E-cigarette synthetic cooling agent WS-23 and nicotine aerosols differentially modulate airway epithelial cell responses

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1. Introduction

Electronic cigarette (e-cig) aerosol exposures are strongly associated with pulmonary dysfunctions, and the airway epithelial cells (AECs) of respiratory passages play a pivotal role in understanding this association. However, not much is known about the effect of synthetic cooling agents such as WS-23 on AECs. WS-23 is a synthetic menthol-like cooling agent widely used to enhance the appeal of e-cigs and to suppress the harshness and bitterness of other e-cig constituents. Using primary human AECs, we compared the effects of aerosolized WS-23 with propylene glycol/vegetable glycerin (PG/VG) vehicle control and nicotine aerosol exposures. AECs treated with 3 % WS-23 aerosols showed a significant increase in viable cell numbers compared to PG/VG-vehicle aerosol exposed cells and cell growth was comparable following 2.5 % nicotine aerosol exposure. AEC inflammatory factors, IL-6 and ICAM-1 levels were significantly suppressed by WS-23 aerosols compared to PG/VG-controls. When differentiated AECs were challenged with WS-23 aerosols, there was a significant increase in secretory mucin MUC5AC expression with no discernible change in airway inflammatory SCGB1A1 expression.

Electronic cigarettes (e-cigs) or other electronic nicotine delivery systems (ENDS) are marketed as products to aid tobacco smoking cessation. However, recent studies have found that e-cig/ENDS use during smoking cessation led to both a reduced effectiveness in cessation and a higher relapse rate as compared to no product use [1,4,20,34,39]. Menthol usage in e-cigarettes, e-liquids, and other ENDS products is known to enhance the appeal of these products, particularly to young adult users, as it reduces the harshness and bitterness of the products [11,12]. The U.S. Food and Drug Administration (FDA) recently banned synthetic menthol-containing e-cigarettes and tobacco in closed pod systems, dramatically increasing the use of menthol-containing e-cig/ENDS products [10,40]. Reports have suggested that this change may have led to increased presence of menthol and synthetic cooling agents such as WS-23 in e-cig products which may ultimately exposure users to more harm [33]. Chemical analysis of various menthol-flavored e-cig products corroborated the presence of various harmful compounds [13,17,22,33]. Despite efforts to curb the appeal of e-cigarettes, particularly to young adults, the use of menthol and "iced/cooling" flavors has only increased in popularity and has potentially contributed to the increased addictive properties of e-cigarettes [22]. Considering the recent outbreak of e-cigarette or vaping use-associated lung injury (EVALI) [2], the current rate of e-cig use may cause severe comorbid conditions among a larger population. Among various cooling agents analyzed, the synthetic cooling agent WS-23 was reportedly used most prominently [16]. 2-isopropyl-N,2,3-trimethylbutyramide, commonly known as WS-23, was found to be present in most e-liquids marketed in the U.S. in quantities that may exceed consumer exposure safety standards [16].

Although studies on the biological effects of WS-23 are lacking, a recent reports found that there may be cytotoxicity induced by WS-23 exposure in vitro [33] as well as alters the ROS generation by lung...
epithelial cells [43]. Another study found that various flavoring products induced ROS generation and superoxide production in vitro in lung epithelial cell lines and monocytes [31]. Similarly, ROS generation and pro-inflammatory effects were observed to be induced by e-cig aerosol exposure; these effects were further amplified by flavored e-cigs in periodontal fibroblasts [36]. Furthermore, e-cig use affected lung inflammatory responses, and importantly, the aerosols consisting of propylene glycol/vegetable glycerin (PG/VG) vehicle alone were found to induce a potent pro-inflammatory response and immune infiltration in bronchoalveolar lavage fluid (BALF) [38].

