E-Cigarette Aerosol Exposure Induces Reactive Oxygen Species, DNA Damage, and Cell Death in Vascular Endothelial Cells

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ABSTRACT

Cigarette smoking remains one of the leading causes of preventable death worldwide. Vascular cell death and dysfunction is a central or exacerbating component in the majority of cigarette smoking related pathologies. The recent development of the electronic nicotine delivery systems known as e-cigarettes provides an alternative to conventional cigarette smoking; however, the potential vascular health risks of e-cigarette use remain unclear. This study evaluates the effects of e-cigarette aerosol extract (EAE) and conventional cigarette smoke extract (CSE) on human umbilical vein endothelial cells (HUVECs). A laboratory apparatus was designed to produce extracts from e-cigarettes and conventional cigarettes according to established protocols for cigarette smoking. EAE or conventional CSE was applied to human vascular endothelial cells for 4–72 h, dependent on the assay. Treated cells were assayed for reactive oxygen species, DNA damage, cell viability, and markers of programmed cell death pathways. Additionally, the antioxidants α-tocopherol and n-acetyl-l-cysteine were used to attempt to rescue e-cigarette induced cell death. Our results indicate that e-cigarette aerosol is capable of inducing reactive oxygen species, causing DNA damage, and significantly reducing cell viability in a concentration dependent fashion. Immunofluorescent and flow cytometry analysis indicate that both the apoptosis and programmed necrosis pathways are triggered by e-cigarette aerosol treatment. Additionally, anti-oxidant treatment provides a partial rescue of the induced cell death, indicating that reactive oxygen species play a causal role in e-cigarette induced cytotoxicity.

Key words: anti-oxidants; cardiovascular system; cytotoxicity; e-cigarettes; endothelial cells; oxidative stress.

Over the past 50 years, the scientific and medical communities have amassed a wealth of data on the harmful effects of tobacco cigarettes. Cigarette smoking damages every major organ system and remains one of the leading causes of preventable death (U.S. Department of Health and Human Services, 2014). The World Health Organization estimates that cigarette smoking leads to around 6 million deaths per year worldwide and projects that number will increase to 8 million by the year 2030. Yet, approximately one-fifth of the world’s population persists in cigarette smoking (WHO, 2015).

Recently, the development of the electronic nicotine delivery systems known as e-cigarettes has provided an alternative to conventional cigarettes. In a 2015 study, researchers noted that e-cigarettes have already become the most popular alternative to conventional cigarettes and are routinely used by about 1 in 5 American tobacco product users. It is of particular interest that 1 in 10 current e-cigarette users have no history of smoking tobacco cigarettes (Weaver et al., 2015). Another recent study indicates that e-cigarettes have become the most commonly used tobacco product among middle and high schoolers with 3.9% of middle schoolers and 13.4% of high schoolers responding that they currently use e-cigarettes (Arrazola et al., 2015). These statistics are troubling as there is currently no consensus on the health consequences of e-cigarette usage.
As e-cigarettes appear to contain fewer known toxic and carcinogenic compounds than conventional cigarettes, e-cigarette use has been advocated as a harm reduction strategy for cigarette smokers (Cahn and Siegel, 2011; Goniewicz et al., 2014). However, current literature reviews on the question of e-cigarette safety are inconclusive and cite significant methodological problems and conflicts of interest in many studies (Callahan-Lyon, 2014; Pisinger and Dissing, 2014; West and Brown, 2014). Moreover, the rapid development of new models of e-cigarettes and new flavors of e-cigarette liquid provides a challenge to meaningful research and regulation (Zhu et al., 2014).

Many of the pathologies associated with conventional cigarette smoking possess a cardiovascular component (U.S. Department of Health and Human Services, 2014), and the core of these pathologies is death and dysfunction at the level of vascular cells (Messner and Bernhard, 2014; Morris et al., 2015; Sobus and Warren, 2014). The mechanism of cigarette smoke induced cardiovascular cytotoxicity/genotoxicity has been well characterized: cigarette smoke increases the oxidative burden on the cell, dysregulates cellular metabolism, alters nitrogen oxide levels, and results in the production of toxic compounds such as peroxynitrate and peroxynitrite (Pryor and Stone, 1993; Irani, 2000; Smith and Fischer, 2001).

