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

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# The effects of dual IQOS and cigarette smoke exposure on airway epithelial cells: implications for lung health and respiratory disease pathogenesis

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**The use of multiple electronic nicotine delivery products is on the rise. While the potential harm associated with dual- or poly-product usage is not yet clear, this study suggests that dual cigarette smoke and IQOS may be detrimental to lung health.** https://bit.ly/3IfQ28R

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## Abstract

*Background* Cigarette smoking remains a 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 (cigarette smoke (CS)+IQOS) is detrimental to lung epithelial cells when compared with CS or IQOS exposure alone.

*Methods* Human airway epithelial cells (BEAS-2B) were exposed to either CS, IQOS or their dual combination (CS+IQOS) at concentrations of 0.1%, 1.0%, 2.5% and 5.0%. Cytotoxicity, oxidative stress, mitochondrial homeostasis, mitophagy and effects on epithelial–mesenchymal transition (EMT) signalling 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 with IQOS alone ($p<0.01$). Dual exposure significantly increased oxidative stress and perturbed mitochondrial homeostasis when compared with CS or IQOS alone ($p<0.05$). Additionally, dual exposure induced EMT signalling as shown by increased mesenchymal (α-smooth muscle actin and N-cadherin) and decreased epithelial (E-cadherin) markers when compared with CS or IQOS alone ($p<0.05$).

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

## Introduction

Lung disease-related mortality has risen to an alarming level globally [1]. Cigarette smoke (CS) is the leading cause of lung disease and is responsible for a significant burden of public health, contributing to 8 million deaths worldwide each year [2]. It is one of the most significant threats posed by the tobacco epidemic. Although cigarette smoking rates have declined in the 21st century, the introduction of e-cigarettes and their widespread use 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 over 2800 hospitalisations [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 adolescents 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%) US middle and high school students reported using e-cigarettes within a 30-day period, while over 2 million youths were estimated to be current e-cigarette users [9]. In recent years, the heat-not-burn (HnB) or heated tobacco product 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 authorised the sale of IQOS as the first HnB device from PMI, which was designated as a "modified risk tobacco product (MRTP)" [17].

Although 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 fibroblasts 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-cigarettes 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 CS 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 scarce 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 the 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 signalling, which is known to initiate pathological lung changes as observed in various obstructive lung diseases.

## Methods

### *Chemicals and reagents*

All chemicals were of analytical grade and purchased from Sigma (Burlington, MA, USA) and Thermo Scientific (Waltham, MA, USA). Additional information is provided in the supplementary material.

### *Cell culture*

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

### *Preparation of CS, IQOS and dual CS+IQOS extract*

CS or IQOS aerosol extracts were prepared by bubbling smoke from one cigarette (Marlboro Red, India) or from IQOS HEETS (Tobacco flavour) per 5 mL PBS (pH 7.4). The extract was filtered and normalised to nicotine content after high-performance liquid chromatography (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 utilised in this study (0.1–5.0%) are based on many seminal studies [27–30] and from our own preliminary data and a previous publication [13]. Serum cotinine (stable nicotine metabolite) levels among 1 pack per day smokers may vary greatly from 16 to 1180 $ng \cdot mL^{-1}$ [31], while immediate levels of nicotine after smoking one cigarette in venous and arterial blood can fluctuate between 5 and 100 $ng \cdot mL^{-1}$ [32]. Also, the number of cigarettes smoked or IQOS used varies greatly in humans. To avoid this variation in our experiments, we performed our studies based on one standardisation,

*i.e.* approximate nicotine content in either one cigarette or in one IQOS HEETS (supplementary figure S1). Accordingly, nicotine levels from 0.1% to 5.0% extract are within the 0.4–200 ng·mL$^{-1}$ range.

### Nicotine estimation by HPLC

The concentration of nicotine extracted in PBS was estimated using the Dionex UltiMate 3000 ultra-HPLC system (Thermo Scientific) equipped with a photodiode array detector, as described in the supplementary material.

### 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. Cells were then exposed to different concentrations of CS, IQOS or CS+IQOS extract for 24 or 48 h and media replaced with 100 µL growth arrest media containing MTT (5 mg per 10 mL) for 4 h. After incubation, media were removed, 200 µL dimethyl sulfoxide was added and absorbance was measured at 570 nm, as previously described [33].

### Assessment of intracellular ROS generation

Intracellular ROS generation was assessed using the 2′,7′-dichlorodihydrofluorescein diacetate (DCFDA) assay (Invitrogen CM-H2DCFDA; Thermo Scientific). After CS, IQOS or CS+IQOS extract exposure for 6 and 24 h, cells were stained with DCFDA dye and analysed using an Attune NxT flow cytometer (Invitrogen), as described previously [34].

### Assessment of mitochondrial superoxide generation

Mitochondrial superoxide generation was analysed using MitoSOX Red (Invitrogen). After CS, IQOS or CS+IQOS extract exposure for 6 and 24 h, cells were stained with MitoSOX Red and imaged using confocal laser scanning microscopy (TCS SP8; Leica, Wetzlar, Germany), as described previously [34].

### Assessment of mitochondrial membrane potential

Mitochondrial membrane potential was measured using JC-1 dye. After CS, IQOS or CS+IQOS extract exposure for 6 and 24 h, cells were stained with JC-1 dye and analysed using flow cytometry, as described previously [34].

### Quantitative assessment of mitochondrial morphology

Mitochondrial morphology and shape were determined using MitoTracker Deep Red (Invitrogen). After CS, IQOS or CS+IQOS extract exposure for 6 and 24 h, cells were stained and then imaged using confocal laser scanning microscopy (Leica TCS-SP8), as described previously [35].

### Assessment of mitophagy

MitoTracker Deep Red FM and LysoTracker Green DND 26 (Invitrogen) 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 confocal laser scanning microscopy.

### Immunoblotting

After 24 h of CS, IQOS or CS+IQOS extract exposure, immunoblotting for cell lysate was performed as described previously [33]. Primary antibodies against DRP1, OPA1, E-cadherin, N-cadherin, α-smooth muscle actin (α-SMA), vimentin, α-tubulin and β-actin (1:1000) were used and procured from ABclonal (Woburn, MA, USA).

### 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 the Prime Script 1st Strand cDNA Synthesis Kit (Takara Bio, Shiga, Japan), as per the kit protocol. PowerUP SYBR Green Master Mix was used to perform quantitative real-time PCR using an Applied Biosystems real-time PCR machine (QuantStudio 5; Thermo Scientific). *GAPDH* was used as a housekeeping gene to calculate gene expression data.

