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RESEARCH ARTICLE



Effect of E-Cigarette aerosol exposure on airway inflammation in a murine model of asthma

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ABSTRACT

Objective: The popularity of electronic cigarettes (E-Cigs) smoking is increasing worldwide including patients with asthma. In this study, the effects of E-Cigs aerosol exposure on airway inflammation in an allergen-driven murine model of asthma were investigated.

Materials and methods: Balb/c mice were randomly assigned to; control group (received fresh air, Ovalbumin (Ova) sensitization and saline challenge), E-Cig group (received E-Cig aerosol, Ova sensitization, and saline challenge), Ova S/C group (received fresh air, Ova sensitization and Ova challenge) and E-Cig + Ova S/C group. Bronchoalveolar lavage fluid (BALF) and lung tissue were evaluated for inflammatory cells and inflammatory mediators, respectively.

Results: Exposure to E-Cig aerosol significantly increased the number of all types of inflammatory cells in BALF ($p < 0.05$). Further, E-Cig aerosol reduced levels of transforming growth factor (TGF)- $\beta 1$ and matrix metalloproteinase (MMP)-2 in lung tissue homogenate ($p < 0.05$). Combined E-Cig aerosol and Ova S/C increased the airway recruitment of inflammatory cells, especially neutrophils, eosinophils, and lymphocytes ($p < 0.05$), increased the level of interleukin (IL)-13, and reduced the level of TGF- $\beta 1$ ($p < 0.05$).

Conclusions: E-Cig aerosol exposure induced airway inflammation in both control mice and allergen-driven murine model of asthma. The inflammatory response induced by E-Cig was slightly higher in allergen-driven murine model of asthma than in healthy animals.

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E-Cigarette; airway inflammation; murine model of asthma; inflammatory mediators; inflammatory cells

Introduction

Asthma is a chronic airway inflammatory disease involving airways hyper-responsiveness, eosinophilia, and increased mucus production (Lambrecht et al. 2019). About 334 million people had asthma in 2014 (Cai et al. 2017) and it is estimated that by 2025 further 100 million people will be affected (Dharmage et al. 2019). Asthma arises from a complex interaction between genetic, epigenetic, and environmental factors (Kabesch and Tost 2020). Tobacco smoking is a well-known factor that provokes adult onset asthma (Coumou et al. 2019). Patients with asthma who are active smokers are more prone to asthma exacerbations, tend to have more severe asthma symptoms, are less likely to respond to treatment, and have a higher mortality rate compared with nonsmokers (Chaudhuri et al. 2008; Shimoda et al. 2016; Hromis et al. 2018; Ioachimescu and Desai 2019). Unfortunately, the WHO estimated that over 1.1 billion people smoked (World Health Organization 2019) accounting for 11.5% of global deaths in 2015 (Collaborators 2017). Among patients with asthma, 21% reported being current smokers (Thomson 2017) and about 50% of them reported that smoking worsen their respiratory symptoms (Silverman et al. 2003).

Electronic cigarettes (E-Cigs), also known as electronic nicotine delivery devices, are battery powered devices that heat a liquid to produce an aerosol that is inhaled by the user (Leigh et al. 2016; Larcombe et al. 2017). The E-Cigs liquid commonly contains humectants, a flavoring agent and a variable concentration of nicotine (Morean et al. 2016). E-Cigs are a new smoking method that was introduced to market in early 2000s, and became a trend, especially among adolescent and young adults (Grana et al. 2014). The prevalence of E-Cig use is rapidly increasing, rising from 0.3% in 2010, to 6.8% in 2013, 7.4% in 2015, and 8.5% in 2017 in USA (Levy et al. 2019). Although E-Cigs are portrayed as a safer alternative to tobacco smoking and are extensively marketed as an effective method for smoking cessation, there is a lot of debates about their safety (Grana et al. 2014). Three toxic metals were identified in E-Cig vapor; chromium, nickel, and lead (Williams et al. 2013). Further, E-Cig cartridge solution contains toxic nitrosamines and diethylene glycol as well as noxious alkaloids, including anabasine and myosmine (Lim and Kim 2014). Propylene glycol and glycerol, the most commonly utilized humectants in E-Cig liquids, when heated and aerosolized, cause irritation to eyes and respiratory system (Grana et al. 2014; Hahn

et al. 2014). E-Cig users reported a twofold increase in respiratory symptoms including cough, phlegm, and bronchitis and until 2019, seven deaths were attributable to E-Cig use (Gotts et al. 2019). Osei et al. (2019) reported an increased odds of asthma among E-Cigs users who never smoked cigarettes. However, both short- and long-term health consequences of E-Cig remain largely unexplored, especially in asthma patients, because of the relative novelty of this product. Therefore, the aim of the current study was to evaluate the effect of E-Cig aerosol exposure on airway inflammation, inflammatory mediators, and matrix metalloproteinase in an allergen-driven murine model of asthma.

