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Early View

Research letter

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The effects of electronic cigarette vapor on the lung: direct comparison to tobacco smoke

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Short message: Electronic cigarettes are as toxic as tobacco cigarettes and can cause significant lung damage

Key words: Electronic cigarettes, tobacco smoke, nicotine, lung, emphysema, vasculature, endothelium

Electronic cigarettes (E-cig) usage in the US has drastically increased in the past five years due to age restrictions on conventional cigarettes, aggressive marketing, and perception that E-cig are a healthy alternative. E-cig contain nicotine, water, glycerol, propylene glycol, and optional flavoring. On inhalation, the device heats the ingredients into a vapor [1]. While tobacco cigarette smoke is known to cause a deleterious effect on the cardiovascular system, angiogenesis, and skin capillary perfusion by causing direct injury to blood vessel walls, increased platelet aggregation, microvascular thrombosis [2–4], and inflammation [5], the consequences of E-cig vapor exposure on the lung are still largely unexplored [6, 7]. Recently, Lerner and colleagues reported that vapors produced by E-cig and E-juices with flavorings induced toxicity, oxidative stress, and inflammatory response in human bronchial airway epithelial cells (H292) and fetal lung fibroblasts (HFL1) as well as mouse lung [8]. Garcia-Arcos and colleagues showed that the aerosolized nicotine-containing e-cigarette fluid increased airway hyper-reactivity, distal airspace enlargement, mucin production, cytokine and protease expression in mice, implying the potential dangers of nicotine inhalation during E-cig use [9]. The inflammatory response to E-cig use involved increased neutrophil activation and mucus production [10] and decreased mucociliary clearance [11]. In human embryonic and mouse neural stem cells and human pulmonary fibroblasts [12], as well as skin and lung cells [13], cytotoxicity of E-cig vapor was correlated with the number and concentration of chemicals used to flavor fluids. We recently showed in the skin flap survival model *in vivo* that nicotine-containing E-cig vapor is just as harmful to the microcirculation as tobacco cigarette smoke (CS) [4].

In the present study, we examined whether long-term exposure to E-cig vapor or nicotine produce the same damaging effect on the lung structure and vasculature as tobacco smoke in a rat model *in vivo*.

Six-week old male Sprague Dawley rats (Envigo Laboratories) were divided into four groups of eight animals per group and exposed for five weeks to: Group 1- room air (RA); Group 2 - Nicotine (NIC) - subcutaneous injections of (−)-Nicotine di-tartrate (Sigma Aldrich) 2 mg/kg twice daily. The amount of nicotine for injections were based on the previous studies to produce stable plasma nicotine levels of approximately 25 ng/ml, that is compatible with plasma levels of habitual smokers [14, 15]; Group 3 - Blu® E-cigs (Classic Tobacco Flavor, containing 12 mg/ml of nicotine) vapor produced in an electronic cigarette smoking machine TE-2E (Teague Enterprises, Davis, CA; www.teague-ent.com). The coil temperature of the electronic cigarettes was within the normal range usually used by vapers in the range of 200°C- 250°C. Rats in E-cig group were exposed to 48 mg of nicotine/per day [4]. Our experimental design by subjecting rats to E-cig vapor is the major improvement versus to aerosolized electronic cigarettes liquid used in Garcia-Arcos et al study in mice [9]. Group 4 –cigarette smoke (CS) exposure in the smoking chamber TE-10z (Teague Enterprises, Davis, CA) by burning five Kentucky 3R4F reference cigarettes (Tobacco Research Institute, University of Kentucky, Lexington, KY) (4h per day: 2h and 2h with 1-hour rest period) as described [16]. Total suspended particulate (TSP) levels in the CS chamber were maintained at 60 mg/m^3 and nicotine levels at 48-50 mg/m^3. Based on plasma nicotine levels (data from our own group [4]), the whole-body exposures of rats to E-cig and tobacco cigarettes were comparable to the use of E-cig and tobacco cigarettes

by people [17]. The work was performed with the approval of the Institutional Animal Care and Use Committee (IACUC) at University of Colorado Denver Anschutz Medical Campus.

At the end of the exposure, rats were sacrificed and lungs were inflated with 1% of low melting agarose at 25 cm of H_2O pressure. The mean alveolar airspace enlargement was measured using an automated image analyzer ImageJ software (NIH, Bethesda, MD) and calculated as a percentage of total airspace versus tissue density [16]. While less sensitive than stereological method, the alveolar airspace area measurements (based on our extensive experience) accurately reflect lung morphological changes.

All data represented as mean ± standard error of the mean (SEM). Statistical analysis was performed using two-way analysis of variance test (ANOVA) followed by Tukey's HSD post-test and two-tailed, unpaired Student's t-test. The alveolar airspace enlargement measurements within each exposure group was analyzed using GraphPad Prism© with one-way analysis of variance (ANOVA), and Tukey's multiple comparisons test.