### Statistical analysis

All data are expressed as mean±SEM. The non-parametric unpaired t-test was used to compare two groups. One-way ANOVA with a post-hoc Dunnett's test or Tukey's test was used when more than two groups were compared. Prism version 7 (GraphPad, La Jolla, CA, USA) was used for all statistical analyses. $p<0.05$ 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$^{-1}$ 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$^{-1}$ in CS and IQOS extracts, respectively (supplementary figure S1).

### *Dual exposure displays loss of airway epithelial cell viability*

Cell viability was assessed upon exposure of BEAS-2B cells to equivalent nicotine concentrations of CS, IQOS or CS+IQOS extract for 24 and 48 h (figure 1a). 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 and 48 h (figure 1b and c). Additionally, CS+IQOS dual exposure was found to be more cytotoxic than IQOS exposure alone both at 24 and 48 h.

### *Dual exposure enhances intracellular ROS and mitochondrial superoxide generation*

CS-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 (figure 2a) and mitochondrial superoxide generation (figure 3a). CS and IQOS single exposures (5.0%, p<0.001) and CS+IQOS extract exposure (5.0%, p<0.001) after 6 h caused a significant increase of intracellular ROS when compared with unexposed cells (figure 2b). This indicates that lower percentage of CS+IQOS combination extract showed a potent effect on increasing intracellular ROS when compared with CS 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 (5.0%, p<0.001) at 6 h (figure 3b). Our data suggest that a combination of

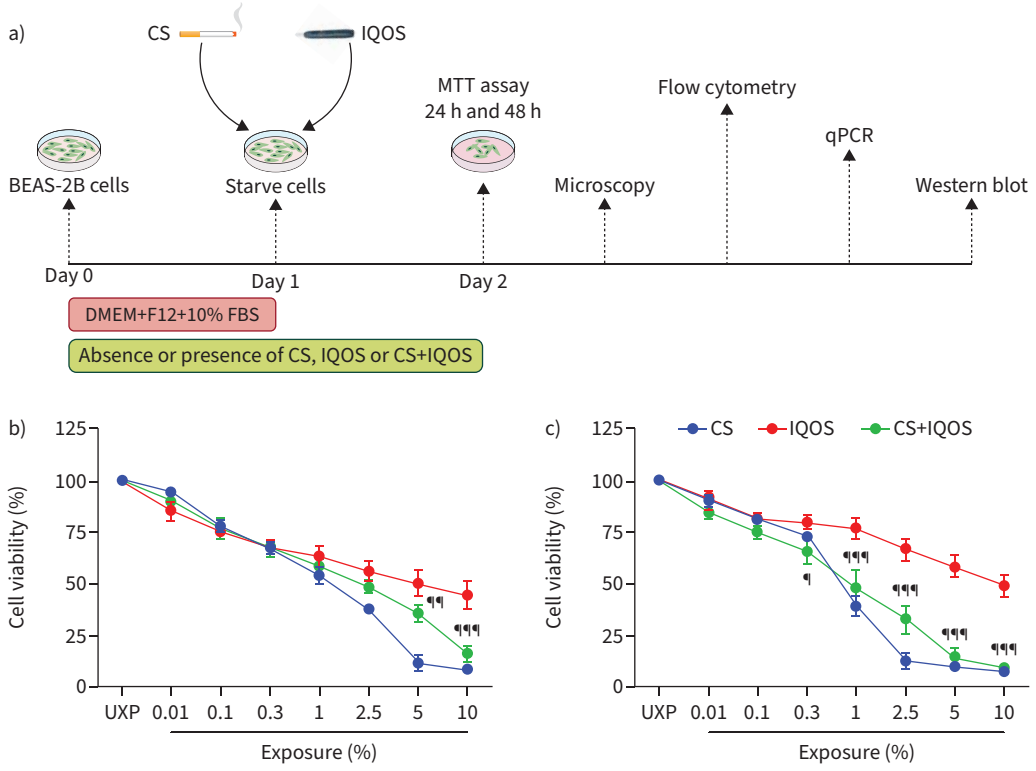


FIGURE 1 Dual exposure displays loss of airway epithelial cell viability. a) Schematic design for *in vitro* study. b, c) Line plots representing percentage viability of BEAS-2B cells upon exposure to cigarette smoke (CS), IQOS or CS+IQOS for b) 24 h and c) 48 h. Data are presented as mean±SEM (n=5). qPCR: quantitative PCR; UXP: unexposed. 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 *versus* IQOS-exposed.