Materials and methods

Animals

Adult male Balb/c mice (6–8 weeks old) were purchased from the animal care unit at Jordan University of Science and Technology (JUST, Irbid, Jordan). Mice were maintained at $24 \pm 1^\circ\text{C}$ in an automatic 12:12 light/dark cycle with free access to water and chaw. Mice were allowed to acclimatize for 1 week before conducting the experiment. All mice were given ovalbumin (Ova) sensitization, and received either (1) fresh air and saline challenge (control group), (2) 1 h of E-Cig aerosol daily for 4 weeks and saline challenge (E-Cig group), (3) fresh air and Ova challenge (Ova S/C group) or (4) 1 h of E-Cig aerosol daily for 4 weeks and Ova challenge (E-Cig + Ova S/C group). The study procedure was approved by Animal Use and Care Committee (ACUC) of JUST. The study was conducted in accordance with the Basic & Clinical Pharmacology & Toxicology policy for experimental and clinical studies (Tveden-Nyborg et al. 2018).

Ova sensitization and challenge

Mice were sensitized by administering a mixture of 2 mg/kg/day of Ova (Sigma-Aldrich, St. Louis, MO) and 2 mg of alum (Imject Alum; Thermo Scientific, Pierce, Rockford, IL) through intraperitoneal (i.p.) injection on days 0, 7 and 14 as described previously (Thanawala et al. 2013). From days 23 to 27, mice were challenged by nebulization of 1% Ova for 50 min (Ova S/C) or saline (control) (Al-Sawalha et al. 2017) as shown in Figure 1.

E-Cig aerosol exposure

Mice in the E-Cig and the E-Cig + Ova S/C groups were exposed to E-Cig aerosol as described previously (Al-Sawalha et al. 2020). The aerosol was generated using a smoking box for one hour session per day for 28 days using a digitally controlled reverse puffing smoke exposure machine. The machine is designed to convert the nicotine-containing juice to aerosol that is delivered into an aerosol box where mice were placed. The aerosol was generated by 1.8 Ω single coil E-Cig (Mini Protank2, KangerTech, Shenzhen, China). The machine was powered with 5.76 W

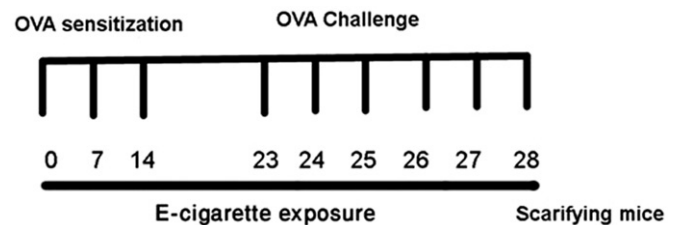


Figure 1. Treatment protocol. All animals were sensitized with Ova and randomly assigned to receive either; fresh air and saline challenge (control group), 1 h of E-Cig aerosol daily for 4 weeks and saline challenge (E-Cig group), fresh air and Ova challenge (Ova S/C group) or 1 h of E-Cig aerosol daily for 4 weeks and Ova challenge (E-Cig + Ova S/C group).

to produce aerosol at a rate of 5 puffs/min, each puffing has a volume of 116.7 mL and lasted for 4 seconds with 10 seconds inter-puff interval (Al-Sawalha et al. 2020; Mayyas et al. 2020). During puffing, a pump is activated to deliver room air to the air inlet port, and once the aerosol is generated, it is puffed through the mouthpiece into a 38x25x25 cm box. The exposure chamber is designed with a fresh air ventilation system and it maintains an air change at a rate of 1.75 L/min. The mean total particulate matter (TPM) in the box per each session was $1338 \pm 342 \text{ mg/m}^3$. The aerosol concentration in the box was 1.33 g/m^3 . The nicotine-containing juice was prepared by mixing propylene glycol (analytical grade) and glycerol in a ratio of 7:3 to mimic many commercially available E-Cig juices. Nicotine was added to the previous mixture to produce a concentration of 18 mg/ml.

Bronchoalveolar lavage fluid (BALF) collection

Mice were sacrificed on day 28 by administering 0.1 ml of xylazine (2%) and 0.1 ml of ketamine (10%) through i.p. injection. BALF was collected as described previously (Al-Sawalha et al. 2017). The collected BALF was centrifuged at 12,000 rpm for 5 minutes. The pellet was resuspended in normal saline and total inflammatory cells were counted using hemacytometer (Hausser Scientific, Horsham, PA). The differential cell count was determined by cytocentrifugation using cytospin (Cytotek, Sakura Finetek, Netherlands) at 1500 rpm for 5 minutes. The obtained slides were stained with Wright Giemsa stain to assess the differential cell count.

