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Comparison of electronic cigarette refill fluid cytotoxicity using embryonic and adult models

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ABSTRACT

Electronic cigarettes (EC) and refill fluids are distributed with little information on their pre- and postnatal health effects. This study compares the cytotoxicity of EC refill fluids using embryonic and adult cells and examines the chemical characteristics of refill fluids using HPLC. Refill solutions were tested on human embryonic stem cells (hESC), mouse neural stem cells (mNSC), and human pulmonary fibroblasts (hPF) using the MTT assay, and NOAELs and IC_{50}s were determined from dose–response curves. Spectral analysis was performed when products of the same flavor had different MTT outcomes. hESC and mNSC were generally more sensitive to refill solutions than hPF. All products from one company were cytotoxic to hESC and mNSC, but non-cytotoxic to hPF. Cytotoxicity was not due to nicotine, but was correlated with the number and concentration of chemicals used to flavor fluids. Additional studies are needed to fully assess the prenatal effect of refill fluids.

1. Introduction

Electronic cigarettes (EC) are nicotine delivery devices that are rapidly gaining acceptance as an alternative to conventional cigarettes with little knowledge regarding their effects on prenatal development or adult health [1–3]. EC have a mouthpiece containing a fluid-filled cartridge, an atomizer used to vaporize the cartridge fluid, and a battery that powers the atomizer [3]. The cartridge fluid usually contains nicotine, flavorings, and a humectant that when heated by the atomizer creates an inhalable aerosol. In some EC, the cartridge and atomizer are combined into a single unit called a "cartomizer" [3,4]. Refill fluid, also known as E-juice or E-liquid, contains flavoring, nicotine, and a humectant(s), such as propylene glycol (PG) and/or vegetable glycerin (VG). Used EC cartridges or cartomizers can be refilled with drops of refill fluid, which is readily available often from third party vendors on the Internet or in shopping malls.

While the detrimental effects of conventional cigarette smoke on both adult and prenatal health are well documented [5–9], little direct work has been done on the health effects of EC products, in spite of a recognized need for such information [10]. It has been proposed that EC are less harmful than conventional tobacco products due to their lower total number of chemicals and lower concentration of carcinogens [11,12]. EC refill fluids are often sold by vendors other than the EC manufacturers, and they have received even less evaluation than EC devices themselves. As a step toward better understanding the health effects of EC, we evaluated the cytotoxicity of 40 samples of EC refill fluid using cells that model both embryonic and adult stages of the life cycle. With the introduction of human embryonic stem cells (hESC) [13], it is now possible to examine effects of consumer products and environmental chemicals on cells that model an early stage of prenatal development [14]. Recent studies have shown that hESC when cultured in vitro have the characteristics of the epiblast cells present in young

Abbreviations: DMEM, Dulbecco's modified Eagle's medium; EC, electronic cigarette; DPBS, Dulbecco's phosphate buffered saline; EDTA, ethylenediaminetetraacetic acid; hESC, human embryonic stem cells; hPF, human pulmonary fibroblasts; HPLC, high pressure liquid chromatography; IC_{50}, concentration that produces a 50% inhibition when compared to a control; mNSC, mouse neural stem cells; MTT, 3-(45-dimethylthiazol-2-yl)-25-diphenyltetrazolium bromide; NOAEL, no observed adverse effect level; PG, propylene glycol; VG, vegetable glycerin.

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Table 1
NOAELs, and IC_{50}s for refill fluid products in the screen.

Inv. no	Refill fluid	Company	Nicotine (mg/ml)	hESC		mNSC		hPF	
				IC_{50}	NOAEL	IC_{50}	NOAEL	IC_{50}	NOAEL
32	Propylene glycol	FS-USA[a]		>1	0.3	>1	0.3	>1	>1
33	Vegetable glycerin	FS-USA		>1	>1	>1	>1	>1	>1
18	Bubblegum	FS-USA	24	>1	0.3	>1	0.3	>1	>1
30	Butterscotch	FS-USA	0	>1	0.3	>1	0.1	>1	0.001
29	Butterscotch	FS-USA	6	>1	0.1	>1	0.1	>1	>1
26	Caramel	FS-USA	0	>1	0.3	>1	0.1	>1	>1
27	Caramel	FS-USA	6	>1	0.3	>1	0.3	>1	0.1
28	Caramel	FS-USA	6	>1	0.3	>1	0.3	>1	0.3
40	Caramel	Global Smoke	18	0.75	0.1	>1	0.3	0.41	0.01
19	Butterfinger	FS-USA	24	0.51	0.1	>1	0.3	>1	>1
23	Menthol Arctic	FS-USA	0	0.45	0.3	>1	>1	0.45	0.3
7	Wisconsin frost	Red Oak	18	0.37	0.1	0.61	0.3	>1	>1
1	Domestic	Red Oak	18	0.37	0.1	0.31	0.1	>1	>1
13	JC original	Johnson Creek	18	0.38	0.03	0.45	0.3	>1	>1
12	French vanilla	Johnson Creek	18	0.34	0.1	0.37	0.1	0.97	0.3
25	Vanilla Tahity	FS-USA	0	0.36	0.1	0.35	0.1	0.19	0.03
17	Tennessee cured	Johnson Creek	18	0.26	0.01	0.32	0.1	>1	0.3
5	Tennessee cured	Red Oak	18	0.32	0.1	0.09	>1	>1	0.03
2	Island	Red Oak	18	0.24	0.01	0.30	0.1	>1	>1
24	Pure nicotine	FS-USA	100	0.23	0.01	0.31	0.1	0.35	0.001
6	Valencia	Red Oak	18	0.22	0.03	0.31	0.1	>1	0.03
14	Mint chocolate	Johnson Creek	18	0.12	0.01	0.28	0.1	>1	0.1
4	Swiss Dark	Red Oak	18	0.11	0.03	0.16	0.03	0.30	0.1
21	Caramel	FS-USA	0	0.1	0.03	0.14	0.03	0.22	0.01
11	Espresso	Johnson Creek	18	0.08	0.01	0.30	0.1	>1	0.3
3	Mercado	Red Oak	18	0.08	0.01	0.09	0.03	0.82	0.3
15	Simply strawberry	Johnson Creek	18	0.06	0.01	0.43	0.3	>1	0.1
8	Arctic Menthol	Johnson Creek	18	0.05	0.01	0.19	0.1	>1	0.3
20	Butterscotch	FS-USA	0	0.06	0.03	0.22	0.03	0.26	0.03
16	Summer peach	Johnson Creek	18	0.04	0.01	0.45	0.1	>1	0.3
9	Black cherry	Johnson Creek	18	0.05	0.01	0.16	0.1	>1	0.3
34	JC original	Johnson Creek	11	0.04	0.01	0.46	0.1	>1	>1
10	Chocolate truffle	Johnson Creek	18	0.03	0.01	0.26	0.03	>1	>1
31	Tennessee cured	Johnson Creek	11	0.03	0.01	0.30	0.1	>1	0.001
22	Cinnamon Ceylon	FS-USA	0	0.01	0.01	0.04	0.01	0.07	0.03
41	Butterscotch[b]	Freedom Smoke FlavourArt	0	–		0.58	0.3	0.26	0.03