The respiratory airflow epithelial cells (AECs) are pivotal to innate immune defense against inhaled toxicants/allergens, and the AEC responses to aerosolized e-cig components are crucial for orchestrating the lung immune responses [8,30]. Any dysregulation in AEC-mediated responses can significantly impact the susceptibility to infection [6]; a recent study has shown that e-cig use induces a reduction in AEC ciliary beating frequency, as well as changes in cytokine and chemokine production [18]. Nevertheless, the impact of synthetic cooling agents such as WS-23 individually has not been investigated. With the continuously increasing usage of the WS-23 cooling agent, coupled with the increased usage of e-cigarettes, particularly in young adults, there is a need to assess the effects of WS-23 aerosols on both AECs and the innate immune response of airways.

In this report, we analyzed the effect of aerosolized WS-23 in vitro using primary airway epithelial cells and compared the responses to those induced by PG/VG vehicle aerosols using the Buxco EVT exposure system (Data Sciences International, St. Paul, MN). Notably, the PG/VG vehicle has been demonstrated to have an aberrant effect on lipid homeostasis and downregulate innate immune responses in AECs, particularly against viral infection [27], therefore, we focused on the analyzing the effects of e-cig aerosols containing WS-23 reconstituted in PG/VG vehicle with and without nicotine. As such, we compared the effect of WS-23 against PG/VG vehicle and found that WS-23 may be reducing the expression of interleukin (IL)-6 and ICAM-1, thereby dysregulating the AEC innate immune responses.

2. Materials and methods

2.1. Human airway epithelial cell culture

Primary human AECs were seeded in clear TC-treated 6-well plates (Corning Costar® using bronchial epithelial cell growth media (BEGM, Lonza, or UNC MLI Cell Culture Core), and e-cig aerosol treatments were started 24 h after seeding. To mimic the epithelial lining of conducting airways, primary AECs were also grown in air-liquid interface (ALI) cultures as described previously [7] and cells were differentiated into ciliated and secretory cells for a minimum of 21 days before treatments.

2.2. E-liquid reagents and E-cig aerosol exposures

E-liquid synthetic cooling and flavoring agent WS-23 (CAS#51115-67-4, from FlavorJungle, Bellingham, WA) was used with or without Nicotine (Sigma-Aldrich, Inc.), and PG/VG (1:1, propylene glycol: vegetable glycerin) was used as a vehicle control. There were four treatment groups, where cells were treated with PG/VG, 3 % WS-23 in PG/VG, 2.5 % Nicotine in PG/VG, or 2.5 % Nicotine + 3 % WS-23 prepared in PG/VG. Human AECs were exposed to e-liquid aerosols using the Buxco E-cigarette, Vapor, and Tobacco (EVT) exposure system (Data Sciences International, St. Paul, MN, USA) as described before [42]. Briefly, cells were exposed to e-cig aerosols for 15 min per day with a puff topography of 2 puffs/minute (3 s/puff, 55 ml/puff). Smok® X-Priv mod kit was used for smoke delivery installed with Prince® V12 triple mesh coils with 90 watts coil wattage. For the submerged cultures, cells were exposed in 6-well plates with minimal media over the cells to allow a direct contact of e-cig aerosols and media was replenished immediately after the 15 m exposure. Following exposure, cells were incubated at 37 °C and 5 % CO2 for another 24 h and cells and media supernatant were harvested after 48 and 72 h of exposure. Cell viability was assessed by the trypan blue exclusion method. Briefly, trypsinized cells were resuspended in phosphate-buffered saline (PBS), and samples were mixed 1:1 with 0.4 % trypan blue solution (catalog no. 302643; Sigma-Aldrich), and live/dead cells were counted using TC20 automated cell counter (Bio-Rad Inc).

2.3. Inflammatory gene expression analysis by qRT-PCR

Total RNA extraction was performed using the RNeasy Mini kit (Qiagen) according to the manufacturer’s instructions, and cDNAs were synthesized using the Applied Biosciences High-Capacity RNA-to-cDNA® Synthesis Kit (Thermo Fisher Scientific, Inc), per manufacturer’s instructions. Expression levels of ICAM-1 and IL6 mRNAs were quantified using SYBR Green-based primers and the iTaq master mix (Bio-Rad Inc) in the Bio-Rad CFX Real-Time PCR detection system (Bio-Rad Inc). Relative quantification data were obtained using the delta-delta (ΔΔCt) method by normalizing to the respective beta-actin and/or GAPDH mRNA levels as described recently [7].