Specific studies of the cardiovascular effects of e-cigarettes tend to conclude that they are less hazardous than conventional cigarettes when used by healthy individuals; however, reviewers point out that these studies are few in number and often short term with no follow-ups (Benowitz and Burbank, 2016; Lippi et al., 2013; Morris et al., 2015; Nelluri et al., 2016). It has been recently demonstrated that e-cigarette aerosol can induce cell death and oxidative stress both in vitro across multiple cell lines (Farsalinos et al., 2013; Lerner et al., 2015; Putzhammer et al., 2016; Romagna et al., 2013; Schweitzer et al., 2015; Teasdale et al., 2016) and in vivo in the serum of smokers and e-cigarette users (Carnevale et al., 2016; Hom et al., 2016; Schweitzer et al., 2015).

The purpose of this study was to better characterize the effects of e-cigarette aerosol on human vascular endothelial cells. We developed a laboratory apparatus capable of creating extract from conventional cigarette smoke and e-cigarette aerosol. The aerosol from a panel of 4 brands of tobacco flavored e-cigarettes was compared with smoke from conventional tobacco cigarettes in its ability to induce reactive oxygen species formation, DNA damage, and cell death in human umbilical vein endothelial cells (HUVECs). To look more closely at the specific mechanisms of e-cigarette aerosol induced cell death, we investigate the presence of key proteins and cellular hallmarks of the apoptotic and necrotic pathways of programmed cell death. To determine the role of oxidative stress in e-cigarette induced cell death, we attempted to prevent e-cigarette induced cell death with anti-oxidant treatment. This work contributes to the growing body of literature on the potential vascular harm of e-cigarette aerosol and substantially advances our understanding of the mechanisms of e-cigarette aerosol induced cell death.

MATERIALS AND METHODS

Sample Selection

In this study, we chose to examine high revenue generating, tobacco flavored, cigarette-like e-cigarettes. Manufacturers were chosen from a list of companies that make up >1% of the multi-outlet market share (which consists of sales from grocery and food stores, drug stores, club stores, big box stores, dollar stores, mass merchandisers, and military commissaries) (Maier, 2015). The final panel consisted of tobacco flavored e-cigarettes of the brands Blu (Imperial Tobacco), Vuse (R.J. Reynolds), Green Smoke (Altria), and NJoy (NJoy). For comparison to conventional tobacco cigarettes, we used 3R4F research reference cigarettes from the University of Kentucky Center for Tobacco Reference Products. Figures 1A and B shows comparative schematics of a conventional tobacco cigarette (Figure 1A) and the model of e-cigarette used in this study (Figure 1B). All samples of each brand were acquired at the same time and stored in the dark at room temperature in airtight plastic bags. 3R4F reference cigarettes were stored in airtight plastic bags at 4°C and pre-equilibrated in a humidifier at room temperature at 60 ± 3% humidity for at least 30 min before use.

Cell Culture

HUVECs (American Type Culture Collection) were maintained at 37°C, 5% CO2 in EGM-2 cell culture medium (Lonza). Cells were kept sub-confluent and used at passages 4-7. Cells were split for experiments using 0.25% Trypsin/EDTA (Gibco) and all samples were allowed to acclimate at least 12 h prior to treatment.
Cigarette Smoke and E-Cigarette Aerosol Extraction and Preparation