[a] FS-USA, Freedom Smoke USA.
[b] This was not a part of the original screen.

implantation embryos [15,16]. Although some toxicological work has been done previously using hESC [17,18], adaptation of these cells to standard toxicological studies has been slow because they grow in colonies that are difficult to count and plate accurately. We recently developed a method that is amenable to studying hESC in 96-well plate assays, such as the MTT assay. In the current study, we have taken advantage of this method to perform dose–response cytotoxicity experiments using: (1) hESC, which model the epiblast stage of development [15,16], (2) mouse neural stem cells (mNSC) isolated from the brain of a newborn, and (3) human pulmonary fibroblasts (hPF), which represent an adult cell from one of the initial points of contact for inhaled EC aerosol. The purpose of our study was to compare the sensitivity of embryonic and adult cells to a range of EC refill products and to test the hypothesis that embryonic cells are more sensitive to EC product exposure than adult lung cells. The study included two humectants, 29 different flavors of refill fluid, products from four vendors, five concentrations of nicotine, and six samples that may have caused adverse health effects in users. HPLC spectral analysis was also done to determine if chemicals varied between products with the same flavor or between bottles of the same product.

2. Materials and methods

2.1. Sources of refill fluids

A convenience sampling procedure was adopted to select products for analysis. Products were manufactured by Freedom Smoke USA (Tucson, AZ), Global Smoke (Los Angeles, CA), Johnson Creek (Johnson Creek, WI), and Red Oak (a subsidiary of Johnson Creek). These manufacturers were chosen as they represent popular domestic companies whose products are readily available to e-cigarette users on the Internet. Thirty-six bottles of refill fluid containing various flavorings and nicotine concentrations were evaluated (Table 1). Thirty-four refill bottles were purchased from the manufacturers via the Internet, the Global Smoke product was purchased at a local mall (Riverside, CA), and one bottle was sent to us by a user who thought the refill sample had made her ill. The bottle from the user had been opened when we received it, and we cannot eliminate the possibility that the contents were modified. The bottles that we purchased were chosen to give a range of manufacturers, humectants, nicotine concentrations, and flavors. All bottles were given an inventory number.

2.2. Culturing hESC, mNSC, and hPF

H9-Oct4-GFP hESC, obtained from the Stem Cell Core at the University of California, Riverside, and H9 hESC obtained from WiCell (Madison, WI) were cultured in a 5% CO_2 incubator at 37 °C and 95% relative humidity using methods previously described in detail [19]. hESC were maintained on Matrigel (Fisher Scientific, Bedford, MA) coated 6-well plates (Falcon, Fisher Scientific, Chino, CA) containing complete mTeSR®1 Medium (Stem Cell Technologies, Vancouver, BC, Canada) and were used for experimentation when wells were 60–80% confluent. Each day, cultures were observed for normal morphology, and medium was changed. To subculture or prepare hESC for experiments, wells were washed with Dulbecco's phosphate buffered saline (DPBS) (GIBCO, Invitrogen, Carlsbad, CA), colonies were enzymatically detached using Accutase (eBioscience, San Diego, CA), and large cell clumps were mechanically dispersed using sterile glass beads. For MTT experiments, cell concentration was adjusted spectrophotometrically to produce 20,000 cells/well using a BioMate 3S Spectrophotometer (Thermo Fisher Scientific, Chino, CA).

mNSC were cultured in Dulbecco's modified Eagle's medium (DMEM) (Lonza, Walkersville, MD) containing 10% fetal bovine serum, 5% horse serum, 1% sodium pyruvate (Lonza, Walkersville, MD) and 1% penicillin–streptomycin (GIBCO, Invitrogen, Carlsbad, CA). The cells were cultured in Nunc T-25 tissue culture flasks (Fisher