Measurement of inflammatory mediators in the lungs

Lung tissues were homogenized using a tissue homogenizer (Tissue Master-125, Omni International, Kennesaw, GA) in ice cold homogenization buffer containing protease inhibitor cocktail (Sigma-Aldrich Corp., MI, USA) (Al-Sawalha et al. 2017). The homogenates were centrifuged at 15,000 rpm for 15 min at 4°C to remove insoluble debris and the supernatant was used to measure levels of cytokines and inflammatory mediators using by ELISA technique. Interleukin (IL)-10 (R&D Systems, MN, USA), IL-13 (Abcam, Cambridge, UK), transforming growth factor- β 1 (TGF- β 1) (Invitrogen/ThermoFisher, MA, USA), High mobility group

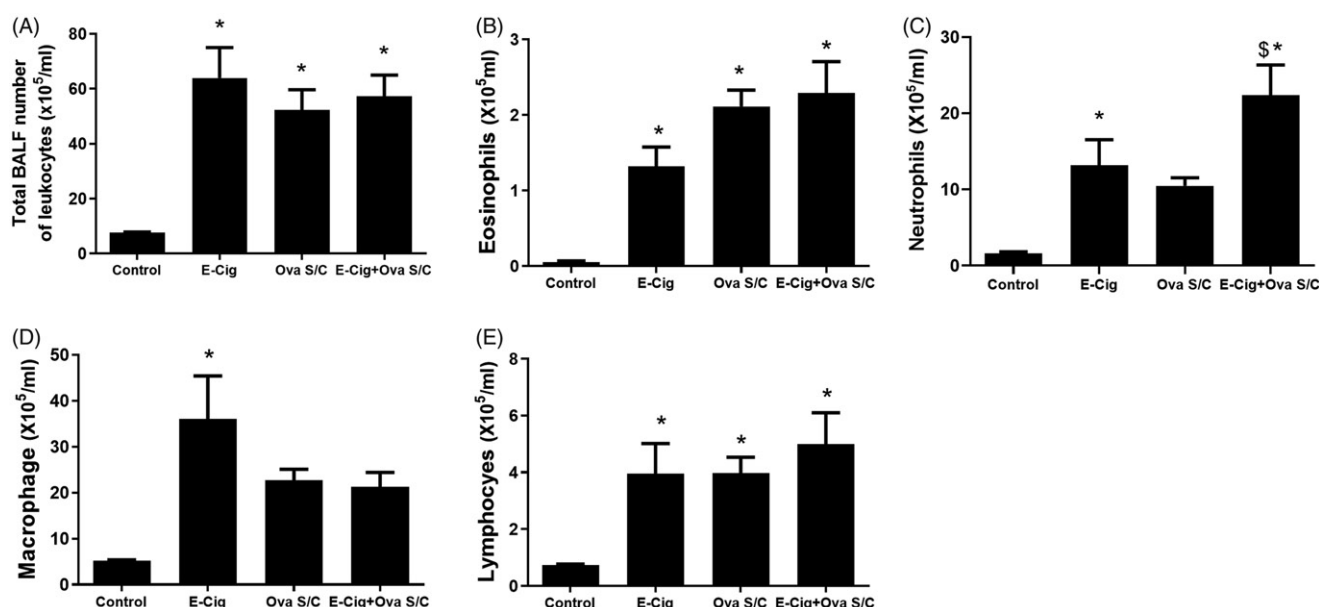


Figure 2. Effect of E-Cig aerosol exposure on inflammatory cells in BALF. BALF was collected from mice that were exposed to fresh air and saline challenge (control group), 1 h of E-Cig aerosol daily for 4 weeks and saline challenge (E-Cig group), fresh air and ovalbumin challenge (Ova S/C group), or E-Cig aerosol and ovalbumin challenge (E-Cig and Ova S/C group). BALF was collected 24 h after the last Ova challenge and last E-Cig aerosol exposure. BALF was analyzed for (A) total inflammatory cells, (B) eosinophils, (C) neutrophils, (D) macrophages and (E) lymphocytes. *indicates significant difference from control group, \$indicates significant difference from Ova S/C. Values represent the mean $\pm$ SEM of data from 7 to 10 mice in each group. $p < 0.05$ was considered significant.

box 1 protein (HMGB-1) (MyBioSource, CA, USA), matrix metalloproteinase-2 (MMP-2) (MyBioSource, CA, USA) and MMP-9 (R&D Systems, MN, USA) levels were measured following the manufacturers' instructions. The absorbance was measured at 450 nm using a plate reader (ELx800 plate reader, Bio-tek instruments, Winooski, USA).

