

Evaluation of Differential Oral Cell-specific Responses to the E-cigarette Component Nicotine

Abstract

Objectives: The recent introduction of electronic cigarettes (EC) or e-cigarettes, also known as the electronic nicotine delivery device (ENDD), has been promoted as a safer alternative to tobacco products and smoking. Many groups have advocated for the use of ECs or ENDDs as a tool to reduce carcinogenic potential, while simultaneously promoting strategies and protocols for smoking and nicotine cessation. Based upon this information, the main objective of this study was to determine the biological effects of the most basic aerosol component of all ECs and ENDDs (nicotine) on cells and tissues specifically derived from the oral cavity. The working hypothesis was that no discernable effects would be apparent at the concentrations typically associated with EC and ENDD use.

Experimental Methods: In brief, oral cell lines were obtained, which included normal, non-cancerous Human Gingival Fibroblasts (HGF-1) and two oral squamous cell carcinomas (SCC25, CAL27). Cells were exposed to nicotine at concentrations equivalent to those found in e-cigarette mixtures (5.77×10^{-5} M) for five day proliferation and viability assays.

Results: The results of this study strongly suggest that nicotine may have negative effects on both cellular viability and cellular proliferation among cancerous and non-cancerous cells. Moreover, these effects appear to become more pronounced over time, suggesting that **short-term exposure** to vaping solutions comprised of water with small amounts of nicotine may be sufficient to induce these effects – at least in this experimental or *in vitro* setting.

Conclusions: In summary, these data provide further evidence that nicotine administration may present significant risks to cell viability and growth over time. In addition, this study demonstrated that these effects were evident in both cancerous and non-cancerous cells – a finding that may suggest more research in this area is needed to determine the mechanisms that might be shared between these differing cell types, which may also suggest more caution may be needed when advertising or marketing ECs or ENDDs are low- or no-risk alternatives to cigarette smoking.

Key words: Nicotine, e-cigarette, oral response

Abbreviations: United States (US), electronic cigarettes (EC), electronic nicotine delivery device (ENDD), polycyclic aromatic hydrocarbons (PAH), nicotine-derived nitrosamines (NDN), American Tissue Culture Collection (ATCC), human gingival fibroblast (HGF-1),

37 Dulbecco's Modified Eagle's Medium (DMEM). fetal bovine serums (FBS), smokeless
38 tobacco extract (STE).

39 **1. Introduction**

40 Tobacco use and alcohol consumption are the major contributing risk factors identified with
41 the development and oncogenesis of oral and pharyngeal cancers in the United States (US)
42 [1,2]. In recent years there has been an overall decline in tobacco use and smoking in the US,
43 which has resulted in a corresponding decline the incidence of tobacco-induced diseases that
44 include lung, oral and pharyngeal cancers [3,4]. Despite these positive advances, some
45 evidence has demonstrated that smoking and tobacco use among specific demographic
46 (minority) groups and those within lower socioeconomic groups may not be experiencing
47 these same declines [5,6].

48 The recent introduction of electronic cigarettes (EC) or e-cigarettes, also known as the
49 electronic nicotine delivery device (ENDD), has been promoted as a safer alternative to
50 tobacco products and smoking [7,8]. The removal of combustion from the process of tobacco
51 smoking is designed to reduce production, and therefore intake, of multiple carcinogenic
52 compounds that include polycyclic aromatic hydrocarbons (PAH) and nicotine-derived
53 nitrosamines (NDN) [9-11]. Many groups have advocated for the use of ECs or ENDDs as a
54 tool to reduce carcinogenic potential, while simultaneously promoting strategies and
55 protocols for smoking and nicotine cessation [12,13].

56 These advances have led to widespread use of ECs and ENDDs by some smokers, which
57 have widely become known as vaping [14,15]. These increases have led to a great expansion
58 in the range of types and flavors of ECs, which may contain carcinogenic and deleterious
59 compounds that may negative impact the overall health benefits originally anticipated by this
60 process [16-18]. In addition, the increased consumer choices have also led to uptake and use
61 by non-smokers and younger adults and teenagers who believe that ECs and ENDDs pose
62 only limited health risks [19,20].