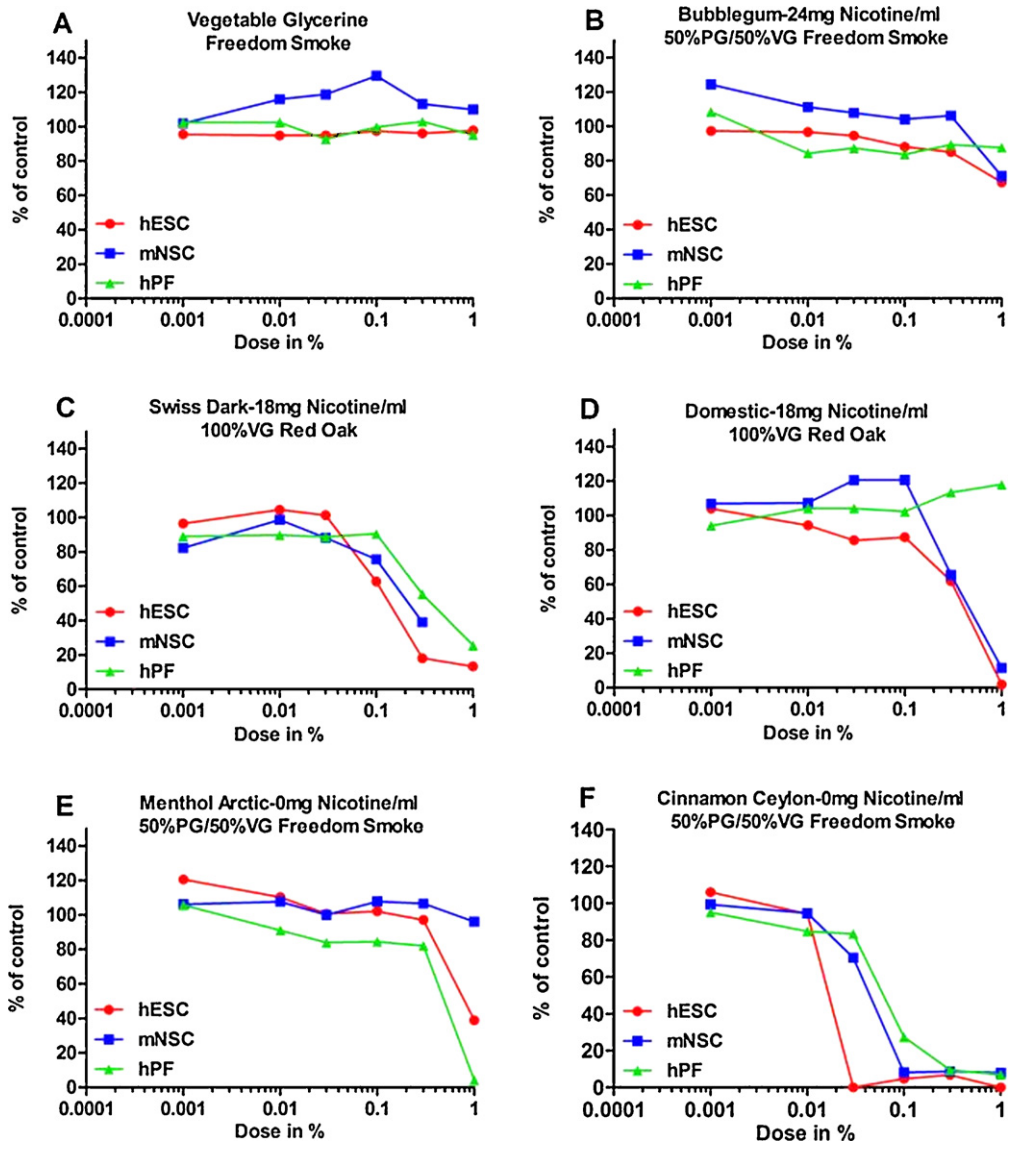

Fig. 1. Dose–response curves showing representative examples of data obtained in the MTT cytotoxicity assay. Absorbance (percentage of the control) from the MTT assay is plotted as a function of the refill fluid dose. (A) Vegetable glycerin (non-cytotoxic), (B) Bubblegum (non-cytotoxic), (c) Swiss Dark (moderately cytotoxic), (D) Domestic (moderately cytotoxic to the stem cells), (E) Menthol Arctic (moderately cytotoxic the hPF), (F) Cinnamon Ceylon (highly cytotoxic).

Scientific, Tustin, CA), medium was replaced on alternate days, and when confluency reached about 80%, cells were used in an experiment. To detach cells for testing, wells were washed with DPBS then treated with 0.05% trypsin EDTA/DPBS (GIBCO, Invitrogen, Carlsbad, CA) for 1 min at 37 °C. For the MTT assay, cells were plated at 2500 cells/well in 96-well plates.

Human pulmonary fibroblasts (hPF) (ScienCell, Carlsbad, CA) were cultured using the suppliers protocol in complete fibroblast medium containing 2% fetal bovine serum, 1% fibroblast growth serum, and 1% penicillin/streptomycin. hPF were grown on poly-L-lysine (15 μl/10 ml) coated T-25 flasks, which were prepared and incubated overnight prior to use. hPF were examined microscopically daily, and medium was changed every other day. hPF were cultured in 5% CO_2 at 37 °C and 95% relative humidity until 85% confluent, at which time they were used for MTT testing. For sub-culturing and experimental set up, cells were washed with DPBS and detached with 0.01% trypsin diluted in DPBS for 1 min at 37 °C.

2.3. Testing refill solutions for cytotoxicity using hESC, mNSC and hPF in the MTT assay

Thirty-five refill products were evaluated for cytotoxicity in 96-well plates using the MTT assay with hESC, mNSC and hPF. The 96-well plates were laid out to have negative controls in columns 1 and 2, followed by various doses of refill solution (0.001%, 0.01%, 0.03%, 0.1%, 0.3%, and 1%) in ascending order from left to right, followed by two additional negative controls in columns 10 and 11. The latter two controls were used to determine if any of the 1% doses produced vapor that impaired cell survival in adjacent wells lacking refill solution.

