Comparative effects between electronic and cigarette smoke in human keratinocytes and epithelial lung cells

F. Cervellati a, X.M. Muresan a, C. Sticozzi a, R. Gambari a, G. Montagner a, H.J. Forman b,c, C. Torricelli d, E. Maioli d, G. Valacchi a,*

a Department of Life Sciences and Biotechnology, University of Ferrara, Ferrara, Italy
b Davis School of Gerontology, University of Southern California, USA
c Life and Environmental Sciences Unit, University of California at Merced, USA
d Department of Life Sciences, University of Siena, Siena, Italy

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Abstract
Information about the harmful effects of vaping is sparse and inconsistent, therefore, since the use of electronic cigarettes (e-CIGs) has become increasingly popular as a tool to limit tobacco smoking, it is urgent to establish the toxicity of the commercial e-CIGs.

Skin (HaCaT) and lung (A549) cells, the main targets of cigarette smoke (CS), were exposed to e-CIG vapor and CS using an in vitro system. The cytotoxic effect of the exposure was analyzed in both cell types by ultrastructural morphology, Trypan Blue exclusion test and LDH assay. In addition, pro-inflammatory cytokines were measured by the Bio-Plex assay.

The cytotoxic components of e-CIG were restrained to the flavoring compound and, to a lesser extent, to nicotine although their effects were less harmful to that of CS. Humectants alone exhibited no cytotoxicity but induced the release of cytokines and pro-inflammatory mediators.

Based on our results, we can state that exposure to e-CIG vapors results in far less toxic than exposure to CS. In fact, besides the deleterious effect of flavor and nicotine, even the humectants alone are able to evoke cytokines release. This study will hopefully promote the development of safer e-CIGs to help people quit smoking.

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1. Introduction
Developed in China in 2004, the electronic cigarette (e-CIG) has become increasingly popular worldwide. Marketers of the e-CIG describe it as an aid to help people quit smoking. They claim that while using an e-CIG simulates tobacco cigarette smoking, the odor and risks associated with tobacco smoke are eliminated as no combustion products and no tobacco toxins are inhaled (Caponnetto et al., 2012).

In fact, in addition to variable doses of nicotine and different flavors, the base liquid typically includes propylene glycol (PG) or glycerol (also called vegetable glycerin or VG) and/or polyethylene glycol 400 (PEG400), all of which are widely used as additives in foods and personal care products, such as toothpaste (Fluhr et al., 2008).

Thus, the components of e-CIG vapors, inhaled in the act now called vaping, are assumed to be less harmful than the thousands of known and unknown toxicants in tobacco smoke. Nonetheless, this assumption does not entirely rule out potentially deleterious effects of inhaling the vapor of the nicotine/flavor mixture. Indeed, while The World Health Organization (WHO) has not excluded the e-CIG might be useful as a smoking cessation aid, it has stated that current research does not warrant the conclusion that the e-CIG is as safe and effective, in reducing nicotine-related withdrawal symptoms, as nicotine-replacement products or gum (Barbeau et al., 2013). There is no specific legislation on the use of e-CIGs in Europe and currently member countries set their own regulations. Some countries, such as Belgium and Denmark, banned the sale of e-CIGs while Germany and Austria classified the e-CIG as a medical product. In the Netherlands, the use and purchase of e-CIGs is legal, but advertisement of them is banned. The European Union however, is currently debating banning all smokeless tobacco throughout Europe. Taking these products off the market however, would force thousands of users, who positively experienced vaping (Barbeau et al., 2013), to return to cigarette smoking.
with the known deleterious effects. Therefore, it is urgent to establish the safety or the toxicity of the components of the vapors from commercial e-CIGs in order to provide legislators, manufacturers and smokers with the essential scientific information required to make informed decisions. In the current study, we compared the in vitro cytotoxicity of cigarette smoke and e-CIG vapors on cells from lung and skin, the organs directly targeted by tobacco cigarettes (Sticcozzi et al., 2012; Valacchi et al., 2011).