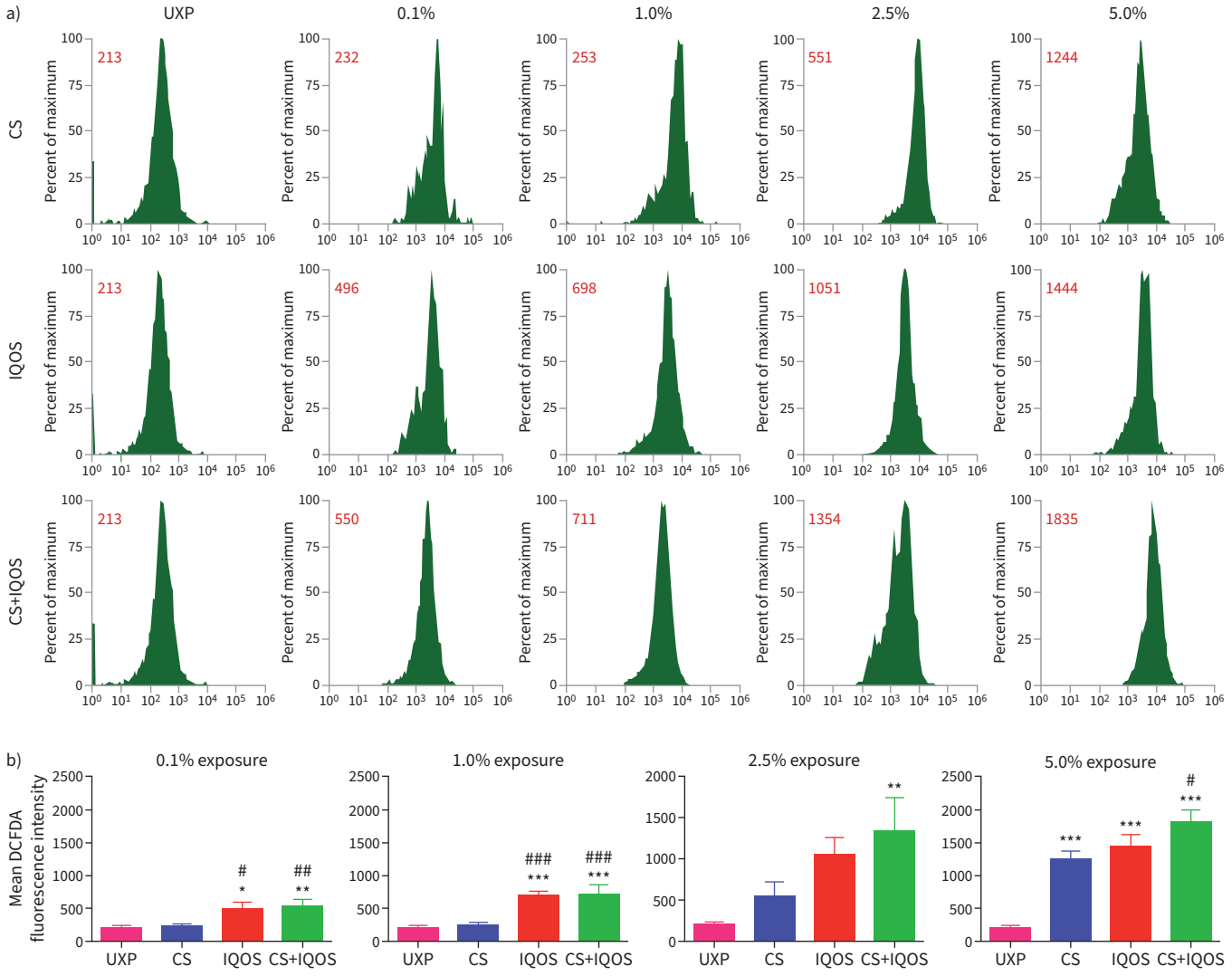


**FIGURE 2** Dual exposure enhances intracellular reactive oxygen species (ROS) in bronchial epithelial cells. a) Pictorial representation of histogram plots for 2′,7′-dichlorodihydrofluorescein diacetate (DCFDA) assay to assess intracellular ROS generation in BEAS-2B cells upon exposure to cigarette smoke (CS), IQOS or CS+IQOS for 6 h using flow cytometry. b) Bar graphs representing mean fluorescence intensity changes upon DCFDA staining. Data are presented as mean±SEM (n=5). 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 *versus* unexposed (UXP). #: p<0.05; ##: p<0.01; ###: p<0.001 *versus* CS-exposed.

CS and IQOS had a significant effect on increasing mitochondrial superoxide generation compared with exposure to either CS or IQOS alone (at 5.0% concentration). CS, IQOS or CS+IQOS exposure after 24 h also showed increased intracellular ROS generation (supplementary figure S2a and b), but to a lesser extent when compared with 6 h exposure. In contrast, mitochondrial superoxide generation (supplementary figure S3a and b; 24 h) increased to a similar extent as that of 6 h exposure.

### *Dual exposure results 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 (figure 4a). We found that all smoke extract exposures caused a dose-dependent decrease in mitochondrial membrane potential evidenced by increasing JC-1 monomer percentage when compared with unexposed cells (figure 4b). Exposure to CS+IQOS extract showed enhanced mitochondrial membrane potential at 0.1% (p<0.05), 1.0% (p<0.01) and 5.0% (p<0.001) when compared with CS exposure alone at the respective concentrations. Our data strongly indicate that the combined exposure to CS and IQOS has a significantly more harmful effect on airway epithelial cells compared with exposure to either CS or IQOS alone.