63 These shifts in consumer perception and behavior have prompted great interest in more
64 expansive scientific review to evaluate and determine the safety of all EC components on
65 human cells and tissues [21,22]. Based upon this information, the main objective of this study
66 was to determine **any changes in cellular proliferation or viability to** the most basic aerosol
67 component of all ECs and ENDDs (nicotine) on cells and tissues specifically derived from
68 the oral cavity.

69

70 **2. Methodology**

71 *2.1 Cell culture*

72 Tissues derived from the oral cavity were obtained from American Tissue Culture Collection
73 (ATCC). These included a normal (non-cancerous) human gingival fibroblast cell line, HGF-
74 1 (CRL-2014), which was cultured in accordance with the recommended protocol. In brief,
75 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) with the addition of

10% fetal bovine serum (FBS) in a humidified tissue culture incubator that was supplemented with 5% CO₂. Other cell lines included CAL27 (CRL-2095) and SCC25 (CRL-1628), which are both squamous cell carcinomas of the tongue, which can be maintained using the same procedures as HGF-1 cells. All experimental assays were performed in 96-well tissue culture-treated plates with the addition of 1.2×10^4 cells per well.

2.2 Cell viability

Cell viability was determined at the initiation of cell plating and during cell culture at each passage prior to experimentation. Cells were culturing until 75-85% confluence and split 1:3 to maintain appropriate levels of cell viability per the manufacturer recommendation. Baseline measurements of cell viability were taken at the start of each experiment or time point zero (T0) and then at each subsequent 24 hour time point, such as day one (T1), day two (T2), day three (T3), day four (T4) and day five (T5). Viability was assessed using the Trypan Blue exclusion assay, which was quantified using the TC20 Cell Counter from Bio-Rad (Hercules, CA) as previously described [23-26]. All measurements were done in triplicate and each individual assay involved n=8 wells – yielding n=24 replicates wells per experimental condition.

2.3 Cell proliferation

Growth of cells was determined by two separate methods in this study. First, live cell number and total cell number were obtained from three experimental wells at each time point to determine viability. However, the Trypan Blue method is preferred for assessment of viability but may not be preferable to accurately evaluate total proliferation – in order account for all cells within each experimental well, as previously described [23-26]. To verify these results, all experimental wells for each day were then fixed with formalin and processed using Gentian Violet, as previously described [23-26]. Cell number was then obtained using a BioTek ELx808 Microplate Absorbance Reader to determine approximate cell number and cell confluence.

2.4 Materials

Purified liquid nicotine was obtained from Acros organics, L-Nicotine 99+% (CAS: 54-11-5; Geel, Belgium). Viability and proliferation assay were performed in the appropriate complete media with (experimental) and without the addition of liquid nicotine diluted to produce an EC equivalent of 5.77×10^{-5} M in each experimental well.

2.5 Statistics

Differences between the control and experimental cell viability averages were determined using two-tailed t-tests, which is appropriate for use with parametric data with a sample size of at least twenty (n=20). Statistical significance was set at $\alpha=0.05$ and descriptive statistics were given where appropriate, along with the appropriate p-value.

3. Results

In order to determine the relative change in viability in this experimental assay, baseline viability was measured at the initiation of cell culture and for five consecutive days (Figure 1). These results demonstrated high viability for all cell lines, ranging from 86 to 94%. More specifically, viability for HGF-1 cells was initially measured as 88%, which ranged over five days between 88% - 91% resulting in an average which was consistently at or near the average of 88.8%. Similarly, SCC25 cell viability was observed at 86% and ranged between 86% and 88% - very close to the average of 86.8%. CAL27 cells exhibited an initial viability of 94%, with fluctuations observed between 93% and 95 that were near approached the overall average of 94%.