To set up an experiment with hESC, wells were coated with Matrigel, and then 50 μl of either mTeSR or mTeSR with varying doses of refill solution were added to each well. 50 μl of cell suspension in mTeSR (20,000 cells/well) were added to each well. Experiments with mNSC and hPF were set up in a similar manner except that mNSC were plated directly onto non-coated plates at 2500 cells/well and hPF were plated on poly-L-lysine coated plates (20 μl/10 ml) at 20,000 cells/well. After incubation at 37 °C, 5% CO_2 and 95% relative humidity for 48 h, the MTT assay was performed.

The MTT assay measures conversion of a yellow tetrazole (MTT) to a purple formazan that can be quantified spectrophotometrically at 570 nm [20]. Conversion to the colored formazan occurs in healthy cells with active mitochondria. After plates incubated 48 h, MTT (Sigma–Aldrich, St. Louis, MO) (5 mg/ml in DPBS with calcium and magnesium) (Fisher Scientific, Chino, CA) was added to each well, and the plates were rocked at least 5 min to disperse MTT, then incubated for 2 h at 37 °C, 95% relative humidity, and 5% CO_2. Plates were then drained of solution, and 100 μl of dimethyl sulfoxide (DMSO) (Fisher, Chino, CA) were added and mixed evenly with a pipette to form a uniformly colored solution. Absorbance was read at 570 nm using a Victor2 (PerkinElmer, Waltham, MA, USA) or Epoch (Biotek, Winooski, VT) microplate reader.

2.4. HPLC analysis of Butterscotch and Caramel flavored refill solution

Three Butterscotch (#20, #29, #30) and five Caramel (#21,#26, #28, #40, #27) flavored refill products (Table 1) were analyzed by HPLC. After performing the MTT assays, one additional Butterscotch flavored sample (#41) was received from

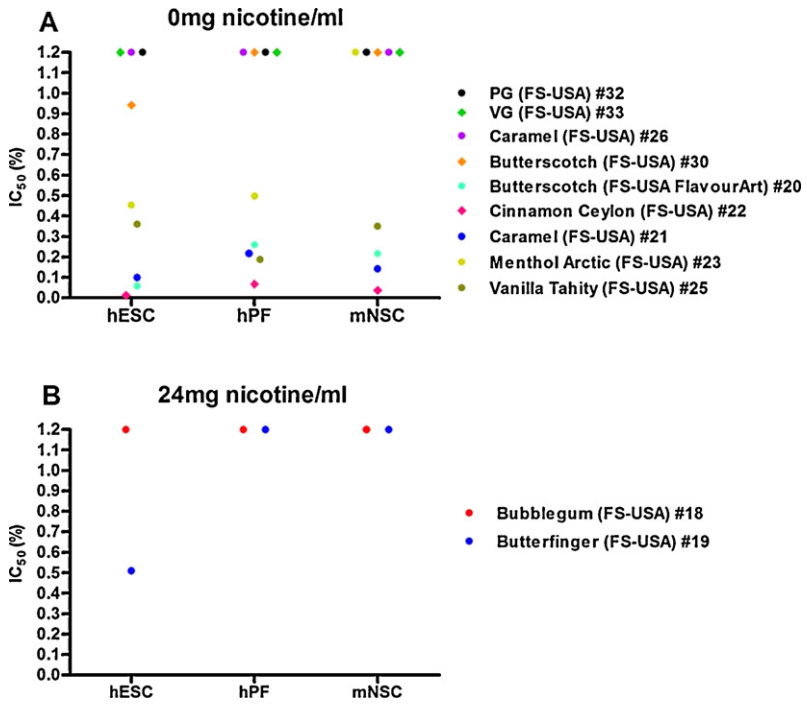

Fig. 2. Relationship between cytotoxicity and nicotine. The IC_{50}s (dose in percent) are plotted for each cell type for each product in a category. Points plotted at 1.2 were non-cytotoxic in the MTT assay. (A) IC_{50}s for cells treated with refill fluid containing 0 mg of nicotine. (B) IC_{50}s for cells treated with refill fluid containing 24 mg of nicotine/ml. There was no correlation between nicotine concentration and cytotoxicity.

Freedom Smoke USA, analyzed using HPLC, and tested for cytotoxicity using mNSC and hPF. Phosphoric acid (85%) and HPLC grade chemicals (triethylamine, water, and acetonitrile) were purchased from Fischer Scientific (Fair Lawn, NJ). Sodium hydroxide was purchased from EM Scientific (Gibbstown, NJ). Samples were analyzed using a Hewlett Packard Series 1100 HPLC, consisting of a quaternary pump, degasser, column thermostat and manual injector. A 200 mm × 4.6 mm Thermo Scientific Hypersil ODS C18 column with a particle size of 5 μm was used at 35 °C with a flow rate of 0.8 mL/min. The diode array detector signal was set to 260 nm with a bandwidth of 40 nm and a reference signal of 380 nm and bandwidth of 10 nm. The injection volume was 5 μL. An isocratic method was used with a buffered mobile phase consisting of 76.9% water, 23% acetonitrile, and 0.1% triethylamine. The pH of the mobile phase was adjusted daily to 7.6 using phosphoric acid and sodium hydroxide. A 5% stock solution of refill fluid in non-buffered mobile phase, consisting of 77% water and 23% acetonitrile was produced for each sample. The working concentration of refill fluids was 0.5%. Three-dimensional spectra were analyzed for each sample to determine the number of peaks and their elution time and relative height.

