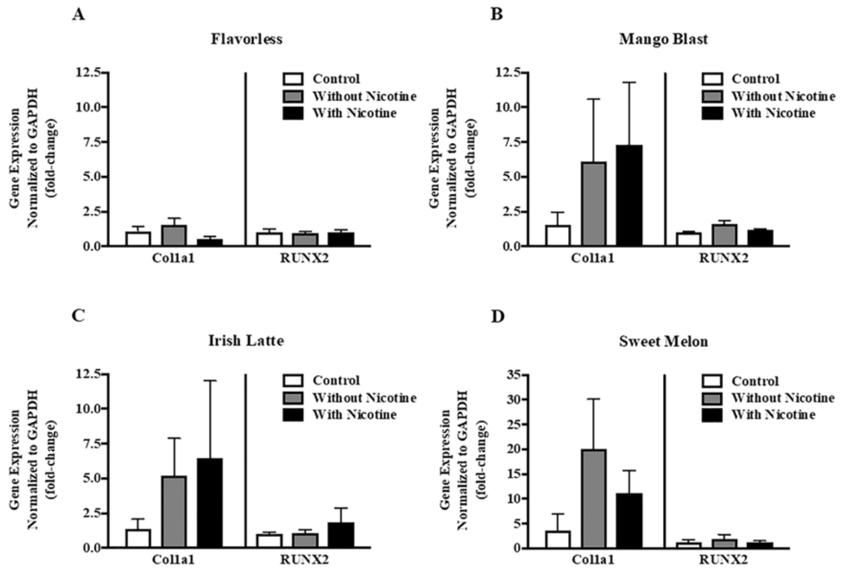
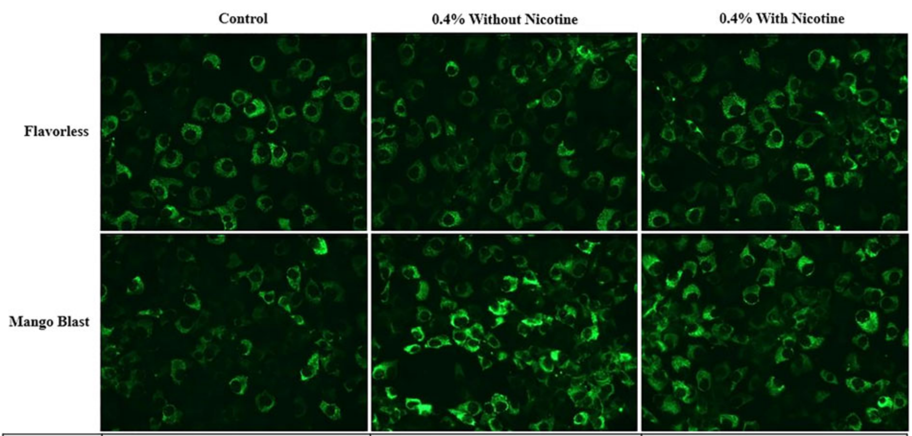


FIGURE 3 Effect of e-liquids on mRNA expression. MG-63 cells were treated for 48 h with culture medium only, 0.4% e-liquid treatment without nicotine or 0.4% e-liquid treatment containing 0.1 mg/mL nicotine. A-D, E-liquids used. A, Vapor Emporium Flavorless, Lotus brand. B, Mango Blast. C, Irish Latte. D, Sweet Melon. Col1a1 and RUNX2 mRNA expression was measured by quantitative reverse transcription-polymerase chain reaction and normalized to GAPDH. Each bar represents the mean ± SEM of at least three independent experiments



E-liquid	Control	0.4% Without Nicotine	0.4% With Nicotine
Flavorless	1.57 ± 2.22	1.94 ± 1.01	1.74 ± 1.33
Mango Blast	1.90 ± 0.71	*6.37 ± 1.36	*7.93 ± 2.70

FIGURE 4 Immunofluorescence detection of cytosolic collagen type I protein. MG-63 cells were treated for 48 h with Lotus brand Mango Blast or Mister-E-liquid Clear (Flavorless). A panel of images representative of one experiment with culture medium control, 0.4% e-liquid treatment without nicotine and 0.4% e-liquid treatment containing 0.1 mg/mL nicotine. Images were analyzed and quantified as the percentage area of the image within the intensity threshold using ImageJ software. Values presented are the mean ± SEM of three independent experiments. *Significant difference from culture medium only control ($P < 0.05$)