Statistical analysis

The normality of the data was checked by D'Agostino & Pearson omnibus and Shapiro–Wilk tests. Kruskal–Wallis test and One-way ANOVA followed by Tukey's multicomparison test were used to compare the inflammatory cells and inflammatory markers wherever is appropriate. GraphPad Prism® software was utilized for data analysis. $p < 0.05$ was considered statistically significant. Data were presented as mean $\pm$ SEM.

Results

Effect of E-Cig aerosol exposure on airway inflammatory cells in allergen driven murine model of asthma

To evaluate the effect of E-Cig aerosol exposure on airway inflammatory cells, mice were exposed to E-Cig aerosol (1 hour per day) or fresh air for 28 days and inflammatory cells were examined in the BALF preparation (Figure 2). Exposure to E-Cig aerosol significantly increased the number of inflammatory cells ($63.98 \pm 11.05 \times 10^5/\text{ml}$) compared to the control group ($7.78 \pm 0.15 \times 10^5/\text{ml}$, $p > 0.001$, Figure 2(A)). Similarly, the number of inflammatory cells increased in Ova S/C group ($52.33 \pm 7.41 \times 10^5/\text{ml}$, $p < 0.001$) compared to the control group. In addition, the total number of

inflammatory cells was slightly higher in E-Cig group than Ova S/C group but the difference was not statistically significant ($p > 0.05$). Moreover, the combined Ova S/C and E-Cig aerosol exposure increased in the total inflammatory cells ($57.28 \pm 7.76 \times 10^5/\text{ml}$) to a level similar to that induced by each treatment alone (Figure 2(A)).

The inflammatory response in the allergen driven murine model of asthma is characterized by increased airway recruitment of eosinophils. Figure 2(B) shows that mice in Ova S/C group showed elevated ($p > 0.001$) numbers of eosinophils ($2.11 \pm 0.22 \times 10^5/\text{ml}$) compared to the control group ($0.06 \pm 0.01 \times 10^5$). Significant elevation ($p < 0.01$) in the number of eosinophils ($1.32 \pm 0.26 \times 10^5/\text{ml}$) was also observed in E-Cig aerosol exposed group. Further, E-Cig aerosol exposure in mice that were sensitized and challenged with Ova showed a significant increase in the number of eosinophils ($2.29 \pm 0.42 \times 10^5/\text{ml}$) to a level comparable to that induced by Ova S/C group (Figure 2(B)) without significant differences between E-Cig aerosol/Ova S/C, E-Cig aerosol and Ova S/C groups ($p > 0.05$).

Moreover, the airway recruitment of other inflammatory cells was affected by Ova S/C and by E-Cig aerosol exposure. Ova S/C significantly increased the number of lymphocytes ($3.97 \pm 0.57 \times 10^5/\text{ml}$ in Ova S/C versus $0.74 \pm 0.03 \times 10^5/\text{ml}$ in the control group, $p = 0.048$) and resulted in a trend of increased number of macrophages ($22.73 \pm 2.36 \times 10^5/\text{ml}$ in Ova S/C group versus $5.22 \pm 0.18 \times 10^5/\text{ml}$ in the control group, $p = 0.09$) but did not affect the recruitment of neutrophils ($10.47 \pm 1.08 \times 10^5/\text{ml}$ in Ova S/C versus $1.58 \pm 0.19 \times 10^5/\text{ml}$ in the control group, $p < 0.05$) (Figure 2(C–E)).

Moreover, the airway recruitment of other inflammatory cells was affected by Ova S/C and by E-Cig aerosol exposure. Although ova S/C did not affect the recruitment of

Table 1. Effect of E-Cig aerosol exposure on inflammatory mediators in allergen driven murine model of asthma.

Inflammatory mediator	Control	ECIG	Ova S/C	ECIG + Ova S/C
IL-13 (pg/mg)	1.25 ± 0.274	0.93 ± 0.26	2.7 ± 0.70	4.60 ± 1.43*#
IL-10 (pg/mg)	4.73 ± 2.68	4.64 ± 0.62	1.03 ± 0.72	1.46 ± 0.78
HMGB1 (pg/mg)	17.46 ± 2.3	12.96 ± 1.32	15.66 ± 1.54	16.56 ± 2.56
TGF-β (pg/mg)	4996.00 ± 1131.00	1436.00 ± 289.20*	3218.00 ± 6282.00	1560.00 ± 693.70*

*indicates significant difference from control group.

#indicates significant difference from ECIG group.

