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RESEARCH ARTICLE

Cytotoxicity evaluation of electronic cigarette vapor extract on cultured mammalian fibroblasts (ClearStream-LIFE): comparison with tobacco cigarette smoke extract

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Abstract

Context: Electronic cigarettes (ECs) are used as alternatives to smoking; however, data on their cytotoxic potential are scarce.
Objective: To evaluate the cytotoxic potential of 21 EC liquids compared to the effects of cigarette smoke (CS).
Methods: Cytotoxicity was evaluated according to UNI EN ISO 10993-5 standard. By activating an EC device, 200 mg of liquid was evaporated and was extracted in 20 ml of culture medium. CS extract from one cigarette was also produced. The extracts, undiluted (100%) and in five dilutions (50%, 25%, 12.5%, 6.25% and 3.125%), were applied to cultured murine fibroblasts (3T3), and viability was measured after 24-hour incubation by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide assay. Viability of less than 70% was considered cytotoxic.
Results: CS extract showed cytotoxic effects at extract concentrations above 12.5% (viability: 89.1 ± 3.5% at 3.125%, 77.8 ± 1.8% at 6.25%, 72.8 ± 9.7% at 12.5%, 5.9 ± 0.9% at 25%, 9.4 ± 5.3% at 50% and 5.7 ± 0.7% at 100% extract concentration). Range of fibroblast viability for EC vapor extracts was 88.5–117.8% at 3.125%, 86.4–115.3% at 6.25%, 85.8–111.7% at 12.5%, 78.1–106.2% at 25%, 79.0–103.7% at 50% and 51.0–102.2% at 100% extract concentration. One vapor extract was cytotoxic at 100% extract concentration only (viability: 51.0 ± 2.6%). However, even for that liquid, viability was 795% higher relative to CS extract.
Conclusions: This study indicates that EC vapor is significantly less cytotoxic compared tobacco CS. These results should be validated by clinical studies.

Keywords

Cytotoxicity, electronic cigarette, fibroblasts, *in vitro*, nicotine, smoking, tobacco harm reduction

History

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Introduction

There is overwhelming evidence that smoking is a major cause of respiratory and cardiovascular disease (Bartecchi et al., 1995). Even low cigarette consumption has significant effects on human health (Bjartveit & Tverdal, 2005). Complete cessation is the goal for all smokers; however, many of them are unwilling or unable to quit. Therefore, harm reduction strategies have been developed, aiming at substituting tobacco cigarettes with other products that deliver less harmful constituents to human organism (Stratton et al., 2001).

Electronic nicotine-delivery devices, commonly called electronic cigarettes (ECs), were invented in China and have been recently introduced to the market worldwide (Henningfield & Zaatari, 2010; Pauly et al., 2007) as an alternative and potentially safer habit. They consist of a battery-part, a cartridge containing liquid and an electrical resistance that gets warm by activation of the battery and evaporates the liquid. The liquid usually contains glycerol, propylene glycol, water, nicotine and a variety of flavors that the user can choose.

It is estimated that millions of people are using EC, and surveys suggest that they may be effective in smoking cessation (Etter, 2010). Although they do not contain or burn tobacco, which seems promising in avoiding delivery of harmful substances, no studies have specifically evaluated their toxicity. This has raised serious public health concerns (Cobb et al., 2010). Our research team has developed a series of protocols called ''ClearStream'' (CLarifying Evidence and Research on the Safety and The Risks of Electronic AtMos; atmos = vapor in Greek), to evaluate the toxicological, environmental and clinical effects of ECs. The purpose of this study (ClearStream-LIFE; LIFE = Living In-vitro Fibroblasts' Exposure) was to evaluate the *in vitro* cytotoxicity of vapor extract of 21 commercially available liquids used for EC and to compare it with the cytotoxicity of cigarette smoke (CS) extract.