2.4. Secretory inflammatory factor analysis by ELISA

The protein levels of ICAM-1 and IL-6 were determined using human ELISA kits against ICAM-1 (LifeSpan Biosciences Inc., Seattle, WA) and IL-6 (BioLegend Inc., San Diego, CA), respectively, as per manufacturers’ instructions.

2.5. Immunocytochemical staining and imaging analysis

For immunocytochemical staining, cells were fixed with 4 % paraformaldehyde (PFA) and washed in 0.05 % v v−1 Brij-35 in PBS (pH 7.4) and immunostained using antibodies to MUC5AC (Millipore Inc., Burlington, MA), SGB1A1 or secretoglobulin 1A1 (Santa Cruz Biotechnology, Santa Cruz, CA) and β-tubulin (Cell Signaling Tech., Danvers, MA) or isotype controls. Briefly, cells were blocked using a solution containing 3 % BSA, 1 % Gelatin, and 1 % normal donkey serum with 0.1 % Triton X-100 and 0.1 % Saponin and were stained with antibodies. The immunolabelled cells were detected using respective secondary antibodies conjugated fluorescent dyes (Jackson Immunoresearch Lab Inc., West Grove, PA) and mounted with 4',6-diamidino-2-phenylindole (DAPI) containing Fluormount-G™ (SouthernBiotech, Birmingham, AL) for nuclear staining. Immunofluorescent images were captured using the BZX700 Microscopy system (Keyence Corp., Japan) and analyzed using NIH Image J software as described recently [29].

2.6. Statistical analysis

Data expressed as mean ± SEM are the representative of three independent experiments (n = 2/gp) and analyzed by GraphPad Prism Software (GraphPad Software Inc.) using one-way analysis of variance (ANOVA) following Tukey’s multiple comparison test. When significant main effects were detected (p < 0.05), student’s t-test was used to determine differences between the groups.

3. Results

3.1. Synthetic cooling agent WS-23 aerosols modulate human airway epithelial cell viability

The early interaction of E-cig aerosols with AECs of conducting airways is pivotal in mounting the appropriate immune response and once differentiated on the ALI culture setting, AECs show minimal response in terms of changes in cell numbers. Therefore, we first analyzed the effects of aerosolized 3 % WS-23 on the cell numbers of AECs in submerged cultures at 48 h and 72 h of treatment where only minimal media was...
present during the exposures to allow a direct contact of e-cig aerosols and mimic the real-life exposures. The estimated deposited dose for the aerosolized e-cig solutions containing 3% WS-23% and 2.5% nicotine was 109.9 and 91.6 μg/cm², respectively, per exposure. The viable cell counts showed no significant change in cell numbers among all the groups tested. However, the WS-23 aerosol-treated cells showed a trend toward reducing viable cell numbers (Fig. 1A). Interestingly, at 72 h of treatment, specifically in cells treated with 3% WS-23 or 2.5% nicotine aerosols, we observed a significant increase in viable cell numbers, but there was no synergistic effect observed in cells treated with WS-23 + nicotine combination (Fig. 1B). It is noteworthy that cells treated with PG/VG aerosols showed a marked reduction in cell numbers at 72 h compared to 48 h of treatment.

3.2. PG/VG vehicle induces an inflammatory response in human AECs independent of nicotine and WS-23

As PG/VG itself has been shown to dysregulate AEC responses [27] we analyzed IL-6 and ICAM-1 mRNA levels upon exposure to PG/VG for 24 h, 48 h, and 72 h. Interestingly, compared to the cells harvested at 24 h, we observed a significant increase in both IL-6 and ICAM-1 mRNA at 48 h of exposure, which was further potentiated at 72 h of exposure time (Fig. 2A–B). These data suggest that PG/VG vehicle without added constituents can dysregulate the AEC inflammatory responses, which may be altered by the addition of nicotine and/or WS-23.