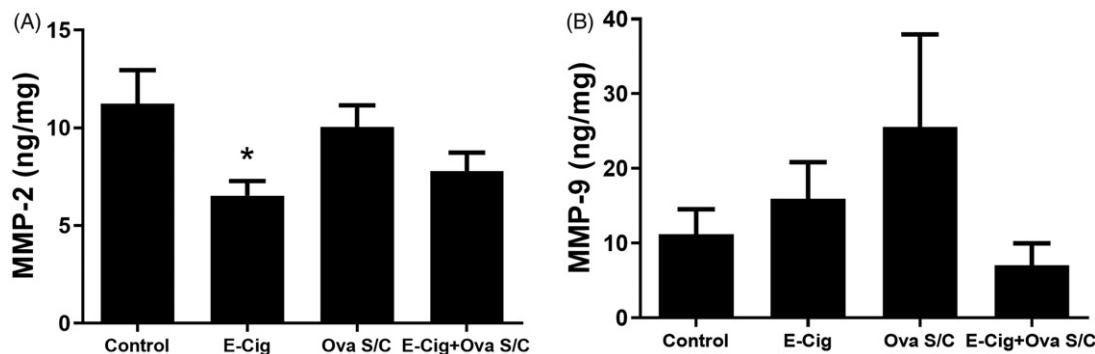


Figure 3. Effect of E-Cig aerosol exposure on MMPs. Lung tissues were collected and homogenized from mice that were exposed to fresh air and saline challenge (control group), 1 h of E-Cig aerosol daily for 4 weeks and saline challenge (E-Cig group), fresh air and ovalbumin challenge (Ova S/C group), or E-Cig aerosol and ovalbumin challenge (E-cig and Ova S/C group). BALF was collected 24 h after the last Ova challenge and last E-Cig aerosol exposure. Lungs tissue were analyzed for (A) MMP-2, and (B) MMP-9. * indicates significant difference from control group. Values represent the mean ± SEM of data from 7 to 10 mice in each group. $p < 0.05$ was considered significant.

neutrophils ($10.47 \pm 1.08 \times 10^5/\text{ml}$ in Ova S/C versus $1.58 \pm 0.19 \times 10^5/\text{ml}$ in the control group, $p < 0.05$), it resulted in a trend of increased number of macrophages ($22.73 \pm 2.36 \times 10^5/\text{ml}$ in Ova S/C group versus $5.22 \pm 0.18 \times 10^5/\text{ml}$ in the control group, $p = 0.09$) and significantly increased the number of lymphocytes ($3.97 \pm 0.57 \times 10^5/\text{ml}$ in Ova S/C versus $0.74 \pm 0.03 \times 10^5/\text{ml}$ in the control group, $p = 0.048$) (Figure 2(D)).

Similar findings were found in E-Cig and simultaneous E-Cig and Ova S/C groups to that of Ova S/C except for the recruitment of neutrophils that was increased significantly with simultaneous E-Cig aerosol exposure and Ova S/C compared to Ova S/C group ($22.37 \pm 3.95 \times 10^5/\text{ml}$ in E-Cig + Ova S/C group $10.47 \pm 1.08 \times 10^5/\text{ml}$ in Ova S/C group, $p = 0.02$) (Figure 2(C)).

Effect of E-Cig aerosol exposure on inflammatory mediators in allergen driven murine model of asthma

To evaluate if the changes in the number of inflammatory cells was coupled with changes in levels of inflammatory mediators, the lung levels of IL-13, IL-10, TNF- α , HMGB1, and TGF- β were measured in lung tissue homogenate (Table 1). Exposure to E-Cig aerosol or Ova S/C did not affect the level of IL-13 compared to the control group. However, simultaneous Ova S/C and E-Cig aerosol exposure significantly increased the level of IL-13 compared to control and to E-Cig group. There were no detectable changes in lungs level of IL-10 or HMGB1 with E-Cig aerosol exposure or Ova S/C. On the other hand, exposure to E-Cig aerosol significantly reduced the level of TGF- β compared to the control group. Moreover, simultaneous Ova S/C and E-Cig

aerosol exposure significantly reduced TGF- β compared to the control group.