2.5. Data analysis

MTT absorbance data were normalized by setting the negative control group (column 2) in each row to 100%. All other wells in each row were expressed as a percentage of the negative control. IC_{50}s were computed with Prism software (GraphPad, San Diego, CA) using the log inhibitor vs. normalized response-variable slope with the top and bottom constraints set to 100% and 0%, respectively. When a sigmoidal curve could not be fit to the data using GraphPad, IC_{50}s were determined by eye to obtain a best fit. No observed adverse effect levels (NOAEL) were determined by reading directly off the dose–response curves.

3. Results

3.1. Dose–response of 35 refill products using the MTT assay

Refill solutions had various effects on cell survival in the MTT assay ranging from no evidence of cytotoxicity to high levels of toxicity (representative graphs are shown in Fig. 1; additional data are shown in Table 1 and Supplement Figs. 1–3). Products listed in Table 1 are grouped in a hierarchy of potency based on their IC_{50}s for hESC, which, in general, were more sensitive to refill solutions than the other two cell types. Table 1 also gives information on the NOAELs for each cell type and refill solution tested.

Refill products were grouped in three major categories: low cytotoxicity (IC_{50} > 1%) (Fig. 1A and B and Supplement Fig. 1), moderate cytotoxicity (IC_{50} between 0.1 and 1%) (Fig. 1C–E and Supplement Fig. 2), and high cytotoxicity (IC_{50} < 0.1%) (Fig. 1F and Supplement Fig. 3). The two humectants most often used in refill solutions, vegetable glycerin (VG) (Fig. 1A) and propylene glycol (PG) (Supplement Fig. 1A; Table 1), were non-cytotoxic for all cell types. An example of a non-cytotoxic refill fluid (Bubblegum #18) is shown in Fig. 1B. Five additional samples, which were Butterscotch or Caramel flavored, were also non-cytotoxic at the highest dose tested (Supplement Fig. 1).

Fifteen refill samples were moderately cytotoxic to hESC, and in general, mNSC responded similarly to these samples (Fig. 1C–E; Table 1, and Supplement Fig. 2). For most refill samples in this group, hESC and mNSC were killed by the 1% dose. In contrast, most (10 of 15) refill samples in this group had little or no effect on hPF (Supplement Fig. 2B–E, G–I, K–L). However, Freedom Smoke Menthol Arctic (Fig. 1E) and Global Smoke Caramel (Supplement Fig. 2 A) produced stronger cytotoxic effects on hPF than on the other two cells.

Twelve refill samples were highly cytotoxic to hESC (Fig. 1F, Table 1, Supplement Fig. 3), and all samples in this group affected mNSC. In contrast, the effect was not as strong for hPF, and 7 of 12 samples in this group did not affect hPF at the highest dose (Supplement Fig. 3B–E, G, H, J, K). Cinnamon Ceylon was the most potent sample tested and the only sample that produced strong cytotoxic effects on all three cell types (Fig. 1F).

3.2. Relationship between nicotine concentration and potency

In the samples studied, nicotine concentration ranged from 0 to 24 mg/ml. The IC_{50}s for samples within each nicotine concentration were compared for the three cell types to determine if nicotine concentration correlated with potency (Fig. 2). Points plotted at 1.2 on the *Y*-axis in Fig. 2 had IC_{50}s greater than 1% and were considered non-cytotoxic.

