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E-cigarette-induced changes in cell stress and mitochondrial function

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Abstract

Inhaling aerosols from electronic nicotine delivery systems, such as e-cigarettes (e-cigs), may pose health risks beyond those caused by nicotine intake. Exposure to e-cig aerosols can lead to the release of exosomes and metabolites into the bloodstream, potentially affecting mitochondrial physiology across the body, leading to chronic inflammatory diseases. In this study we assessed the effects of e-cig use by young healthy human subjects on the circulating exosome profile and markers of cell stress, and also defined the effects of e-cig user plasma on mitochondrial function in endothelial cells (EA. Hy 926) and epithelial cells (A549) via adoptive transfer. E-cig users had altered plasma exosome profiles, with significantly increased levels of cell free mitochondrial DNA (mtDNA), protein carbonyls, and 4-HNE relative to non-users. Plasma from e-cig users decreased maximal mitochondrial respiration and spare capacity of cells, while

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Disclosures

The authors have no relevant disclosures.

Declaration of competing interest

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also increasing metabolic stress, as evidenced by changes in mitochondrial phenotype from basal to stressed in both endothelial and epithelial cells, which was corroborated by electron microscopy demonstrating structural changes in mitochondria. Mitochondrial membrane potential (MMP) and reactive oxygen species (ROS) levels significantly increased in e-cig plasma-subjected cells. Overall, we identified alterations in plasma exosome profiles and increased markers of mitochondrial stress in e-cig users and evidence that circulating factors within plasma from e-cig users drives metabolic stress in endothelial and epithelial cells. Our results imply that e-cig use adversely affects mitochondrial function, leading to stress and potentially chronic inflammation across the body.

Keywords

Tobacco; Electronic (e)-cigarette; Mitochondria; Reactive oxygen species; Metabolic stress; Endothelial cells; Epithelial cells

1. Introduction

Tobacco has been smoked for over 5000 years and its modern day use continues to play a significant role in increasing the likelihood of developing lung and heart diseases [1]. Cigarette smoke is known to cause and accelerate development of chronic diseases and cardiovascular issues through mechanisms including increased oxidative stress, inflammation, lipid oxidation, thrombogenesis, and sympathetic activity [1-6]. Emerging nicotine delivery systems, including e-cigarettes (e-cigs) and vaping devices, have introduced uncertainties regarding their impact on cardiovascular and cardiopulmonary health [7-10]. Recent evidence suggests that these electronic nicotine delivery systems (ENDS) will pose significant risks to users, extending beyond the effects of nicotine alone. One of the mechanisms by which e-cigs will cause disease is through induction of oxidative damage [11-15]. Further, studies have linked e-cig constituents to cardiovascular disease risk through oxidative stress pathways and induction of vascular cell dysfunction [16].

Mitochondria, essential organelles sensitive to cellular stress, are pivotal in signaling stress responses and metabolic changes [2,17]. Dysfunctional mitochondria are associated with activating the integrated stress response (ISR) and metabolic reprogramming, representing hallmarks of mitochondrial dysfunction [18,19]. E-cig aerosols induce stem cell toxicity, resulting in mitochondrial changes and impaired autophagy [17]. The e-cig flavorant cinnamaldehyde disrupts mitochondrial respiration and glycolytic function in bronchial epithelial cells, leading to reduced intracellular ATP levels and impaired cilia motility [20]. Furthermore, JUUL menthol e-cig pods have been found to disrupt mitochondrial respiration by causing proton leakage and decreasing mitochondrial complex I, II, and IV levels [8,21]. After 24 h of exposure, overall mitochondrial respiration decreases, highlighting the detrimental effects of e-cigs on mitochondria and other organs [8,21]. Interestingly, Virginia tobacco pods do not significantly impact mitochondrial respiration but increase the expression of specific mitochondrial complexes [21]. The work above underscores the consequences of different e-cig constituents on lung epithelial cell mitochondria. However, effects on endothelial cell mitochondria remain to be elucidated. To better define the effects

of e-cig aerosols and their associated secreted mediators on cell metabolism and physiology, we conducted detailed studies of plasma from young, healthy e-cig users.