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Materials and methods

Materials

A commercially available tobacco cigarette containing 1 mg of nicotine, 10 mg of tar and 10 mg of carbon monoxide was used for this experiment. Twenty-one commercially available liquids used for EC were obtained from the market in sealed bottles, each containing 10 ml of liquid (manufactured by FlavourArt s.r.l., Oleggio, Italy).The composition of EC liquids, as reported by the manufacturer, was (w/w) 46.17% propylene glycol USP, 44.92% glycerol USP, 8.11% water, 0.8% nicotine USP and <0.5% flavorings. The only difference between liquids composition was the flavorings used (Table 1). Twelve of the flavors were tobacco-like, while the rest were mostly fruit and sweet flavors. Each flavoring (including tobacco-like flavors) is a complex mixture of several physically extracted or chemically produced substances approved for use in food industry, for which no additional information was provided by the manufacturer. A commercially available EC device (510 T, Omega Vape, Manchester, UK) was used for vapor production. The device consists of a 3.7-volt lithium battery, an atomizer with a resistance of 2.2 Ohms wrapped over a fiberglass wick and a cartridge attached to the mouthpiece with a capacity of 1 ml of liquid. Care was taken to have the battery fully charged before each vapor extract was produced. Vacuum produced by inhalation (and by the vacuum pump during the experiment) leads to automatic activation of the battery, delivering 3.7 volts until the battery is discharged. The battery voltage was checked before and after use for the production of each EC extract with a digital voltmeter. A new atomizer was used for each vapor extract production; its resistance was measured with a digital multimeter and it was discarded if the resistance was found to differ by more than 0.1 volt. By applying 3.7 volts to a 2.2 Ohm resistance, the total energy for liquid evaporation in the experiment was 6.2 Watts.

An important issue was to test the function of the atomizer in conditions similar to the experimental setting, in order to ensure that no ''dry puff'' occurs. ''Dry puff'' is a phenomenon that occurs when the wick is insufficiently supplied with liquid, so that the evaporation rate is higher than the liquid supply rate to the wick; this leads to higher temperature of evaporation that is detected by the user as an unpleasant burning taste. This cannot be detected during any laboratory experiment. In addition, it is possible that the unpleasant taste is caused by substances that may form as a result of evaporation and that may or may not be toxic. Since the user detects and then avoids this phenomenon (by lowering device activation time and increasing puff intervals), the value of the experiment would be significantly undermined if ''dry puff'' was reproduced during the laboratory study. The only realistic way we found of testing this was to assign one of the researchers (who is a regular EC user) to test the EC device with three randomly selected atomizers from the pack delivered to the laboratory, using them in the same manner as during the experiment (2-second puffs, one puff every 60 s; see section ''*Production of extracts*''). Testing revealed that ''dry puff'' phenomenon was not reproduced when the EC atomizers were used in a way similar to the experimental setting.

Cell cultures

Cytotoxicity was measured by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay on monolayer-cultured mouse BALB/3T3 fibroblasts derived from Swiss

Table 1. Fibroblast viability in electronic cigarette vapor and cigarette smoke extracts.

