OnLine Journal of Biological Sciences

Original Research Paper

Effects of Electronic Cigarette E-Liquids on the Adhesion and Growth of Osteoblast Cells

[1,2]Ana Carla Lorenz-Vieira, [2]Cesar Augusto Magalhães Benfatti and [1]Mahmoud Rouabhia

[1]Groupe de Recherche en Écologie Buccale, Université Laval, Québec City, QC, Canada
[2]Center for Education and Research on Dental Implants, Post-Graduate Program in Dentistry, Federal University of Santa Catarina, Florianopolis, SC, Brazil

Article history
Received: 22-11-2022
Revised: 01-02-2023
Accepted: 27-03-2023

Corresponding Author:
Mahmoud Rouabhia
Groupe de Recherche en Écologie Buccale, Université Laval, Québec City, QC, Canada
Email: Mahmoud.Rouabhia@fmd.ulaval.ca

Abstract: Electronic cigarettes (E-cigarettes) were designed to replace traditional cigarettes to decrease the harmful effects of smoking. The liquid in e-cigarettes contains different chemicals, such as Propylene Glycol (PG) and Vegetable Glycerin (VG), and may contain nicotine. To be more attractive, e-liquids are supplemented with flavorings. Because vaped e-liquids are shown to be damaging to oral tissues, similar effects could occur in the oral cavity with non-vaped e-liquids. We evaluated the effects of non-vaped e-liquid constituents on osteoblast behavior. For this purpose, human osteoblast cells (MG-63) were exposed to e-liquid containing 70% PG -30% VG with and without tobacco flavor and with or without nicotine at 12, 18 mg/mL, or no nicotine content. The e-liquids were used at various concentrations (0, 1, and 5%). To evaluate the effect of the e-liquids on osteoblast morphology and adhesion, optical microscope observations and viable cell counting using trypan blue exclusion were used. Cell growth was also analyzed using the Methyl Thiazol Tetrazolium (MTT) assay, while cytotoxicity was tested by measuring Lactate Dehydrogenase (LDH) levels after cell exposure for 24 h to the e-liquids. Our results show that e-liquids induced significant morphological changes evidenced by round cell forms, with no contact between cells. Adhesion was significantly reduced, particularly in the presence of nicotine. E-liquids at a concentration of 5% significantly reduced osteoblast growth. This effect was observed with both flavored and non-flavored e-liquid with or without nicotine. The decreased osteoblast adhesion and growth after exposure to e-liquid was confirmed by increased levels of LDH. Overall results indicate the potentially harmful effects of e-liquid non-vaped chemicals on bone cells, which could lead to the impairment of bone regeneration and tissue remodeling processes.

Keywords: Electronic Cigarettes, E-Liquid, Osteoblast, Cell Adhesion, Cell Growth, LDH

Introduction

Cigarette smoking causes harmful effects associated with oropharyngeal and lung cancer, cardiovascular illnesses, and chronic diseases, to name a few. It is also the leading cause of avoidable deaths (National Center for Chronic Disease Prevention and Health Promotion (US) Office on Smoking and Health, 2014). The continuous use of cigarettes is due to the addictive tobacco-derived product that is nicotine (Benowitz *et al*., 2009; Randall *et al*., 2023). More than 7,000 chemical components are found in cigarette smoke, depending on the type and brand of cigarette used and many of these chemicals cause serious health problems for smokers (Onor *et al*., 2017).

The oral cavity is the first body site to come in contact with cigarette smoke; smokers are therefore at increased risk of developing oral lesions that may be associated with cancer development (National Center for Chronic Disease Prevention and Health Promotion (US) Office on Smoking and Health, 2014). Furthermore, tobacco users are more likely to suffer from oral infections, including candidiasis and periodontal disease, and are at a higher risk of undesirable outcomes in dental implant surgeries (Javed *et al*., 2019).

In an effort to counter the adverse effects of cigarette smoke, a new smoking device called an Electronic cigarette (e-cigarette) was developed and advertised for smokers. e-cigarettes are composed of a battery and a cartridge that

holds a liquid to be aerosolized, producing the vapor by an electric heater (Otero *et al*., 2019). The liquid is primarily constituted of Propylene Glycol (PG) and Vegetable Glycerin (VG) and may contain nicotine (Williams *et al*., 2022). To be more attractive to smokers, e-liquids are supplemented with flavorings such as tobacco, menthol, candy, and many others (Breland *et al*., 2017). These flavors could damage the airways of e-cigarette users (Gschwend *et al*., 2023; Ramôa *et al*., 2017; Williams *et al*., 2022).

PG and VG are humectants that mimic cigarette smoke and transport nicotine during vaping. While VG provides visual smoke and improves taste and smell, PG is associated with health issues such as sore throat, headache, and dizziness (Harvanko *et al*., 2019; Guo *et al*., 2022; Noël and Ghosh, 2022). These main e-liquid constituents have been generally considered to be safe ingredients for oral ingestion (FDA, 1980). However, there is no consensus as to their safety when heated and inhaled.

The oral cavity is the first site to come in contact with e-cigarette aerosols. Indeed, these e-liquid products come in contact with the teeth, the gingival tissue, and the microorganisms present in the oral cavity. E-liquids and their aerosol have been shown to display cariogenic properties, as they contain nicotine and sugary flavors (Catala-Valentin *et al.*, 2022; Kim *et al*., 2018). Furthermore, these humectants (PG and VG) have been found to not only increase the adhesion of *Streptococcus mutans* to e-cigarette users' teeth but also the formation of biofilm (dental plaque) (Rouabhia and Semlali, 2021). E-cigarettes may also be associated with periodontal inflammation due to the e-liquid's negative chemical interaction with epithelial tissues (Holliday *et al*., 2021). Studies have shown that exposure to e-cigarette aerosol decreases the adhesion and proliferation of gingival fibroblasts and epithelial cells (Alanazi *et al*., 2018; Beklen and Uckan, 2021; Rouabhia *et al*., 2017) and that e-cigarette flavors could cause significant damage leading to high oxidative stress, DNA damage, and increased destructive inflammatory cytokine secretion, contributing to tissue damage (Javed *et al*., 2019). E-cigarette aerosol damage may affect oral tissues such as soft (gingival mucosa) and hard tissues (bone), as well as the interaction of gingival tissue with oral bone and restorative materials, such as dental implants. A previous study reported an adverse effect of e-cigarette aerosol on the interaction between osteoblasts and dental implants by decreasing F-actin expression (therefore cell growth), which compromises the formation of bone tissue. This deregulation would suggest postoperative complications and even implant failure (Rouabhia *et al*., 2019; Youssef *et al*., 2022).

Several studies suggested the possible deleterious effects of e-cigarette aerosol on the oral cavity. These harmful effects can be attributed to vaped e-liquid that has been heated. However, non-heated chemicals such as PG or VG may also present certain toxic effects on human cells (Komura *et al*., 2022; Morshed *et al*., 1998).

Woodall *et al.* (2020) recently reported that the exposure of human epithelial cells to PG or VG decreased glucose transport and metabolism in these cells, leading to the deregulation of epithelial barrier behaviors. It is suggested that these harmful effects could also occur with osteoblasts.

We thus evaluated the effects of non-aerosolized e-cigarette liquids on osteoblast adhesion and growth. Specifically, we investigated the impact of different flavored and non-flavored e-liquids with and without nicotine on osteoblast adhesion, morphology, growth, and the release of lactate dehydrogenases.