3.3. WS-23 aerosols alter innate inflammatory response kinetics of human AECs

We next analyzed the effects of WS-23 aerosols on AEC mRNA expression of inflammatory factors, IL-6, and ICAM-1, which are important modulators of AEC innate responses [5,29]. At 48 h of treatment, IL-6 mRNA levels were significantly reduced by WS-23 or nicotine aerosol exposure, as the WS-23, WS-23 + nicotine, and nicotine alone aerosol treated groups presented with 2.0-fold or higher reduction in IL-6 mRNA levels, compared to PG/VG-treated controls (Fig. 3A). In contrast, cells treated with WS-23 or nicotine aerosols showed only a trend towards increased ICAM-1 mRNA expression; however, WS-23 + nicotine combined treatment induced a significant increase (p = 0.0121) in ICAM-1 expression (Fig. 3B). Thus, ICAM-1 mRNA levels were marginally increased by WS-23- or nicotine-only aerosol treatments, and the combined treatment with WS-23 and nicotine (WS-23 + nicotine) potentiated the significant increase in ICAM-1 mRNA levels.

After 72 h treatment, we observed a similar trend in the expression levels of IL-6 mRNA. Cells treated with WS-23, WS-23 + nicotine, and nicotine alone aerosols presented with a 1.5-, 1.7-, and 2.0-fold decrease in IL-6 mRNA levels, respectively, compared to PG/VG-treated controls after 72 h post-treatment (Fig. 4A). Surprisingly, ICAM-1 mRNA levels showed a trend toward reduced expression with WS-23, WS-23 + nicotine, and nicotine treatments causing a 1.8-, 1.75-, and 1.88-fold reduction in ICAM-1 expression, respectively, when compared to the PG/VG-treated controls (Fig. 4B).

We next corroborated these results by investigating the changes in protein levels of secretory IL-6 and ICAM-1 in culture media supernatants harvested at 72 h treatment. Interestingly, IL-6 secretory levels were on an average 2292 pg/ml in PG/VG control culture media; and 1932 pg/ml in WS-23; 2835 pg/ml in WS-23 + nicotine; and 2338 pg/ml in nicotine treated groups (Fig. 4C). There was a significant reduction in ICAM-1 protein levels upon all three treatments. The PG/VG control treatment presented with 15,105 pg/ml of ICAM-1, whereas the WS-23, WS-23 + nicotine, and nicotine alone treatments presented an average of 11,196, 10,381, and 11,388 pg/ml, respectively (Fig. 4D). Thus, aerosolized synthetic cooling agent WS-23 alters the innate airway inflammatory responses of human AECs by altering IL-6 and ICAM-1 expression.

3.4. WS-23 aerosol exposure modulates the goblet cell differentiation in AECs

Next, we analyzed the effects of WS-23 aerosols on a differentiated AEC population cultured on an air-liquid interface to assess the effects on the mucoinflammatory responses of AECs. Groups of transwells were treated with aerosolized PG/VG vehicle, WS-23, nicotine, and WS23 + nicotine. After 72 h treatment, there were significant changes in MUC5AC mucin expression by both WS-23 or nicotine aerosol exposure (Fig. 5A). The WS-23 or nicotine alone aerosol treated groups presented with increased expression of a secretory mucin MUC5AC (MFI) compared to PG/VG-treated controls, but there was no synergistic effect of WS-23 + nicotine combination exposure (Fig. 5B). In contrast, there was no significant change in another airway inflammatory protein SCGB1A1 expression in WS-23, WS-23 + nicotine, or nicotine aerosols treated groups (Figs. 5C and 5D). Thus, WS-23 and nicotine aerosols modulate the airway mucosecretory protein expression by affecting the MUC5AC mucin levels, which could impact the respiratory physiology and needs further validation using in vivo exposure models.