	Dilutions						
Extracts	100%[a]	50%[b]	25%[c]	12.5%[d]	6.25%[e]	3.125%[f]	*p**
Tuscan[g]	94.5 ± 2.8	99.8 ± 5.7	104 ± 1.5	101.4 ± 4.1	100.7 ± 5.9	98.6 ± 3.8	0.216
Black fire[g]	96.3 ± 9.9	93.4 ± 2.5	94.4 ± 1.6	104.6 ± 2.9	95.3 ± 4.3	97 ± 3.2	0.159
Ozone[g]	90.7 ± 9.9	95.9 ± 9.1	96.2 ± 4.3	94.9 ± 6	96.7 ± 5.1	97 ± 4.9	0.879
Reggae night[g]	81.3 ± 5.1	90.3 ± 3.7	89.5 ± 4.2	89.7 ± 3.4	90.2 ± 5.7	91.6 ± 4.2	0.132
Vanilla	100 ± 2.4	98.5 ± 3.5	100.3 ± 2.0	100.1 ± 0.8	104.1 ± 3.1	98.3 ± 3.3	0.183
7foglie[g]	81.4 ± 2.9	87.5 ± 1.5	89.4 ± 4.0	87.1 ± 8.3	89.6 ± 12.1	93.2 ± 10.7	0.587
Max blend[g]	96.2 ± 6.0	97 ± 6.9	102.1 ± 7.4	111.8 ± 4.5	114.3 ± 1.7	115.5 ± 5.3	0.003
Virginia[g]	78.4 ± 14.4	86.1 ± 13.5	91.3 ± 15.6	96.4 ± 16.2	106.3 ± 9.7	104.4 ± 10.7	0.478
Perique black[g]	79.3 ± 1.5	89.8 ± 2.4	94.7 ± 1.2	95.3 ± 5.2	95.1 ± 2.4	93.9 ± 3.4	<0.001
Layton blend[g]	101.1 ± 1.0	103.7 ± 0.8	102.7 ± 2.8	100.6 ± 2.1	103.4 ± 5.5	97.9 ± 4.2	0.295
Hypnotic[g]	93.8 ± 10.8	95.2 ± 14.0	106.2 ± 6.5	97.4 ± 5.1	100.6 ± 7.4	98.5 ± 3.9	0.579
Hazelnut	88.7 ± 1.4	90.1 ± 5.6	93.5 ± 6.7	91.5 ± 1.5	115.3 ± 8.0	117.8 ± 13.4	0.001
Shade[g]	83.6 ± 5.1	92.5 ± 3.9	94.6 ± 5.0	97.8 ± 5.9	101.5 ± 2.5	101.9 ± 1. 3	0.002
RY4[g]	88.4 ± 8.1	96.1 ± 3.7	98.7 ± 6.4	95.8 ± 7.4	98.9 ± 6.3	98.9 ± 5.9	0.378
Strawberry	85.8 ± 2.8	95.4 ± 2.3	97.5 ± 1.5	104.0 ± 6.2	99.6 ± 1.4	107.5 ± 1.2	<0.001
Managua	79.1 ± 2.4	79.9 ± 3.3	79.1 ± 3.1	85.8 ± 2.0	86.4 ± 1.7	88.5 ± 3.5	0.002
Burley	102.2 ± 3.4	95.8 ± 2.9	97.6 ± 1.3	97.3 ± 3.4	106.2 ± 8.3	100.5 ± 6.2	0.171
Apple	95.2 ± 1.2	87.4 ± 2.7	100.8 ± 8.2	95.6 ± 3.9	101.8 ± 3.1	106.6 ± 15.6	0.106
Licorice	95.4 ± 3.9	93.9 ± 2.8	96.5 ± 2.6	98.5 ± 4.4	98.9 ± 2.0	99.6 ± 2.5	0.252
Chocolate	87.6 ± 2.2	89.6 ± 0.6	93.2 ± 1.3	93.4 ± 1.5	93.7 ± 1.9	98.9 ± 1.2	<0.001
Coffee	51.0 ± 2.6	85.9 ± 11.8	92.0 ± 8.9	101.5 ± 3.1	112.2 ± 3.6	114.5 ± 1.1	<0.001
CS	5.7 ± 0.7	9.4 ± 5.3	5.9 ± 0.9	72.8 ± 9.7	77.8 ± 1.8	89.1 ± 3.5	<0.001

Values are presented as mean ± standard deviation. Viability is expressed as percent, compared to untreated cells.
CS = cigarette smoke.
For electronic cigarette liquid extracts, dilutions represent (w/v): [a]1%, [b]0.5%, [c]0.25%, [d]0.125%, [e]0.0625% and [f]0.03125%.
**p* value for comparison between different extract concentrations in each liquid and in tobacco cigarette (ANOVA).
[g]Tobacco flavors.

albino mouse embryos (NIH 3T3 Batch 2 051163, NIH AIDS Research & Reference Reagent Program), according to UNI ISO 10993-5 standard. Cells were grown in Dulbecco's basal medium (Euroclone), supplemented with fetal bovine serum (Euroclone), penicillin–streptomycin 0.1 mg/ml (Euroclone), kanamycin 0.1 mg/ml (SIGMA, St Louis, MO), non-essential amino acid 0.1 mg/ml (SIGMA) and 4 mM glutamine (Euroclone). The doubling time of this cell line was 16–20 h.

Production of extracts

Vapor extract was produced by simulating EC use. The EC device was connected to a flask containing culture medium through a sealed tube. Horizontal orientation of the device was chosen, because this is the orientation of the device during real EC use. The other end of the tube was inside the flask, just above the culture medium level. A vacuum pump was connected to the flask; vacuum from the pump automatically triggered the EC device. The vapor was allowed to flow into the flask, over the medium. The EC cartridge was filled with 400 mg of liquid, and a number of inhalation simulations were performed in order to consume 200 mg of liquid, therefore having a theoretical concentration of 1% (w/v) into the culture medium of the flask (denoted as 100% EC extract). Weighting of the EC cartridge was performed before and during the experiment by a precision scale (Mettler, model AB104-S, precision of 0.1 mg), in order to make sure that the quantity of liquid consumed did not exceed 200 mg. Each inhalation simulation lasted 2 s, with 60 s between inhalations. The medium inside the flask was kept swirling during the experiment. CS extract was produced by using a similar method. Inhalation simulations, consisting of 2-second puffs every 60 s, were performed until one cigarette was consumed. The resulting solution was denoted as 100% CS extract. Immediately after preparation, all EC vapor and CS extracts were used in cell cultures.