Materials and Methods

Source of the E-Liquids

E-liquids were purchased from local retailers (Québec City, QC, Canada). The selected e-liquids were chosen as per their availability and use by consumers. The e-liquids we used contained either 70% PG-30% VG, 70% PG-30% VG + Favor, 70% PG-30% VG + Favor + nicotine at 12 mg/mL, or 70% PG-30% VG + Favor + nicotine at 18 mg/mL (Table 1). The flavor tested in this study was artificial tobacco flavoring (smooth Canadian tobacco). Different concentrations (0, 1, and 5%) of each e-liquid were used to perform the experiments.

Cell Culture

Human osteosarcoma cells, namely, MG-63 with osteoblastic properties (ATCC, CRL-1427™), were selected for this study. The cells were cultured in Dulbecco Vogt's Modified Eagle's (DME) medium and Ham's F-12 (H) supplemented with 24.3 µg/mL of adenine, 10 µg/mL of human epidermal growth factor, 0.4 µg/mL of hydrocortisone, 5 µg/mL of bovine insulin, 5 µg/mL of human transferrin, 2×10^{-9}M 3, 3', 5' triiodo-L-thyronine, 100 µg/mL of penicillin, 25 µg/mL of gentamicin and 10% fetal calf serum. Each incubation was performed at 37ºC in a humid 5% CO_2 atmosphere to maintain cell growth. The culture medium was refreshed every 24 h until 80-90% confluence was attained.

Effect of E-Liquid on Osteoblast Adhesion and Morphology

Osteoblastic cells were seeded at a density of 2×10^5 cells/well in 6-well plates. The culture medium was supplemented with one or the other e-liquid at three concentrations (0, 1, or 5%), for a total volume of 2 mL per well. The cells were incubated at 37°C in 5% CO_2 for 24 h. Following this exposure period, cell adhesion was qualitatively assessed by photographing the adherent and non-adherent cells using a digital camera (model Coolpix 995, Nikon). This was confirmed by a quantitative measurement by means of a Trypan Blue (TB) exclusion assay. Briefly, the cells were detached from each culture well-using trypsin; they were then washed twice with culture medium and used for cell viability analyses.

Table 1: The different e-liquids tested in this study

Group (G)	E-liquid constituents
G1	70% PG -30% VG
G2	70% PG -30% VG + flavor
G3	70% PG -30% VG + flavor + nicotine at 12 mg/mL
G4	70% PG-30% VG + flavor + nicotine at 18 mg/mL

PG: Propylene Glycol; VG: Vegetable Glycerin

Each cell pellet was suspended in 1 mL of culture medium and a 20 mL volume of each cell suspension was suspended with 20 mL of TB, followed by incubation in ice for 5 min. The cell mixture was then placed into a hemacytometer and the viable cells (non-labeled in blue) and dead cells (labeled in blue) were counted using an inverted optical microscope (Nikon Optiphot). The experiments were repeated at least 3 independent times in duplicate.

Effect of E-Liquid Constituents on Osteoblast Growth

Osteoblast cells were first seeded (2×10^5 cells/well) in each well of 12-well plates in 1 mL of culture medium, then incubated for 48 h at 37°C in 5% CO_2 before being exposed to e-liquid. For each culture, the medium was refreshed and supplemented with either 0, 1, or 5% of each e-liquid group (G1, G2, G3, and G4). Cell exposure to the e-liquid was for 24 h at 37°C in a 5% CO_2 humid atmosphere. To evaluate the toxicity of e-liquids on osteoblast cells, we used an MTT [(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium-bromide)] assay, as previously reported (Denizot and Lang, 1986). Briefly, 1% (v/v) of MTT solution (5 mm/mL) was added to each well; the plates were then wrapped in aluminum foil and incubated for 3 h at 37°C. At the end of the incubation, the supernatant was removed, the culture was rinsed once with warm PBS and 500 mL of 0.04 M HCl in isopropanol was added to each well, followed by incubation at room temperature under agitation for 15 min in the dark. Following this incubation, 120 mL of the reaction solution were transferred in quadruplet to a 96-well plate, with absorbance read at 550 nm using a spectrophotometer (model 680; bio rad laboratories, Mississauga, Canada).

Effect of E-Liquid Constituents on Lactate Dehydrogenase (LDH)

Osteoblasts were seeded at 2×10^5 cells/well in 6-well plates and exposed thereafter for 24 h to e-liquid solutions at 0, 1, and 5% concentrations. Following incubation, the cell culture medium was collected and used to measure cell toxicity using an LDH cytotoxicity assay (Promega, Madison, WI, USA) (Melo *et al*., 2002). Briefly, 50 mL of supernatant was transferred to a 96-well flat-bottom plate, supplemented with 50 mL of reconstituted substrate mix, then incubated in the dark at room temperature for 30 min. The assay promotes a conversion reaction of L-lactate and NAD to pyruvate and NADH through the oxidation of NADH to NAD^+, thereafter releasing LDH into the cell culture medium due to the rupture of the plasma membrane (Gleitz *et al*., 1996). The reaction was stopped by adding 50 mL of an acid solution to each well. Subsequently, 100 mL of each reacted solution was transferred (in quadruplicate) to a new 96-well flat-bottom plate and the absorbance was read using an X-mark microplate spectrophotometer (Bio-Rad, Mississauga, on, Canada) at a 490 nm wavelength. A positive control referring to the cell incubation in the presence of 1% Triton × 100 was prepared to generate maximum LDH release, while a negative control referring to the cell incubation without e-liquid was included in the experiments to generate minimum LDH release. The experiment was repeated at least three independent times, in duplicate.

Statistical Analysis

Results were expressed as means ± Standard Deviation (SD), considering that each experiment was performed at least three times. Data were analyzed by comparing the differences between the control (0% e-liquid) and test substances in the other concentrations (1 and 5%). Data normality was assessed by the Shapiro wilk test and Levene's test of variance, with the p-value, considered significant at <0.05. A One-way Analysis of Variance (ANOVA) parametric test was used to determine the statistical significance between the values having a normal distribution. For the data with non-normal distribution, a non-parametric Kruskal Wallis test was performed. In a post hoc analysis, Tukey and Bonferroni's adjustment of the p-value was applied to compare differences between intra and inter-groups. These statistical analyses were all performed using SPSS version 21.0 (IBM Corp., Armonk, NY, USA).

Results

E-Liquid Modulated Osteoblast Morphology and Deregulated Cell Adhesion

In this study, we included different types of e-liquids based on their compositions. These e-liquids included 70% PG -30% VG with or without flavor and with or without nicotine. Each e-liquid was tested at different concentrations (0, 1, and 5%), with 0% considered as the control (only culture medium). After 24 h of exposure to the e-liquid, osteoblast morphology and adhesion were analyzed. The cell exposure to the 70% PG -30% VG e-liquid revealed shape changes and a reduced adhesion with the high concentration (5%) of this e-liquid (Fig. 1). Figure (1A), cell morphology was changing, showing round cells with no contact evidenced between cells, compared to what was observed in the control (Fig. 1A). These observations were confirmed quantitatively (Fig. 1B). Cell viability assessed by trypan blue exclusion showed a significant ($p<0.001$) reduction in the number of viable cells following their contact with e-liquid containing 70% PG 30% VG. Indeed, the viable cell number decreased

from 20.2±2.9 x 10^4 cells in the control to 18.6±2.9 × 10^4 cells in the presence of e-liquid at 1% and 10.4±2.5 × 10^4 cells at the 5% concentration (Fig. 1B).