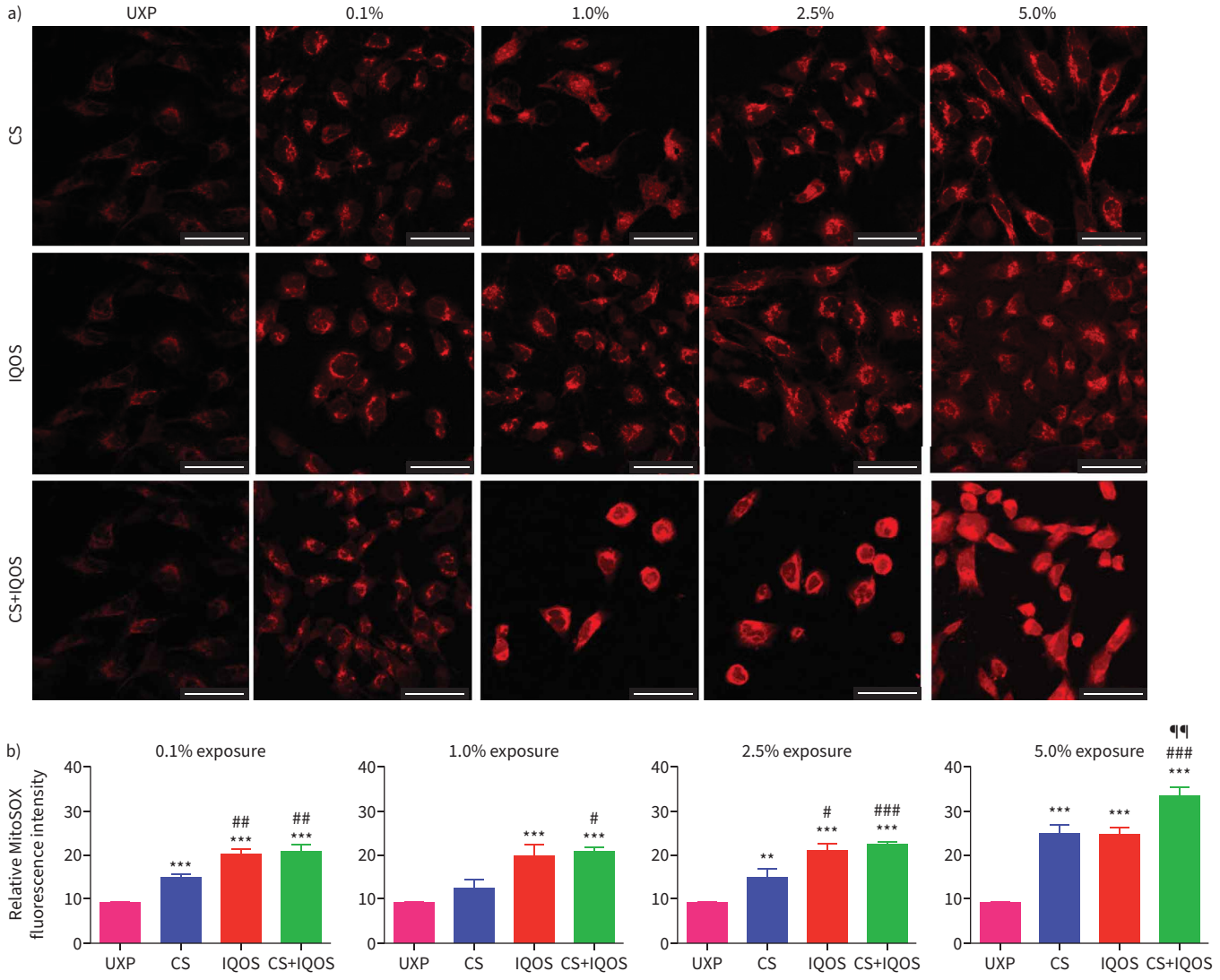


**FIGURE 3** Dual exposure enhances mitochondrial superoxide generation in bronchial epithelial cells. a) Representative images for the MitoSOX assay to assess mitochondrial superoxide generation in BEAS-2B cells upon exposure to cigarette smoke (CS), IQOS or CS+IQOS for 6 h using confocal laser scanning microscopy (×630 original magnification). Scale bar: 50 µm. b) Bar graphs representing mean fluorescence intensity changes upon MitoSOX staining. Data are presented as mean±SEM (n=5). One-way ANOVA followed by post-hoc Tukey's test was used to define statistical significance. **: p<0.01; ***: p<0.001 *versus* unexposed (UXP). #: p<0.05; ##: p<0.01; ###: p<0.001 *versus* CS-exposed. ¶¶: p<0.01 *versus* IQOS-exposed.

### *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 (figure 4c), we used MitoTracker red fluorescent dye, which measures mitochondrial length (aspect ratio: figure 4d) and branching (form factor: figure 4e) in live cells. IQOS exposure alone significantly altered mitochondrial length (figure 4d) and branching (figure 4e) when compared with CS alone, while dual CS+IQOS exposure significantly reduced mitochondrial length when compared with unexposed or CS or IQOS alone (figure 4d). Furthermore, the effect of dual CS+IQOS on mitochondrial branching was profound at all concentrations tested when compared with unexposed or CS or IQOS alone (figure 4e), indicating mitochondrial fission in these cells.

To further corroborate mitochondrial dynamics, we performed immunoblotting for DRP1 and OPA1 after 24 h of CS, IQOS or CS+IQOS exposure (figure 4f). Exposure to CS and IQOS alone at 5.0% resulted in

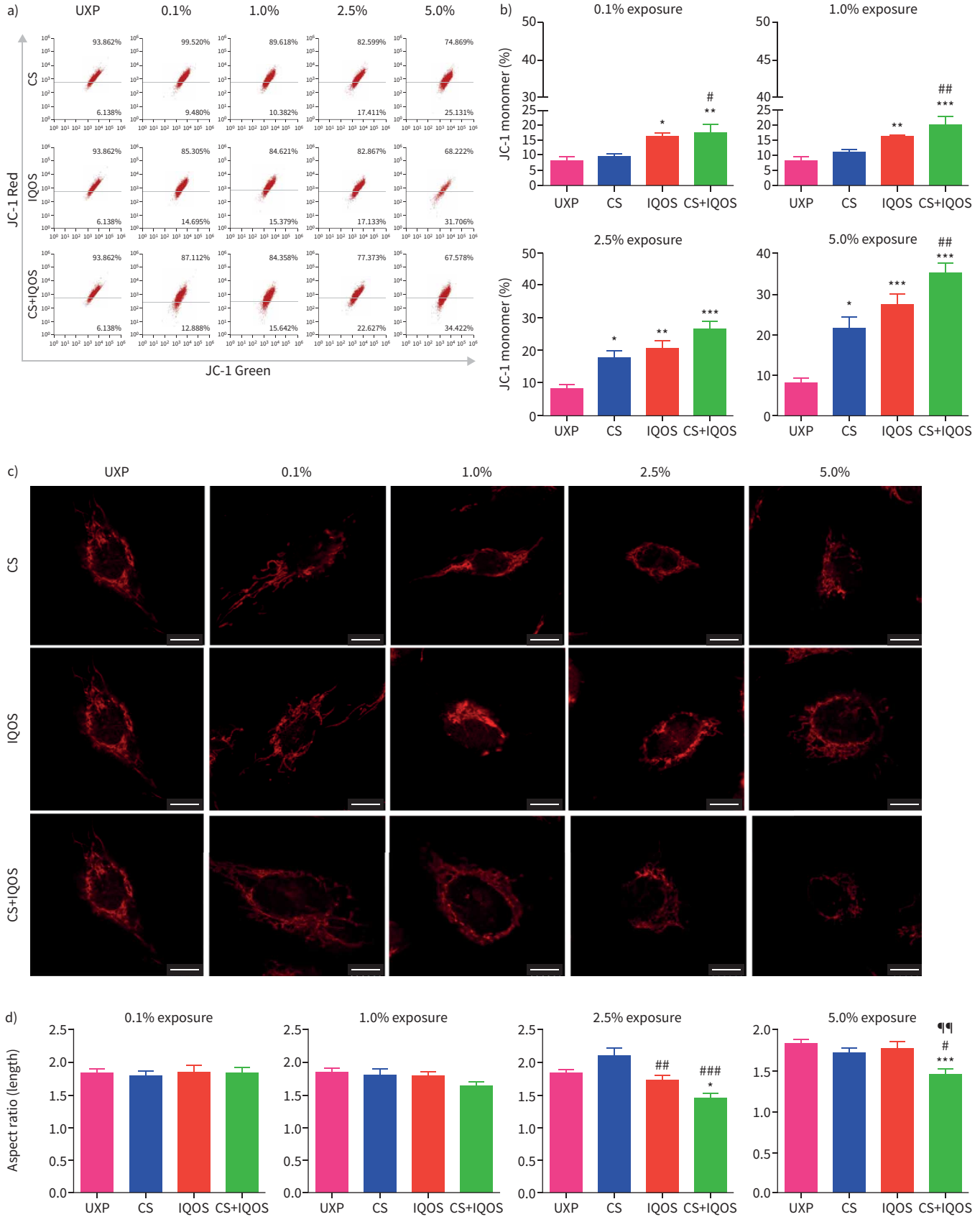


FIGURE 4 Continued.