Treatment and exposure

Cells were seeded in 96-well plate with Dulbecco's basal medium plus 10% fetal bovine serum and maintained in culture for 24 h (5% CO_2, 37 °C, >90% humidity) in order to form a semi-confluent monolayer. In each well, 100 µl of a cell suspension of 1×10^5 cells/ml was dispensed. A different plate was prepared for each extract testing. On the next day, each plate was examined under the microscope to ensure that cell attachment was even across the plate. Then, the medium was aspirated and replaced by medium containing the CS and EC liquid extracts in one undiluted (100%) and five diluted samples (50%, 25%, 12.5%, 6.25% and 3.125%). For the EC extract, 100% EC extract equals to a vapor extract concentration of 1%. Three different wells were treated with each dilution, and columns 2 and 11 were used to culture cells with normal medium (without extract, untreated cells); then, they were incubated for 24 h at 37 °C. Subsequently, cells were tested for viability by MTT assay. Untreated cells were used as controls.

MTT assay

The assay was performed according to the method developed by Mossman (1983). After incubation, the culture medium was removed and replaced with 10 µl of 1 mg/ml MTT. The cells were then incubated for 2 h. MTT is cleaved by mitochondrial dehydrogenases of viable cells, leading to the formation of purple crystals, representing formazan metabolism, which are insoluble in aqueous solutions. The solution was then removed and replaced with 200 µl/well of isopropanol to extract and solubilize the formazan. It was incubated for 30 min at room temperature under medium speed shaking. Then, the solution was measured spectrophotometrically. The absorbance at 570 nm was measured with a microplate reader (Tecan, model Sunrise Remote), and background subtraction was adjusted with absorbance readings at 690 nm. The absorbance values were normalized by setting the negative control group (untreated cells) in each row to 100%. Subsequently, the viability of the treated cells was expressed as a percent of untreated cells.

Quality check of assay

According to UNI ISO 10993-5 standard, a test meets acceptance criteria if the left (column 2) and the right (column 11) mean of the blanks do not differ by more than 15% from the mean of all blanks; this criterion was met in all our experiments. Sodium lauryl sulfate (SLS; SIGMA) was used as positive control in order to demonstrate an appropriate test system response. Historically, inhibitory concentration 50 (IC_{50}) of SLS is 0.093 mg/ml with 95% CI of 0.070–0.116 mg/ml (Spielmann et al., 1991). A test meets acceptance criteria if IC_{50} for SLS is within the 95% CI; in our experiment, IC_{50} for SLS was 0.100 mg/ml. Finally, the absolute value of optical density, OD_{570}, obtained in the untreated wells indicates whether the 1×10^4 cells seeded per well have grown exponentially with normal doubling time during the 2 days of the assay. In our experiments, OD_{570} of untreated cells were $\geq$0.2, meeting the acceptance criteria of UNI ISO 10993-5.

Statistical analysis

All data are reported as mean ± standard deviation. One-way analysis of variance (ANOVA) was used for comparison of percent viability between different extract concentrations of the same liquid. If statistically significant differences were found, post-hoc analysis was performed with Bonferroni test to determine which extract concentrations had different effects on viability. No observed adverse effects level (NOAEL) was defined as the lowest extract concentration that showed statistically significant lower viability compared to the 3.125% extract concentration. The difference in percent viability between CS extract and each EC vapor extract was also assessed with one-way ANOVA. Linear regression analysis was used to determine whether tobacco flavoring was associated with a statistically significant difference in viability. IC_{50} (the concentration of extract that produced 50% viability) was estimated from regression plots. According to UNI ISO 10993-5 standard, viability of less than 70% by MTT assay was considered cytotoxic. All analyses were performed with commercially available software (SPSS v18, Chicago, IL), and a two-tailed P value of ≤ 0.05 was considered statistically significant.