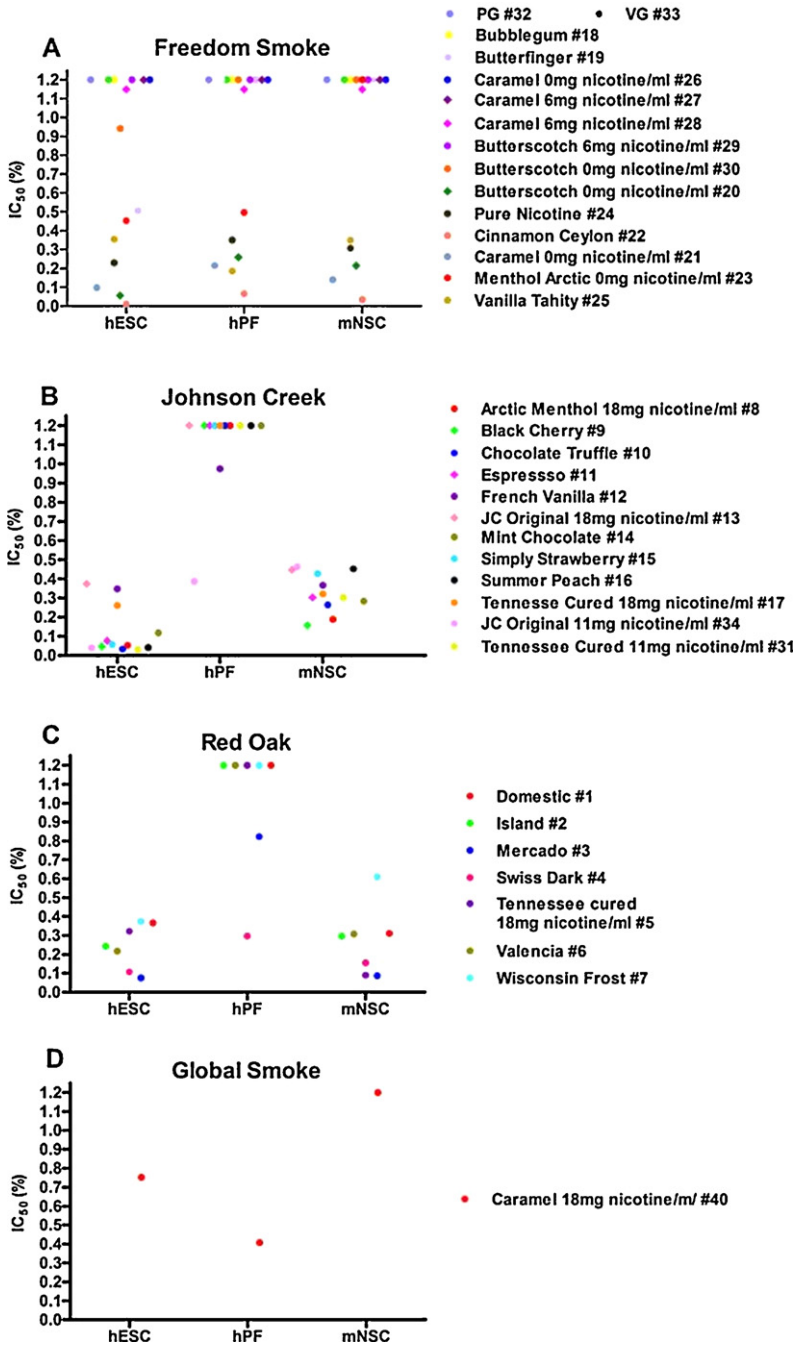

Fig. 3. Relationship between brand and cytotoxicity: The IC_{50}s (dose in percent) are plotted for each cell type for each product in a category. Points plotted at 1.2 were non-cytotoxic in the MTT assay. (A) IC_{50}s for cells treated with Freedom Smoke products. (B) IC_{50}s for cells treated with Johnson Creek products. (C) IC_{50}s for cells treated with Red Oak products. (D) IC_{50}s for the Global Smoke product.

Nine refill samples, including PG and VG, contained no nicotine and fell into all three categories of potency (low, moderate, and high cytotoxicity) (Fig. 2A), indicating cells did not survive better in samples lacking nicotine. Two samples contained 24 mg nicotine/ml, and were either non-cytotoxic or moderately cytotoxic (Fig. 2B), indicating high levels of nicotine were not correlated with high cytotoxicity.

3.3. Relationship between company of origin and potency

Graphs comparing potency among refill products from four companies and comparing sensitivity of each cell type to each product are shown in Fig. 3. Most samples ($N = 15$) came from Freedom Smoke USA, and potency ranged from non-cytotoxic to highly cytotoxic (Fig. 3A). Cinnamon Ceylon was the only sample that was highly cytotoxic to all cell types.

The cytotoxic response was very different for the Johnson Creek samples ($N = 12$), most of which were highly or moderately cytotoxic to hESC and mNSC, with mNSC being slightly less sensitive than the hESC. In contrast, all but one sample was non-cytotoxic to hPF (Fig. 3B). A similar pattern was seen for Red Oak products (Fig. 3C), which were moderately or highly cytotoxic to hESC and mNSC, while most were non-cytotoxic to hPF.

The IC_{50}s for the sample obtained from Global Smoke ranged from non-cytotoxic (mNSC) to moderately cytotoxic (hESC and hPF) (Fig. 3D).