Cigarette smoke extract (CSE) and e-cigarette aerosol extract (EAE) were extracted via a laboratory apparatus (Figure 1C) designed to function within WHO standard operating procedures for intense tobacco smoking: WHO TobLabNet SOP1 (2 s, 55 ml puffs, 2±/min) (WHO, 2012). Either conventional tobacco cigarettes or electronic cigarettes were placed in a plastic tube and PM-992 Parafilm (Bemis) was used to create an airtight seal (Figure 1C1). Puffs of 55 ml volume were taken with the smoke or aerosol being pulled through 2 midget impingers (Figs. 1C2 and 3). The first impinger contained EGM-2 and the second impinger caught any overflow from the first. Downstream of the impingers, an Erlenmeyer flask (Figure 1C4) containing desiccant was used as a moisture trap to protect the vacuum pump. Puffing was performed via opening and closing a 12 volt solenoid valve (US Solid) controlled by a Basic Stamp 2 microcontroller (Parallax) (Figs. 1C5 and 6). The volume of the puff was controlled with an airflow regulator (Figure 1C7). To prevent operator exposure to smoke or aerosol, all extractions were performed in a chemical safety hood.

Pre and post extraction, e-cigarette cartridges were weighed on an analytical balance (Fisher). The weight of the consumed e-liquid was used to determine the weight of consumed nicotine. For 3R4F research reference cigarettes the amount of nicotine consumed by smoking one cigarette was considered to be 0.7 mg (Roemer et al., 2014). The contents of the first and second impinger were collected and the volume of EGM-2 was adjusted so that the final concentrations of consumed nicotine were equivalent across all samples. Prior to being introduced to cell culture, all extracts were filtered through a 0.22 μm syringe filter (Millipore). Extracts were either used immediately or aliquoted into single use tubes and kept at –80°C for up to 1 week.

Neutral Red Uptake Cell Viability Assay

Endothelial cell viability assays were performed according to the protocol of Repetto et al. (2008). Treatment was performed by applying either EAE or CSE (in EGM-2 endothelial cell culture medium) at a range of 31.25-500 μM consumed nicotine across 7 wells of a multi-well plate. The cells were allowed to incubate with extract for either 24 or 72 h. After treatment, cells were rinsed with Tris buffered saline (TBS) and incubated with 0.4 mg/ml Neutral Red (Sigma-Aldrich) in cell culture medium for 2 h at 37°C. Following incubation, cells were rinsed in TBS to remove excess dye and the remaining dye was solubilized with 50% ethanol/1% acetic acid. The relative quantity of neutral red was measured by reading absorbance at 540 nm (baseline 630 nm).

TUNEL Assay

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) was performed with a TMR-Red in situ Cell Death Detection Kit (Roche) per manufacturer’s protocols. Treatment was carried out over 24 h at either 25 or 500 μM consumed nicotine. Positive control cells were treated with 5 U/ml DNase I (Thermo) for 10 min. Following treatment, cells were fixed in 10% formalin, permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate solution, and incubated with TUNEL reagent for 2 h at 37°C in the dark. Samples were mounted with Vector mounting medium containing DAPI and imaged via fluorescence microscopy.

Antibody Staining

Antibody staining was performed via conventional methods. Cells were incubated for 24 h with either EAE or CSE before being fixed in 10% formalin. Cells were washed in TBS, permeabilized with TBS with 0.1% Tween-20 (Sigma-Aldrich), and incubated overnight at 4°C with primary antibodies (rabbit anti-human cleaved caspase-3 [Clvd Casp-3], Cell Signaling, 1:250; rabbit anti-human MLKL phospho s358, Abcam, 1:250). The next day, cells were washed with TBS and incubated for 1 h at room temperature in the dark with secondary antibody (Alexa-594 conjugated donkey anti-rabbit IgG, Invitrogen, 1:500). Samples were mounted with DAPI and imaged via fluorescence microscopy.

ROS Detection Assay

Cells were analyzed for reactive oxygen species generation using a ROS-ID Total ROS Detection Kit (Enzo Life Sciences) per manufacturer’s protocol. Cells were treated with ROS detection reagent at the same time as 500 μM consumed nicotine EAE or CSE and incubated for 4 h at 37°C in the dark. Following incubation, ROS level was imaged via fluorescence microscopy and quantified as level of fluorescence per cell.