Results

Fibroblast viability measurements for each EC liquid and CS extracts at different dilutions are displayed in Table 1. From the 21 samples examined, only ''Coffee'' exhibited a cytotoxic effect; this was observed at the highest extract concentration only. Figures S1–S7 (supplemental material) display fibroblast viability for all EC liquids together with the respective viability for CS extract. The range of fibroblast viability for all EC liquids was 88.5–117.8% at 3.125%, 86.4–115.3% at 6.25%, 85.8–111.7% at 12.5%, 78.1–106.2% at 25%, 79.0–103.7% at 50% and 51.0–102.2% at 100% extract concentration. CS extract exhibited significant cytotoxicity at extract concentrations $>12.5\%$. The viability rate of CS extract at each dilution was $89.1 \pm 3.5\%$ at 3.125%, $77.8 \pm 1.8\%$ at 6.25%, $72.8 \pm 9.7\%$ at 12.5%, $5.9 \pm 0.9\%$ at 25%, $9.4 \pm 5.3\%$ at 50% and $5.7 \pm 0.7\%$ at 100% ($p < 0.001$ compared to every EC liquid extract at 100%, 50% and 25% concentration). Viability rate of ''Coffee'' flavor, the only EC liquid that showed cytotoxic potential (according to ISO 10993-5 definition), was $114.5 \pm 2.0\%$ at 3.125%, $112.2 \pm 3.6\%$ at 6.25%, $101.5 \pm 3.1\%$ at 12.5%, $92.0 \pm 8.9\%$ at 25%, $85.9 \pm 11.8\%$ at 50% and $51.0 \pm 2.6\%$ at 100% extract concentration. Figure 1 displays the relative difference in viability between CS extract and ''Coffee'' extract at each dilution; statistically significant higher fibroblast viability was observed for ''Coffee'' extract at all extract concentrations. IC_{50} and NOAEL for each EC and for the CS extracts are displayed in Table 2. IC_{50} could not be determined for EC vapor extracts, since viability was >50% at all extract concentrations. For the majority of EC liquids (13 of 21), viability was not statistically different between extract concentrations, thus NOAEL for these samples was defined as 100% concentration. Twelve of the EC liquids tested were flavors mimicking tobacco. However, they were not associated with a statistically significant difference in fibroblast viability.

Discussion

This is the first study that has evaluated the cytotoxic effects of vapor produced from commercially available EC liquids. The main result of our study is that the vapor from only 1 of the 21 EC liquids examined had cytotoxic effects on cultured fibroblast according to protocol definition. CS extract had significant cytotoxic effects, and fibroblast viability was significantly lower at all extract concentrations compared to EC vapor extracts. It is important to note that, we tested the EC liquids by simulating the way they are used by every user, that is, by activating a commercially available EC device and producing vapor, which was subsequently tested. In addition, we used standardized protocols and procedures such as UNI ISO 10993-5 standard and MTT-assay, with cytotoxicity defined according to UNI ISO 10993-5 standard as viability <70% compared to untreated cells. Moreover, we used cells that have been commonly used in studies evaluating tobacco cigarette cytotoxicity (Lu et al., 2007; Yu et al., 2006). Finally, we performed a cytotoxic study on CS extract using the same methodology to generate the test article. This is particularly important since EC are marketed for the smokers only as an alternative option. Therefore, the main scientific question is whether the EC is less harmful compared to regular tobacco cigarette, and this was evaluated in our study.

CS is a complex suspension that contains more than 4000 chemicals according to EPA report (1992). Several of these are linked to cancer or cardiovascular and lung disease from *in vitro* studies, including tobacco-specific nitrosamines (Hecht & Hoffmann, 1988; Wu et al., 2003), polycyclic aromatic hydrocarbons (Besaratinia et al., 2002; Zedeck, 1980), metals like cadmium and lead (Ronco et al., 2005) and

