The Effects of Dual IQOS and Cigarette Smoke Exposure on Airway Epithelial Cells: Implications for Lung Health and Respiratory Disease Pathogenesis

Pritam Saha, Siddhi Jain, Ipsita Mukherjee, Samir R Panda, Amir A. Zeki, VGM Naidu, Pawan Sharma


This manuscript has recently been accepted for publication in the ERJ Open Research. It is published here in its accepted form prior to copyediting and typesetting by our production team. After these production processes are complete and the authors have approved the resulting proofs, the article will move to the latest issue of the ERJOR online.

Copyright ©The authors 2023. This version is distributed under the terms of the Creative Commons Attribution Non-Commercial Licence 4.0. For commercial reproduction rights and permissions contact permissions@ersnet.org
The Effects of Dual IQOS and Cigarette Smoke Exposure on Airway Epithelial Cells: Implications for Lung Health and Respiratory Disease Pathogenesis

Pritam Saha¹*, Siddhi Jain¹*, Ipsita Mukherjee¹*, Samir R Panda¹, Amir A. Zeki², VGM Naidu¹$, Pawan Sharma³$

¹Department of Pharmacology & Toxicology, National Institute of Pharmaceutical Education and Research Guwahati, Changsari, Guwahati, Assam, 781101, India.

²University of California, Davis School of Medicine, UC Davis Lung Center, Department of Internal Medicine, Division of Pulmonary, Critical Care, and Sleep Medicine, Davis, CA.

³Center for Translational Medicine, Division of Pulmonary, Allergy and Critical Care Medicine, Jane & Leonard Korman Respiratory Institute, Sidney Kimmel Medical College, Thomas Jefferson University, Philadelphia, PA, USA 19107.

* Authors contributed equally

$Correspondence

Pawan Sharma, PhD
Center for Translational Medicine
Thomas Jefferson University
1020 Locust Street, Philadelphia, PA, USA 19107
Email: Pawan.Sharma@jefferson.edu

VGM Naidu, PhD
National Institute of Pharmaceutical Education and Research, Guwahati
Changsari, Guwahati
Assam, 781101, India
Email: vgmnaidu@gmail.com
ABSTRACT

**Background:** Cigarette smoking (CS) remains the primary cause of chronic lung diseases. After a steady decline, smoking rates have recently increased especially with the introduction of newer electronic nicotine delivery devices, and it is also emerging that dual or poly product usage is on the rise. Additionally, with the introduction of IQOS (a heated tobacco product) globally, its impact on human health needs to be investigated. In this study we tested if dual exposure (CS+IQOS) is detrimental to lung epithelial cells when compared to CS or IQOS exposure alone.

**Methods:** Human airway epithelial cells (BEAS-2B) cells were exposed to either CS or IQOS or their dual combinations at concentrations 0.1, 1.0, 2.5 and 5.0%. Cytotoxicity, oxidative stress, mitochondrial homeostasis, mitophagy, and effects on epithelial mesenchymal transition (EMT) signaling were assessed.

**Results:** Both CS and IQOS alone significantly induced loss of cell viability in a concentration-dependent manner which was further enhanced by dual exposure compared to IQOS alone \((p<0.01)\). Dual exposure significantly increased oxidative stress and perturbed mitochondrial homeostasis when compared to CS or IQOS alone \((p<0.05)\). Additionally, dual exposure induced EMT signaling as shown by increased in mesenchymal (α-SMA and N-cadherin) and decreased epithelial (E-cadherin) markers when compared to CS or IQOS alone \((p<0.05)\).

**Conclusion:** Collectively, our study demonstrates that dual exposure enhances pathogenic signaling mediated by oxidative stress and mitochondrial dysfunction leading to EMT activation which is an important regulator of small airway fibrosis in obstructive lung diseases.

**Keywords:** Cigarette smoke, IQOS, Dual, EMT, Airway epithelial cells
INTRODUCTION

Lung disease-related mortality has risen to an alarming level globally [1]. Cigarette smoke is by far the leading cause of lung disease burden and is one of the most significant public health threats the world has faced in the form of the tobacco epidemic, that causes 8 million deaths worldwide each year [2]. Though cigarette smoking rates have declined in the 21\textsuperscript{st} century, the introduction of e-cigarettes and their widespread usage by the public has led to an increased uptake of conventional cigarette smoking globally [3]. The 2019 vaping epidemic in the USA which caused 68 deaths and >2,800 hospitalizations [4], is a stark reminder that smoking in all its forms remains harmful to human populations [5], still remains incurable [6], and can also affect other vital organs in the body [7].

Currently e-cigarette use is at an all-time high both in young and adults [8], while the vaping epidemic continues with the public being largely unaware of the detrimental effects of using new nicotine-containing products. Moreover, tobacco companies continue to introduce newer products with taglines such as ‘safer alternatives’ or ‘less harmful’ that are designed to have public appeal. Vaping is causing a new generation that is becoming addicted to nicotine, paving the way for the increase in the use of other, more harmful tobacco products [8]. In 2021, an estimated 3.6 million (>13\%) U.S. middle and high school students reported using e-cigarettes within a 30-day period while > 2.0 million youths were estimated to be current e-cigarette users [9]. In recent years, the heat-not-burn (HnB) or heated-tobacco-product (HTP) has been introduced worldwide [10, 11]. The HnB is a hybrid between the traditional cigarette and e-cigarette and heats the rolled tobacco sheet at 350 °C rather than burning it at 600 °C to generate a nicotine-containing aerosol, which can be inhaled by users [12, 13]. IQOS, which is a HnB product from Phillip Morris International (PMI), currently dominates the world market in more than 50 countries [14]. Since 2014, HnB sales have grown exponentially (~13,000\%) [15],
and these products have been gaining traction both among cigarette smokers and e-cigarette users globally [11, 16]. In 2020, the US Food and Drug Administration authorized the sale of IQOS as the first HnB device from PMI, which was designated as a modified risk tobacco product (MRTP) [17].