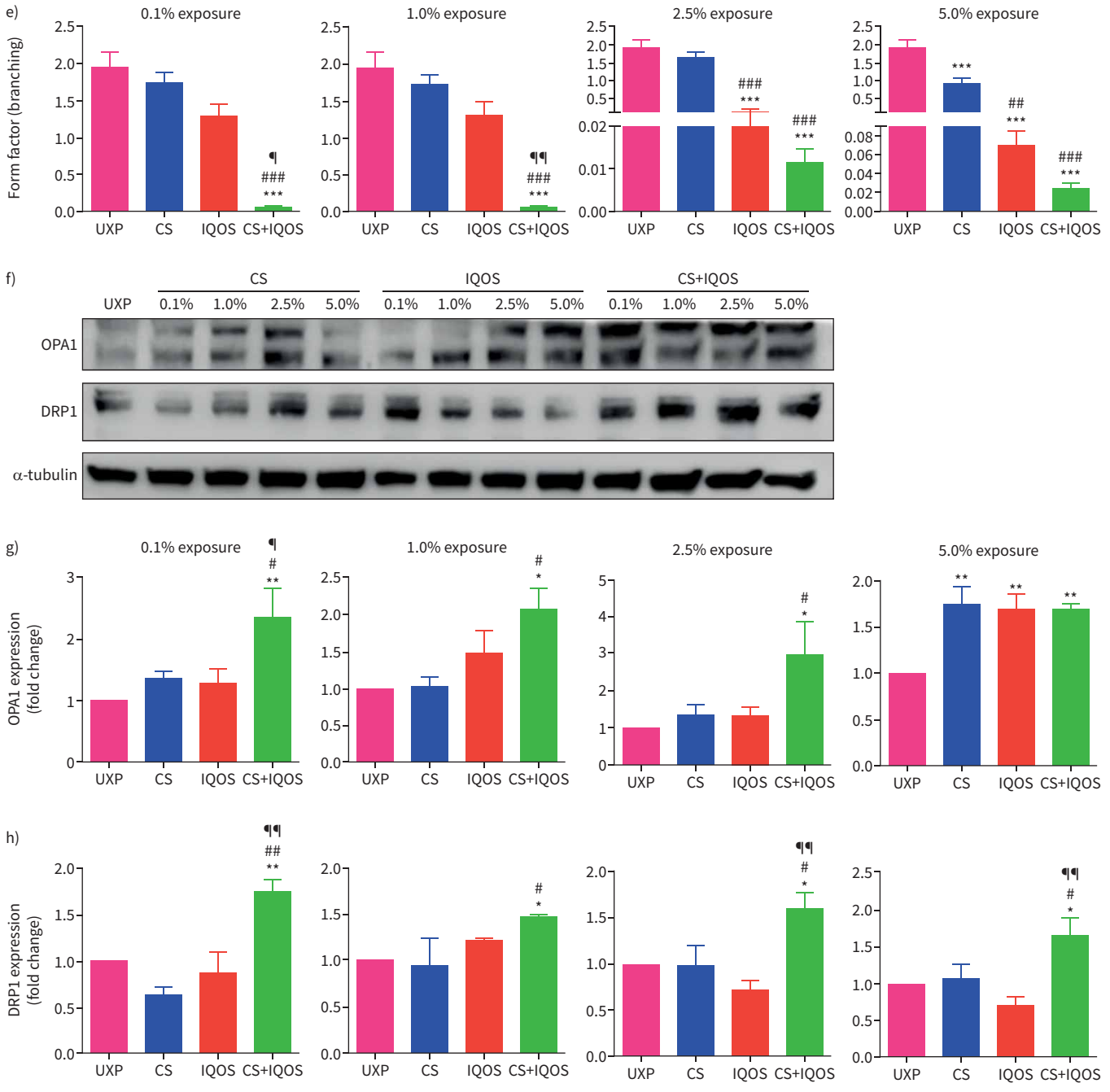


**FIGURE 4** Dual exposure disrupts mitochondrial morphology and homeostasis. a) Pictorial representation of dot plots showing mitochondrial membrane potential of BEAS-2B cells upon exposure to cigarette smoke (CS), IQOS or CS+IQOS for 24 h using JC-1 dye. b) Bar graphs representing percentage of JC-1 monomer. c) Representative images of BEAS-2B cells showing changes in mitochondrial morphology upon exposure to CS, IQOS or CS+IQOS for 24 h using confocal laser scanning microscopy (×630 original magnification). Scale bar: 50 µm. d, e) Bar graphs representing d) aspect ratio (mitochondrial length) and e) form factor (mitochondrial branching) analysed using ImageJ software. f) Representative protein expression of OPA1 and DRP1 upon exposure to CS, IQOS or CS+IQOS for 24 h. g, h) Bar graphs representing densitometric analysis of g) OPA1 and h) DRP1. Data are presented as mean±SEM (n=5). One-way ANOVA followed by post-hoc Tukey's test was used to define statistical significance. *: p<0.5; **: p<0.01; ***: p<0.001 *versus* unexposed (UXP). #: p<0.05; ##: p<0.01; ###: p<0.001 *versus* CS-exposed. ¶¶: p<0.01; ¶¶¶: p<0.001 *versus* IQOS-exposed.

significant upregulation of OPA1 (p<0.01) (figure 4g). Interestingly, lower percentage of CS or IQOS exposure did not have any significant effect on OPA1 protein expression. On the contrary, CS+IQOS dual exposure led to a dose-dependent increase in OPA1 expression (0.1%, 1.0% and 2.5%, p<0.05; 5.0%,

p<0.01) compared with unexposed cells (figure 4g). In addition, DRP1 expression levels also significantly increased in a concentration-dependent manner with dual CS+IQOS exposure when compared with unexposed cells, or exposure to CS or IQOS alone (figure 4h). Our data demonstrate that dual exposure to CS and IQOS caused significant alterations in mitochondrial dynamics compared with single exposure to either CS or IQOS.