Anti-Oxidant Rescue Experiments

Cells were exposed simultaneously to 500 μM consumed nicotine EAE and either 50 μM α-tocopherol (α-Toc) (Sigma-Aldrich) or 5 mM n-acetyl-l-cysteine (NAC) (Enzo Life Sciences) for either 24 or 72 h. Following treatment, the cells were collected and analyzed for extent and type of cell death using an Apoptosis & Necrosis Quantitation Kit Plus (Biotium) per manufacturer’s protocol. Cells were spun down, washed in TBS, spun down again, washed in annexin V (Ax V)-binding buffer, and then incubated with Ax V/ethidium-III homodimer staining solution at room temperature in the dark. Following incubation, samples were run on a Guava EasyCyte Mini flow cytometer (Millipore).

Statistical Analysis

All statistical analyses were performed in R. Significant differences among groups were analyzed via ANOVA followed by Tukey honest significant difference testing. A P value ≤ .05 was considered statistically significant.

RESULTS

E-Cigarette Aerosol Displays Reduced and Delayed Cytotoxicity as Compared with Cigarette Smoke

In order to determine the cytotoxic effects of e-cigarette aerosol as compared with conventional cigarette smoke, we made extracts from each brand of e-cigarette in our panel as well as 3R4F research reference cigarettes. As nicotine drives smoking behavior (Picciotto and Kenny, 2013), we adjusted the concentration of the extracts so that the same amount of nicotine was consumed by our smoking/vaping apparatus per final volume of cell culture medium. Figure 2 shows the percentage of cell viability after exposure to either EAE or CSE across a range of concentrations from 31.25 to 500 μM consumed nicotine. At 24 h, CSE displayed acute cytotoxicity across all concentrations while EAE displayed a limited cytotoxicity (up to 13% cell death) across the highest 2 concentrations (Figure 2A). When the experiment was extended out to 72 h, CSE still displayed acute cytotoxicity...
and a greater cytotoxic effect (up to 22% cell death) was evident at higher concentrations of EAE (Figure 2B). EAE was less cytotoxic than CSE at all treatment concentrations in both experiments. Neither experiment showed any significant variation within or among brands of e-cigarette.

Both E-Cigarette Aerosol and Cigarette Smoke Cause DNA Damage

As e-cigarette aerosol caused significant cell death at higher concentrations; we performed a TUNEL assay to determine whether DNA damage, a hallmark of programmed cell death, could be detected. HUVECs were treated with either EAE from one of the e-cigarette in our panel or CSE. Figure 3 shows representative pictures of the results after 24 h of treatment with the extracts. CSE caused DNA damage at low (25 μM) concentrations while EAE did not; whereas both EAE and CSE caused DNA damage at high (500 μM) concentrations.

E-Cigarette Aerosol Triggers Both Apoptotic and Necrotic Cell Death

Historically the TUNEL assay has been used as an indicator of apoptotic cell death. However, it has recently been shown that TUNEL positivity can occur as a result of either apoptosis or programmed necrosis (Hanus et al., 2015). To determine which cell death pathway is triggered by EAE, we used antibodies against either active caspase-3 (an apoptosis marker) or phosphorylated MLKL (a programmed necrosis marker) (He et al., 2016). Figure 4 shows representative pictures of the results after 24 h of treatment with EAE. A low concentration of EAE (25 μM) did not activate either pathway; however, a high dose (500 μM) induced activation of both the apoptotic pathway mediated by caspase-3 (Figure 4A) and the necrotic pathway mediated by MLKL (Figure 4B).