Fig. 1. Synthetic cooling agent WS-23 aerosols induce cell proliferation in AECs following 72 h of treatment. Live cell numbers following (A.) 48 h, and (B.) 72 h treatment with aerosolized PG/ VG (50:50), PG/ VG + 3% WS-23, PG/ VG + 3% WS-23 + 2.5% nicotine, or PG/ VG + 2.5% nicotine using Buxco EVT system. Data shown as mean ± SEM is representative of 3 independent experiments (n = 2/qp) and analyzed by one-way ANOVA.
4. Discussion

The FDA considers flavoring and cooling agents safe when utilized as food additives; however, the risks associated with their inhalation, through vaping, are poorly defined [14]. Little is known about how e-cig constituents affect the respiratory tract, specifically when emerging evidence indicates that the acute effects of e-cig products use on the respiratory system need to be revisited [23,24,27,35,37]. Furthermore, it has been reported that menthol, in concentrations found in e-cig aerosols may disturb cell homeostasis and can trigger oxidative stress via the NF-κB pathway [32] and is implicated in inducing respiratory symptoms such as wheezing [25].

Induction of oxidative stress and chronic mitochondrial dysregulation is central in many pathologic conditions such as chronic inflammatory and aging-associated degenerative diseases [28]. Most importantly, alarmingly high toxic levels of synthetic cooling agents/-coolants such as WS-3 or WS-23 carboxamides are used in the emerging e-cigs products. Still, the risk associated with their inhalation and safety regulations is understudied. These are not only found in mint/menthol-flavored products but also in the fruit-, candy-, and ice flavors, including the popular disposable pod- and mod-based products. Even without nicotine or flavoring agents, e-cig use can induce significant changes in the lung epithelium; one study found that chronic exposure to e-cig aerosols in a mouse model suppresses the innate immune response, particularly against viral infection [27]. Aerosolized e-liquid solvents exposure induces significant changes in the airway epithelium, with or without added flavoring elements. However, the results are highly variable. Contrasting changes in inflammatory factors expression were reported on a significant increase either in expression or with no change in the expression levels of IL-6 and CXCL-8 [18]. This discrepancy could be mainly attributed to the exposure systems used for e-cig aerosols and the addition of nicotine or other flavoring chemicals [18]. There have been significant variations in study design, and the need for further investigation is dire. As newer ENDS emerge and regulations continue to change with evolving consumer preferences, it is crucial to understand the effects of prominently used flavoring compounds, like WS-23. Our results further indicate that PG/VG dysregulates cellular responses with or without nicotine or flavoring agents. Notably, we found that those responses were inhibited by adding additional constituents such as WS-23 and nicotine.

In this study, we used a submerged culture model of primary AECs and exposed them to e-cig aerosols using PG/VG as a vehicle-only control and compared the effects to those with PG/VG and WS-23, nicotine or WS-23 and nicotine exposure. AECs cultured on transwells
over the air-liquid interface best mimic the in-vivo conditions of the respiratory tract, however, these differentiated AECs undergo a slow turnover rate that renders them less sensitive to changes in cell proliferation. Therefore, submerged AEC cultures are ideal for analyzing the toxicity effects on cell numbers and viability. Moreover, there was only minimal media on the submerged cultures to allow for direct contact of e-cig aerosols mimicking the real-life exposures. Nonetheless, compared to ALI-cultured differentiated cells, AECs in submerged culture conditions show minimal changes in mucoinflammatory response [7], therefore, we also determined the AEC mucosecretory responses using the differentiated AECs. As such, to understand the acute effects of E-cig aerosol exposure, we analyzed the early time-points by focusing on cells at 24, 48, and 72 h post-exposure. Our results demonstrated that even at 48 h after exposure, there is a significant reduction in the expression levels of IL-6 and concurrently, a trend of increased expression of ICAM-1, with a significant increase in ICAM-1 levels upon WS-23 + nicotine treatment. These data further corroborate the observations that WS-23 may alter the innate immune responses of AEC. Suppressing the IL-6 levels may hinder the rapid AEC immune response to pathogen presence [19]. Alternatively, the increased levels of ICAM-1 suggest a differential effect on regulatory pathways. It has been reported that e-cig exposure without nicotine may induce a transient increase in secretory ICAM-1 levels; however, the effects of WS-23 on these pathways have not been thoroughly investigated [3]. Furthermore, at the 72-h time point, we observed a significant reduction in the secretory protein levels of ICAM-1. The observed differences in ICAM-1 mRNA and protein expression levels suggest that the repetitive exposures (72 h) cause more significant alteration than the one (24 h) or two (48 h) exposures. Moreover, in addition to the transcriptional modulation, the tested e-cig aerosols also target the pathways regulating ICAM-1 protein expression and stability.