### *Dual exposure induces aberrant mitophagy*

In mitophagy, mitochondria colocalise within lysosomes forming autophagosomes which can be measured using specialised dyes that trace these organelles. To assess the immediate (6 h: supplementary figure S4) and prolonged (24 h: figure 5a) effects of CS, IQOS or CS+IQOS exposure on mitophagy in airway epithelial cells, we used LysoTracker and MitoTracker stains. Exposure to CS (5%), IQOS (5.0%) or CS+IQOS (5.0%) for 6 h displayed increased colocalisation of mitochondria into lysosomes, indicating enhanced mitophagy (p<0.01) compared with unexposed cells (supplementary figure S4b). 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 percentages of CS, IQOS or CS+IQOS exhibited enhanced mitophagy when compared with unexposed cells (figure 5b).

To further corroborate our findings, we measured mRNA expression levels of the mitophagy markers PINK1 and Parkin after 24 h of CS, IQOS or CS+IQOS exposure (figure 5c and d). PINK1 and Parkin signalling plays a key role in mitophagy and mitochondrial motility including the magnitude of response to mitochondrial membrane potential caused by damage/dysfunction induced by various toxicants [36, 37]. IQOS (2.5% and 5.0%, p<0.001) and CS+IQOS (2.5% and 5.0%, p<0.001) exposure increased mRNA expression of PINK1 when compared with unexposed cells. Interestingly, IQOS (5.0%) and CS+IQOS (5.0%) caused a significant difference in PINK1 expression levels (p<0.01) when compared with CS alone, indicating that increased mRNA expression of PINK1 was purely due to IQOS exposure. IQOS (1.0%, p<0.01; 2.5% and 5.0%, p<0.05) and CS+IQOS (1.0%, p<0.001; 2.5%, p<0.01; 5.0%, p<0.001) showed increased Parkin mRNA expression when compared with 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 with CS alone. Furthermore, CS+IQOS dual exposure (5.0%, p<0.01) was significantly different when compared with IQOS alone (5.0%), indicating that dual exposure caused greater mitophagy in airway epithelial cells when compared with CS or IQOS single exposures.

### *Both CS and IQOS single exposures and dual CS+IQOS exposure drive EMT*

EMT plays a crucial role in airway remodelling and disease progression. To assess EMT changes upon exposure to CS, IQOS or CS+IQOS extract, we performed immunoblotting assays (figure 6a). CS (2.5%, p<0.001), IQOS (0.1% and 1.0%, p<0.05; 2.5% and 5.0%, p<0.001) or CS+IQOS (0.1% and 1.0%, p<0.01; 2.5% and 5.0%, p<0.001) showed dose-dependent decreases in the expression of the epithelial marker E-cadherin when compared with unexposed cells (figure 6b). By contrast, the mesenchymal marker N-cadherin was significantly increased upon exposure to CS+IQOS (1.0%, p<0.001; 2.5% and 5.0%, p<0.05) when compared with unexposed cells (figure 6c). IQOS alone (1.0%, p<0.01) and CS+IQOS (1.0%, p<0.001) also increased N-cadherin expression when compared with CS alone. In addition, upon exposure to CS+IQOS (2.5% and 5.0%, p<0.05), the expression of the mesenchymal marker vimentin also increased when compared with unexposed or CS alone (figure 6d). Exposure to CS+IQOS resulted in significantly increased protein expression of α-SMA at all levels of exposure compared with unexposed cells (0.1% and 1.0%, p<0.001; 2.5% and 5.0%, p<0.05) (figure 6e). Of note, IQOS extract alone elicited a dose-dependent increase in the expression of α-SMA. Additionally, CS+IQOS exposure (0.1% and 1.0%, p<0.01) also displayed a significant increase in mesenchymal markers compared with CS and IQOS alone. These results indicate that dual smoke extract exposure causes enhanced changes to proteins critical to EMT in airway epithelial cells compared with CS or IQOS single exposures.

## Discussion

CS is a major risk factor for the development of COPD and can also promote acute lung injury, pulmonary fibrosis, asthma and 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 signalling. Our study findings indicate that the combined exposure of CS and IQOS (dual) has a negative impact on airway epithelial cell functions in vitro, resulting in decreased cell viability, activation of pathogenic EMT signalling, increased oxidative stress, and mitochondrial dysfunction.