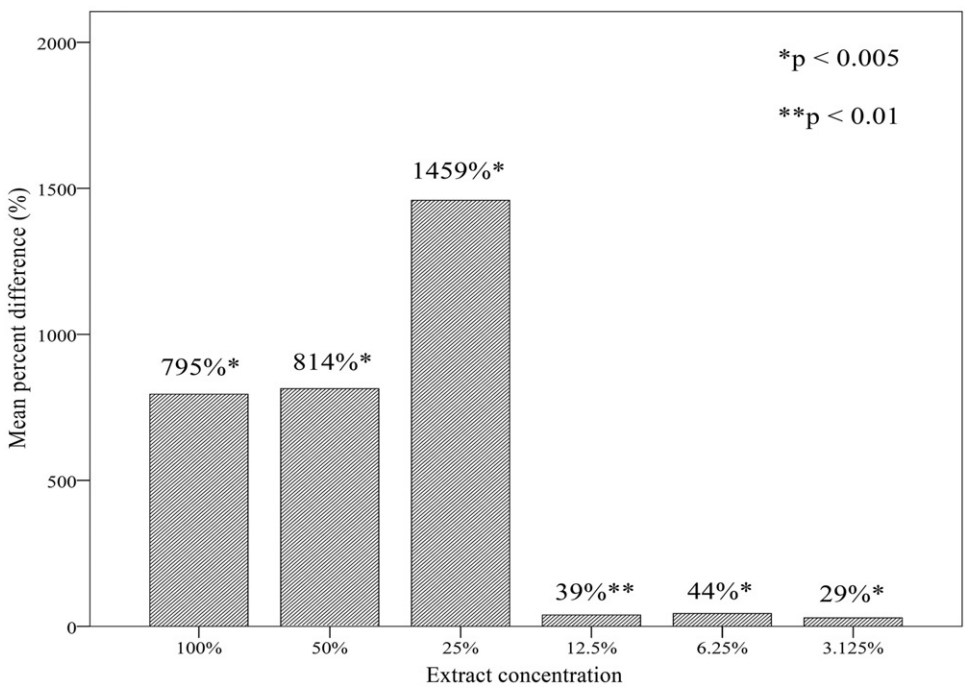

Figure 1. Relative mean differences between cigarette smoke extract viability and electronic cigarette ''Coffee'' vapor extract viability. Coffee was the only electronic cigarette liquid that showed cytotoxic effects according to the definition of UNI ISO 10993-5 standard.

Table 2. Inhibitory concentration 50 (IC_{50}) and no adverse effect level (NOAEL) for each electronic cigarette vapor extract and for the cigarette smoke (CS) extract.

Extracts	IC_{50}	NOAEL
Tuscan[a]	>100%	100%
Black fire[a]	>100%	100%
Ozone[a]	>100%	100%
Reggae night[a]	>100%	100%
Vanilla	>100%	100%
7foglie[a]	>100%	100%
Max blend[a]	>100%	25%
Virginia[a]	>100%	100%
Perique black[a]	>100%	50%
Layton blend[a]	>100%	100%
Hypnotic[a]	>100%	100%
Hazelnut	>100%	6.25%
Shade[a]	>100%	50%
RY4[a]	>100%	100%
Strawberry	>100%	12.5%
Managua	>100%	12.5%
Burley	>100%	100%
Apple	>100%	100%
Licorice	>100%	100%
Chocolate	>100%	3.125%
Coffee	>100%	12.5%
CS	16%	6.25%

[a]Tobacco flavors.

other compounds like acrolein, formaldehyde and phenol (Risner & Martin, 1994; Smith & Hansch, 2000). The major contributors to the *in vitro* cytotoxic effects of smoke are also responsible for the respiratory tract irritation in experimental animals and humans and cause histopathological changes in the upper respiratory tract (Lu et al., 2007). Therefore, in vitro cytotoxicity screening represents an important initial step in the toxicological evaluation of tobacco products.

There may be multiple mechanisms that lead to CS extract-induced cytotoxicity. For example, oxidative stress is an important mechanism that alters the balance between proliferation and apoptosis in fibroblasts (Müller & Gebel, 1998). Genetic damage is also induced by CS extract (Cui et al., 2012). Depletion of antioxidants by several CS extract components like acrolein and aldehydes compromises the defensive mechanisms of fibroblasts and promotes cell damage (Colombo et al., 2012; Ishii et al., 2003). Other chemicals cause direct cell-membrane damage (Thelestam et al., 1980). The end-result is fibroblast apoptosis and death (Kim et al., 2011; Park et al., 2010, 2008). This has important implications in the development of lung disease like emphysema (Baglole et al., 2006; Rennard et al., 2006).