Though overall public awareness of HnB remains low, these products are perceived as being less addictive and more socially acceptable than traditional cigarettes [18]. Recent evidence suggests that IQOS exposure induces macrophage cell death and impairs macrophage function [19], and IQOS also causes myocardial systolic and diastolic dysfunction like traditional cigarette smoking [20]. IQOS exposure in airway cells results in cell death, oxidative stress via reactive oxygen species (ROS), and impaired mitochondrial homeostasis leading to altered immune response, increased mucus, and inflammation [13, 21]. In addition, IQOS exposure in oral fibroblast and keratinocytes enhances their cellular proliferation and migration potential, which may lead to activation of epithelial-mesenchymal transition (EMT) [22]. Of greatest concern and what is now emerging is the increasing use of multiple tobacco products, i.e., combining conventional cigarette smoking with either e-cigarette or IQOS (dual-usage) [23] or combining all three products (poly-usage) [24, 25]. The effects of such combination smoke use on lung health is an area needing investigation.

The harmful effects of cigarette smoke exposure on human health are well established. The current narrative by nicotine companies promotes IQOS as a “safe product” [26] or something that is less harmful while there are little data that have assessed the safety of IQOS alone or in combination with other tobacco products. We were the first to demonstrate that IQOS exposure is similarly detrimental to lung cells in vitro. Moreover, data on dual (CS+IQOS) exposure-specific effects on airway cells are lacking. Therefore, we tested if dual exposure was
detrimental to airway epithelial cells in the lung. In this present work, we report that dual exposure is highly damaging to airway cell homeostasis with significant effects on oxidative stress and mitochondrial function leading to EMT-signaling, which is known to initiate pathological lung changes as observed in various obstructive lung diseases.

**MATERIAL AND METHODS**

*Chemicals and Reagents*

All the chemicals were of analytical grade and purchased from Sigma and Thermo Scientific. Additional information is provided in the Supplementary file.

*Cell Culture*

Human bronchial epithelial cells BEAS-2B purchased from American Type Culture Collection (Rockville, MD) were selected and maintained as per our standard epithelial cell culturing protocols.

*Preparation of Cigarette smoke, IQOS, and Dual extract*

Cigarette smoke (CS) or IQOS aerosol extracts were prepared by bubbling smoke from 1 cigarette (Marlboro Red, India) or from IQOS HEETS (Tobacco flavor) into 25 ml of phosphate buffer saline (PBS, pH 7.4). The extract was filtered and normalized to nicotine content after HPLC estimation. This was used as 100% in concentration to prepare the desired lower dilutions of extract necessary for cytotoxicity assessment and immunoblotting. Both were mixed in equivalent volumes to produce a corresponding percentage of dual extract (CS+IQOS). The concentrations utilized in this study (0.1 to 5.0%) are based on many seminal studies [27-30] and from our own preliminary data and a previous publication [31]. Serum cotinine (stable nicotine metabolite) levels among one pack per day smokers may vary greatly from 16 to 1180 ng/ml [32], while immediate levels of nicotine after smoking one cigarette in venous and arterial blood
can fluctuate between 5 to 100 ng/ml [33]. Also, the number of cigarettes smoked or IQOS used vary greatly in humans. To avoid this variation in our experiments, we performed our studies based on one standardization i.e., ~nicotine content in either one cigarette or in one IQOS-HEETS (as shown in Fig S1). Accordingly, nicotine levels from 0.1 to 5% extract are within the 0.4 to 200 ng/ml range.

*Nicotine Estimation by HPLC*

The concentration of nicotine extracted in PBS was estimated using the Dionex Ultimate 3000 UHPLC system equipped with a PDA (Photodiode array) detector, as described in the Supplementary materials.

*Cell Viability Assay*

BEAS-2B cells were seeded in 96-well plates and allowed to reach confluence, then switched to the corresponding growth arrest media for 4 h. Then, cells were exposed to different concentrations of CS, IQOS, or CS+IQOS extract for 24 or 48 h, and media were replaced with 100 µL of growth arrest media containing MTT (5 mg/10 ml) for 4 h. After incubation, media were removed, 200 µL of DMSO was added, and absorbance was measured at 570 nm, as previously described [34].

*Assessment of Intracellular ROS Generation*

Intracellular ROS generation was assessed using the DCFDA assay (Invitrogen Cat No-C6827). After CS, IQOS, or CS+IQOS extract exposure for 6 and 24 h, cells were stained with DCFDA dye and analyzed using a flow cytometer (Invitrogen Attune NxT), as described previously [35].
Assessment of Mitochondrial Superoxide Generation

Mitochondrial Superoxide generation was analyzed using MitoSOX Red (Invitrogen Cat No-M36008). After CS, IQOS, or CS+IQOS extract exposure for 6 and 24 h, cells were stained with MitoSOX Red and imaged using CLSM (Leica, Germany, TCS SP8), as described previously[35].