Short term exposure of HaCaT cells (keratinocytes) and A549 cells (lung epithelial cells) to tobacco smoke and e-CIG vapors with and without aroma or nicotine were carried out. The results revealed that e-CIG vapors have some toxic effect on cell viability. In particular, the harmful component of the e-CIG seems to be confined to the flavoring compounds rather than to nicotine and humectants. In addition, screening of an array of cytokines released from the cells exposed to e-CIG vapors without additives showed that the basal components alone are able to induce the release of several cytokines and pro-inflammatory mediators, suggesting that even humectants might have a potential, although non-cytotoxic, harmful effect.

2. Methods

2.1. Cell culture

HaCaT cells, (a gift from Dr. F. Virgili), were grown in Dulbecco's modified Eagle's medium High Glucose (Lonza, Milan, Italy), supplemented with 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin and 2 mM l-glutamine. A549 cells were purchased from ATCC (Rockville, MD). Ham's F-12, foetal bovine serum, RPMI-1640, penicillin/streptomycin and l-glutamine were obtained from Lonza (Milan, Italy). Cell suspension containing 5 × 10⁶ viable cells/ml were used. Cells were incubated at 37 °C for 24 h in 95% air/5% CO₂ until 80% confluency.

2.2. CS and e-CIG exposure

Prior to CS and e-CIG exposure of the cells, medium was aspirated and fresh serum-free medium was added. Cells were then exposed for 50 min to CS and e-CIG mixture. Control cells were exposed to filtered air for the same duration (50 min) after changing media. The time and the method of exposure were chosen based on our previous works (Sticcozzi et al., 2010, 2012).

HaCaT cells were exposed to fresh CS in an exposure system that generated CS.

Our exposure system is comparable to a conventional smoking machine, briefly A549 and HaCaT cell cultures were exposed to fresh CS in an exposure system that generated CS by burning one UK research cigarette (12 mg tar, 1.1 mg nicotine) using a vacuum pump to draw air through the burning cigarette and leading the smoke stream over the cell cultures. The overnight serum-starved cells in culture were placed with lids removed in a 37 °C chamber, briefly A549 and HaCaT cell cultures were exposed to fresh CS in an exposure system that generated CS.

2.3. Cell viability

Viability studies were performed at different times after exposure by Trypan blue exclusion test and LDH release assay. After Trypan blue staining, cells were counted by a cell counter (Invitrogen, Monza, Italy). Viable and nonviable cells were recorded separately, and the means of three independent counts were pooled for analysis and expressed as percent of dead cells with respect to total cell number. The LDH release was measured by a two step enzymatic assay. In the first step, NAD⁺ is reduced to NADH/H⁺ by the LDH-catalyzed conversion of lactate to pyruvate; in the second step, the catalyst (diaphorase) transfers H⁺/H₂ from NADH/H⁺ to tetrazolium salt which is reduced to formazan. The amounts of LDH in the culture medium were determined and calculated according to kit instructions (EuroClone Milan, Italy). Prior to each assay, the cells were lysed with 2% (V/V) Triton X-100 in culture media for 30 min at 37 °C to obtain a representative maximal LDH release as the positive control with 100% toxicity. All tests were performed in triplicate and assay was repeated three times with fresh cell cultures independently and we have obtained similar results.

2.4. Ultrastructural study

After CS exposure, HaCaT and A549 cells (1 × 10⁶ cell/ml) were scraped and collected in 0.1 M cacodylate buffer (pH 7.4). After centrifugation, pellets were fixed with 2.5% gluteraldehyde in 0.1 M sodium cacodylate buffer for 4 h at 4 °C. The specimens were dehydrated in graded concentrations of ethanol and embedded in epoxy resin (Agar Scientific, 66A Cambridge Road, Stansted Essex, CM24 8DA, UK), after washing and post-fixation. Cells were then transferred to latex modules filled with resin and subsequently thermally cured at 60 °C for 48 h. Semi-thin sections (0.5–1 m thickness) were cut using an ultra-microtome (Reichard Ultracut S, Austria) stained with toluidine blue, and blocks were selected for thinning. Ultra-thin sections of about 40–60 nm were cut and mounted onto formvar-coated copper grids. These were then double-stained with 1% uranyl acetate and 0.1% lead citrate for 30 min each and examined under a transmission electron microscope, Hitachi H-800 (Tokyo, Japan), at an accelerating voltage of 100 kV.