0.03 µM to 10 mM (Benowitz, 1988; Russell et al., 1980). Here we report a dose-dependent decrease in cell viability in Saos-2 and MG-63 osteoblast cell lines exposed to 23 different e-liquids without nicotine and with 0.01-1.0 mg/mL (6.2 µM-6.2 mM) nicotine compared to cells treated with culture medium only. Interestingly, we report no significant differences between e-liquid treatments with or without nicotine when grouped by flavor categories, suggesting the decrease in viability occurs independently of nicotine. Furthermore, other researchers using a direct unvaped exposure method, with comparable nicotine concentrations and culture conditions, report e-liquids to induce cytotoxicity irrespective of the presence of nicotine in human gingival fibroblasts and oropharyngeal mucosa cells, human embryonic stem cells, adult pulmonary fibroblasts and mouse neural stem cells. In addition, two studies using e-liquid vapor extracts report cytotoxicity occurring independently of nicotine in myocardial and airways related cells (Farsalinos et al., 2013; Leslie et al., 2017). Hence, several studies to date that use different cell types and screen a wide variety of e-liquid brands and flavors report cytotoxicity differences related to flavorings rather than nicotine alone.

The current research demonstrates a spectrum of osteotoxicity that is flavor-dependent and consistent among the brands tested. Key to this conclusion is that treatments with unflavored e-liquids are the least cytotoxic, thereby implying that flavoring agents are a primary contributor to cytotoxicity. The observed trend from least to greatest osteotoxicity is as follows: unflavored, coffee and fruity, menthol and cinnamon (Figure 5). This trend is consistent between the two cell lines, although Saos-2 is more sensitive, particularly to the cinnamon-flavored e-liquids. These findings are similar to others that report flavored e-liquids to have varying degrees of cytotoxicity. For example, fruity flavors, in particular strawberry, show greater cytotoxicity among a variety of flavors tested in airways cells exposed to vaped extracts (Leslie et al., 2017). Using a similar experimental design to the current study, oropharyngeal mucosal cells treated with 10%-25% volume of unvaped e-liquids for 24 hours show fruity flavors to be more cytotoxic than tobacco flavors (Welz et al., 2016). Another study reports that menthol, strawberry and coffee flavors are overly cytotoxic to H292 human bronchial epithelial cells using an air-liquid interface exposure method (Leigh, Lawton, Hershberger, & Goniewicz, 2016). A trend consistently reported, in a variety of cell types, is that cinnamon flavors tend to be the most cytotoxic using both unvaped and vaped exposure methods (Bahl et al., 2012; Behar et al., 2017; Lerner et al., 2015).

Mounting evidence demonstrates that e-liquid flavorings alone can induce adverse cellular effects and points to the need to investigate the chemicals used as flavoring agents. For example, exposure to the e-liquid chemicals vanillin and chocolate 2,5-dimethylpyrazine leads to cell death via cystic fibrosis transmembrane conductance regulator through protein kinase A activation in airways epithelial cells (Sherwood & Boitano, 2016). In relation to our study, Behar et al., 2014 identified chemicals in cinnamon-flavored e-liquids with cinnamaldehyde being the dominant flavoring chemical (Behar et al., 2014). In support of the current study, a recent report demonstrates cinnamaldehyde exposure to be the most cytotoxic among the flavoring chemicals tested in human monocytic cell lines (Muthumalage et al., 2017). Furthermore, treatment with non-cytotoxic concentrations of cinnamaldehyde leads to a proinflammatory response in human lung epithelial cells and fibroblasts (Gerloff et al., 2017) and results in cytoskeletal alterations and genotoxicity in human pulmonary fibroblasts (Behar et al., 2016). Interestingly, cinnamaldehyde also is commonly found in fruit- and sweet-flavored e-liquids (Behar et al., 2016). Hence, the widespread use of cinnamaldehyde in e-liquids warrants further mechanistic studies on its toxic action, including in bone.