Exposure to an E-liquid containing 70% PG -30% VG + flavor at 1 and 5% altered osteoblast shape (Fig. 2A), resulting in the presence of round cells with no cell contact, compared to that observed in the control (cells not exposed to the e-liquid), suggesting deregulation of cell adhesion. These observations were quantitatively confirmed by cell viability measurements. Figure (2B), cell viability decreased following the exposure to flavor-rich e-liquid (70% PG -30% VG). The number of viable cells decreased from 30.5±8 × 10^4 cells in the control to 26.6±9 × 10^4 cells with e-liquid at 1% and 14.9±2.9 × 10^4 cells at the 5% concentration (Fig. 2B). The decrease was significant ($p<0.001$) with 5% e-liquid.

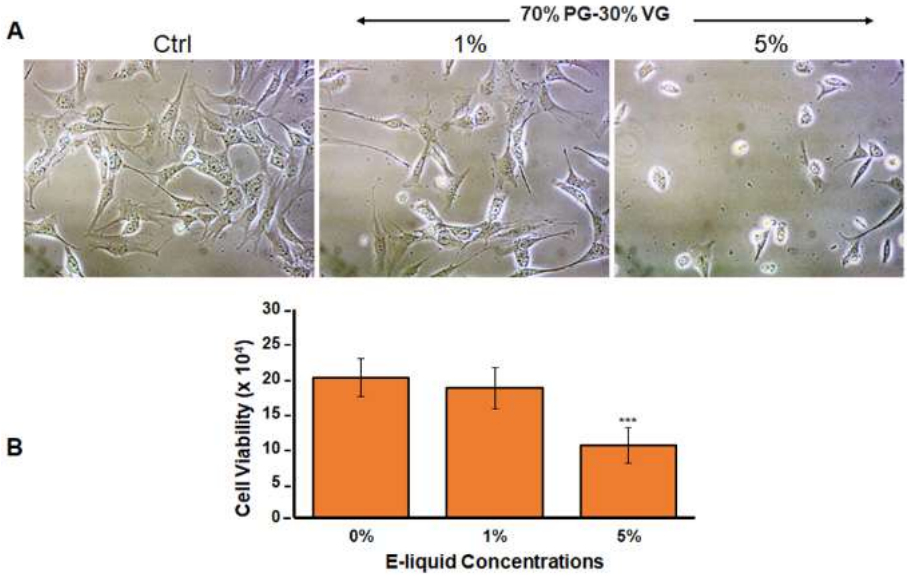

Fig. 1: Unflavored e-liquid altered osteoblast morphology and cell viability. (A) Osteoblast shape after exposure to 70% PG -30% VG e-liquid for 24 h. Representative images were observed under an inverted optical microscope. (B) Cell adhesion was evaluated by trypan blue exclusion assay. Each bar represents the mean ± SD of at least three independent experiments. ***$p<0.001$ when comparing the control and the 5% condition

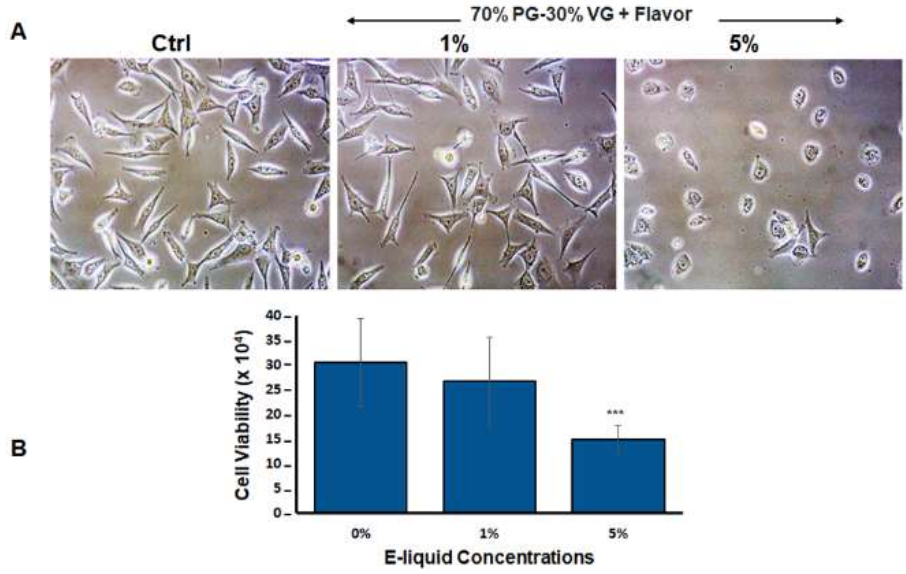

Fig. 2: Exposure to flavored e-liquid altered osteoblast morphology and viability. (A) Cell morphology after exposure for 24 h to e-liquid containing 70% PG -30% VG + flavor. (B) The number of adherent osteoblasts was determined by trypan blue exclusion assay. Results are the mean ± SD of at least three independent experiments. ***$p<0.001$ when comparing the control and the 5% condition

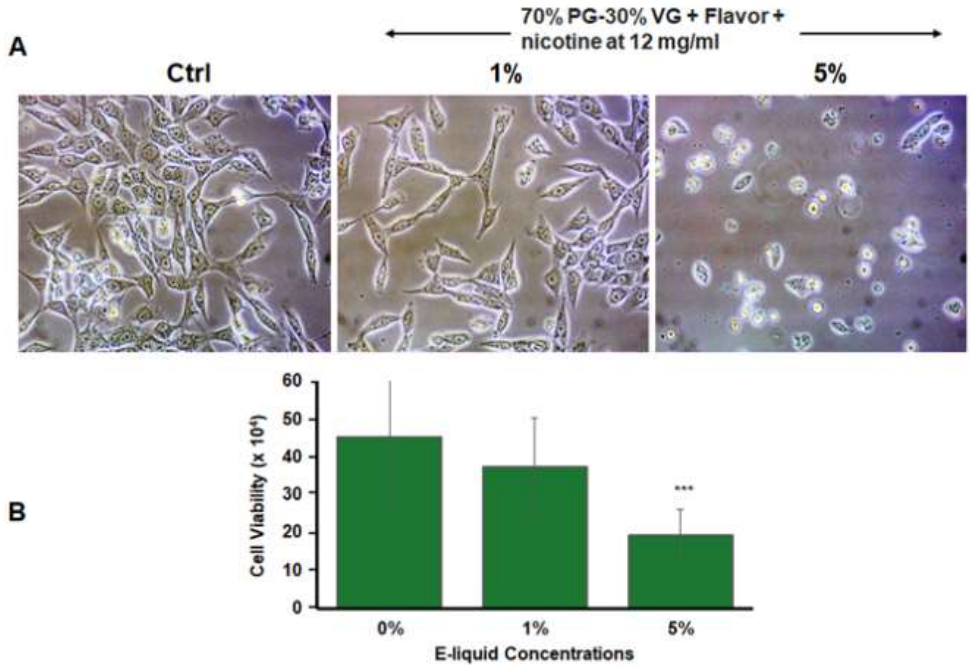

Fig. 3: Flavored nicotine-rich E-liquid altered cell morphology and adhesion. (A) Osteoblast morphology. (B) Live adherent cells. Results are presented as the mean ± SD of at least three independent experiments. ***p<0.001

The exposure to 70% PG -30% VG + flavor + nicotine at 12 mg/mL led to osteoblast damage in terms of cell shape and adhesion. Figure 3A, the cell morphology changed from cuboidal with a small nucleus to round cells with an almost non-visible nucleus, basically with the 5% e-liquid concentration (Fig. 3A). The morphological changes were supported by cell viability assessment. Figure 3B, cell viability decreased significantly ($p<0.001$) after exposure to various concentrations of the e-liquid containing 70% PG -30% VG + flavor + nicotine at 12 mg/mL. Indeed, the number of viable cells dropped from 45.2±17 × 104 cells in the control to 37.5±12 × 104 cells with e-liquid at 1% and 19.4±7 × 104 cells at 5% (Fig. 3B). The decrease was significant ($p<0.001$) with 5% e-liquid.