2.5. Cytokine assay

Cytokines released in culture medium by HACAT and A549 cells were measured by Bio-Plex cytokine assay (Bio-Rad Laboratories, Hercules, CA) as described elsewhere (Borgatti et al., 2008; Gambari et al., 2012).

The Bio-Plex cytokine assay is designed for the multiplexed quantitative measurement of multiple cytokines in a single well using as little as 50 µl of sample. In our experiments, we used the premixed multiplex beads of the Bio-Plex human cytokine Human 27-Plex Panel (Bio-Rad, Cat. n.o. 171-A11127), which included twenty-seven cytokines [IL-1β; IL-1ras, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12 (P70), IL-13, IL-15, IL-17, Basic FGF, Eotaxin, G-CSF, GM-CSF, IFNγ, IP-10, MCP-1 (MCAF), MIP-1α, MIP-1β, PDGF-BB, RANTES, TNF-α, VEGF]. Briefly, 50 µl of cytokine standards or samples (supernatants from treated cells) were incubated with 50 µl of anti-cytokine conjugated beads in 96-well filter plates for 30 min at room temperature with shaking. Plates were then washed by vacuum filtration three times with 100 µl of Bio-Plex wash buffer, 25 µl of diluted detection antibody was added, and plates were incubated for 30 min at room temperature with shaking. After three filter washes, 50 µl of streptavidin–phycoerythrin was added, and the plates were incubated for 10 min at room temperature with shaking. Finally, plates were washed by vacuum filtration three times, beads were suspended in Bio-Plex assay buffer, and sample were analyzed on Bio-Rad 96 plate reader using the Bio-Plex suspension array system and Bio-Plex manager software (Bio-Rad Laboratories, Hercules, CA).
2.6. Statistical analysis

Values are expressed as the mean ± SD values from at least three independent experiments. ANOVA followed by Dunnett’s test was used to determine statistical significance with a threshold of p values less than 0.05.

3. Results

3.1. LDH release and viability

As shown in Figs. 1A and 2A respectively, HaCaT and A549 cells viability does not change in controls over 24 h and, consistently, a steady low release of LDH was observed (Figs. 3A and 4A). In contrast, exposure to CS caused an early (6 h) and progressive decrease in cell viability (Figs. 1B and 2B) and increased LDH release (Figs. 3B and 4B) with a similar trend during the different time points in both cell lines, although keratinocytes seem to be more susceptible to CS induced toxicity after 24 h (Fig. 4B). Exposure to e-CIG vapor in which both flavoring substances and nicotine were absent resulted in no change in either LDH release (Fig. 1E and 2E) or cell viability (Figs. 3E and 4E) over 24 h. In stark contrast to e-CIG without the additives, exposure to e-CIG with flavor caused significant increased of LDH release (Figs. 1D and 2D) and a progressive loss of viability (Figs. 3D and 4D) in both cell types although HaCaT cells seems to be more susceptible than A549 cells. Even more dramatically, e-CIG with nicotine caused rapid (50 min) and marked loss in viability (Figs. 3C and 4C) and enhanced LDH release (Figs. 1C and 2C), exhibiting a quantitative and qualitative response superimposable to that of CS exposure.

3.2. Cellular morphology

Results from TEM are in line with the viability and toxicity data. As shown in Fig. 5, in control conditions, the ultrastructural appearance is unchanged after both 50 min (T0) and 24 h (T24); cells appear closely adherent and cellular organelles and mitochondria are well defined in both cell types. The morphology of the cells exposed to CS shows clear signs of cellular damage and presence of vacuoles. Once the cells were exposed to e-CIG with flavors it is possible to observe an increase in vacuolization and alteration of cytoplasmic membrane. The degeneration of intracellular organelles is worsened in the presence of flavors plus nicotine and particularly evident in HaCaT cells, with a marked vacuolization consequent to the expansion of the mitochondria and the endoplasmic reticulum. The cells treated with e-CIG without nicotine and flavors (humectants alone), remained intact with the same ultrastructural aspect of control cells, even 24 h after treatment.