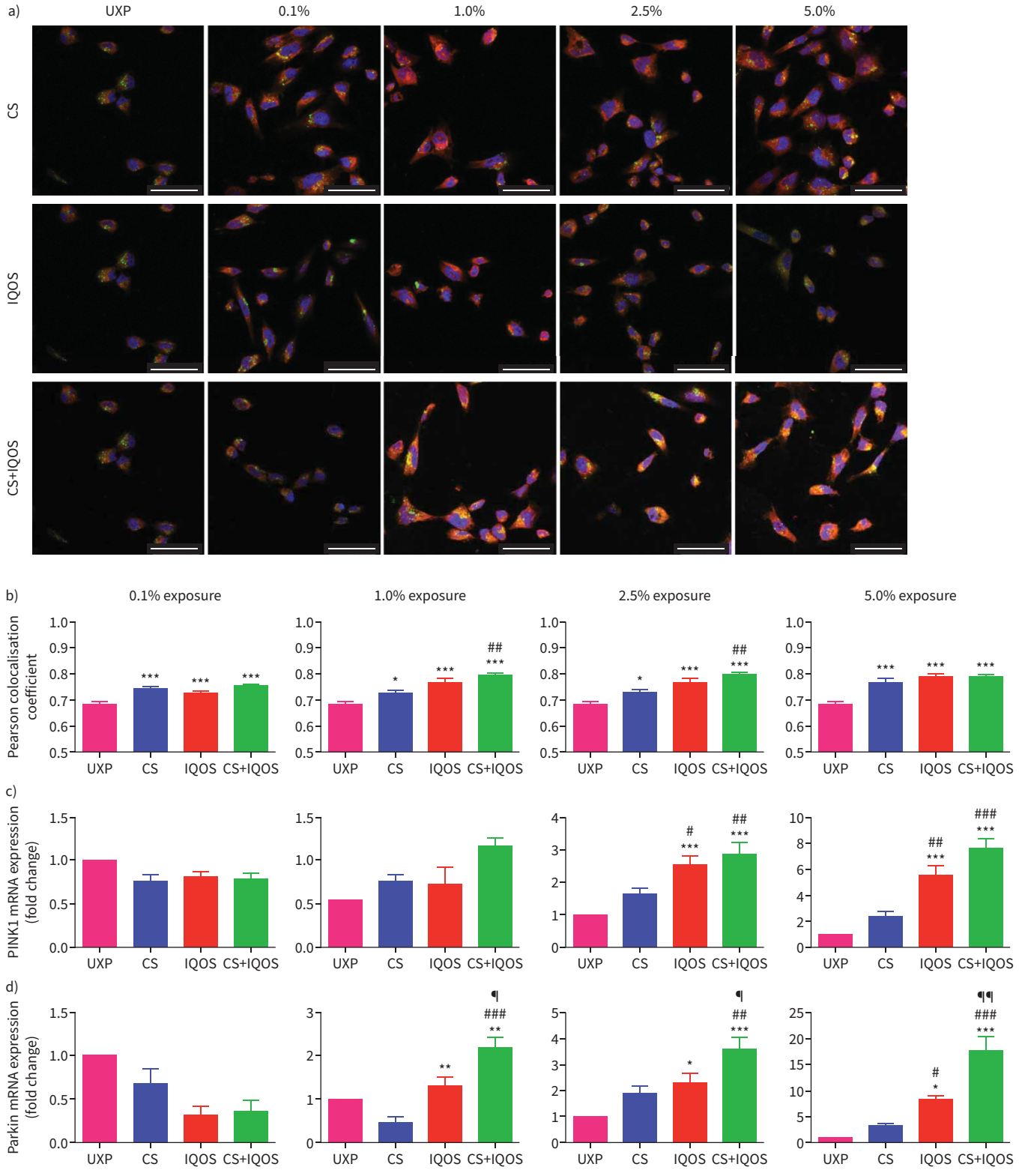


**FIGURE 5** Dual exposure induces aberrant mitophagy. a) Representative images of BEAS-2B cells stained with MitoTracker and LysoTracker to show mitophagy upon exposure to cigarette smoke (CS), IQOS or CS+IQOS for 24 h using confocal laser scanning microscopy (×630 magnification). Scale bar: 50 µm. b) Bar graphs representing Pearson's colocalisation coefficient upon MitoTracker and LysoTracker staining. c, d) Bar graphs representing changes in mRNA gene expression levels of c) PINK1 and d) Parkin upon exposure of BEAS-2B cells to CS, IQOS or CS+IQOS for 24 h. Data are presented as mean±SEM (n=5). One-way ANOVA followed by post-hoc Tukey's test was used to define statistical significance. *: p<0.5; **: p<0.01; ***: p<0.001 *versus* unexposed (UXP). $^{\#}$: p<0.05; $^{\#\#}$: p<0.01; $^{\#\#\#}$: p<0.001 *versus* CS-exposed. $^{¶}$: p<0.05; $^{¶¶}$: p<0.01 *versus* IQOS-exposed.

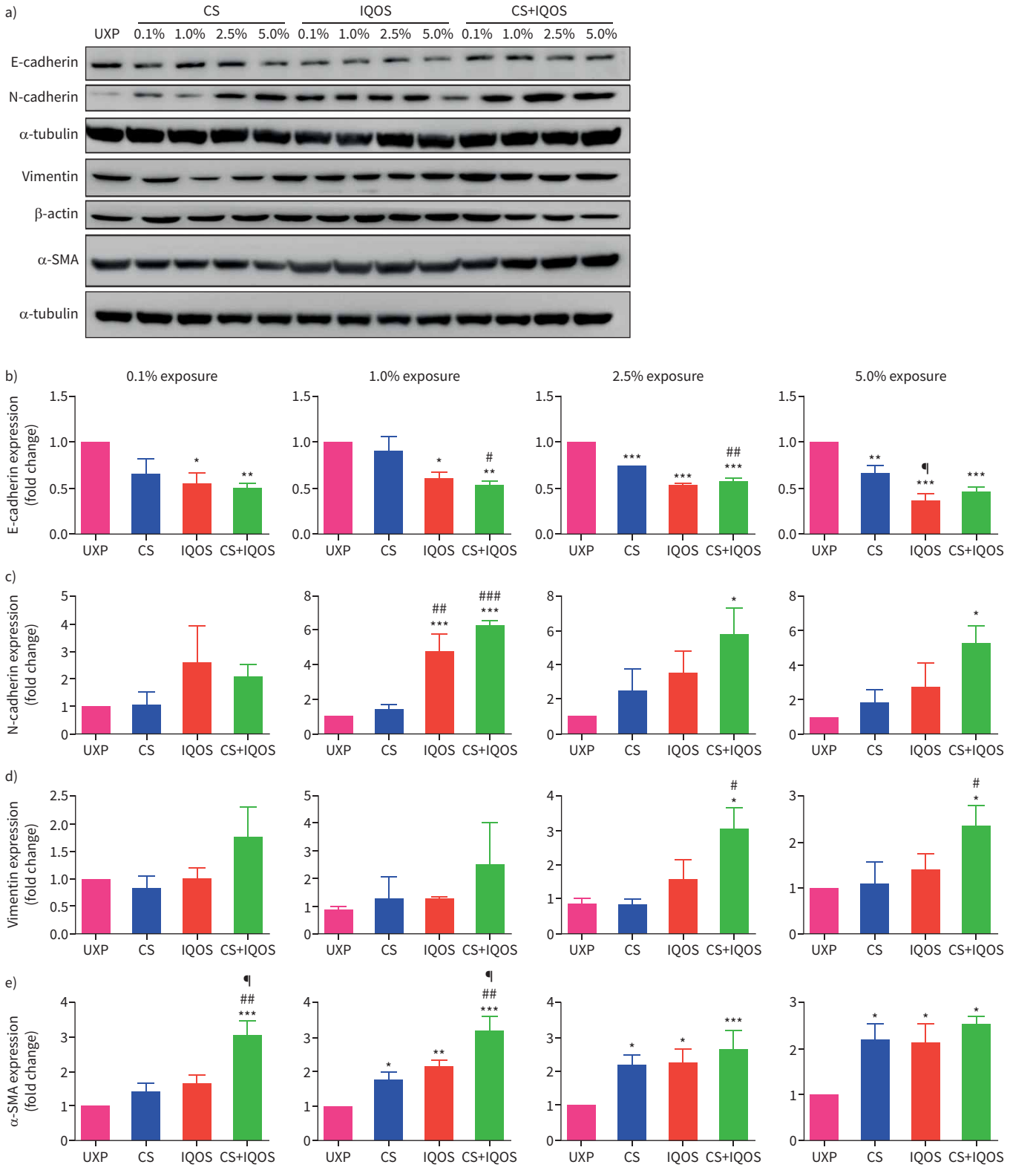


**FIGURE 6** Cigarette smoke (CS) and IQOS exposure alone and dual exposure (CS+IQOS) drive epithelial–mesenchymal transition. a) Representative protein expression of E-cadherin, N-cadherin, vimentin and α-smooth muscle actin (α-SMA) upon exposure to CS, IQOS or CS+IQOS for 24 h. b–e) Bar graphs representing densitometric analysis of b) E-cadherin, c) N-cadherin, d) vimentin and e) α-SMA. Data are presented as mean±SEM (n=5). One-way ANOVA followed by post-hoc Tukey's test was used to define statistical significance. *: p<0.5; **: p<0.01 *versus* unexposed (UXP). #: p<0.05 *versus* CS-exposed. ¶: p<0.05 *versus* IQOS-exposed.