Assessment of Mitochondrial Membrane Potential

Mitochondrial membrane potential was measured using JC-1 dye. After CS, IQOS, or dual extract exposure for 6 and 24 h, cells were stained with JC-1 dye and analyzed using a flow cytometer, as described previously [35].

Quantitative Assessment of Mitochondrial Morphology

Mitochondrial morphology and shape were determined using Mitotracker Deep Red (Invitrogen Cat. No. M22426). After CS, IQOS, and CS+IQOS extract exposure for 6 and 24 h, cells were stained and then imaged using confocal laser scanning microscope (Leica TCS-SP8), as described previously [36].

Assessment of Autophagy Induction

Activation of autophagy was measured using acridine orange dye. After CS, IQOS, or CS+IQOS extract exposure for 6 and 24 h, cells were stained and analyzed using a flow cytometer.

Assessment of Mitophagy

Mitotracker Deep Red FM and Lysotracker Green DND 26 (Invitrogen Cat. No- L7526) were used to assess mitophagy upon CS, IQOS, or CS+IQOS exposure for 6 and 24 h. After exposure, cells were stained and imaged using CLSM.
**Immunoblotting**

After 24 h of CS, IQOS, or CS+IQOS extract exposure, immunoblotting for cell lysate was performed as described[34]. Primary antibodies against DRP1, OPA1, E-cadherin, N-cadherin, α-SMA, vimentin, α-tubulin, and β-actin (1:1000) were used and procured from ABclonal (Woburn, MA).

**RNA Isolation and Gene Expression**

After 24 h of CS, IQOS, or CS+IQOS extract exposure, total RNA was isolated using Trizol reagent (Invitrogen). Later, RNA was quantified and transcribed into cDNA using Prime Script 1st strand cDNA synthesis Kit (Takara Bio Cat.No-6100A), as per kit protocol. PowerUP syber green master mix was used to perform quantitative real-time PCR using an Applied Biosystems real-time PCR machine (Quantstudio 5). GAPDH was used as a housekeeping gene to calculate gene expression data.

**Statistical Analysis**

All data are expressed as mean ± SEM. Non-parametric unpaired t-test was used to compare two groups. A one-way ANOVA with a post-hoc Dunnett's test or Tukey's test was used when more than two groups were compared. The GraphPad Prism-7 software was used for all statistical analyses, and the p-value of less than 0.05 was considered statistically significant.

**RESULTS**

**Estimation of Nicotine Content in CS and IQOS Extracts Using HPLC**

CS and IQOS extracts were prepared freshly, as described in the *Methods* section. The HPLC method was developed and validated to estimate the average nicotine content in both extracts. Nicotine standard concentrations ranging from 0.78 to 12 µg/mL showed the best fit for
linearity with a correlation coefficient ($R^2$) of 0.999. The retention time of nicotine was found to be 4.3 min, with a mean nicotine content of 1.49 and 0.81 µg/mL (Fig. S1) in CS and IQOS extracts, respectively.

*Dual Exposure Displays Loss of Airway Epithelial Cell Viability*

Cell viability was assessed upon exposure of BEAS-2B cells with equivalent nicotine concentrations of CS, IQOS, or CS+IQOS extract for 24 h (Fig. 1B) and 48 h (Fig. 1C). We found a concentration and a time-dependent reduction in cell viability with CS and dual CS+IQOS exposure on airway epithelial cells while IQOS exposure alone only reduced cell viability in a concentration dependent manner both at 24 h and 48 h. Additionally, CS+IQOS dual exposure was found to be more cytotoxic than IQOS exposure alone both at 24 h and 48 h.

*Dual Exposure Enhances Intracellular ROS and Mitochondrial Superoxide Generation*

Cigarette smoke-mediated oxidative stress is a significant factor that drives various molecular and cellular changes in airway epithelial cells. Therefore, to assess the immediate (6 h) and latent (24 h) effect of CS, IQOS, or CS+IQOS extract exposure, we measured intracellular ROS (Fig. 2A) and mitochondrial superoxide generation (Fig. 3A). CS and IQOS single exposures (Fig. 2B, 5.0%, $p<0.001$) and CS+IQOS extract (5.0%, $p<0.001$) exposure after 6 h caused a significant increase of intracellular ROS when compared to unexposed cells. This indicates that lower percentage of CS+IQOS combination extract showed a potent effect on increasing intracellular ROS when compared to IQOS alone (5.0%). On the other hand, CS+IQOS exposure (5.0%, $p<0.001$) showed increased mitochondrial superoxide generation to a similar extent as that of CS or IQOS (Fig. 3B, 5.0%, $p<0.001$) at 6 h. Our data suggest that a CS+IQOS combination showed a significant effect on increasing mitochondrial superoxide generation as compared to CS or IQOS (5.0%) exposures alone. CS, IQOS, or CS+IQOS
exposure after 24 h also showed increased intracellular ROS generation (Fig. S2A-B) but to a lesser extent when compared to 6 h exposure. In contrast, mitochondrial superoxide generation (Fig. S3A-B, 24 h) increased to a similar extent as that of 6 h exposure.