Research evaluating the effect of WS-23 on MUC5AC expressing goblet cells and airway epithelial cells is lacking; however, it has been shown that WS-23 may induce MUC5AC expression in patients with chronic obstructive pulmonary disease (COPD) by binding to transient receptor potential cation channel (TRMP)8, which is upregulated in COPD patients [21,26]. The present data in this study using the ALI cells also corroborate these findings where WS-23 aerosols show a trend towards increased MUC5AC expression in AECs at 72 h post-exposure. The E-cig aerosols containing nicotine, however, showed a significant increase in MUC5AC expression supporting the previous findings that nicotine exposure is a strong inducer of mucin MUC5AC expression [9, 15]. Here, we report that WS-23 may be inducing a similar response independent of nicotine presence and may require further investigation into long-term effects. Interestingly, it has been reported that nicotine can reduce the levels of SCGB1A1 or CCSP (club cell secretory protein) by reducing the transcription factor FOXA2 [41,44]. However, research investigating the effect of WS-23 on SCGB1A1 expression is lacking. In contrast to the previous study [41,44], we found that exposure to both WS-23 and/or nicotine aerosol exposure do not alter the SCGB1A1 expression.
expression compared to PG/VG controls. This may suggest that WS-23 induces an acute effect on MUC5AC mucin expression only without altering the SCGB1A1 expression, and further investigation is required into the long-term changes that may be induced by WS-23 exposure, independent of nicotine. The present study has several limitations with most notable one being the limited early time-course analysis and the lack of a comparative analysis with the cells not treated with any E-cig aerosol. Nonetheless, the objective of the study was to assess the toxic effects of the cooling agent WS-23, a frequently used flavoring e-cig additive on the AECs. And the data presented do suggest of a direct modulation of the AEC mucinflammatory response by WS-23 aerosols and the effect was further augmented by the addition of nicotine. In addition to the in-vivo studies, large-scale in-vitro studies with appropriate controls and longer time-points and using the primary cells from different donors are further warranted to help establish the toxicity of these widely used E-cig flavoring agents.

Our data further corroborate the effects of both nicotine and WS-23 on AECs and suggest that flavoring agents may amplify or reduce the toxic effects of various e-cig components such as those induced by PG/VG vehicle and nicotine or nicotine salt additives. Notably, WS-23 and nicotine presence reduced the inflammatory responses that were strongly induced by PG/VG vehicle independently. These data collectively suggest that aerosolized synthetic cooling agent WS-23 alters the innate airway immune responses of human AECs and thus, potentially could increase the susceptibility to respiratory pathologies.

**CRediT authorship contribution statement**

Conceptualization, H.S.C., and I.R.; Methodology, H.S.C.; assay performance: M.M., and D.D., Software, M.M., and D.D.; Validation, M. M., D.D., and H.S.C.; Formal analysis, M.M., and D.D.; Investigation, M. M., and D.D.; Resources, H.S.C.; Data curation, M.M., and D.D.; Writing – original draft, M.M., and H.S.C.; Writing – review & editing, M. M., S.Y., I.R., D.D., and H.S.C.; Visualization, M.M.; Supervision, D.D., and H.S.C.; Project administration, D.D., and H.S.C.; Funding acquisition, I.R., and H.S.C. All authors have read and agreed to the published
Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

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