The cell exposure to the 70% PG -30% VG + flavor + nicotine at 18 mg/mL also revealed morphological changes with both the 1% and the 5% concentrations of e-liquid. With the 1% concentration, fewer cells were adhering, which reduced the interaction between cells, as few dendrites contributing to this cell interaction were evidenced (Fig. 4A). With 5%, a high number of cells were round and floating, suggesting the negative effect of this e-liquid on cell adhesion (Fig. 4A). Cell viability assessment confirmed these observations. Figure 4B shows a significant reduction ($p<0.001$) in the number of viable cells when exposed to the 70% PG -30% VG + flavor + nicotine at 18 mg/m, 5% concentration.

Overall results show that all the tested e-liquids decreased cell adhesion and that the decrease was greater in the presence of nicotine (12 and 18 mg/mL). The reduced cell adhesion may suggest a toxic effect of e-liquids on osteoblast cell adhesion.

Contact with E-Liquid Decreased Osteoblast Growth

To confirm cell viability and proliferation capacity, we performed an MTT assay in each group. We found that growth reduction was in a dose-dependent manner based on e-liquid concentration (Fig. 5). Indeed, when exposed to the 70% PG -30% VG e-liquid, the cells showed a significant ($p<0.001$) reduction in MTT metabolism (Fig. 5A), as cell absorbance decreased from 1.39±0.28 in the control to 1.34±0.26 with e-liquid at 1% and 0.65±0.17 at 5%. It should be noted that with the 5%, the osteoblast growth decreased twofold as compared to the control.

Following exposure to the 70% PG 30% VG e-liquid containing flavor, the data shown in (Fig. 6B) were similar to the results obtained with the previously described condition, namely, from 1.31±0.32 in the control to 1.28±0.3 at 1% and 0.54±0.16 at 5%. A significant difference ($p<0.001$) was found at 5% compared to that observed in the control and also with the 1% concentration of e-liquid (Fig. 5B), meaning a more significant decrease in cell proliferation due to the exposure to the 70% PG -30% VG + flavor. Figure (6A) shows that when the osteoblasts were exposed to 70% PG -30% VG + flavor + nicotine at 12 mg/mL, their growth decreased. Absorbance also decreased from 1.38±0.35 in the control to 1.35±0.35 with 1% e-liquid and 0.56±0.23 with 5% e-liquid. The 5% concentration thus caused a significant ($p<0.001$) decrease in osteoblast proliferation (Fig. 5C).

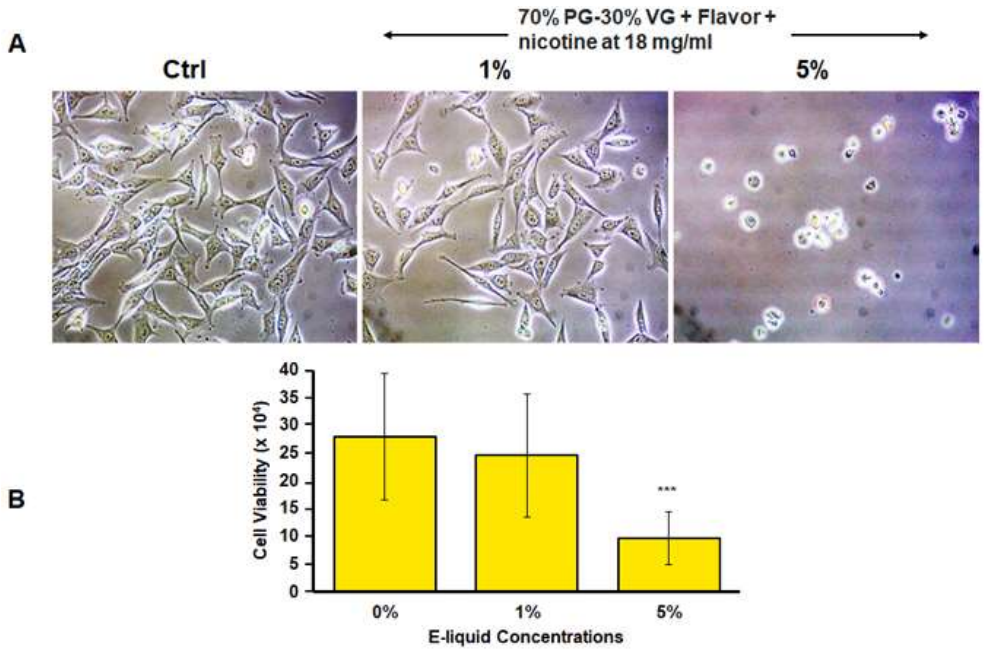

Fig. 4: E-liquid with a high amount of nicotine decreased osteoblast adhesion. (A) Cell morphology was determined by optical microscope observations. (B) Cell viability was assessed by trypan blue exclusion assay. The cell viability results are presented as the mean ± SD of at least three independent experiments. ***p<0.001