**Dual Exposure Resulted in Impaired Mitochondrial Transmembrane Potential**

As mitochondrial superoxide generation was similar at 6 and 24 h, we assessed mitochondrial membrane potential after 24 h of CS, IQOS, or CS+IQOS exposure (Fig. 4A). We found that all smoke extract exposures caused a dose-dependent decrease in mitochondrial membrane potential evidenced by increasing in %JC-1 monomer (Fig. 4B) when compared to unexposed cells. Exposure of CS+IQOS extract (2.5%, \( p<0.01 \) and 5.0%, \( p<0.001 \)) showed enhanced mitochondrial membrane potential when compared alone CS exposure (2.5%, \( p<0.05 \) and 5.0%, \( p<0.01 \)). Our data indicate that CS and IQOS combination has a greater detrimental effect on airway epithelial cells than single CS or IQOS extract exposures.

**Dual Exposure Disrupts Mitochondrial Morphology and Homeostasis**

Enhanced oxidative stress and impaired mitochondrial membrane potential can impose aberrant mitochondrial morphological changes resulting in dysregulated mitochondrial fission and fusion dynamics, causing cell death. To assess morphological changes upon 24 h of CS, IQOS, and CS+IQOS exposure, we used mitotracker red fluorescent dye, which measures mitochondrial length (aspect ratio, Fig. 4D) and branching (form factor, Fig. 4E) in live cells. IQOS (2.5 and 5%, \( p<0.001 \)) and CS+IQOS (2.5 and 5.0%, \( p<0.001 \)) extract exposure displayed a dose-dependent alteration on mitochondrial branching and length, indicating mitochondrial fission when compared to unexposed cells. Interestingly, CS+IQOS exposure showed significant detrimental effects on mitochondrial branching (5.0%, \( p<0.001 \)) and length (5.0%, \( p<0.001 \)) when compared to alone CS or IQOS exposure.
To further corroborate mitochondrial dynamics, we performed immunoblotting for DRP1 and OPA1 after 24 h of CS, IQOS, or CS+IQOS exposure (Fig. 4F). Exposure of CS and IQOS alone at 5.0% resulted in significant upregulation of OPA1 (Fig. 4G, \(p<0.001\)). Interestingly, lower percentage of CS or IQOS exposure did not have any significant effect on OPA1 protein expression. By contrast, CS+IQOS dual exposure dose-dependently increased OPA1 expression (0.1 and 1.0%, \(p<0.05\); 2.5%, \(p<0.001\); and 5.0%, \(p<0.01\)) as compared to unexposed cells. DRP1 expression levels also significantly increased with CS+IQOS exposure including at lower extract concentrations (0.1, \(p<0.05\); 1.0, 2.5, and 5.0%, \(p<0.001\)) as compared to unexposed cells. Our data show that dual smoke exposure caused alterations in mitochondrial dynamics which were of greater magnitude as compared to CS or IQOS single exposures.

**Dual Exposure Induces Aberrant Mitophagy**

In mitophagy mitochondria co-localize within lysosomes forming autophagosomes which can be measured using specialized dyes that trace these organelles. To assess the immediate (6 h; Fig. S4) and prolonged (24 h; Fig. 5A) effects of CS, IQOS, or CS+IQOS exposure on mitophagy in airway epithelial cell, we used Lysotracker and Mitotracker stains. Exposure of CS (5%), IQOS (5.0%), or CS+IQOS (5.0%) for 6 h displayed increased co-localization of mitochondria into lysosomes indicating enhanced mitophagy (Fig. S4B, \(p<0.01\)) as compared to unexposed cells. However, lower percentage extract exposure did not show significant mitophagy except for CS (2.5%; \(p<0.01\)) and CS+IQOS (2.5%; \(p<0.001\)). We also evaluated the prolonged effects of smoke extracts and found that all the percentage of CS, IQOS, or CS+IQOS exhibited enhanced mitophagy (Fig. 5B) when compared unexposed cells.

To further corroborate our findings, we measured mRNA expression levels of the mitophagy markers PINK1 (Fig. 5C) and Parkin (Fig. 5D) after 24 h of CS, IQOS, or CS+IQOS
exposure. PINK1 and Parkin signaling plays a key role in mitophagy and mitochondrial motility including the magnitude of response to mitochondrial ΔΨm caused by damage/dysfunction induced by various toxicants [37, 38]. IQOS (2.5%, p<0.05 and 5%, p<0.01) and CS+IQOS (2.5%, p<0.05 and 5.0%, p<0.001) exposure increased mRNA expression of PINK1 when compared to unexposed cells. Interestingly, IQOS (5.0%) and CS+IQOS (5.0%) caused a significant difference in PINK1 expression levels (p<0.05) when compared to CS alone indicating that increased mRNA expression of PINK1 was purely due to IQOS exposure. IQOS (2.5%, p<0.05 and 5.0%, p<0.01) and CS+IQOS (2.5%, p<0.01 and 5.0%, p<0.001) showed increased Parkin mRNA expression when compared to unexposed cells. There was also a significant difference in Parkin expression levels upon IQOS (5.0%, p<0.05) and CS+IQOS (5.0%, p<0.001) exposure compared to CS alone. Furthermore, CS+IQOS dual exposure (5.0%, p<0.001) was significantly different when compared to IQOS alone (5.0%), indicating that dual exposure caused greater mitophagy in airway epithelial cells when compared to CS or IQOS single exposures.