Effect of E-Cig aerosol exposure on matrix metalloproteinases in allergen driven murine model of asthma

The effect of E-cig aerosol exposure on MMPs was also evaluated in lung tissue homogenates. Exposure to E-Cig aerosol significantly reduced the level of MMP-2 compared to the control group ($6.53 \pm 0.75 \text{ ng/mg}$ in E-Cig group versus $11.23 \pm 1.72 \text{ ng/mg}$ in the control group, $p = 0.035$). On the other hand, simultaneous Ova S/C and E-Cig aerosol exposure did not affect the level of MMP-2 compared to Ova S/C group ($7.8 \pm 0.93 \text{ ng/mg}$ in E-Cig + Ova S/C group versus $10.05 \pm 1.11 \text{ ng/mg}$ in Ova S/C group, $p > 0.05$) (Figure 3(A)). In addition, E-Cig aerosol exposure or Ova S/C did not affect the level of MMP-9 in the lung homogenates ($11.20 \pm 3.33 \text{ ng/mg}$, $15.93 \pm 4.90 \text{ ng/mg}$, $25.53 \pm 12.43 \text{ ng/mg}$, and $7.02 \pm 2.95 \text{ ng/mg}$ in control, E-Cig, Ova S/C, and E-Cig + Ova S/C respectively, $p > 0.05$) (Figure 3(B)).

Discussion

The current study evaluated the effect of E-Cig exposure on airway inflammation; the recruitment of airway inflammatory cells and levels of various inflammatory markers in allergen driven murine model of asthma.

Asthma is characterized by airway recruitment of inflammatory cells, especially eosinophils (Busse and Lemanske 2001). In the current study, Ova S/C resulted in increased number of inflammatory cells in the airways consistent with previous studies (Kim et al. 2011, 2012; Thanawala et al.

2013). The Ova sensitization and saline challenge did not induce any airway inflammation in the current study consistent with previous studies that used this protocol in the control group (Callaerts-Vegh et al. 2004; Nguyen et al. 2008). Therefore, Ova sensitization and saline challenge protocol was used in all other groups that did not receive Ova challenge.

The exposure to E-Cig aerosol increased the recruited airway inflammatory cells compared to unexposed mice, consistent with a previous study with 3-days E-Cig aerosol exposure (Glynos et al. 2018). Previous studies showed a trend of increased airway inflammation by E-Cig aerosol exposure in mice that did not reach a significant level (Lerner et al. 2015; Garcia-Arcos et al. 2016). This inconsistency could be explained by the difference in the exposure duration as well as the mouse strain as it could affect the response to allergens (Whitehead et al. 2003; Antunes et al. 2009). The current study showed that simultaneous E-Cig aerosol exposure and Ova S/C resulted in a significant increase in total inflammatory cells in BALF compared to control. Previous study revealed that intra-tracheal instillation of E-Cig cartridge solution, for 10 weeks, to Ova sensitized and challenged mice increased the recruited inflammatory cells compared to unexposed as well as Ova sensitized and challenged mice (Lim and Kim 2014). However, the current study did not reveal any increase in the number of inflammatory cells in BALF by simultaneous E-Cig aerosol exposure and Ova S/C in comparison with Ova sensitized and challenged mice alone, inconsistent with a previous report (Lim and Kim 2014). This inconsistency could be due to the variations in the exposure duration as well as the route of E-Cig administration as the concentration of E-Cig components in the airways would be higher with intra-tracheal instillation. Future studies should examine the effect of E-Cig exposure in other allergen-induced murine model of asthma such as house dust mite.

Several inflammatory cells contribute to asthma pathogenesis. Eosinophils secrete pro-inflammatory cytokines, chemokines, lipid mediators, and cytotoxic granular proteins (Walsh 2001, Kim et al. 2012). E-Cig exposure increased the number of eosinophils in BALF compared to control. Hwang et al. (2016) reported no effect of E-Cig aerosol exposure on the total inflammatory cells and numbers of eosinophils compared to control female CD-1 mice. sex and strain difference could affect the response (Werley et al. 2016). In addition, Ova S/C increased the number of recruited eosinophils, consistent with previous reports (Yim et al. 2010; Liu et al. 2013). The current study revealed that simultaneous E-Cig aerosol exposure and Ova S/C increased eosinophils count compared to control. Similar finding was reported by Lim and Kim (2014) upon instillation of E-Cig intra-tracheally.

In the current study, Ova S/C did not affect the number of recruited neutrophils compared to control, consistent with previous studies (Hofstra et al. 1998; Li et al. 2017). E-Cig aerosol exposure, alone and with simultaneous Ova S/C, significantly increased the number of recruited neutrophils compared to control. Neutrophils contribute to epithelial

damage, airways obstruction and remodeling by the production of reactive oxygen species, lipid mediators, and proteases (Sampson 2000). Schweitzer et al. (2015) reported a trend toward increase in neutrophils with E-Cig aerosol exposure to fresh air and several other studies reported significant increase in neutrophils count with cigarette smoke exposure (Martorana et al. 2005; Larcombe et al. 2017). The increased number of recruited neutrophils is seen in severe form of asthma as well as during an asthma exacerbation (Kelly and Jarjour 2003). Therefore, E-Cig exposure shifts the airway inflammation to a more severe form.