Both E-Cigarette Aerosol and Cigarette Smoke Generate ROS

The cytotoxic effects of cigarette smoke are closely associated with its ability to induce oxidative stress through ROS (Morris et al., 2015). To determine whether EAE could induce oxidative stress...
FIG. 5. ROS generation and flow cytometry analysis of apoptosis and necrosis after EAE exposure. A, Endothelial cells exposed to EAE for as little as 4 h display significant levels of ROS. Simultaneously to 500 μM EAE exposure, cells were treated with ENZO Oxidative Stress Detection Reagent, which fluoresces green in the presence of ROS. After 4 h of incubation, fluorescent signal was quantified and averaged over cell number. Bars represent mean ± SEM of 3 samples. Statistical significance is indicated by *, **P < .05; ***P < .01; ****P < .001. B and C, Treatment with antioxidants limits EAE-induced cell death. Endothelial cells were simultaneously treated with 500 μM
stress, and at what level as compared with cigarette smoke, we used a fluorescent based assay for total ROS generation. We chose a treatment concentration of 500 μM as it was capable of causing EAE induced cell death in our previous assays. Figure 5A shows the relative level of ROS generated by either 500 μM EAE or 500 μM CSE 4 h after exposure to extracts. EAE treatment generated significant levels of ROS (~4.5-fold upregulation over control) though still less than the levels generated by CSE at the same treatment concentration (~7.8-folds over control).

Anti-Oxidant Treatment Prevents E-Cigarette Aerosol Induced Cell Death

As significant levels of ROS were generated in EAE treated endothelial cells prior to the induction of cell death, we hypothesized that ROS plays a causative role in EAE induced cell death. We subjected cells to 72 h of treatment with both 500 μM EAE and either 1 of 2 anti-oxidants that have been previously demonstrated to protect endothelial cells from oxidative stress: α-Toc and NAC (Bielli et al., 2015). Figure 5B summarizes the results of flow cytometry analysis (Figure 5C) using Ax V as an apoptosis marker and ethidium III homodimer as a necrosis marker. EAE treatment significantly increased the number of cells that stain with either Ax V (1.43 ± 0.05% vs 10.69 ± 0.08%), ethidium III homodimer (1.39 ± 0.08 vs 5.9 ± 0.21%), or both (1.98 ± 0.23 vs 10.17 ± 0.13%). Treatment with either anti-oxidant significantly decreased the number of positively stained cells, α-Toc (Ax V: 6.75 ± 0.17%, ethidium III homodimer: 1.31 ± 0.06%, both: 2.86 ± 0.35%) proved significantly more effective at preventing both forms of cell death than NAC (Ax V Ax V: 9.20 ± 0.42%, ethidium III homodimer: 3.06 ± 0.11%, both: 4.83 ± 0.09%). The reduction in the number of Ax V positive cells was significantly weaker than the reduction in ethidium-III homodimer positive cells or doubly positive cells in both anti-oxidant treatments. Notably, α-Toc treatment is capable of reducing the number of ethidium-III homodimer positive cells or doubly positive cells in EAE treated samples to levels indistinguishable from control cells. These data indicate that sufficient anti-oxidant treatment is capable of preventing the necrotic cell death induced by e-cigarette aerosol. Anti-oxidant treatment can reduce but does not prevent e-cigarette aerosol induced apoptotic cell death.

DISCUSSION

In this study, we have demonstrated that e-cigarette aerosol is capable of inducing reactive oxygen species, DNA damage, and cell death in HUVECs. In all cases, the effects of e-cigarette aerosol were less than those of cigarette smoke applied at the same consumed nicotine concentration. No significant differences were noted within or among different brands of e-cigarette. By demonstrating that e-cigarette aerosol can cause cleavage of caspase-3 and Ax V positivity as well as phosphorylation of MLKL and ethidium III homodimer positivity, we have provided strong evidence that the cytotoxic effect of e-cigarette aerosol is mediated by both apoptosis and programmed necrosis. Further, our partial prevention of e-cigarette aerosol induced cell death by treatment with anti-oxidants indicates that the increased levels of ROS observed play a causal role in e-cigarette aerosol induced cytotoxicity. That we have been able to use anti-oxidant treatment to prevent e-cigarette induced necrosis but not apoptosis provides evidence that e-cigarette aerosol induced cytotoxicity is a multifactorial process. This raises the possibility that distinct components of e-cigarette aerosol (nicotine, fine particles, flavor compounds, etc.) may be linked to distinct toxic effects, providing an avenue for potentially improving these systems by eliminating harmful components.