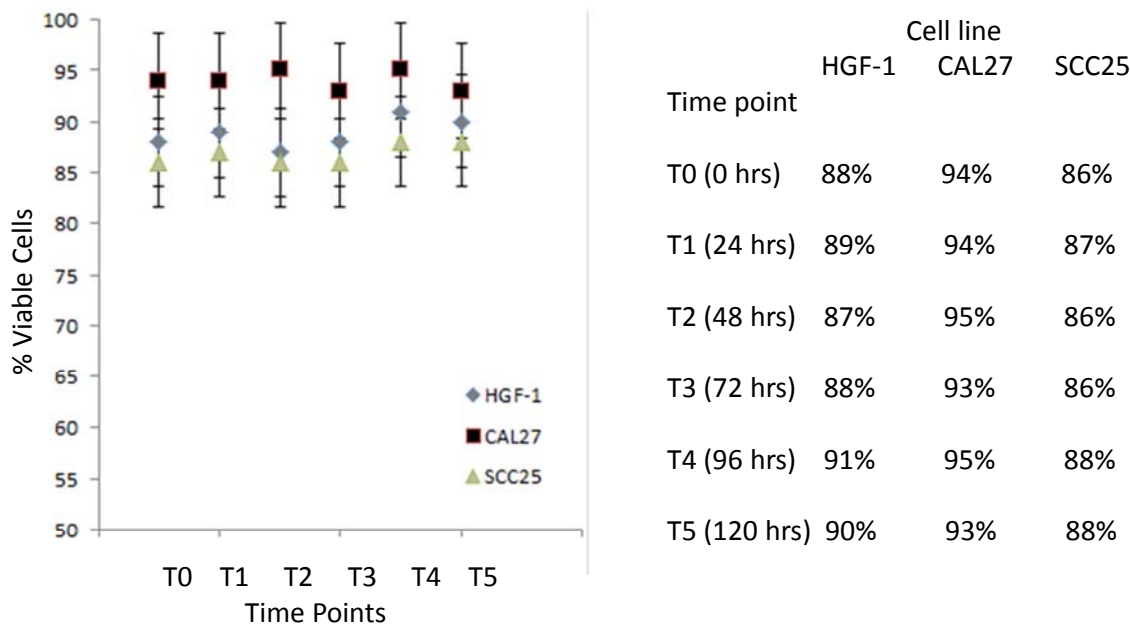


Figure 1. Baseline measurement of cell viability prior to experimentation. Cell viability as determined by Trypan Blue exclusion assay revealed high percentages among all three cell lines; HGF-1 (88.8%), CAL27 (94%), SCC25 (86.8%), which remained relatively consistent (no significant change) over the course of several days, $p>0.3$.

After establishment of the baseline viability, as well as the average range and consistency of these measurements, cells were plated in 96-well assay plates with and without the addition of nicotine at the concentration of 5.77×10^{-5} M (Figure 2). These results revealed a slight, non-significant decline in cell viability among all cell lines. More specifically, cell viability among HGF-1 cells was reduced from 89% to 85%, while CAL27 was reduced from 94% to 88% and SCC25 declined from 87% to 82%, $p>0.1$.

Further analysis of cell viability over time revealed a more significant overall declining trend that was observed among the experimental (nicotine) wells between day two (T2) and day

five (T5) (Fig 2A). In brief, viability among HGF-1 cells was inhibited by 33.1% over the course of the experiment with the greatest declines observed between day three (T3) and day five (T5), $p < 0.05$. In addition, viability among the experimental CAL27 cells declined by an average of 49.3% with the greatest decline observed between day one (T1) and day two (T2), $p < 0.05$. Finally, viability among the experimental SCC25 cells exhibited a similar dramatic reduction in viability of 47.2% with the greatest reduction observed between day one (T1) and day two (T2), similar to the observations with CAL27 cells. These data demonstrated a small reduction in cell viability at the onset of this assay with sustained, dramatic temporal reductions in cell viability that persisted throughout the course of this assay (Fig. 2B).