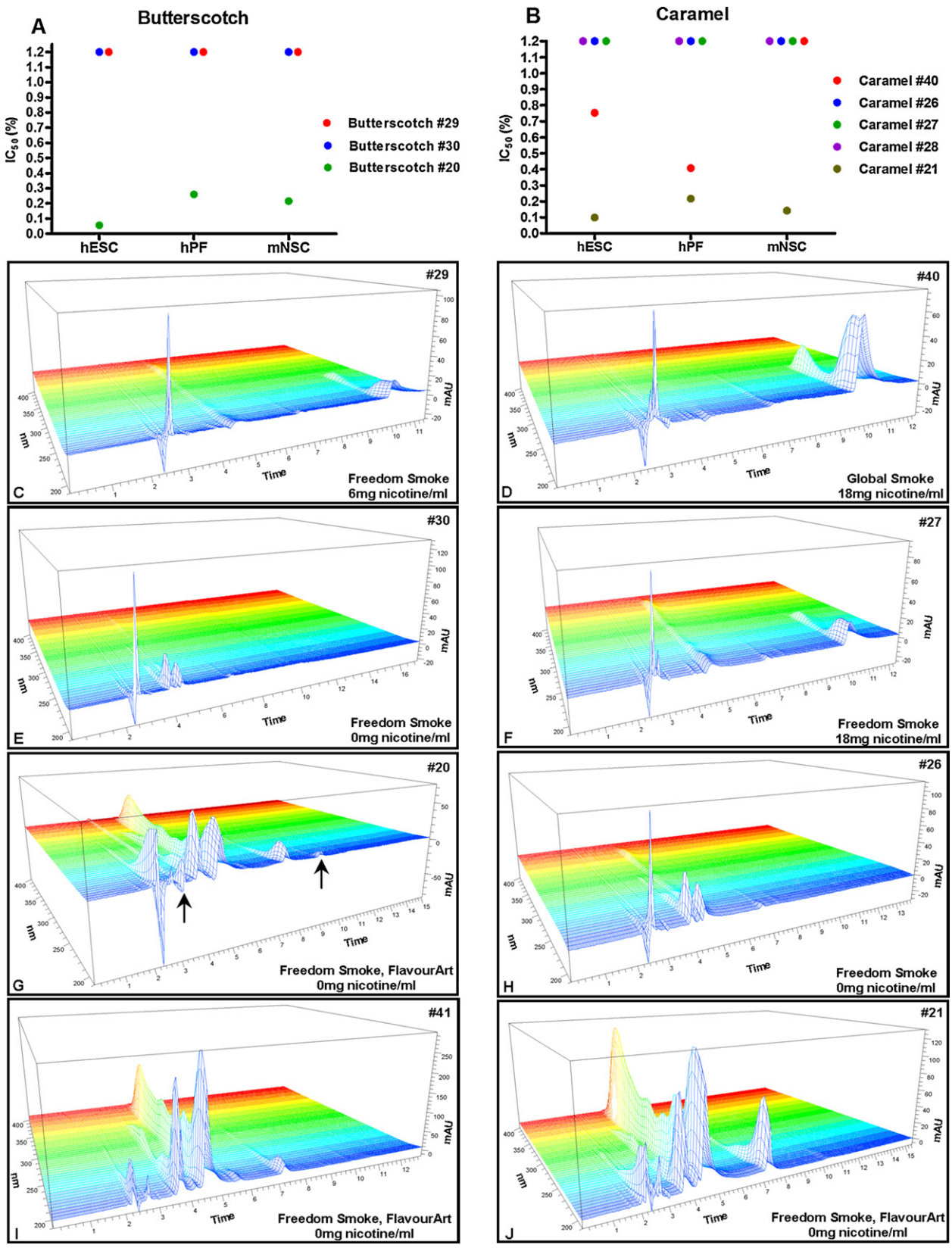

Fig. 4. Relationship between flavors and cytotoxicity: (A) IC_{50}s for cells treated with Butterscotch flavored refill fluid. (B) IC_{50}s for cells treated with Caramel flavored refill fluid. For A and B, the IC_{50}s (dose in percent) are plotted for each cell type for each product in a category. Points plotted at 1.2 were non-cytotoxic in the MTT assay. (C, E, G, I) Three-dimensional HPLC spectra for four samples of Butterscotch flavored refill fluid. (D, F, H, J) Three-dimensional HPLC spectra for four samples of Caramel flavored refill fluid. *X* axis = time (minutes), *Y* axis = absorbance (mAu), *Z* axis = wavelength in nm. First peaks are humectants, peak between 10 and 11 min in some spectra is nicotine, and peaks between the humectants and nicotine are flavoring peaks. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

3.4. Relationship between flavors and potency

The IC_{50}s for the three Butterscotch and five Caramel samples included in the study ranged from non-cytotoxic to highly cytotoxic (Fig. 4A and B). To determine if chemical differences in these samples could account for the cytotoxic differences, three-dimensional HPLC spectra of the Butterscotch and Caramel samples were analyzed.

HPLC spectra for the three Butterscotch samples from Freedom Smoke USA differed from each other (Fig. 4C, E, G). The first peaks to elute were the humectants, the peak eluting between 10 and 11 min is nicotine, and the peaks between the humectant and nicotine are flavorings. The two Butterscotch samples that were non-cytotoxic (Fig. 4C and D) had few flavoring peaks with low heights. In contrast, Butterscotch (FlavourArt #20) (Fig. 4G), which was cytotoxic to all cell types (Fig. 4A), had a complex spectrum with more flavor peaks that were higher than in the non-cytotoxic samples. The highly cytotoxic sample (bottle #20) was received from a user and therefore could have been altered after manufacture. A bottle (#41) which had an identical label to that in Fig. 4G (bottle #20) was received directly from the vendor after the 35-sample study was completed and was tested in the MTT assay and by HPLC (Fig. 4I). The original sample (Fig. 4G, bottle #20) had two minor peaks (arrows) that were not present in the new sample (Fig. 4I, bottle #41). Moreover, the two major peaks in the new sample (Fig. 4I) were 4–5 times higher than the corresponding peaks in the original sample (Fig. 4G), indicating a much higher concentration of these chemicals in bottle #41. The refill solution in both bottles was moderately to highly cytotoxic (Fig. 4A and Supplement Fig. 4).

Spectra for four Caramel samples (Fig. 4D, F, H, J) were different from each other (#27 is not shown). Global Smoke #40 contained mainly humectant (PG) and nicotine with virtually no flavoring peaks (Fig. 4D). Freedom Smoke USA #27 contained humectant (VG), nicotine, and a small flavoring peak (Fig. 4F). Freedom Smoke USA #26 had humectant (VG) and two flavoring peaks of small height (Fig. 4H). Freedom Smoke USA FlavourArt #21 contained three flavoring peaks that were 5–6 times higher than peaks in the other three samples (Fig. 4J). The Caramel product that had the largest number of peaks and the highest peaks (Freedom Smoke, #21) (Fig. 4J) was also the most cytotoxic (Fig. 4B).

4. Discussion

Understanding the health effects of EC refill fluid is important as these products have become widely distributed without much prior testing. Refill fluid is handled by users, manufacturers, and potentially by children living in homes where EC are used. As a step to understanding how EC products affect human health, we compared the cytotoxicity of 35 refill fluid samples using embryonic and adult cells. Refill products varied significantly in their potency over the dose range tested. In general, stem cells from embryos (hESC) and newborns (mNSC) were more sensitive to refill solutions than differentiated adult lung fibroblasts, as shown clearly in the Johnson Creek/Red Oak data. Of 35 products tested, only Caramel #40 and Menthol Arctic #23 had stronger effects on hPF than on the stem cells. These data support our hypothesis that cells from embryos and newborns are more sensitive to EC products than adult cells and are consistent with the concept that embryos are usually more sensitive to environmental chemicals than adults [21]. Our data further demonstrate the importance of using multiple cell types, including embryonic cells, when evaluating EC products. The cytotoxic effects that some refill fluids produced on stem cells could translate into embryonic loss or developmental defects during pregnancy. While it is not yet known what dose of refill fluid reaches an embryo or fetus when a pregnant woman receives dermal, oral, or pulmonary exposure, our data indicate that further work should be done on the effects of these products during pregnancy.