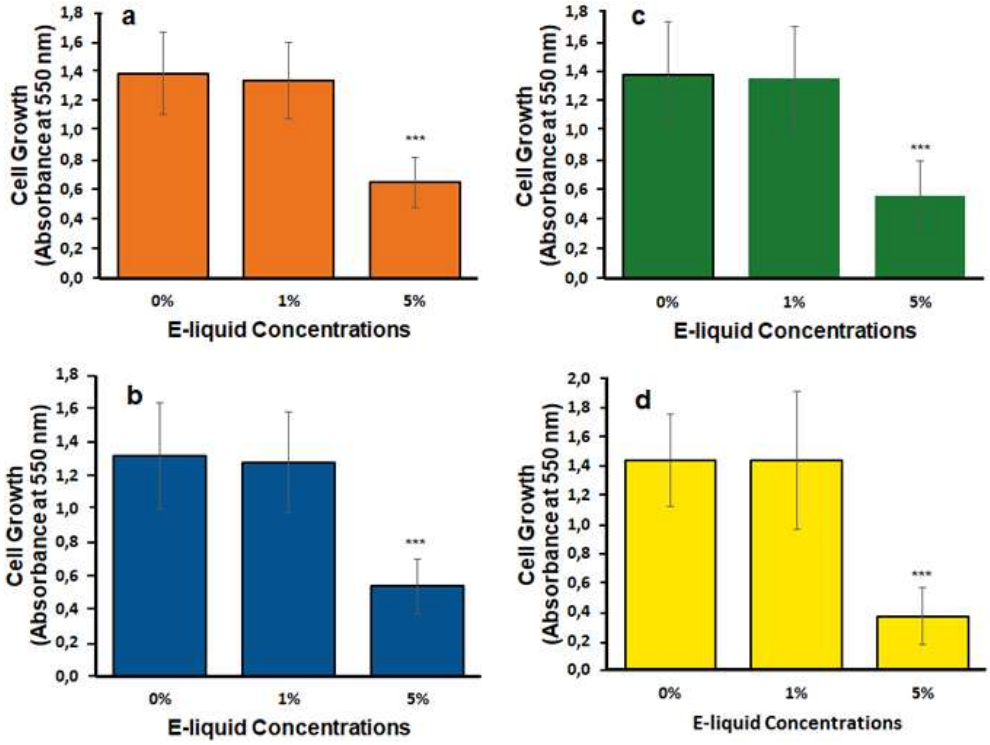

Fig. 5: Each tested e-liquid decreased osteoblast cell growth. Osteoblasts were seeded and cultured for 24 or 48 h prior to exposure to e-liquids. The cells were then fed fresh medium with or without e-liquid, with or without flavor, and with or without nicotine at 12 or 18 mg/mL. Cells were cultured for 24 h and subjected thereafter to an MTT colorimetric assay. Results are the mean ± SD of at least three independent experiments. (a) E-liquid containing 70% PG -30% VG only. (b) E-liquid containing 70% PG -30% VG + flavor. (c) E-liquid containing 70% PG -30% VG + flavor + nicotine at 12 mg/mL. (d) E-liquid containing 70% PG - 30% VG + flavor + nicotine at 18 mg/mL. ***p<0.001

Similar to the other groups, after exposure to e-liquid 70% PG -30% VG + flavor + nicotine at 18 mg/mL, a statistical difference was found with the 5% e-liquid concentration ($p<0.001$) compared to the data of the control and the 1% concentration. However, at 5%, the ability of cell proliferation was more compromised, with a decrease in absorbance from 1.44±0.3 in the control to 0.4±0.2 at 5% (Fig. 5D).

A comparison of the results of the different groups at 1% and 5% showed interesting effects. A statistical difference in cell growth inhibition was found with the 5% e-liquid in the group containing nicotine at 18 mg/mL (G4), compared to the growth evidenced in the group containing 70% PG 30% VG only (G1) ($p = 0.006$). A significant difference was also found when comparing both groups containing nicotine (G3 and G4) ($p<0.001$) (Fig. 6). Moreover, our findings with the 5% concentration of e-liquid suggest that the higher concentration (18 mg/mL) of nicotine had a greater effect on osteoblast growth.

E-Liquid Induced Greater LDH Levels in Osteoblasts

The observed adhesion and growth reduction could be due to a toxic effect brought on by the e-liquids. We, therefore, measured the LDH levels after osteoblasts were exposed to e-liquids for 24 h. Results show (Fig. 7) a significant ($p<0.01$) increase in LDH activity in the culture medium when the cells were exposed to e-liquid at a concentration of 5%, compared to that observed in the control and 1% solutions. No statistical difference was found when comparing the groups at a concentration of 1% ($p = 0.147$) and 5% ($p = 0.059$); however, we did note an increased level of LDH in the e-liquid with tobacco flavor, from 0.82±0.09 in the control to 1.47±0.52 at 5%, as well as in the e-liquid group containing 18 mg/mL of nicotine, which showed an increased value from 0.82±0.09 in control to 1.43±0.35 at 5%. The high amount of LDH evidence confirms the toxic effect of e-liquids in reducing osteoblast adhesion and growth (Fig. 7).

E-Liquid Induced Greater LDH Levels in Osteoblasts

The observed adhesion and growth reduction could be due to a toxic effect brought on by the e-liquids. We, therefore, measured the LDH levels after osteoblasts were exposed to e-liquids for 24 h. Results show (Fig. 7) a significant ($p<0.01$) increase in LDH activity in the culture medium when the cells were exposed to e-liquid at a concentration of 5%, compared to that observed in the control and 1% solutions. No statistical difference was found when comparing the groups at a concentration of 1% ($p = 0.147$) and 5% ($p = 0.059$); however, we did note an increased level of LDH in the e-liquid with tobacco flavor, from 0.82±0.09 in the control to 1.47±0.52 at 5%, as well as in the e-liquid group containing 18 mg/mL of nicotine, which showed an increased value from 0.82±0.09 in control to 1.43±0.35 at 5%. The high amount of LDH evidence confirms the toxic effect of e-liquids in reducing osteoblast adhesion and growth (Fig. 7).

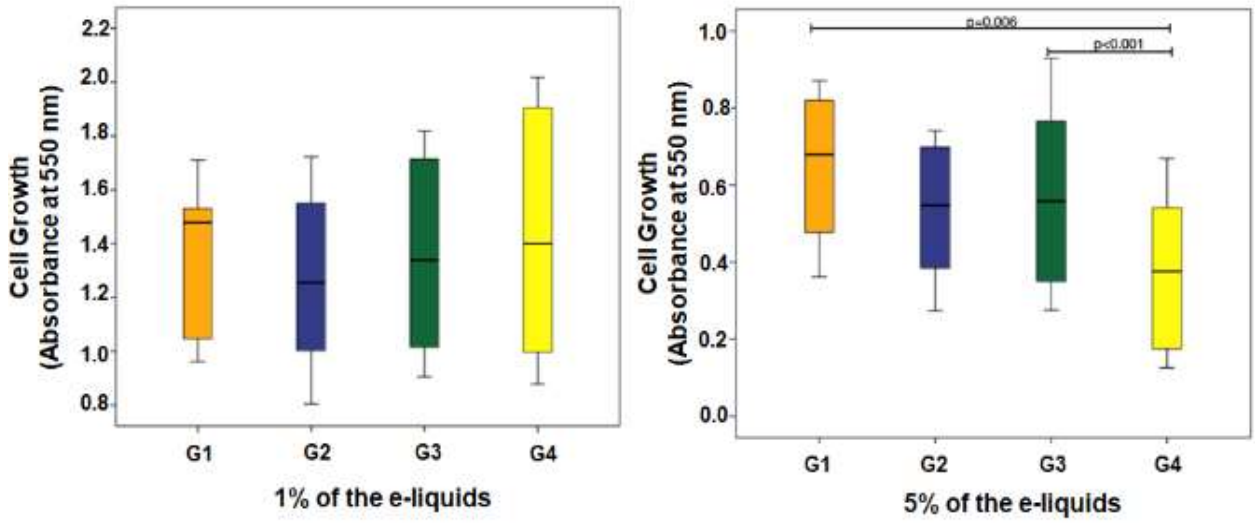

Fig. 6: Comparative effect of 1 and 5% e-liquid on osteoblast growth. After contact for 24 h with each e-liquid at various concentrations, the cell growth rates were compared