Both Cigarette Smoke and IQOS Single Exposures and Dual Exposure Drive EMT

EMT plays a crucial role in airway remodeling and disease progression. To assess EMT changes upon exposure to CS, IQOS, or CS+IQOS extract, we performed immunoblotting assays (Fig. 6A). CS (2.5%, p<0.001), IQOS (2.5 and 5.0%, p<0.001), or CS+IQOS (2.5 and 5.0%, p<0.001) showed dose-dependent decreases in the expression of the epithelial marker E-cadherin (Fig. 6B) when compared to unexposed cells. By contrast, the mesenchymal marker N-cadherin (Fig. 6C) was significantly increased upon exposure to CS+IQOS (1.0%, p<0.001; 2.5% and 5.0%, p<0.05) when compared to unexposed cells. IQOS alone (1%, p<0.01) and CS+IQOS (1.0%, p<0.001) also increased N-cadherin expression when compared to CS alone. In addition,
upon exposure to CS+IQOS (2.5 and 5.0%, p<0.05), the expression of the mesenchymal marker vimentin (Fig. 6D) also increased when compared to unexposed or CS alone. There was increased protein expression of α-SMA (Fig. 6E; p<0.01) upon exposure of CS+IQOS at all percentages of exposure compared to unexposed cells. Interestingly, IQOS alone showed a dose-dependent increase the expression of α-SMA. Additionally, CS+IQOS exposure (2.5 and 5.0 %, p<0.001) also displayed a significant increase in mesenchymal markers compared to alone CS and IQOS. These results indicate that dual smoke extract exposure causes enhanced changes to proteins critical to EMT in airway epithelial cells as when compared to CS or IQOS single exposures.

**DISCUSSION**

Cigarette smoke is a major risk factor for the development of chronic obstructive pulmonary disease (COPD) and can also promote acute lung injury, pulmonary fibrosis, asthma, lung cancer while also negatively impacting other vital organ functions [7]. With the advent of e-cigarettes and smokeless devices (IQOS), the uptake in CS has increased and is further attributed to dual- or poly-product usage which itself may have detrimental effects on human health. Our current study focused on the cellular effects of dual exposure. We assessed various assays relevant to airway epithelial cell homeostasis and pathogenic signaling. We discovered that dual (CS+IQOS) exposure *in vitro* negatively impacts airway epithelial cell functions leading to loss of cell viability, pathogenic EMT-signaling, oxidative stress, and mitochondrial dysfunction.

Globally, cigarette smoking was on the decline in 21st century. As a result, tobacco companies began developing alternatives to CS that could increase their market shares. Among the various products that have emerged, IQOS was introduced as being safer than conventional cigarettes. Studies indicate that those addicted to conventional cigarettes start using IQOS to help
them quit smoking, however, many end up using both IQOS and CS [39]. Given that the harmful effects of IQOS or dual (CS+IQOS) exposure on lung physiology are not known, we investigated this important question. We found that dual CS+IQOS exposure increased the loss of cell viability when compared to IQOS exposure alone. Additionally, even lower concentrations of CS+IQOS exposure were sufficient to cause significant oxidative stress compared to CS or IQOS exposure alone. We also found that dual CS+IQOS exposure perturbed mitochondrial homeostasis when compared to CS or IQOS exposure alone. Collectively, CS+IQOS exposure lead to changes that initiated pathogenic EMT signaling, a key player in many serious pulmonary diseases.

We found that the nicotine content in an IQOS HEETS stick was almost half of the nicotine concentration present in a conventional cigarette. There are few studies that suggest that IQOS extract is less cytotoxic than traditional cigarette smoke in various lung cells [13, 40] while there is also an evidence to suggest that IQOS is equitoxic to traditional CS. We found that IQOS exposure was slightly less toxic than CS in causing loss of cell viability; this could be due to the differences in chemical composition between IQOS and CS.

Oxidative stress plays a pivotal role in numerous chronic lung pathologies [41]. Cigarette smoking is known to generate various toxic chemicals like carbon monoxide (CO), hydrogen cyanide (HCN), nitrogen oxides, formaldehyde, acrolein, benzene, nitrosamines, nicotine, phenol, polyaromatic hydrocarbons (PAHs), and many others [42], while IQOS generates several toxic chemicals such as carbonyls, acrolein, 3-methylbutanal, diacetyl, and 2,3-pentanedione, heavy metals, flavoring agents, acrolein [43]. All these chemicals released from CS and IQOS can generate mitochondrial ROS, the initial response to activate oxidative stress. As reported earlier [44], we also found that IQOS induces ROS and mitochondrial superoxide production but
Interestingly these responses were significantly higher in our experiments with IQOS when compared to CS alone. In addition, CS+IQOS exposure results in a significant elevation in ROS and mitochondrial superoxide generation as compared to CS or IQOS exposure alone. Our findings reveal that dual exposure induced excessive oxidative stress, which is a trigger for pathogenic signaling in obstructive lung diseases.