As shown in Figure 1a, exposure of rats to subcutaneous injections of nicotine (NIC), E-cig vapor or CS in the smoking chamber for five weeks led to significant ($p<0.01$) emphysematous lung destruction, when compared to RA controls. The mean units ± SEM of alveolar airspace area (Fig. 1b, black bars) for RA control, NIC, E-cig vapor and CS groups were 71 ± 5.1, 83 ± 2.7, 86 ± 2.0, and 84 ± 3.3, respectively. There were statistically significant differences in alveolar airspace enlargements between RA controls and experimental E-cig, NIC and CS groups (p<0.01).

In emphysema, along with the airway space enlargement, there is also visible loss of peripheral of vasculature [18]. To assess capillary vessel (less than $100\mu m$) density, lung sections were stained for von Willebrand factor. Three fields per slide (total 24 fields per eight animal group) were counted by 2 independent investigators in a blind manner. As shown in Figure 1b (open bars) the capillary count was significantly (*$p<0.01$) decreased in all three treatment groups. The differences were also significant between NIC alone and E-cig and CS groups (#$p<0.02$).

Our results clearly demonstrate that E-cigs are as damaging to pulmonary structures as traditional tobacco cigarettes. The emphysematous changes seen in cigarette smoke exposed rat lungs are also abundantly apparent in E-cig- and in nicotine-treated rat lungs (Fig. 1). Other than a common ingredient, nicotine, E-cigs and tobacco cigarettes are fundamentally different. The E-cig contain three main ingredients - nicotine, propylene glycol and glycerin - and functions through vaporization of a nicotine-containing fluid, whereas the tobacco cigarette beside nicotine contains over 7,000 chemical compounds and involves the combustion of tobacco. Surprisingly, in a rat model, both produce very similar devastating effects on the lungs.

While in our study, serum levels of nicotine and cotinine were higher in CS group than in E-cig group [4], the amount of lung tissue destruction was similar across both exposure groups. Nicotine and cotinine plasma levels in the CS-exposed rats were comparable with those found in smokers and our values corroborated prior published nicotine and cotinine levels in rats exposed to tobacco smoke [19].

It is possible that the particles within the vapor with a hydrodynamic diameter of 2.5µm or less (known as "fine particulate matter"), rather than the nicotine itself or in conjunction with nicotine, have a drastic negative effect on lung morphology. Fine particles are concerning because they can penetrate lung tissue and blood stream causing serious health effects. Recent study [20] demonstrated that the fine particulate matter within E-cig vapor alters platelet function to the same extent as the particulate matter within tobacco smoke. Just as seen with

conventional cigarettes [16], the exposure to E-cig vapor causes decreased density of lung vasculature (Fig. 1a,b) meaning that as seen in emphysema patients [18], both airway and vascular cells are affected resulting in alveolar airspace enlargement and disappearance of peripheral vasculature.

There are some limitations of our study. First, there is no standard electronic cigarette or "vaping" machine. To best match nicotine content of the E-cig vapor to cigarette smoke [17], we used AirCheck 52 pump to determine the levels of nicotine at multiple settings within the TE-2 and TE-10 chambers. A second limitation was that we only evaluated Blu® electronic cigarettes. Third, common to all translational research, equating results from experimentation with rats may not necessarily translate into similar results in humans. While our study was designed to imitate the exposure levels of rats to the use of E-cig and tobacco cigarettes by people, they may not replicate the exact smoking experience for human users. However, until a randomized control trial can be performed in humans, this rat model will likely be one of the most appropriate to reference when counseling their patients on E-cig smoking cessation.

In summary, our findings in an experimental model clearly indicate that E-cigs are just as toxic as tobacco cigarettes and that longtime exposure to nicotine vapor can cause significant lung damage. It is not a safe alternative to tobacco smoke.

The FDA now has regulatory authority over e-cigs and can regulate product and e-liquid design features, such as nicotine content and delivery, voltage, e-liquid formulations, and flavors. The administration just announced its strategy that includes forcing cigarette manufacturers to lower the amount of nicotine in their products to "non-addictive levels." However, it is not clear what non-addictive levels are and if they indeed may impact pulmonary toxicity.

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Figure Legend

Figure 1. The effects of E-cigarette vapor (E-cig), nicotine (NIC) and cigarette smoke (CS) exposure on the lung structure and blood vessel count in comparison to the room air (RA) exposed controls. a) Lung morphology and lung vasculature (visualized by staining for von Willebrand factor) after five weeks of exposure. Arrows indicate capillary vessels. b) Alveolar air space enlargements (black bars; % of total airspace versus tissue density /per visual field (n=8 rats, 3 fields/per slide)). Significant differences in alveolar airspace enlargements were found between treatment groups and RA control, *p≤0.01. Open bars show capillary vessel count per field (n=8 rats, 3 fields/per slide). *p≤0.01 – indicates differences in capillary vessel counts between RA controls and all tree treatment groups); #p≤0.02 indicates differences of NIC alone versus E-cig and CS groups.

Figure 1