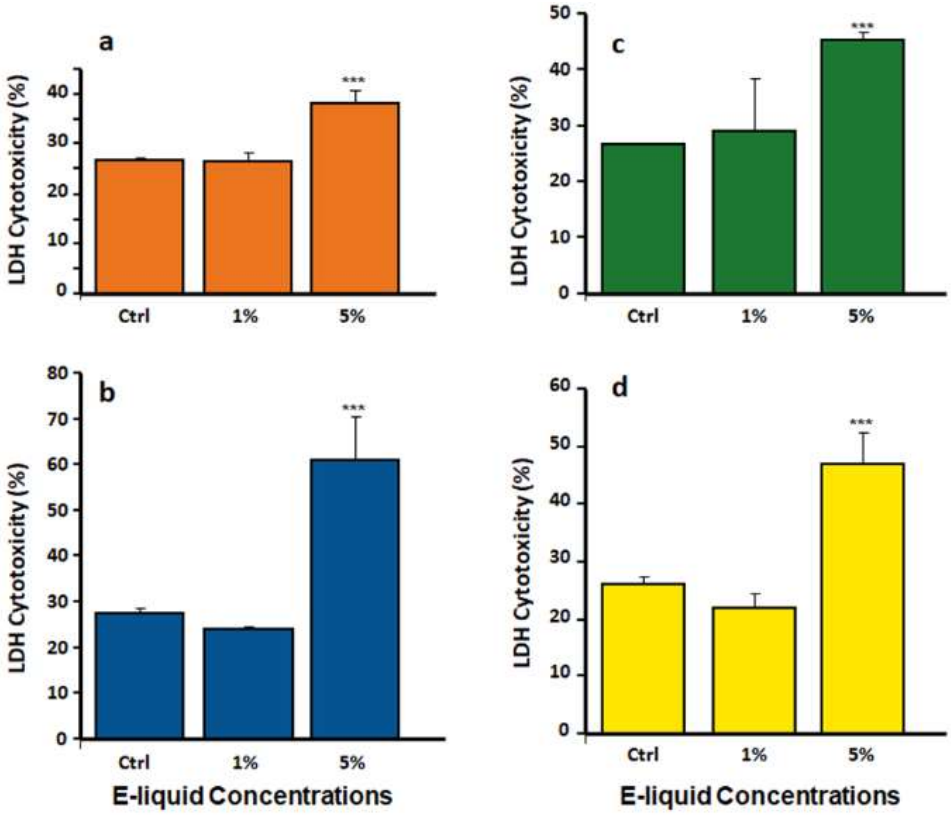

Fig. 7: The exposure of osteoblasts to e-liquids increased the release of LDH. Following exposure of the osteoblasts to the different e-liquids, the supernatants were used to measure LDH release using the LDH-cytotoxicity assay kit. (a) 70% PG -30% VG. (b) 70% PG -30% VG + flavor. (c) 70% PG -30% VG + flavor +nicotine at 12 mg/mL. (d) 70% PG -30% VG + flavor + nicotine at 18 mg/mL. Data are presented as percentages of the levels of LDH. ***p<0.001

Discussion

Combustible cigarettes are known to induce significant damage in the oral cavity, increase the onset of periodontal diseases and result in implant failures, among other health concerns (Javed *et al*., 2019; Pesce *et al*., 2022) and are also a risk factor for osteoporosis and bone fractures (Sparks *et al*., 2022; CDC, 2022). To overcome these adverse effects, e-cigarettes have been proposed as an alternative to counter the negative effects of combustible cigarettes. Several studies have reported damage to gingival cells and tissues (Alanazi *et al*., 2018; Rouabhia *et al*., 2017), which may compromise oral and periodontal health. The effects of e-cigarettes on the oral cavity could be linked to the chemicals in the vaped e-liquid. However, no studies have investigated the interaction of osteoblasts with different e-liquids prior to aerosolizing. In this study, we demonstrate that non-vaped e-liquid significantly reduces osteoblast adhesion. This observation is supported by a previous study from our group showing that vaped e-liquid also negatively affects osteoblast adhesion (Rouabhia *et al*., 2019).

Osteoblast cells are an essential cell type involved in bone formation and regeneration following damage. They also produce and mineralize bone matrix through a sequence of events associated with cell adhesion and proliferation (Caetano-Lopes *et al*., 2007). Our results show for the first time that direct exposure to e-liquids can change osteoblast morphology and viability, thereby compromising cell adhesion. Cell damage was identified initially by morphological analysis, in which cells began to show subtle changes in their shape at a 1% e-liquid concentration and displayed a tendency to cluster and rupture their cytoplasm at 5%. This study supports the findings of Shaito *et al.* (2017) showing that the exposure of bone marrow-derived Mesenchymal Stem Cells (MSCs) to cigarette and e-cigarette aerosol extracts deregulates cell adhesion and morphology.

Cell adhesion is a key osteogenic differentiation process, leading to calcium deposition and bone formation (Salasznyk *et al*., 2007). The deregulation of osteoblast adhesion could lead to a decrease in cell viability. We investigated cell viability after exposure of osteoblasts to each e-liquid, demonstrating a decrease of viable osteoblasts that confirmed the cell morphology changes. Similar results were reported with osteoblasts exposed to e-cigarette

aerosols (Rouabhia *et al*., 2019). The effect of e-liquids on cell adhesion was confirmed by evaluating cell growth. Our results indicate that the non-vaped e-liquids caused a significant reduction in osteoblast viability and growth.

Furthermore, the presence of flavor in combination with a higher concentration of nicotine (18 mg/mL) caused greater cell death. These results are in agreement with those reporting harmful effects of flavors and nicotine (Pennings *et al*., 2023; Rowell *et al*., 2017). The presence of nicotine in the e-liquid to be vaped could cause cytotoxicity in gingival epithelial cells, leading to periodontal tissue destruction (Beklen and Uckan, 2021). Nicotine could also inhibit tumor necrosis factor expression through the activation of the cholinergic anti-inflammatory pathway, thus delaying bone healing (Chen *et al*., 2011). The presence of nicotine impairs bone healing by possibly preventing cellular maturation and proliferation, thus interrupting bone tissue repair (Scolaro *et al*., 2014).

Another e-liquid component linked to e-cigarette toxicity is flavoring agents (Herbert *et al*., 2023; Noël and Ghosh, 2022; Shen *et al*., 2016). In their analysis of e-liquid cytotoxicity on human MG-63 and Saos-2 osteoblast-like cells, (Otero *et al*., 2019) reported a decrease in cell viability, suggesting a toxic effect independent of the presence of nicotine. This study demonstrated that the presence of flavors, such as cinnamon, was toxic as previously reported (Herbert *et al*., 2023; Sassano *et al*., 2018; Wavreil and Heggland, 2020). The effect of e-liquid on osteoblast behavior was also supported by an increased level of LDH, with a significant increase observed in the presence of nicotine and flavor. Tobacco flavor was shown to cause less toxicity than fruity flavors in human pharyngeal cells when the e-liquid was used in a 10% solution (Welz *et al*., 2016). The toxicity of the e-liquid has been linked to chemicals generated when e-cigarettes are heated (Kuehl *et al*., 2022; Williams *et al*., 2022). The non-vaped e-liquid constituents could also lead to toxic effects. Indeed, the exposure of airway cells to PG and VG solutions was shown to decrease GLUT-mediated glucose transport and ATP production in these airway cells (Woodall *et al*., 2020). PG and VG were also found to decrease barrier function and increase epithelial permeability (Woodall *et al*., 2020). These observations are in agreement with our findings showing that the e-liquid constituents, namely PG, VG, flavor, and nicotine, contributed to reducing osteoblast behaviors.

Conclusion

This study demonstrates for the first time that e-liquid constituents, including propylene glycol, vegetable glycerin, and tobacco flavor, are toxic to osteoblasts and that this toxicity increases when the e-liquid contains nicotine. Our findings indeed confirm the potentially detrimental effects of non-vaped e-liquids on osteoblast behavior, bone regeneration, and remodeling processes.

Acknowledgment

We would like to thank Dr. Karin Apaza-Bedoya for her collaboration in the statistical analysis of the data and Dr. Débora M. Pasin for her critical review of this manuscript.

Author's Contributions

Ana Carla Lorenz-Vieira: Performed the experiments; analyzed the data; interpreted results of experiments. Drafted the manuscript; revised and approved the final version of the manuscript.

Cesar Augusto Magalhães Benfatti: Contributed to the conception and designed of this study; interpreted results of experiments. Drafted the manuscript; revised and approved the final version of the manuscript.