Mitochondrial membrane potential is also essential in determining oxidative stress-induced cellular apoptosis. We know that tobacco smoke induces mitochondrial depolarization-mediated cell death [45]. In our study, we see a dose-dependent increase in mitochondrial depolarization with CS+IQOS exposure as compared to CS or IQOS alone, resulting in altered mitochondrial homeostasis. Also, excessive ROS and mitochondrial superoxide generation is a hallmark of oxidative stress leading to aberrant mitochondrial function in chronic lung diseases [46]. Mitochondrial morphology is a key determinant of mitochondrial health. The aspect ratio defines centerline length and average width, whereas the form factor denotes the absolute length of the mitochondrial [47]. The literature indicates that CS leads to mitochondrial fission and fragmentation associated with increased mitochondrial count and decreased average mitochondrial size [48-50]. Other studies also indicate that IQOS and CS significantly reduce mitochondrial and lysosomal activity on various lung-derived cells [40]. In line with the published literature, our data show that IQOS exposure caused a dose-dependent alteration in mitochondrial morphology, with a significant reduction in the aspect ratio and the form factor. Further, dual CS+IQOS exposure accentuated the harmful effects in epithelial cells as compared to CS or IQOS alone. To further corroborate our findings, we also measured the expression of mitochondrial fission and fusion proteins: DRP1 and OPA1, respectively. To our surprise, dual exposure significantly augmented the expression of both DRP1 and OPA1 when compared to
IQOS or CS alone, thus confirming an imbalance of mitochondrial dynamics that can lead to lung pathology.

Mitophagy regulates homeostasis to maintain a healthy mitochondrial population and provides a cytoprotective role against cellular stressors. Mitochondrial dysfunction in various disease states is associated with dysregulated mitophagy [51]. PINK1 and Parkin function in a common signalling pathway known to regulate mitochondrial homeostasis and quality control, including mitophagy [52]. The multistep activation of this pathway, as well as an unexpected convergence between the post-translational modifications of ubiquitylation and phosphorylation, has added breadth to our understanding of cellular damage responses during various exposures. In our study, we found that IQOS-exposure selectively increases PINK1 and Parkin gene expression when compared to CS alone, while dual exposure further augments this effect. These findings demonstrate how IQOS alone and in combination with CS can have a differential effect on PINK1-Parkin mediated signaling which is a key regulator of mitochondrial health and may play a critical pathogenic role in the development of chronic lung diseases [53]. Cigarette smoke-mediated EMT activation also plays an important role in pathological signaling leading to irreversible lung damage [54]. Our results showed that dual exposure significantly augmented EMT signaling as compared to CS or IQOS alone. The preponderance of data in our study show that simultaneous mechanisms known to be involved in the pathogenesis of common chronic lung diseases (such as COPD, asthma, and pulmonary fibrosis, etc.), are enhanced during both single but especially dual smoke exposures.

Our current study had some limitations as we only investigated effects on a single airway epithelia cell line, thus, future studies using fully differentiated primary human airway epithelial cell culture systems, immune cells, and integrative mouse models along with clinical studies are
warranted to fully establish the harm associated with combined use of IQOS with other products. Further, correlative metabolomic and lipidomic analysis in the future will help to identify distinct targets that may reveal new pathogenic mechanisms induced by CS and IQOS combination. As summarized in Fig. 7, our findings indicate that dual use of CS+IQOS may pose greater risks to airway resident cells including epithelial cell homeostasis which is likely to be detrimental to overall lung health.

**Abbreviations:**  **BEAS-2B:** Human Bronchial epithelial cells;  **COPD:** Chronic Obstructive Pulmonary Disease;  **CSE:** Cigarette smoke extract;  **DCFH-DA:** 2’-7’-Dichlorodihydrofluorescein diacetate;  **EMT:** Epithelial-mesenchymal transition;  **HnBs:** Heat Not Burn;  **HTP:** Heated-tobacco-product;  **h:** Hours;  **mins:** Minutes;  **PMI:** Phillip Morris International;  **MRTP:** Modified risk tobacco product.

**Conflict of Interest:** The authors have no conflict of interest to declare.

**Acknowledgments:** The authors would like to acknowledge the fellowship support from Department of Pharmaceuticals, Ministry of Chemical and Fertilizers. The research was supported by the grant from North East Centre for Biological Science Healthcare Engineering, IIT Guwahati (NECBH/2019-20/188) to VGMN.

**Author Contributions:** PS and VGMN conceived and designed research; Pr.S, SJ, IM and SRP performed experiments, analyzed data, interpreted results of experiments, and writing original draft of manuscript; AAZ, VGMN and PS interpreted key results, edited and revised the manuscript, and provided overall direction. All authors approved the final version of the manuscript.
REFERENCES

17. FDA Authorizes Marketing of IQOS Tobacco Heating System with ‘Reduced Exposure’ Information. 2020.


42. Harris JE. Cigarette smoke components and disease: cigarette smoke is more than a triad of tar, nicotine, and carbon monoxide. Smoking and tobacco control monograph 1996: 7: 59-75.


**FIGURE LEGENDS**

**Figure 1:** *Dual exposure displays loss of airway epithelial cell viability.* (A) Schematic design for *in vitro* study. Line plot representing percentage viability of BEAS-2B cells upon exposure of CS, IQOS, or CS+IQOS for (B) 24h and (C) 48h. Values are represented as mean ± SEM (n=5). A one-way ANOVA followed by post-hoc Dunnett’s test was used to define statistical significance. $p<0.05$, $$p<0.01$, $$$p<0.001$ vs IQOS-exposed.