Other types of cells play important roles in the development of asthma. Lymphocytes play an essential role in asthma pathogenesis (Busse and Lemanske 2001). Ova S/C increased lymphocytes count in BALF compared to control, consistent with previous studies (Al-Sawalha et al. 2017; Li et al. 2017). E-Cig exposure, alone and with simultaneous Ova sensitization and challenge, increased lymphocytes count compared to control. Hwang et al. (2016) reported no significant increase in BALF lymphocytes with E-Cig exposure utilizing female CD-1 mice. Macrophages, another important airway inflammatory cell type, are abundantly distributed in the conducting airways (Balhara and Gounni 2012). Airway macrophages are involved in the development of asthma though producing cytokines, chemokines, leukotrienes, and growth factors to recruit inflammatory cells to the lung (Moreira and Hogaboam 2011). E-Cig aerosol exposure alone, but not with simultaneous Ova S/C, increased the number of macrophages in BALF compared to control, consistent with previous reports with 3 days and 2 weeks of E-Cig exposure (Sussan et al. 2015; Glynos et al. 2018). On the other hand, inconsistent results by Lerner et al. (2015) where they reported a non-significant increase in the number of macrophages with E-Cig exposure utilizing C57BL/6J mice. Sex and strain differences could affect response to exposures (Werley et al. 2016).

Cytokines play an imperative role in the inflammatory process of asthma, as they act on multiple targets to induce the activation and proliferation of inflammatory cells, chemotaxis, and apoptosis among others (Chung and Barnes 1999). T-helper 2 (TH2) cells produce IL-13, a pleiotropic cytokine that plays an essential role in mucus production, airway inflammation, airway remodeling, and hyper-responsiveness (Hoshino et al. 2003; Larche et al. 2003; O'Byrne et al. 2004). The current study showed that Ova S/C did not increase the level of IL-13 compared to the control group, consistent with a previous report (Al-Sawalha et al. 2017). This finding is inconsistent with several previous studies that showed a significant increase in IL-13 upon Ova S/C (Henderson et al. 2002; Nguyen et al. 2008; Zeki et al. 2009). However, in those studies, IL-13 was measured in BALF not in lung tissue homogenate. The exposure to E-Cig aerosol did not result in a significant increase in IL-13 level compared to control, inconsistent with Lerner et al. (2015) finding. This variation in the results could be due to the use of a flavored commercially available E-Cig liquid in Lerner et al.'s study in comparison with the unflavored prepared E-Cig liquid in the current study. The used flavors

were linked to airway toxicity (Chun et al. 2017; Glynos et al. 2018; Gotts et al. 2019). On the other hand, simultaneous E-Cig aerosol exposure and Ova S/C increased IL-13 level significantly compared to control, consistent with a previous study (Lim and Kim 2014).

It has been shown that the gene expression and protein level of IL-10, an anti-inflammatory cytokine, were reduced substantially in patients with asthma (Durrant and Metzger 2010). The current study revealed that the level of IL-10 was not affected by Ova S/C, consistent with previous reports (Virag et al. 2004; Ghonim et al. 2015; Ku and Lin 2015). E-Cig aerosol exposure, either alone or with simultaneous Ova S/C, did not affect the level of IL-10. Further studies are needed to examine the effect of E-Cig aerosol exposure on the level of other anti-inflammatory cytokines such as IL-8.

High mobility group box 1 (HMGB1), a potent pro-inflammatory cytokine, is implicated as a vital mediator of asthma (Shim et al. 2012). Ova S/C did not affect the level of HMGB-1 compared to control, inconsistent with previous finding (Ma et al. 2015). This inconsistency could be explained by the different concentration of Ova in the challenge phase and hence the degree of inflammation might be affected. The level of HMGB-1 was not affected by E-Cig aerosol exposure, either alone or with simultaneous Ova S/C, compared to control. Ahmad et al. (2019) reported increased level of HMGB-1 with aerosolized nicotine exposure compared to saline exposure. However, they administered 100 mg/ml of nicotine in normal saline to rats compared to 18 mg/ml nicotine in propylene glycol and glycerol and administered to Balb/c mice in the present study. Heijink et al. (2015) reported that exposure to conventional cigarettes increased level of HMGB-1 compared to air when measured at 2 hours, but not 16 hours, after last exposure. This discrepancy in results could be due to measuring the level of HMGB-1 24 hours after last E-Cig aerosol exposure.