We did not find any significant cytotoxic effects by any of the EC vapor extracts studied, except for ''Coffee'' at the highest extract concentration. Liquids consist mainly of glycerol, propylene glycol, water and nicotine; a wide variety of flavors are also available. Both glycerol and propylene glycol are classified by Food and Drug Administration and Flavor and Extracts Manufacturer Association (FEMA) as additives that are ''generally recognized as safe'' for use in food (FDA, 2012a,b-revised; FEMA GRAS numbers 2525 and 2940, respectively). Glycerol is also present in tobacco cigarettes and it is the main source of acrolein, produced by pyrolysis due to combustion. Acrolein has well-established cytotoxic effect on fibroblasts (Cattaneo et al., 2000; Jia et al., 2009). It is unlikely that acrolein can be produced by EC use because the temperature of liquid evaporation is considerably lower compared to combustion when smoking tobacco cigarette. Propylene glycol is a solvent used in oral, intravenous and topical pharmaceutical products. One study showed moderate cytotoxic effect on skin fibroblasts (Ponec et al., 1990). However, an animal study found that exposure to significant amounts of propylene glycol in air had no adverse effects on the respiratory system (Robertson et al., 1947). Propylene glycol is also present in tobacco cigarettes and is pyrolyzed to acetaldehyde during smoking, which has significant cytotoxic effects (Cattaneo et al., 2000; Krokan et al., 1985). Considering the fact that almost half of EC liquids content we examined was propylene glycol, the results of our study indicate that it is unlikely for propylene glycol to be pyrolyzed to acetaldehyde by EC use or to have any significant cytotoxic effect by itself. Concerning nicotine, there are studies showing that, at levels commonly found in cigarettes, it does not induce cell death (Laytragoon-Lewin et al., 2011) and may even have anti-apoptotic effects (Argentin & Cicchetti, 2006, 2004). It should be mentioned, however, that these effects have been suggested to facilitate the growth of tumors already initiated (Davis et al., 2009). Nicotine is not classified as a carcinogen by the International Agency for Research on Cancer (WHO-IARC, 2004), and the results of this study show that nicotine does not produce cytotoxic effects at the level present in the liquids tested.

Regarding the cytotoxicity observed for ''Coffee'', the manufacturer indicated that this flavor is a complex mixture of several natural and synthetic substances. Most of the natural substances come from roasted coffee beans. This processing of coffee beans may itself lead to production of some toxic elements, like ochratoxin A degradation products, which have cytotoxic and apoptotic properties (Cramer et al., 2008). Hegele et al (2009) found that coffee beans extract contains significant amounts of hydrogen peroxide, inducing cell death *in vitro*. It is possible that these substances are also present in the flavor used for preparing the ''Coffee'' EC liquid. However, we cannot exclude that the process of vapor formation from heating of the ''Coffee'' EC liquid may lead to production of other substances that have cytotoxic properties. It should be mentioned that the cytotoxic effect of this EC liquid extract was found only at the highest extract concentration, and, even at this concentration, fibroblast viability was 795% higher compared to CS extract.

Only one study has been published evaluating the cytotoxic effects of EC liquids (Bahl et al., 2012). Some of the liquids tested were found cytotoxic, mostly in embryonic cells and to a lesser extend in adult cells. This discrepancy in results may be attributed to several fundamental differences between the study by Bahl et al. and the study herein. The most crucial difference is that Bahl et al. tested the EC liquids in liquid form. It should be emphasized that the approach used by Bahl et al. does not deliver the EC liquid in the designated manner, which is less relevant than vapor generation of the liquid *via* activation of the electronic device. Herein, we simulated the exact mode of function of the EC and tested the extract of the resulting vapor. This may have significant implications on the results. Second, it is possible that not all liquid constituents evaporate at the same manner or in similar

concentrations. Furthermore, the concentrations of various constituents (for example, flavorings) may be different in vapor compared to liquid, and this may influence the results.

From a public health perspective, the field of tobacco harm reduction is particularly important. Smoking can produce subclinical dysfunction even at a young age (Farsalinos et al., 2013); therefore, attempts to quit smoking should be performed as soon as possible. However, quitting rates are relatively low with currently approved means (Rigotti et al., 2010). Until recently, only products containing tobacco were available in tobacco harm reduction (smokeless tobacco, like snus). Epidemiological studies have shown that use of such products is promising regarding cancer and cardiovascular disease risk reduction (Janzon & Hedblad, 2009; Lee & Hamling, 2009). Likewise, EC may have an important role in harm reduction. Unlike other products, EC contain no tobacco. In addition, the fact that nicotine is administered by a method that resembles tobacco cigarette use (hand-to-mouth movement, visible ''smoke'' exhaled) make them unique in dealing both with the chemical and psychological (behavioral) addiction to smoking. Several studies have characterized the chemicals contained in EC, with results showing that they do not contain any toxic substances (Ellicott, 2009; Tytgat, 2007; Valance & Ellicott, 2008). Even in studies where nitrosamines were detected (Laugesen, 2008; Westenberger, 2009), the levels were similar to a nicotine patch and 500 to 1400-fold lower compared to tobacco cigarettes (Stepanov et al., 2006). The results of this study are in line with these findings, showing significantly higher cytotoxicity of CS extract compared to EC vapor extracts.