3.3. Cytokine release

Despite the lack of cytotoxicity of the basal component of e-CIG vapors, a possible irritant/inflammatory effect could not be excluded. Therefore, we performed a quantitative measurement using the Bio-plex technology (Borgatti et al., 2008; Gambri et al., 2012) of multiple cytokines released by both keratinocytes and lung cells after vapors exposure. The results concerning the released cytokines/chemokines displaying concentrations ≥ 5 pg/ml in both HaCaT and A549 cells were taken in consideration in order to minimize the background, and are reported in Table 1. As expected, the pattern of cytokine/chemokine release is different in the two cell lines. Interestingly, increases of cytokine/chemokine release was found in the two cell lines when PDGF-BB, basic FGF,
IL-8, IL-12, IL-17, GM-CSF, IP-10, MCP-1 and MIP-1β were analyzed. Increased release of IL-1α, IL-10, G-CSF, IFN-γ, RANTES, TNF-α and VEGF was found only in HaCaT. IL-6 release decreased both in HaCaT and A549 cells. The highest increase in HaCaT cells (1002.8 ± 26.6 pg/ml) was that of IL-8 (6055.6 ± 807 pg/ml) and IP-10 (3345 ± 106 pg/ml). The highest increase in A549 cells was that of basic FGF (240.9 ± 10.6 pg/ml). These results indicate that the basal components of e-CIG vapor, although non-toxic, contain...
some pro-inflammatory stimuli leading to a change in the secretome pattern depending from the employed cells lines. We also found fluctuations in cytokines release after other e-CIG and CS exposures but, because of the concomitant cell death, interpretation of such changes as active release or loss due to cell disruption was not possible (data not shown).

Fig. 4. Cell viability in A549 cells after exposure to air (A), cigarette smoke (B), electronic cigarette with nicotine (C), electronic cigarette with flavor (D) and only vaping (E). Data are expressed as percentage of control (averages of five different experiments); * all time points vs control; ° CS vs e-CIG w/nicotine; ° CS vs e-CIG w/flavor; ° CS vs e-CIG w/o flavor; ° e-CIG w/nicotine vs e-CIG w/flavor; ° e-CIG w/nicotine vs e-CIG w/o flavor – w/o nicotine; ° e-CIG w/flavor vs e-CIG w/o flavor – w/o nicotine.

Fig. 5. Ultrastructural study of A549 cells (left column) and HaCaT cells (right column) after exposure to different conditions at T0 (immediately after the exposure) and T24 (24 h after the exposure). Bars = 2 μm.
4. Discussion