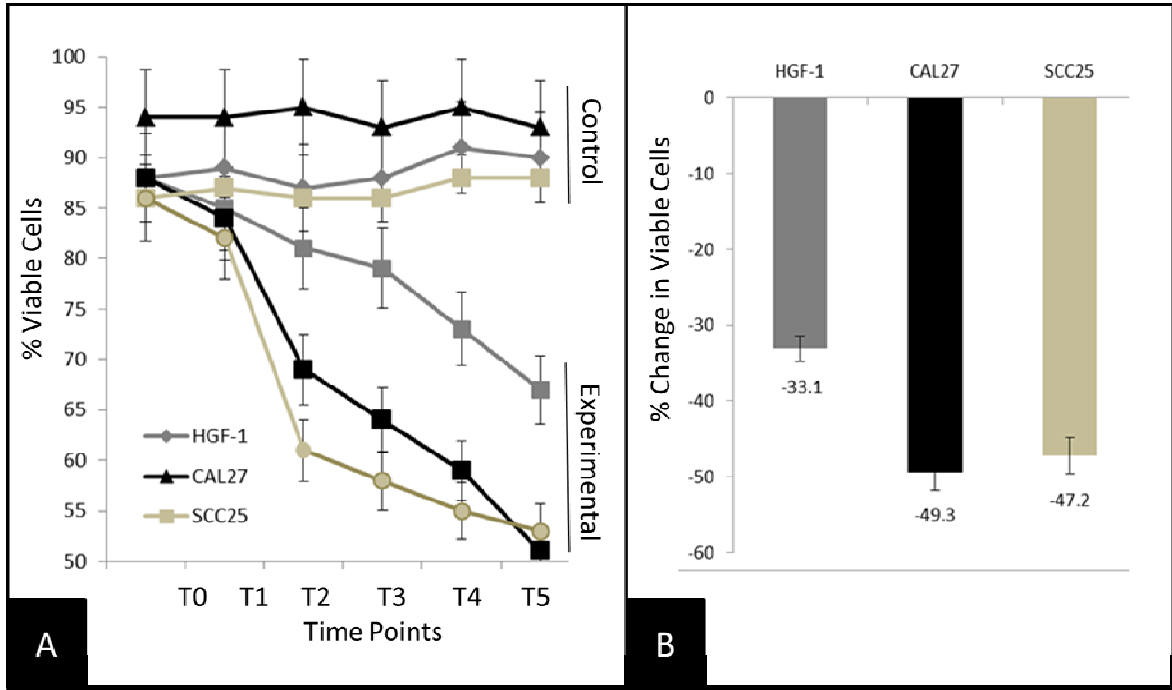


Figure 2. Cell viability following nicotine administration. A) Results from the 96-well proliferation assays revealed differential reductions in cell viability that varied over time and between cell lines. B) Viability was significantly reduced by the assay endpoint T5 (120 hours) in all cell lines between 33.1% (HGF-1), 47.2%(SCC25), and 49.3% (CAL27), $p < 0.01$.

To assess any changes in growth and proliferation in this experimental assay, baseline measurements of cell growth and proliferation were measured over five consecutive days (Figure 3). These results demonstrated that all cell lines were found to have similar absorbance readings (approximating confluence) at the onset of this experiment (T0), evidence that cell number was equivalent at the start of each trial. Each cell line exhibited growth over the course of the five day assay – although growth rates differed significantly.

For example, HGF-1 cells exhibited a nearly three-fold increase in overall cell number over the course of five days (T0 – T5), which nearly approximates an average doubling time of 48 – 72 hours. SCC25 cells exhibited a nearly four-fold increase in overall cell number, while CAL27 exhibited a greater than four-fold increase.