Limitations

There are some limitations applicable to this study. Cytotoxicity studies on cultured cells have been developed in order to reduce the use of experimental animals. Extrapolating these results to the human *in vivo* toxicity should be done with caution. There is no consensus on the methodology of preparing and testing EC vapor extracts, and this is the first study that has attempted to evaluate the cytotoxic potential of EC vapor. However, we provided a comparative measure of toxicity with CS extract, which has well-established *in vivo* toxic effects. We did not use automated whole smoke exposure systems such as VitroCell or RM20s Borgwaldt systems, which offer more *in vivo*-like exposures since the cells are present inside the chamber where CS is delivered (Fukano et al., 2006; Maunders et al., 2007). Moreover, we did not use the standardized ISO method for CS extract (35 ml of air aspirated in 2-second per puff). This was done because we wanted to produce CS extract with the same method as EC liquid extract; aspiration of 35 ml air from the EC device produced very small amount of vapor, which was minimal compared to the amount generated by real EC use. Therefore, we preferred to use the same methodology in both EC and CS extract production. It should be mentioned that the ISO method for CS production significantly underestimates real smokers' exposure (Djordjevic et al., 2000).

We compared vapor extract from 200 mg of liquid with CS extract that was generated from one cigarette, both dissolved in 20 ml of culture medium. These are not similar exposure levels. In fact, there is no established method for comparing the amount of EC liquid and number of tobacco cigarettes. A practical and pragmatic way of comparing the two would be to measure how much liquid is consumed by users after using the EC device for similar time to that needed to smoke one cigarette. We have measured this as part of another protocol and we have found that the average EC liquid consumption was 60 mg. Therefore, we should have used the smoke extract of at least three cigarettes dissolved in 20 ml of culture medium in order to have a comparable exposure level to that of EC liquid extract we used. Unfortunately, this measurement was performed after the completion of this study. If three cigarettes had been used in this protocol, it is probable that the cytotoxicity of CS extract and the resulting differences in cell viability compared to effects induced by the EC liquid extracts would have been even higher than what was observed. However, this is an assumption and cannot be inferred unless explicitly tested.

It should be emphasized that our results do not necessarily apply to all EC liquids marketed. Nicotine is extracted from tobacco; therefore, if liquids contain non-pharmaceutical grade nicotine, several tobacco impurities may be present and adversely affect the results. The same applies for all other liquid constituents (Cahn & Siegel, 2011). We did not find an association between EC tobacco flavors and fibroblast viability. This was probably due to the fact that substances approved for food industry were used even for these flavors (according to manufacturer's report). However, it is possible to use natural tobacco extract to mimic tobacco flavor, and some companies may use or produce themselves such extracts for use in EC liquids. The cytotoxicity potential of these extracts is currently unknown, and they are not approved for use in food industry. In any case, regulation is needed and specific standards should be implemented in order to ensure that quality products are available in the market. Although no standards have been implemented by public health authorities, several industry associations like Electronic Cigarette Industry Trade Association and American E-Liquid Manufacturing Standards Association have developed such standards.

Finally, another important issue not addressed in this study is the effect of different, modified EC devices that deliver higher voltage and wattage to the resistance. This would accelerate the rate of evaporation; and if the resistance is not sufficiently supplied with liquid, it might possibly result in overheating and production of toxic chemicals. We tested the EC device used in the experiment to make sure that no ''dry puff'' phenomenon occurs, but it remains to be examined whether this phenomenon is associated with the production of toxic substances.

Conclusions

In conclusion, from the 21 commercially available EC liquids we tested in vapor form, only one was found to have cytotoxic effects on cultured mammalian fibroblast cells according to ISO 10993-5 definition. Overall, EC vapor extracts showed by far higher fibroblast viability compared to CS extract. This supports the concept that EC may be less harmful compared

to tobacco cigarettes and could be useful products in tobacco harm reduction. However, more research is needed, both in the laboratory with different cell lines and in clinical level, in order to better understand and evaluate the effects of EC use on human health.

Declaration of interest

No author has any financial interest in the outcome of this study.

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