Globally, cigarette smoking was on the decline in 21st century. As a result, tobacco companies began developing alternatives to traditional cigarettes 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, but many end up using both IQOS and conventional cigarettes [38]. 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 with IQOS exposure alone. Additionally, even lower concentrations of CS+IQOS exposure were sufficient to cause significant oxidative stress compared with CS or IQOS exposure alone. We also found that dual CS+IQOS exposure perturbed mitochondrial homeostasis when compared with CS or IQOS exposure alone. Collectively, CS+IQOS exposure led to changes that initiated pathogenic EMT signalling, 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 CS in various lung cells [13, 39], while there is also evidence to suggest that IQOS is equitoxic to traditional CS [39]. 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 [40]. Cigarette smoking is known to generate various toxic chemicals like carbon monoxide, hydrogen cyanide, nitrogen oxides, formaldehyde, acrolein, benzene, nitrosamines, nicotine, phenol, polyaromatic hydrocarbons and many others [41], while IQOS generates several toxic chemicals including various carbonyls, namely formaldehyde, acetaldehyde, propionaldehyde, methacrolein, acrolein, butyraldehyde, valeraldehyde, glyoxal and methyl glyoxal [42]. All these chemicals released from CS and IQOS can generate mitochondrial ROS, the initial response to activate oxidative stress. As reported previously [43], 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 with CS alone. In addition, CS +IQOS exposure results in a significant elevation in ROS and mitochondrial superoxide generation compared with CS or IQOS exposure alone. Our findings reveal that dual exposure induced excessive oxidative stress, which is a trigger for pathogenic signalling in obstructive lung diseases.

Mitochondrial membrane potential is also essential in determining oxidative stress-induced cellular apoptosis. We know that tobacco smoke induces mitochondrial depolarisation-mediated cell death [44]. In our study, we see a dose-dependent increase in mitochondrial depolarisation with CS+IQOS exposure compared with 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 [45]. 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 mitochondria [46]. The literature indicates that CS leads to mitochondrial fission and fragmentation associated with increased mitochondrial count and decreased average mitochondrial size [35, 47, 48]. Other studies also indicate that IQOS and CS significantly reduce mitochondrial and lysosomal activity on various lung-derived cells [39]. 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. Furthermore, dual CS +IQOS exposure accentuated the harmful effects in epithelial cells compared with 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 with 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 [49]. PINK1 and Parkin function in a common signalling pathway known to regulate mitochondrial homeostasis and quality control, including mitophagy [50]. 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 compared with 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 signalling, which is a key regulator of mitochondrial health and may play a critical pathogenic role in the development of chronic lung diseases [51]. CS-mediated

EMT activation also plays an important role in pathological signalling leading to irreversible lung damage [52]. Our results showed that dual exposure significantly augmented EMT signalling compared with CS or IQOS alone. The preponderance of data in our study shows that simultaneous mechanisms known to be involved in the pathogenesis of common chronic lung diseases (such as COPD, asthma, 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 epithelial 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 the combined use of IQOS with other products. Furthermore, 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 summarised in figure 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.

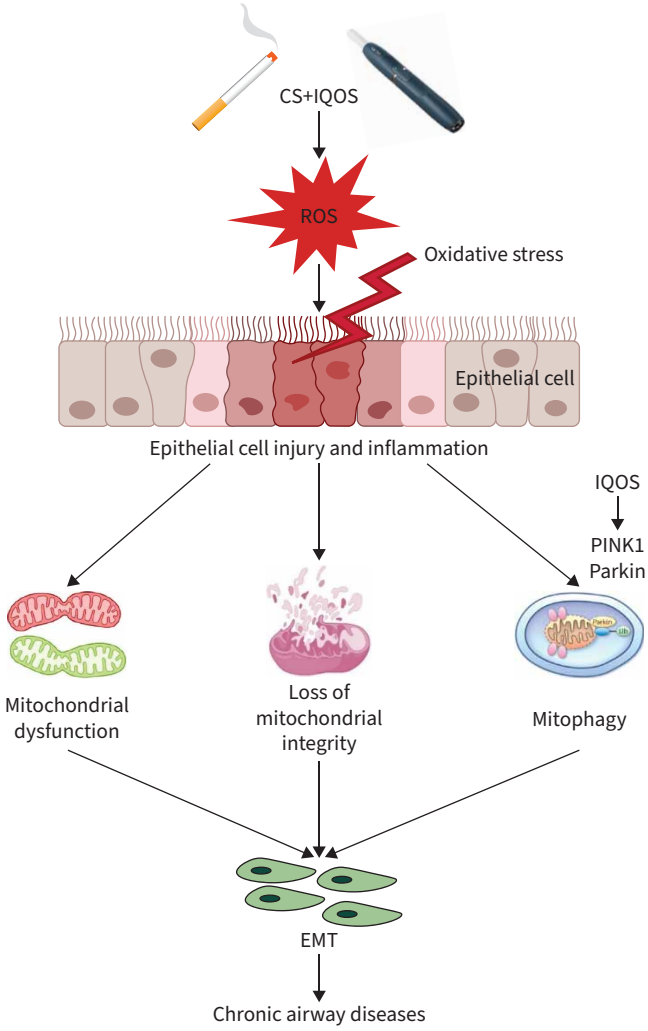


**FIGURE 7** Mechanisms by which dual cigarette smoke (CS) and IQOS exposure can promote airway disease pathology. This schematic depicts how cigarette smoking and IQOS exposure can induce pathogenic signalling in airway epithelial cells. These changes are predominantly driven by mitochondrial reactive 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 epithelial–mesenchymal transition (EMT) signalling, an important mediator of airway fibrosis in chronic airways disease.

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