The expression of RUNX2 is essential for the development, maturation and maintenance of osteoblasts (Ducy et al., 1999). Here we report no detectable change in RUNX2 expression in MG-63 cells exposed to any of the coffee or fruity e-liquids tested with or without nicotine when compared to culture medium only. One possible explanation for these results is the concentration of nicotine used. For example, Kim et al., report a decrease in RUNX2 expression in alveolar bone marrow-derived mesenchymal stem cells when exposed to 2 mM nicotine, a higher concentration than the 0.62 mM used in this study (Kim et al., 2012). Another study reports RUNX2 mRNA to be repressed in human osteoblasts cultured with 0.1-10 µM nicotine, but only after chronic continuous exposure (Marinucci et al., 2014). Another variable to consider is that RUNX2 expression varies depending on the state of osteoblast maturation and mineralization in culture (Prideaux et al., 2014). MG-63 cells are less differentiated (pre-osteoblastic phenotype) compared to Saos-2 cells (Czekanska et al., 2012). Hence, an interesting follow-up study would be to examine RUNX2 mRNA in Saos-2 cells exposed to e-liquids.

In contrast to RUNX2, Col1a1 mRNA expression is upregulated in MG-63 cells treated with highlighted coffee and fruity e-liquids with or without nicotine, but not in the flavorless e-liquid, when compared

FIGURE 5 Diagram depicting osteotoxicity gradient. Position on the diagram represents the relative osteotoxicity between flavor categories, as defined in Table 3. Osteotoxicity is shown from least to greatest as read from left to right. For each of the brands tested: the flavorless e-liquid is the least cytotoxic; fruit-flavored and coffee-flavored e-liquids are mildly cytotoxic; menthol-flavored e-liquids are more cytotoxic than the aforementioned; and cinnamon-flavored e-liquids are the most cytotoxic

to culture medium only. Upon further analysis using Lotus brand Mango Blast, with or without nicotine, there is also a significant increase in collagen type I protein expression. Others report a biphasic effect of nicotine on collagen type I mRNA levels in MG-63 cells, with increasing expression observed at nicotine concentrations less than 100 μM and decreasing expression at concentrations of 1 mM and higher (Rothem et al., 2009). The nicotine-containing treatment in our collagen type I RNA and protein experiments had a concentration of 620 μM (0.1 mg/mL), which falls in between these previous reports, although it is below the 1 mM concentration reported to result in downregulation. Consistent with the biphasic nature of nicotine, when human oral fibroblasts are exposed to unvaped e-liquid containing 6.2 mM nicotine for 24 hours there is a decrease in collagen type I protein (Sancilio et al., 2017). In addition to nicotine, the flavorings alone may alter osteoblast gene expression. For example, Col1a1 mRNA expression was increased in adult osteopenic ovariectomized mice fed a diet of dried mango but not in mice fed dried grape or apricot (Rendina et al., 2013). This study implies natural dried mango has chemical properties that could modulate osteoblast functionality. It remains to be determined whether chemicals used to create artificial mango flavors, like those used in e-liquids, could induce the same responses in osteoblasts. Collectively, this study supports further investigation into the cellular mechanisms by which e-liquids alter osteoblast gene expression, such as the induction of oxidative stress by e-liquid exposure (Bitzer et al., 2018; Lerner et al., 2015; Muthumalage et al., 2017).

There are several challenges to e-cigarette research and studying e-liquid cytotoxicity (Orr, 2014). The lack of manufacturing standards and content labeling on e-liquid bottles creates obstacles for toxicological evaluations. The vast number of e-cigarette models and e-liquids available on the market compound the issue. Another challenge is the lack of standardized in vitro testing that is physiologically relevant to the vaping experience (Lerner et al., 2015; Neilson et al., 2015; Romagna et al., 2013). Thus, using unvaped e-liquids allows for fast screening and provides a way to compare studies from different laboratories and identify cytotoxic e-liquids that warrant further chemical and biological characterization. Standardization in the manufacturing of e-cigarette products, consistent and reliable disclosure of chemical content in e-liquids, and stringent testing procedures are needed for robust toxicological assessments and chemical analyses of e-liquids.

We conclude that the degree of osteotoxicity is flavor-dependent and occurs independently of nicotine and that flavored e-liquids reveal collagen type I as a potential target in osteoblasts. This study provides insight into the potential impact of e-cigarette use on bone health and points to the need for further studies to assess the impact of e-liquid flavorings in bone.

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