Although there have been few studies suggestive of harmful effects from vaping, the results thus far have been inconsistent. This is likely due to a lack of standardized assessment, but also to varying chemical composition of commercial refill fluids among brands. A recent study showed that using an e-CIG for 5 min has immediate adverse physiologic effects similar to those seen with tobacco smoking (Vardavas et al., 2012). In another recent study, where serum cotinine, lung function, exhaled carbon monoxide and nitric oxide were assessed and compared between e-CIG users and tobacco cigarette smokers, the results showed that e-CIGs generated smaller changes in lung function but had a nicotinergic impact similar to that from tobacco cigarettes (Flouris et al., 2013). Although it is generally believed that the diseases caused by smoking are more likely caused by products of combustion rather than by nicotine, nicotine inhalation through e-CIGs can be completely avoided by simply using nicotine-free cartridges. Our results are in agreement with the study by Bahl et al. performed in vitro on embryonic and adult cells who found that cytotoxicity was not due to nicotine or humectants, but was correlated with the number and concentration of chemicals used to flavor fluids (Bahl et al., 2012). Nevertheless, the absence of cytotoxicity of humectants on skin and lung cells does not exclude possible harmful effects on other cell populations, such as pulmonary macrophages, especially after long term exposure. Indeed, an isolated case of lipoid pneumonia associated with vaping and ascribed to the glycerin vapors has been reported in a 42-years-old woman who had used e-CIGs for a long time (Vardavas et al., 2012). Indeed, an isolated case of lipoid pneumonia has been recently found in vapors from an e-CIG leading brand, where the presence of silicate particles and metal elements, which significantly affect the toxicity of smoke inhaled for 13 weeks, was found (McCauley et al., 2012). On the other hand, the glycerin and propylene glycol are commonly used as humectants also in tobacco cigarettes, to prevent excessive drying of the tobacco filler and in water pipe tobacco, to increase smoke development. In a previous in vivo study, performed in a rat model, the addition of glycerin and propylene glycol to tobacco cigarettes was found not to significantly affect the toxicity of smoke inhaled for 13 weeks (Hek et al., 2002). However, despite the likely safety of humectants, the presence of silicate particles and metal elements, which have been recently found in vapors from an e-CIG leading brand, may have some cytotoxic effects (Williams et al., 2013). The authors of this last study recommend the manufacturers a high selection of the materials used in e-CIGs and stringent quality control procedures, since the metals are thought to derive from the cartomizer (atomizer and cartridge), rather than from the fluid. A comparative study on the effects of e-CIG vapor and cigarette smoke on indoor air quality, (McCauley et al., 2012) provided evidence that vaping is safer than smoking and that “secondhand” vapor is much less dangerous than secondhand smoke (Guignab-Cagmat et al., 2012). A recent study analyzed the emission of some e-cigarette pollutants in indoor air and found that the concentrations of elements and metals showed a significant increase for particulate matter and aluminum, while the amounts of other potentially toxic elements, such as lanthanum, cerium, cadmium, arsenic and thallium, were in the range of outdoor air levels (Schober et al., 2013). Cahn and Siegel provided a table of the components of several brands of e-CIGs (Cahn and Siegel, 2011). While some of the components other than flavoring agents and nicotine are generally regarded as safe, including propylene glycol and glycerin, information on some components does not clearly rule out potential toxicity. Although the present results and the bulk of the literature indicate that e-CIG without additives are likely devoid of cytotoxicity, such results probably underestimated their whole harmful potential since toxicity tests do not routinely evaluate possible inflammatory and irritant effects of vaping. The current study showed, for the first time, that a panel of pro-inflammatory cytokines/chemokines (PDGF-BB, basic FGF, IL-8, II-12, IL-17, GM-CSF, IP-10, MCP-1 and MIP-1β) are increasingly released into the medium, following exposure to e-CIG vapors without additives, by keratynocytes and lung cells, indicating that even the nontoxic components of e-CIG might have a potential dangerous effect. The release of other cytokines, such as IL-1ra, IL-10, G-CSF, IFN-γ, RANTES, TNF-α and VEGF increased instead increased only in the keratinocyte cell line. We do not know whether all e-CIGs contain inflammatory stimuli, but it would be prudent if such studies were carried out while the use of e-CIGs is becoming more common so that brands can be developed that are truly innocuous. Moreover, the experimental system employed might be useful to identify molecules able to decrease the pro-inflammatory effects of eCIG. Interestingly, for each brand a differential chemical strategy to block pro-inflammatory effects might be proposed.

In closing, our results indicate that e-CIGs are not really safe, although they are far less noxious than tobacco cigarettes, thus the ideal remains that people should stop both smoking and vaping. In fact even the only vaporing is able to stimulate cytokines release that play a role in inflammation. Nevertheless, this study also suggests that, if the refill accessories are properly chosen, vaping could be a safer alternative to smoking for smokers who are unable or unwilling to stop. Of course, before extrapolating these data to the real world more studies are needed; for instance the use of different kinds of cells and possibly the use of animal models need to integrate and maybe confirm the data herein presented.
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Conflict of Interest

The authors declare that there are no conflicts of interest.

Transparency Document

The Transparency document associated with this article can be found in the online version.

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