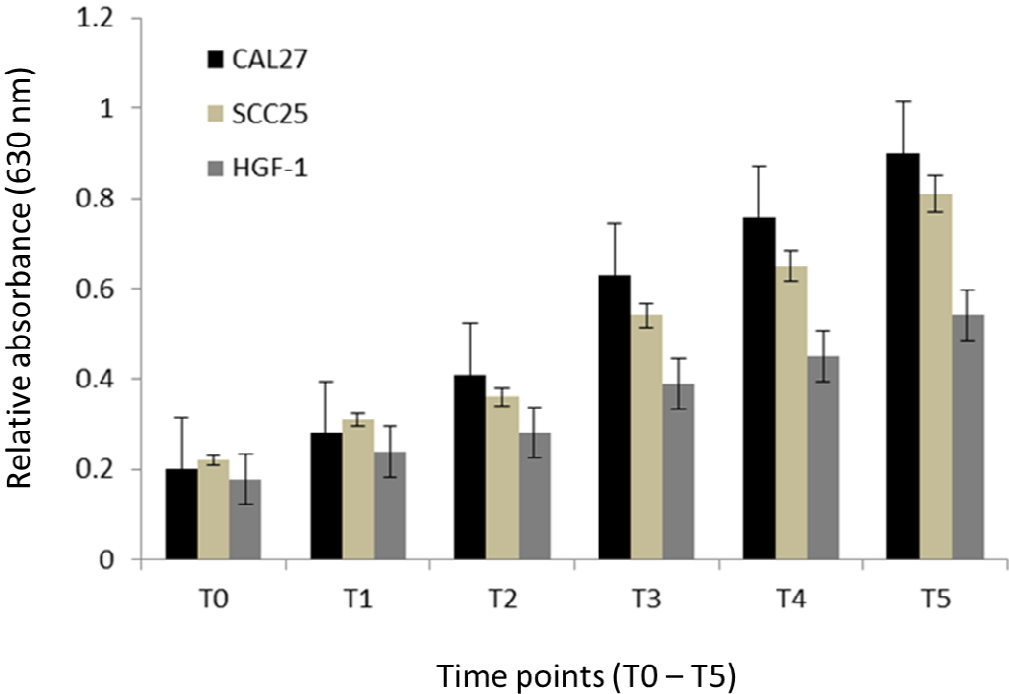


Figure 3. Baseline measurement of cell proliferation prior to experimentation. Cell proliferation (growth) as determined by fixation, staining and absorbance reading (630 nm) revealed growth among all three cell lines. Average doubling time for the normal, non-cancerous HGF-1 cells was between 48-72 hrs., while doubling time among the oral cancer cell lines CAL27 and SCC25 was between 24 – 48 hours.

The establishment of baseline growth and proliferation provided sufficient data to commence the subsequent experimental assay, with and without the addition of nicotine at the concentration of 5.77×10^{-5} M (Figure 4). These data demonstrated that the addition of nicotine was sufficient to alter cellular growth and proliferation. In general, each cell line was capable of producing a slight increase in cell growth between plating at time point zero (T0) and the first time point at 24 hours (T1). However, none of the cell lines exhibited any further growth, while each exhibited evidence of a further decline in overall cell number.

More specifically, under nicotine administration it was noted that CAL27 proliferation was significantly lower (-14.3%) compared to the negative control cells grown without nicotine at the initial time point (T1) of 24 hours, $p < 0.05$. The inhibition of cellular proliferation was also observed on subsequent days with declines ranging from -43.9% (T3) to -90.1% (T5),

p<0.01. It should be noted that the absorbance at the final time point revealed fewer than half the number of cells that were originally plated.

SCC25 cells responded similarly to nicotine administration with a significantly lower amount of growth observed at day one (T1) of -32.2% compared with the negative controls. This inhibition was also observed at all subsequent time points (T2-T5), with reductions ranging from -55.6% (T3) to -86.4% (T5), p<0.01. The final absorbance reading of these cells was also lower than the initial starting point of the assay.

Data generated from the normal, non-cancerous oral gingival fibroblast cell line HGF-1 revealed that administration of nicotine had similar effects on this cell line. For instance, proliferation on day one (T1) was initially reduced by 16.7%, p<0.05. This inhibitory effect was sustained with significant reductions of 25.1%% to 75.9% observed between T2 and T5, p<0.01. Finally, it should be noted that the absorbance reading in the experimental assay for this cell line was also lower than the initial starting point of the assay – similar to the results observed with the oral cancer cell lines.