Mahmoud Rouabhia: Contributed to the conception and designed of this study; performed the experiments; analyzed the data; interpreted results of experiments. Revised the drafted manuscript and approved the final version of the manuscript.

Funding Information

This study was supported the NSERC, grant no. RGPIN-2019-04475, as well as the fonds Émile-Beaulieu de l'Université Laval, grant no. FO123458.

References

Alanazi, H., Park, H. J., Chakir, J., Semlali, A., & Rouabhia, M. (2018). Comparative study of the effects of cigarette smoke and electronic cigarettes on human gingival fibroblast proliferation, migration, and apoptosis. *Food and Chemical Toxicology*, *118*, 390-398. https://doi.org/10.1016/j.fct.2018.05.049

Beklen, A. R. Z. U., & Uckan, D. (2021). Electronic cigarette liquid substances propylene glycol and vegetable glycerin induce an inflammatory response in gingival epithelial cells. *Human & Experimental Toxicology*, *40*(1), 25-34. https://doi.org/10.1177/0960327120943934

Benowitz, N. L., Hukkanen, J., & Jacob III, P. (2009). Nicotine chemistry, metabolism, kinetics and biomarkers. *Nicotine Psychopharmacology*, 29-60. https://doi.org/10.1007/978-3-540-69248-5_2

Breland, A., Soule, E., Lopez, A., Ramôa, C., El-Hellani, A., & Eissenberg, T. (2017). Electronic cigarettes: What are they and what do they do?. *Annals of the New York Academy of Sciences*, *1394*(1), 5-30. https://doi.org/10.1111/nyas.12977

Caetano-Lopes, J., Canhao, H., & Fonseca, J. E. (2007). Osteoblasts and bone formation. *Acta Reumatológica Portuguesa*, *32*(2), 103-110. https://doi.org/10.1016/s0169-6009(08)80210-3

Catala-Valentin, A., Bernard, J. N., Caldwell, M., Maxson, J., Moore, S. D., & Andl, C. D. (2022). E-cigarette aerosol exposure favors the growth and colonization of oral *Streptococcus mutans* compared to commensal Streptococci. *Microbiology Spectrum*, *10*(2), e02421-21. https://doi.org/10.1128/spectrum.02421-21

CDC. (2022). Centers for Disease Control and Prevention., 2018. Health effects of cigarette smoking. https://www.cdc.gov/tobacco/data_statistics/fact_sheets/health_effects/effects_cig_smoking/index.htm

Chen, Y., Guo, Q., Pan, X., Qin, L., & Zhang, P. (2011). Smoking and impaired bone healing: Will activation of cholinergic anti-inflammatory pathway be the bridge?. *International Orthopaedics*, *35*, 1267-1270. https://doi.org/10.1007/s00264-011-1243-5

Denizot, F., & Lang, R. (1986). Rapid colorimetric assay for cell growth and survival: Modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. *Journal of Immunological Methods*, *89*(2), 271-277. https://doi.org/10.1016/0022-1759(86)90368-6

FDA, (1980). HHS. Select Committee on GRAS substances (SCOGS).

Gleitz, J., Tosch, C., & Peters, T. (1996). Continuous enzyme-linked fluorometric detection of L-(+)-lactate released from rat brain vesicles under anoxic conditions. *Journal of Neuroscience Methods*, *67*(2), 97-102. https://doi.org/10.1016/S0165-0270(96)00006-4

Gschwend, G., Jenkins, C., Jones, A., Kelso, C., & Morgan, J. (2023). A Wide Range of Flavoring-Carrier Fluid Adducts Form in E-Cigarette Liquids. *Chemical Research in Toxicology*. https://doi.org/10.1021/acs.chemrestox.2c00200

Guo, X., Ehindero, T., Lau, C., & Zhao, R. (2022). Impact of glycol-based solvents on indoor air quality-Artificial fog and exposure pathways of formaldehyde and various carbonyls. *Indoor Air*, *32*(9), e13100. https://doi.org/10.1111/ina.13100

Harvanko, A., Kryscio, R., Martin, C., & Kelly, T. (2019). Stimulus effects of propylene glycol and vegetable glycerin in electronic cigarette liquids. *Drug and Alcohol Dependence*, *194*, 326-329. https://doi.org/10.1016/j.drugalcdep.2018.08.039

Herbert, J., Kelty, J. S., Laskin, J. D., Laskin, D. L., & Gow, A. J. (2023). Menthol flavoring in e-cigarette condensate causes pulmonary dysfunction and cytotoxicity in precision cut lung slices. *American Journal of Physiology-Lung Cellular and Molecular Physiology*, *324*(3), L345-L357. https://doi.org/10.1152/ajplung.00222.2022

Holliday, R., Chaffee, B. W., Jakubovics, N. S., Kist, R., & Preshaw, P. M. (2021). Electronic cigarettes and oral health. *Journal of Dental Research*, *100*(9), 906-913. https://doi.org/10.1177/00220345211002116

Javed, F., Rahman, I., & Romanos, G. E. (2019). Tobacco-product usage as a risk factor for dental implants. *Periodontology 2000*, *81*(1), 48-56. https://doi.org/10.1111/prd.12282

Kim, S. A., Smith, S., Beauchamp, C., Song, Y., Chiang, M., Giuseppetti, A., ... & Kim, J. J. (2018). Cariogenic potential of sweet flavors in electronic-cigarette liquids. *PloS One*, *13*(9), e0203717. https://doi.org/10.1371/journal.pone.0203717

Komura, M., Sato, T., Yoshikawa, H., Nitta, N. A., Suzuki, Y., Koike, K., ... & Takahashi, K. (2022). Propylene glycol, a component of electronic cigarette liquid, damages epithelial cells in human small airways. *Respiratory Research*, *23*(1), 216. https://doi.org/10.1186/s12931-022-02142-2

Kuehl, P. J., McDonald, J. D., Weber, D. T., Khlystov, A., Nystoriak, M. A., & Conklin, D. J. (2022). Composition of aerosols from thermal degradation of flavors used in ends and tobacco products. *Inhalation Toxicology*, *34*(11-12), 319-328. https://doi.org/10.1080/08958378.2022.2103602

Melo, J. B., Agostinho, P., & Oliveira, C. R. (2002). Amyloid beta-peptide 25-35 reduces [3H] acetylcholine release in retinal neurons. Involvement of metabolic dysfunction. *Amyloid*, *9*(4), 221-228. https://doi.org/10.3109/13506120209114097

Morshed, K. M., Jain, S. K., & McMartin, K. E. (1998). Propylene glycol-mediated cell injury in a primary culture of human proximal tubule cells. *Toxicological Sciences*, *46*(2), 410-417. https://doi.org/10.1006/toxs.1998.2521

National Center for Chronic Disease Prevention and Health Promotion (US) Office on Smoking and Health. (2014). The Health Consequences of Smoking—50 Years of Progress: A Report of the Surgeon General. Centers for Disease Control and Prevention (US).