Over the last several years, e-cigarette use has risen dramatically among both youths and adults, making it vital that we understand the potential health consequences of e-cigarette aerosol exposure (Arrazola et al., 2015; Weaver et al., 2015). However, the general consensus is that the current data is not adequate to make sound, evidence based judgments. This decision stems not only from a lack of available data but also from concerns regarding the representative nature and reproducibility of the data collected (Callahan-Lyon, 2014; Pisinger and Døssing, 2014; West and Brown, 2014). The question of conflict of interest is particularly important in e-cigarette research (Etter, 2015). In the 2014 review by Pisinger and Døssing, it was estimated that 94% of authors publishing on the subject stated conflicts of interest, though the significance and interpretation of this issue have recently become matters of contention (Kosmider and Anastasi, 2016; which was responded to in Pisinger, 2016). Moreover, the varying toxicity profile of different e-cigarette liquids, and the fast rate of development of new flavors, provides an extra level of challenge to addressing the health consequences of e-cigarette aerosol (Allen et al., 2015; Barrington-Trimis et al., 2014; Zhu et al., 2014).

In order to insure the relevance and representative nature of our results we have used a panel consisting of one design of tobacco flavored e-cigarette from 4 different manufacturers whose products make up a significant portion of sales in multiple markets (Maier, 2015). This approach ensures that the products we are testing are products actively being purchased by a significant portion of consumers. E-cigarette liquid aerosolization can result in chemical transformation and the formation of harmful substances (Hutzler et al., 2014). Therefore, assaying pre-aerosolized e-cigarette liquid, or e-cigarette aerosol generated in non-standard fashions, may lead to variations in toxicity analysis. In order to minimize variation and increase the relevance of our study, we developed a laboratory apparatus (Figure 1C) capable of puffing either a conventional cigarette or e-cigarette. While e-cigarette puffing topography varies from conventional cigarette puff topography, there is no current standard operating procedure for e-cigarette puffing (Behar et al., 2015; Farsalinos et al., 2013; Robinson et al., 2015). Consequently, we employed the WHO standard operating procedure for cigarette smoking for both e-cigarettes and conventional cigarettes (WHO, 2012).

There is little consistency in the composition of e-cigarette liquid in the market and high variations in the concentration used in studies across the literature (Cheng, 2014). Since nicotine cravings drive smoking behavior (Picciotto and Kenny, 2013), we...
have created and diluted extracts based on equivalent concentrations of consumed nicotine. This strategy allows us to normalize between e-cigarettes with higher or lower nicotine concentrations as well as between e-cigarette and conventional tobacco cigarettes. Although it is at times difficult to choose appropriately physiologically relevant concentrations for in vitro studies, we chose to keep the amount of aerosol used to ~1 mM consumed nicotine concentration. The range of concentrations used across similar in vitro studies of e-cigarette aerosol cytotoxicity runs from about 2 μM to 25 mM (Farsalinos et al., 2013; Lerner et al., 2015; Romagna et al., 2013; Schweitzer et al., 2015; Teasdale et al., 2016). This means that we are using some of the lowest concentrations of e-cigarette aerosol of any similar study at the time of this writing. For most of our experiments we rely on 25 μM consumed nicotine as a low concentration and 500 μM consumed nicotine as a high concentration. The concentration of 25 μM is particularly significant, as it is extremely close to the projected consumed nicotine concentration of the average smoker in the developed world per day (based on the data gathered by Ng et al., 2014). 500 μM was selected as it was the concentration at which we saw consistent, reproducible harm from e-cigarette aerosol across all methods used in this study.