**Figure 2:** *Dual exposure enhances the intracellular ROS in bronchial epithelial cells.* (A) Pictorial representation of histogram plot for DCFDA assay to assess intracellular ROS generation in BEAS-2B cells upon exposure of CS, or IQOS, or CS+IQOS for 6 h using flow cytometer (Attune Nxt, Invitrogen, Singapore). (B) Bar graph representing mean fluorescence intensity changes upon DCFDA staining. Values are represented as mean ± SEM (n=5). A one-way ANOVA followed by post-hoc Tukey’s test was used to define statistical significance. *p<0.05, **p<0.01, ***p<0.001 vs unexposed and #p<0.05, ##p<0.01, ###p<0.001 vs CS-exposed.

**Figure 3:** *Dual exposure enhances the mitochondrial superoxide generation in bronchial epithelial cells.* (A) Representative images for MitoSOX assay to assess mitochondrial
superoxide generation in BEAS-2B cells upon exposure of CS, or IQOS, or CS+IQOS for 6h using confocal microscope (Leica, Germany) under 630X magnification. (B) Bar graph representing mean fluorescence intensity changes upon MitoSOX staining. Values are represented as mean ± SEM (n=5). A one-way ANOVA followed by post-hoc Tukey’s test was used to define statistical significance. **p<0.01, ***p<0.001 vs unexposed, #p<0.05, ##p<0.01, ###p<0.001 vs CS-exposed, and $$$p<0.01 vs IQOS-exposed.

**Figure 4:** Dual exposure disrupts the mitochondrial morphology and homeostasis. (A) Pictorial representation of dot plot showing mitochondrial membrane potential of BEAS-2B cells upon exposure of CS, IQOS, or CS+IQOS for 24h using JC-1 stain. (B) Bar graph representing percentage of JC-1 monomer. (C) Representative images of BEAS-2B cells showing changes in mitochondrial morphology upon exposure of CS, IQOS, or CS+IQOS for 24h and images were acquired using confocal microscopy under 630X magnification. Bar graph representing (D) aspect ratio (mitochondrial length) and (E) form factor (mitochondrial branching) analysed using the Image-J software. (F) Representative protein expression of OPA1 and DRP1 upon exposure of CS, IQOS, or CS+IQOS for 24h. Bar graph represents densitometric analysis of (G) OPA1 and (H) DRP1. Values are represented as mean ± SEM (n=5). A one-way ANOVA followed by Tukey’s test was applied to determine the statistical significance. *p<0.5, **p<0.01, ***p<0.001 vs. unexposed, #p<0.05, ##p<0.01, ###p<0.001 vs CS-exposed, and $$$p<0.01, $$$p<0.001 vs IQOS-exposed.

**Figure 5:** Dual exposure surges the aberrant mitophagy. (A) Representative images of BEAS-2B cells stained with mitotracker and lysotracker to show mitophagy upon exposure of CS, IQOS, or CS+IQOS for 24h and images were acquired using confocal microscopy under 630X magnification. (B) Bar graph represents Pearson’s co-localisation coefficient upon mitotracker
and lysotracker staining. Bar graph indicating changes in mRNA gene expression levels of (C) PINK1 and (D) Parkin upon exposure of BEAS-2B cells with CS, IQOS, or CS+IQOS for 24h. Values are represented as mean ± SEM (n=5). A one-way ANOVA followed by Tukey’s test was applied to determine the statistical significance. *p<0.5, **p<0.01, ***p<0.001 vs. unexposed, #p<0.05, ##p<0.01, ###p<0.001 vs CS-exposed, and $p<0.05, $$$p<0.01$ vs IQOS-exposed.

**Figure 6:** Cigarette smoke and IQOS exposure alone and Dual exposure drives EMT. (A) Representative protein expression of E-cadherin, N-cadherin, vimentin, and α-SMA upon exposure of CS, IQOS, or CS+IQOS for 24h. Bar graph represents densitometric analysis of (B) E-cadherin, (C) N-cadherin, (D) vimentin, and (E) α-SMA. Values are represented as mean ± SEM (n=5). A one-way ANOVA followed by Tukey’s test was applied to determine the statistical significance. *p<0.5, **p<0.01 vs. unexposed, #p<0.05 vs CS-exposed, and $p<0.05$ vs IQOS-exposed.

**Figure 7:** Mechanisms by which dual cigarette smoke and IQOS exposure can promote airway disease pathology. This schematic depicts how cigarette smoking and IQOS exposure can induce pathogenic signaling in airway epithelial cells. These changes are predominantly driven by mitochondrial reactive oxygen species (ROS) generation and oxidative stress which leads to mitochondrial dysfunction. IQOS-exposure in epithelial cells can selectively enhance mitophagy gene expression alone or in combination with CS (dual exposure). This sequence of events leads to disruption of epithelial cell integrity and results in induction of EMT signaling an important mediator of airway fibrosis in chronic airways disease.
**F)**

<table>
<thead>
<tr>
<th></th>
<th>Unexp</th>
<th>0.1%</th>
<th>1%</th>
<th>2.5%</th>
<th>5%</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CS</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>IQOS</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Dual</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OPA1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DRP1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-tubulin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**G)**