TGF- β , a pro-fibrotic cytokine, is involved in initiation, maintenance, and resolution of inflammation in asthma patients (Halwani et al. 2011). Further, over-expression of TGF- β suppresses the allergic response in a murine model of asthma (Nakao 2001; Halwani et al. 2011). In the present study, Ova S/C did not alter the level of TGF- β 1 compared to control, inconsistent with previous studies (Wei et al. 2015; Wang et al. 2016). However, levels of other isoforms of TGF- β , including TGF- β 2 and TGF- β 3 should be measured in future studies. E-Cig exposure, either alone or with simultaneous Ova S/C, reduced level of TGF- β significantly compared to control. Song et al. (2014) reported a reduction in the level of TGF- β 1 in mice exposed to conventional cigarette smoke compared to the control group. Reduction in level of TGF- β 1 has been associated with exaggerated inflammation as revealed by TGF- β 1 knockout mice that died soon after birth due to extensive multi-organ inflammation (Halwani et al. 2011). Therefore, the reduced level of TGF- β by E-Cig aerosol could partly explain the increased airway inflammation.

MMPs are proteolytic enzymes that degrade extracellular matrix proteins and their levels are elevated in the airways of patients with asthma, especially during exacerbations

(Kelly and Jarjour 2003; Gueders et al. 2006). In the current study, Ova S/C did not affect the levels of MMP-2 and MMP-9 compared to control, consistent with unaltered mRNA transcripts of MMP-2 and MMP-9 from cells of bronchial secretions from patients with asthma (Cataldo et al. 2004). However, previous studies showed elevated activities of MMP-2 and MMP-9 in murine model of asthma (Lee et al. 2008; Choi et al. 2009; Shin et al. 2012).

It has been shown that conventional cigarettes smoke exposure reduced the level of MMP-2 in human lung fibroblasts *in vitro* (La Rocca et al. 2007). This reduction was coupled with a significant increase in tissue inhibitors of matrix metalloproteinases (TIMP)-2, which indicates that tobacco smoking disturbs proteinase/antiproteinase balance in favor of tissue fibrosis (La Rocca et al. 2007). However, the current study showed that E-Cig aerosol exposure selectively reduced the level of MMP-2, but not MMP-9, compared to control. Reduced levels of MMP-2, as observed in the current study, may have a deleterious effect on the lung. MMP-2 knockout mice showed affected lung alveolization (Kheradmand et al. 2002). In addition, Hardy et al. (2018) showed that low levels of MMP-2, below the baseline, is associated with increased lung inflammation by increasing immune cell infiltration as low levels of MMP-2 increased the transcription of inflammatory mediators' genes as TNF- α . Further, MMP-2 causes the cleavage of monocyte chemo-attractant protein-3 (MCP-3) and hence attenuates the recruitment of macrophages and lymphocytes pointing to its anti-inflammatory effect (McQuibban et al. 2000). Therefore, reduced level of MMP-2 increased the attraction of monocytes to the site of inflammation. However, Wang et al. (2019) reported that E-Cig aerosol exposure, for 2 hours/day for 3 consecutive days, did not affect the level of MMP-2 in male mice. This inconsistency could be due to the difference in the exposure duration. A lack of an effect of E-Cig aerosol exposure on MMP-9 levels was also reported by previous studies (Lee et al. 2018; Wang et al. 2019). Future studies should examine the effect of E-Cig aerosol on the activity of MMP-2 and MMP-9 as well as the level of tissue inhibitors of metalloproteinases (TIMPs).

Among the limitations of the current study is that the used vaping topography parameters might not reflect the human use that showed wide-range of variability (Brown and Cheng 2014). Thus, the current findings need to be confirmed using conditions the mimic human use. In addition, factors such temperature, humidity, oxygen, and CO₂ levels during exposure were not monitored in the chamber. Monitoring such factors in future investigations is strongly recommended for better interpretations of the study findings.

In conclusion, the current study showed that E-Cig aerosol exposure significantly increased the number of recruited inflammatory cells to the airways, level of TGF- β , MMP-2 compared to the control group. Simultaneous E-Cig aerosol exposure and Ova S/C increased the airway recruitment of inflammatory cells, especially eosinophils, and lymphocytes compared to the control group and neutrophils compared to control and Ova S/C groups. Simultaneous E-Cig aerosol

exposure and Ova S/C increased the level of IL-13 compared to the control and E-Cig group and reduced the level of TGF- β compared to control. The levels of IL-10, HMGB1, and MMP-9 were not affected. The results indicate that E-Cig aerosol exposure induced airway inflammation in the tested murine model of asthma. This highlights the need to conduct further studies to examine the level of airway inflammation and inflammatory markers in E-Cig smokers. The safety of E-Cigs use in patients with airway inflammation should be considered carefully before recommending these products for smoking cessation.

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