Our findings support and extend previous studies of e-cigarette induced cytotoxicity. Previous in vitro studies have focused on a range of cell types, with some of the most notable being embryonic fibroblasts and stem cells (Palpant et al., 2015; Romagna et al., 2013), oral/buccal cells (Sancilio et al., 2016), lung epithelial cells (Cervellati et al., 2014; Lerner et al., 2015) and cardiovascular cells (Farsalinos et al., 2013; Putzhammer et al., 2016; Schweitzer et al., 2015; Teasdale et al., 2016). In all cases where e-cigarette aerosol was compared with cigarette smoke, e-cigarette aerosol was found to be significantly less harmful, which is consistent with our results. Of these studies, only Sancilio et al. attempted to look deeper the mechanism of e-cigarette aerosol induced cell death. They were able to positively confirm apoptosis through Ax V positivity and Bax expression. Interestingly, Sancilio et al. did not see the induction of necrosis in their study. This lack of necrosis in their study may indicate that programmed necrosis is a cell type specific response to e-cigarette aerosol induced oxidative stress. Of the cardiovascular cell specific studies, Farsalinos et al. used MTT assays to detect reductions in cell viability in response to e-cigarette aerosol while Schweitzer et al. demonstrated the ability of e-cigarette liquid to increase oxidative stress and inhibit endothelial barrier function. Putzhammer et al. employed similar methods to the present study to analyze ROS generation and cytotoxicity in HUVECs, but their results proved highly variable. Our study extends on their work by exploring the nature of e-cigarette aerosol induced cytotoxicity and its relationship to oxidative stress. Teasdale et al. measured oxidative stress response in coronary artery endothelial cells and noted no effect of e-cigarette aerosol. However, they used a concentration of aerosol much lower than the other studies (2.16 μM), which our study indicates is below the threshold of e-cigarette aerosol induced cytotoxicity. As no single cell type can fully represent the highly heterogeneous nature of the cardiovascular system, there is a need for more comprehensive studies in the future.

In vivo studies of e-cigarette aerosol’s effect on the cardiovascular system have shown a broad spectrum of potentially negative effects (Carnevale et al., 2016; Hom et al., 2016; Schweitzer et al., 2015). The findings of Carnevale et al. are particularly interesting as they have shown upregulation of oxidative stress related marker NOX-2 and dysregulation of lipid peroxidation marker 8-isoPGF2α in human serum after both conventional tobacco cigarette smoking and e-cigarette vaping. They were also able to show a reduction of serum α-Toc in the same subjects. A similar effect measured in mouse plasma by assaying levels of 8-OHdG was reported in Schweitzer et al., 2015. Our study supports these findings by demonstrating that there is an increase of reactive oxygen species in endothelial cells as a response to e-cigarette aerosol, that HUVECs die through both programmed necrosis and apoptosis, and that endothelial cell necrosis can be prevented by the addition of α-Toc. The suggestion by Schweitzer et al. is that the majority of the consequences of e-cigarette aerosol exposure can be attributed to the effects of nicotine. Although nicotine has long been associated with cardiovascular disease (Benowitz and Burbank, 2016), nicotine independent effects of e-cigarette aerosol have not been noted. For instance, the work of Hom et al., demonstrates specific nicotine dependent and nicotine independent effects of e-cigarette aerosol on platelet activation in vivo. The nature and magnitude of the effect of nicotine in e-cigarette induced cytotoxicity remains unclear. Our study indicates that nicotine induced oxidative stress does not appear to be the sole cause of e-cigarette induced cell death in HUVECs. However, we cannot rule out effects of nicotine unrelated to oxidative stress or effects of e-cigarette aerosol unrelated to nicotine. Both of these topics deserve additional study if we are to fully understand the potential cardiovascular risks of e-cigarette use. While there is still a great deal of work to be done to understand the long-term health consequences of e-cigarette aerosol, our data contribute to the growing consensus that it is simultaneously significantly safer than cigarette smoke but far from safe.

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REFERENCES


Pisinger, C. (2016). Reading the conflict of interest statement is as important as reading the result section. Prev. Med. 85, 115.