It is possible that our data underestimate the cytotoxicity of refill fluids to lung cells. In a preliminary trial, vapors from 10% doses of some refill fluids killed control cells in adjacent wells. To avoid vapor effects, assays were performed at a maximum concentration of 1%. This would be 100 times less than a user would receive on their skin or inhale into their mouth/lungs. The NOAELs and IC_{50}s should therefore be interpreted with this dose range in mind. Exposure of lung cells to full strength aerosol, which is heated, may have stronger effects than reported in our study, and even samples we found to have low cytotoxicity with lung fibroblasts may be cytotoxic in vivo at full strength.

The potency of refill products varied greatly, demonstrating the importance of evaluating multiple products. Some products had no effect at the doses tested, while others killed all cells at doses lower than a user may receive. Cinnamon Ceylon (#22) was the most potent of the refill fluids tested and strongly inhibited survival of all cell types. Refill fluid users have expressed caution about cinnamon flavored products on Internet blogs and have mentioned mouth, throat, and lung problems when using cinnamon flavored refill fluid (http://www.e-cigarette-forum.com/forum/health-safety-e-smoking/212870-do-you-vape-cinnamon-flavors-read.html).

Cytotoxicity studies on EC products are rare. When various European refill fluid aerosols were tested in the MTT assay using mouse 3T3 fibroblasts, only 1 out of 15 products showed cytotoxicity at the highest doses tested [22,23]. We found more cytotoxic samples in our set of 35 refill products; however, the European study is not directly comparable to ours due to differences in products, sample preparation, experimental design, and method of analysis.

Several major conclusions can be drawn from our study. First, hESC were generally more sensitive to refill fluids than the other two cell types, and mNSC were generally more sensitive than hPF. Secondly, no company emerged as having all non-cytotoxic or all cytotoxic refill products. However, an interesting pattern was observed for samples from Johnson Creek and Red Oak, which were generally cytotoxic to stem cells and non-cytotoxic to lung fibroblasts. Third, there was no correlation between cytotoxicity and nicotine concentration for the dose range used. Fourth, each refill product needs individual evaluation to determine cytotoxicity, preferably using multiple cell types. Fifth, the refill fluid provided to us by a user who thought the sample had made her ill was moderately to highly cytotoxic, as was a duplicate bottle purchased directly from the vendor. Sixth, within a particular flavor, cytotoxicity was highly variable, even when the flavor came from a single manufacturer, as was seen with the Butterscotch and Caramel samples from Freedom Smoke. For example, one Butterscotch sample (#41) received directly from the company was highly toxic, while two other Butterscotch flavors (#29 and #30) from the same company had low toxicity. HPLC analysis showed that increased cytotoxicity within a flavor was correlated with an increase in the number and height of the flavoring peaks (Fig. 4C, E, G, I). In addition, two different bottles from the same manufacturer with identical Butterscotch labels (#20 and 41) had slightly different chemical composition and significantly different amounts of the two major flavoring chemicals (Fig. 4G and I). Since one of these bottles was supplied to us by a user, we cannot eliminate the possibility that the two additional peaks were added after manufacture. However, the bottle we purchased from the company had much higher concentrations of the two major flavoring peaks. Since it is unlikely the user could have removed flavoring from the bottle, the difference in amount of flavoring between bottles #20 and #41 probably represents a true difference in the contents of a single product from this company. Similar differences in the amount of added flavorings were seen in caramel flavored refill fluid from Freedom Smoke (e.g., bottles

#26 and #21) These data show that users cannot assume that the chemicals or the concentration of the chemicals used to create a particular flavor will be identical in all products having the same flavor. We are currently identifying the chemicals in those products that were cytotoxic, so that in the future refill products can be improved by using only non-cytotoxic flavorings at relatively low concentration.

Our data may help refill fluid users identify and avoid products that could pose health risks to themselves and their offspring. For example, Cinnamon Ceylon (#22) was highly potent for the three cell types and would likely present more risk than flavors such as Bubblegum (#18) which had low cytotoxicity for all cells. However, even products we found to be non-cytotoxic may produce different, possibly stronger, effects when used repeatedly at full strength doses. As related examples, PG, which is "generally regarded as safe" and was non-cytotoxic for all cell types in the MTT assay, increased respiratory, throat and nasal symptoms, and cause vocal cord inflammation with prolonged inhalation by theater workers [24], and chronic exposure to PG in indoor air may induce or exacerbate allergic symptoms, asthma, and rhinitis [25].

Lung fibroblasts were relatively robust and often not affected by doses of refill fluid that were cytotoxic to the two stem cell groups. However, lungs contain progenitor cells and stem cells that are critical to lung tissue regeneration and repair [26,27]. Further studies are needed to determine how lung stem cells and other lung cell types respond to refill fluid and if chronic exposure to inhaled refill fluid affects lung health. A recent human study showed that 5 min of EC inhalation significantly altered several measures of lung physiology [28]. The MTT assay used in our study measured cytotoxicity, while the latter study by Vardavas et al. measured physiological responses that do not include cell death, but could be important to the overall lung health.

5. Conclusions

Embryonic and neonatal stem cells were generally more sensitive to refill products than adult lung fibroblasts. Refill fluid users should be aware that: (1) the low doses and one time exposure used in our study may underestimate cytotoxicity, and (2) within a flavor, such as Butterscotch or Caramel, chemical composition and cytotoxicity were variable. The latter point demonstrates that it cannot be assumed that a specific flavor, such as Butterscotch, will always be non-cytotoxic. The results of this study, while preliminary, may be helpful to individuals who are considering using EC, to EC users who are trying to identify refill brands that have low cytotoxicity, to refill fluid suppliers concerned with user safety, to health care workers and physicians who advise EC users, and to policy makers involved in health and environmental issues relating to EC regulation.

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Conflict of interest

None declared.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.reprotox.2012.08.001.

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