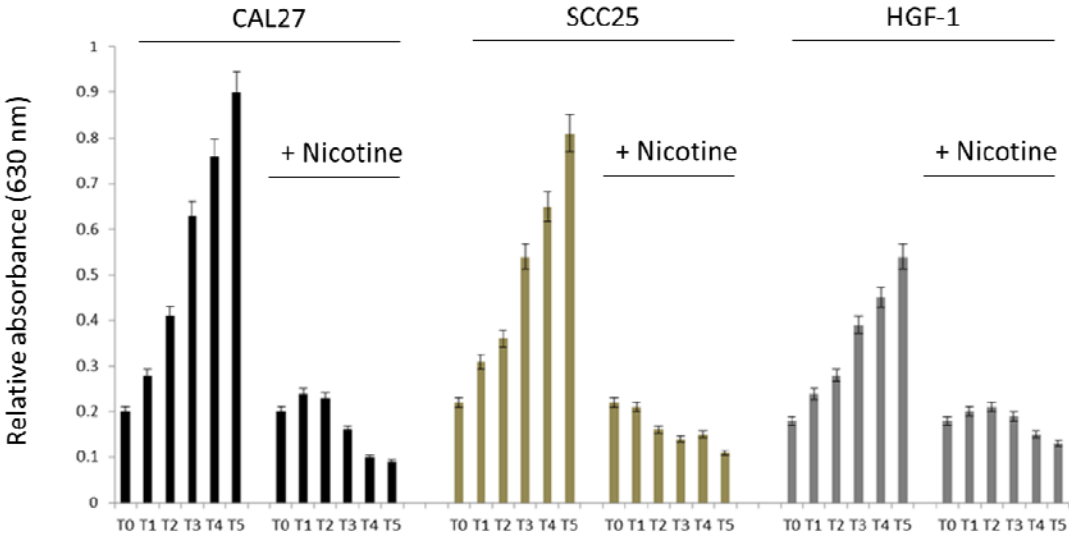


Figure 4. Cell proliferation following nicotine administration. The addition of nicotine to the experimental wells significantly inhibited cell growth over the course of the assay. Although CAL27 cells were initially able to proliferate (T1, T2), the rate of growth was severely inhibited by nicotine administration with more significant reductions in cell numbers observed between T3 and T5. Similar results were observed with SCC25 cells and the HGF-1 normal cell controls.

4. Discussion

Based upon the premise that ECs and ENDDs effectively remove most of the carcinogens and other harmful products of smoking, such as PAHs and NDNs, the working hypothesis was

that no discernable effects would be apparent in these experimental nicotine assays at the concentrations typically associated with vaping and electronic cigarette use. However, the results of this study strongly suggest that nicotine may have negative effects on both cellular viability and cellular proliferation among cancerous and non-cancerous cells. Moreover, these effects appear to become more pronounced over time, suggesting that short-term exposure to vaping solutions comprised of water with small amounts of nicotine may be sufficient to induce these effects – at least in this experimental or *in vitro* setting.

These data are consistent with other recently published studies that also demonstrate smokeless tobacco extract (STE) or nicotine-containing vapor is sufficient to induce cell damage and cell death at similar concentrations to those used in this study [27-29] . Moreover, these reports have revealed that some oral tissues including fibroblasts may exhibit a greater range of cellular behaviors that are negatively impacted by nicotine administration, such as impaired wound healing and migration properties [30-32]. This may suggest that nicotine administration may not be as “safe” as initially suggested by consumer marketing and advertising.

Some limitations that must be considered in this study include the route and duration of nicotine administration. Cells were not exposed directly to e-cigarette vapor, but rather had liquid nicotine added to a concentration that would be comparable to that found in an average EC or ENDD [33]. This would represent a much higher concentration than would typically be available in the blood stream and therefore bioavailable. However, it should be noted that the average EC or ENDD intake for young adults and teenagers is much higher than that of an average smoker, presumably due to the mistaken assumption that ECs remove the carcinogenic risk associated with traditional smoking and tobacco products [34-36].

Another limitation of this study was the use of only nicotine as the experimental condition. Most ECs and ENDDs include a preservative in their nicotine suspension, such as glycerol [37,38]. Future studies will include concomitant administration of both nicotine and glycerol in order to more accurately assess the potential effects on cell growth and cell viability. Finally, the most popular ECs and ENDDs now include other additives to mask the flavor of pure nicotine suspensions, which may introduce other harmful and damaging additives that were not part of this testing protocol.

5. Conclusions

In summary, these data provide further evidence that nicotine administration may present significant risks to cell viability and growth over time. In addition, this study demonstrated that these effects were evident in both cancerous and non-cancerous cells – a finding that may suggest more research in this area is needed to determine the mechanisms that might be shared between these differing cell types. Finally, these data also suggest the possibility that short-term use and administration of nicotine may not only inhibit growth and reduce viability but may also induce cell death – a troubling finding that appears to have a growing

body of corroborating evidence that may suggest more caution may be needed when
advertising or marketing ECs or ENDDs are low- or no-risk alternatives to cigarette smoking.

Ethical Disclaimer:

As per international standard or university standard written ethical permission has been
collected and preserved by the authors.

Consent: NA

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