0.1 % Exposure  
1 % Exposure  
2.5 % Exposure  
5 % Exposure

**H)**
Cigarette + IQOS → ROS → Oxidative stress → Epithelial cell

Epithelial Cell Injury and Inflammation

Mitochondrial Dysfunction → Loss of Mitochondrial Integrity → Mitophagy

IQOS → PINK1 → Parkin

Epithelial Mesenchymal Transition → Chronic Airway Diseases
Supplementary Information (SI) Methods

Chemicals and reagents

Foetal Bovine Serum (South American origin, Gibco, Life Technologies, 10270-106), Phosphate Buffer Saline (HIMEDIA, M1866), 0.25% Trypsin-EDTA 1X (Gibco, Life Technologies, 25200-056), F12 media (Gibco, Life Technologies, 11765-054), Antibiotic-Antimycotic 100X (Gibco, Life Technologies, 15240-062), MTT (Sigma Aldrich, M2128), DMSO (Merck, SK5S650642), Alexa Fluor 488 anti-rabbit (Invitrogen, A11034), Alexa Fluor 594 anti-mouse (Invitrogen, A11032), Prolong gold Antifade with DAPI(Invitrogen, 8961S), Piperine (Sigma), Dexamethasone (HIMEDIA), Methacholine (TCI, M0073), TPER (Thermo Scientific, 78510), RIPAs (Thermo Scientific, 89901), Protease and phosphatase Inhibitor (Thermo Scientific, A32961), Protein Assay Dye Reagent Concentrate (BIO-RAD, 5000006), Trans-Blot Turbo 5X Transfer buffer (BIO-RAD, 10026938), Ponceau S solution (Sigma Aldrich, P7170), Bovine serum albumin (HIMEDIA, MB083), primary antibodies E-cadherin (Abclonal, A18135), N-cadherin (Abclonal, A0433), Vimentin (Abclonal, A2584), α-SMA (Abclonal, A17910), OPA1 (Abclonal, A9833), DRP1 (CST, 8480S), β-tubulin (CST, 8480S), α-tubulin (CST, 8480S), β-actin (Abclonal, AC026), TRIZol reagent (Ambion, Life Technologies, 15596018), cDNA synthesis kit (Takara, 6110A), PowerUp™ SYBER™ Green Master Mix (A25742), primers from Integrated DNA technologies- PINK1 (F- 5’ AGAGAGGTCCCAAGCAACTA 3’, R- 5’ GCAGGTCAGGGATAGTTC 3’), PARKIN (F- 5’ CCCAGTGACCATGATAGTGT 3’, R- 5’ CAGTCCAGTCATTCCGAC 3’), and GAPDH (F- 5’ TCATTTCCTGGTATGACAACGA 3’, R- 5’ AGGGGAGATTCGTGTTG 3’). All the analytical grade chemicals were used and were purchased from Sigma and Thermo.
**Nicotine Estimation by HPLC**

The concentration of nicotine extracted in phosphate buffer saline was estimated by using Dionex Ultimate 3000 UHPLC system equipped with (photodiode array) PDA detector. The chromatographic analysis was performed on Hypersil Gold C8 column (150 X 4.6mm, 5µm) using 0.1% TFA in water and acetonitrile (9:1) as mobile phase with a flow rate of 0.5mL/min. The detection wavelength was 260nm.

**Supplementary Figure legends**

**Figure S1:** HPLC chromatogram of nicotine from Cigarette smoke and IQOS extract recorded at 260nm.

**Figure S2:** (A) Pictorial representation of histogram plots for the DCFDA assay to measure intracellular ROS generation in BEAS-2B cells upon exposure of CS, or IQOS, or CS+IQOS for 24h. (B) Bar graph representing mean fluorescence intensity changes upon DCFDA staining. Values are represented as mean±SEM (n=4). A one-way ANOVA followed by post-hoc Tukey’s test was used to define statistical significance. *p<0.05, **p<0.01, ***p<0.001 vs unexposed.

**Figure S3:** (A) Representative images for MitoSOX assay to assess mitochondrial superoxide generation in BEAS-2B cells upon exposure of CS, or IQOS, or CS+IQOS for 24h. (B) Bar graph representing mean fluorescence intensity changes upon MitoSOX staining. Values are represented as mean±SEM (n=3). A one-way ANOVA followed by post-hoc Tukey’s test was used to define statistical significance. *p<0.05, **p<0.01, ***p<0.001 vs unexposed, #p<0.05, ###p<0.001 vs CS exposed.
Figure S4: (A) Representative images of BEAS-2B cells stained with mitotracker and lysotracker to show mitophagy upon exposure of CS, IQOS, or CS+IQOS for 6h. (B) Bar graph represents Pearson’s co-localisation coefficient between mitotracker and lysotracker staining. Values are represented as mean±SEM (n=3). A one way ANOVA followed by Tukey’s test was applied to determine the statistical significance. *p<0.5, **p<0.01, ***p<0.001 vs. unexposed.
A) Unexp  0.1%  1%  2.5%  5%

CS

IQOS

CS+IQOS

B) 0.1 % Exposure  1 % Exposure  2.5 % Exposure  5 % Exposure

Relative MitoSox fluorescence intensity

Unexp  CS  IQOS  CS+IQOS  Unexp  CS  IQOS  CS+IQOS  Unexp  CS  IQOS  CS+IQOS  Unexp  CS  IQOS  CS+IQOS