Noël, A., & Ghosh, A. (2022). Carbonyl Profiles of Electronic Nicotine Delivery System (ENDS) Aerosols Reflect Both the Chemical Composition and the Numbers of e-liquid Ingredients-Focus on the *in vitro* Toxicity of Strawberry and Vanilla Flavors. *International Journal of Environmental Research and Public Health*, *19*(24), 16774. https://doi.org/10.3390/ijerph192416774

Onor, I. O., Stirling, D. L., Williams, S. R., Bediako, D., Borghol, A., Harris, M. B., ... & Sarpong, D. F. (2017). Clinical effects of cigarette smoking: Epidemiologic impact and review of pharmacotherapy options. *International Journal of Environmental Research and Public Health*, *14*(10), 1147. https://doi.org/10.3390/ijerph14101147

Otero, C. E., Noeker, J. A., Brown, M. M., Wavreil, F. D., Harvey, W. A., Mitchell, K. A., & Heggland, S. J. (2019). Electronic cigarette liquid exposure induces flavor-dependent osteotoxicity and increases expression of a key bone marker, collagen type I. *Journal of Applied Toxicology*, *39*(6), 888-898. https://doi.org/10.1002/jat.3777

Pennings, J. L., Havermans, A., Krüsemann, E. J., Zijtveld, D., Huiberts, E. H., Bos, P. M., ... & Talhout, R. (2023). Reducing attractiveness of e-liquids: Proposal for a restrictive list of tobacco-related flavourings. *Tobacco Control*. https://doi.org/10.1136/tc-2022-057764

Pesce, P., Menini, M., Ugo, G., Bagnasco, F., Dioguardi, M., & Troiano, G. (2022). Evaluation of periodontal indices among non-smokers, tobacco and e-cigarette smokers: A systematic review and network meta-analysis. *Clinical Oral Investigations*, *26*(7), 4701-4714. https://doi.org/10.1007/s00784-022-04531-9

Ramôa, C. P., Eissenberg, T., & Sahingur, S. E. (2017). Increasing popularity of waterpipe tobacco smoking and electronic cigarette use: Implications for oral healthcare. *Journal of Periodontal Research*, *52*(5), 813-823. https://doi.org/10.1111/jre.12458

Randall, C. A., Sun, D., & Randall, P. A. (2023). Differential Effects of Nicotine, Alcohol and Coexposure on Neuroimmune-Related Protein and Gene Expression in Corticolimbic Brain Regions of Rats. *ACS Chemical Neuroscience*, *14*(4), 628-644. https://doi.org/10.1021/acschemneuro.2c00413

Rouabhia, M., & Semlali, A. (2021). Electronic cigarette vapor increases *Streptococcus mutans* growth, adhesion, biofilm formation and expression of the biofilm-associated genes. *Oral Diseases*, *27*(3), 639-647. https://doi.org/10.1111/odi.13564

Rouabhia, M., Alanazi, H., Park, H. J., & Gonçalves, R. B. (2019). Cigarette smoke and e-cigarette vapor dysregulate osteoblast interaction with titanium dental implant surface. *Journal of Oral Implantology*, *45*(1), 2-11. https://doi.org/10.1563/aaid-joi-D-18-00009

Rouabhia, M., Park, H. J., Semlali, A., Zakrzewski, A., Chmielewski, W., & Chakir, J. (2017). E-cigarette vapor induces an apoptotic response in human gingival epithelial cells through the caspase-3 pathway. *Journal of Cellular Physiology*, *232*(6), 1539-1547. https://doi.org/10.1002/jcp.25677

Rowell, T. R., Reeber, S. L., Lee, S. L., Harris, R. A., Nethery, R. C., Herring, A. H., ... & Tarran, R. (2017). Flavored e-cigarette liquids reduce proliferation and viability in the CALU3 airway epithelial cell line. *American Journal of Physiology-Lung Cellular and Molecular Physiology*, *313*(1), L52-L66. https://doi.org/10.1152/ajplung.00392.2016

Salasznyk, R. M., Klees, R. F., Williams, W. A., Boskey, A., & Plopper, G. E. (2007). Focal adhesion kinase signaling pathways regulate the osteogenic differentiation of human mesenchymal stem cells. *Experimental Cell Research*, *313*(1), 22-37. https://doi.org/10.1016/j.yexcr.2006.09.013

Sassano, M. F., Davis, E. S., Keating, J. E., Zorn, B. T., Kochar, T. K., Wolfgang, M. C., ... & Tarran, R. (2018). Evaluation of e-liquid toxicity using an open-source high-throughput screening assay. *PLoS Biology*, *16*(3), e2003904. http://doi.org/10.1371/journal.pbio.2003904

Scolaro, J. A., Schenker, M. L., Yannascoli, S., Baldwin, K., Mehta, S., & Ahn, J. (2014). Cigarette smoking increases complications following fracture: A systematic review. *JBJS*, *96*(8), 674-681. https://doi.org/10.2106/JBJS.M.00081

Shaito, A., Saliba, J., Husari, A., El-Harakeh, M., Chhouri, H., Hashem, Y., ... & El-Sabban, M. (2017). Electronic cigarette smoke impairs normal mesenchymal stem cell differentiation. *Scientific Reports*, *7*(1), 14281. https://doi.org/10.1038/s41598-017-14634-z

Shen, Y., Wolkowicz, M. J., Kotova, T., Fan, L., & Timko, M. P. (2016). Transcriptome sequencing reveals e-cigarette vapor and mainstream-smoke from tobacco cigarettes activate different gene expression profiles in human bronchial epithelial cells. *Scientific Reports*, *6*(1), 1-17. https://doi.org/10.1038/srep23984

Sparks, N. R., Walker, L. M., Sera, S. R., Madrid, J. V., Hanna, M., Dominguez, E. C., & Zur Nieden, N. I. (2022). Sidestream Smoke Extracts from Harm-Reduction and Conventional Camel Cigarettes Inhibit Osteogenic Differentiation via Oxidative Stress and Differential Activation of intrinsic Apoptotic Pathways. *Antioxidants*, *11*(12), 2474. https://doi.org/10.3390/antiox11122474

Wavreil, F. D., & Heggland, S. J. (2020). Cinnamon-flavored electronic cigarette liquids and aerosols induce oxidative stress in human osteoblast-like MG-63 cells. *Toxicology Reports*, *7*, 23-29. https://doi.org/10.1016/j.toxrep.2019.11.019

Welz, C., Canis, M., Schwenk-Zieger, S., Becker, S., Stucke, V., Ihler, F., & Baumeister, P. (2016). Cytotoxic and genotoxic effects of electronic cigarette liquids on human mucosal tissue cultures of the oropharynx. *Journal of Environmental Pathology, Toxicology and Oncology*, *35*(4). https://doi.org/10.1615/JEnvironPatholToxicolOncol.2016016652

Williams, M., Luo, W., McWhirter, K., Ikegbu, O., & Talbot, P. (2022). Chemical Elements, Flavor Chemicals and Nicotine in Unused and Used Electronic Cigarettes Aged 5-10 Years and Effects of pH. *International Journal of Environmental Research and Public Health*, *19*(24), 16931. https://doi.org/10.3390/ijerph192416931

Woodall, M., Jacob, J., Kalsi, K. K., Schroeder, V., Davis, E., Kenyon, B., ... & Baines, D. L. (2020). E-cigarette constituents propylene glycol and vegetable glycerin decrease glucose uptake and its metabolism in airway epithelial cells in vitro. *American Journal of Physiology-Lung Cellular and Molecular Physiology*, *319*(6), L957-L967. https://doi.org/10.1152/ajplung.00123.2020

Youssef, M., Marzouk, T., Abdelsalam, H., Malmstrom, H., Barmak, A. B., Fraser, D., & Tsigarida, A. (2022). The effect of electronic cigarette use on peri-implant conditions in males. A systematic review and meta-analysis. *Oral Surgery, Oral Medicine, Oral Pathology and Oral Radiology*. https://doi.org/10.1016/j.oooo.2022.08.010