Plasma and its constituents are critical elements of the circulatory system, serving various indispensable functions. When individuals inhale e-cig aerosols, they rapidly cross pulmonary epithelium and endothelium to enter the bloodstream, and thus come into contact with cells throughout the body. Interactions between e-cig aerosol chemicals and pulmonary epithelial and vascular endothelial cells can lead to release of cellular content, including exosomes, into the circulation. Exosomes are small membrane-enclosed vesicles released by cells into the extracellular environment, and can carry a wide array of bioactive molecules, including proteins, lipids, and nucleic acids [22]. Exosomes are crucial in intercellular communication, immune responses, and tissue healing [23]. Surprisingly, little research has been done to explore exosome profiles in individuals using emerging tobacco products, including e-cigs [24].

Exosomes and plasma components released through the interactions of e-cig aerosol chemicals with cells within the vasculature may induce mitochondrial dysfunction, cellular stress, and ultimately drive chronic inflammation leading to a multitude of diseases [25].

Here we aim to elucidate the relationships between e-cig use, plasma components (including exosomes), and mitochondrial cell biology, to add critical insights into the health risks associated with e-cig use.

2. Materials and methods

2.1. Human subjects and specimens

A longitudinal cohort investigation of 21 subjects who exclusively used e-cigs and 10 non-users (non-smokers/non-vapers) was carried out [26]. The inclusion criteria encompassed individuals actively using e-cigs without any prior lung disease and normal lung function, falling within the age range of 18–30. Exclusion criteria included use of combustible tobacco within the prior year, and use of any cannabis product more than once per month. Confirmation of e-cig use was accomplished through in-person interviews and assessment of plasma cotinine levels. Active e-cig use was defined as the consumption of ≥ 0.5 –1 mL of e-liquid per day or 3.5–7 mL per week for ≥ 6 months. Plasma was collected using EDTA plasma isolator tubes (BD Biosciences) and preserved at -70°C until use. This study was approved by the Institutional Review Board of the University of California San Diego (Project 160204) and conducted in accordance with the principles outlined in the Declaration of Helsinki. All participants provided written informed consent.

2.2. Plasma exosome profile

The size of exosomes and macrovesicles in the plasma of e-cig users and non-users were quantified using the NanoSight NS300 system (Malvern Technologies, Malvern, UK), configured with a 488 nm laser. Plasma samples were diluted 1:100 in particle-free PBS (Gibco, Waltham, MA, USA) to an acceptable concentration per the manufacturer's recommendations. The samples were analyzed under constant flow conditions (flow rate = 50) at 25°C . Data were analyzed using NTA 3.1.54 software with a detection threshold of

5. For the validation cohort, 15 s × 60 s videos were captured with a camera level 16 and a detection threshold 7.

2.3. Quantification of mtDNA in plasma

Absolute quantification of mitochondrial DNA (mtDNA) in plasma by droplet digital PCR (ddPCR) the protocol published in our prior study [27]. We targeted sequences from the mitochondrial genome's NADH dehydrogenase 1 (ND1) gene, specifically a 69 base pair region between positions 3484 and 3553. The primers and probes used were:

Forward: 5'-CCCTAAAACCCGCCACATCT-3'

Reverse: 5'-GAGCGATGGTGAGAGCTAAGGT-3'

Probe: 5' (FAM) CCATCACCTCTACATCACCGCCC (BHQ-1) 3'

A 20 µL ddPCR reaction mixture was prepared, which included 10 µL of 2x ddPCR supermix for probes, 1 µL of 20x primer/probe mix (final concentrations of 900 nM forward primer, 900 nM reverse primer, and 250 nM probe), 1 µL of plasma sample, and 8 µL of water. This mixture and 70 µL of droplet-generating oil were placed in a plastic cartridge, sealed, and processed with a droplet generator machine (Bio-Rad Laboratories, Hercules, CA). The resulting droplets were transferred to a 96-well ddPCR plate and incubated at 95 °C for 10 min. Thermal cycling was then carried out at 94 °C for 30 s and 60 °C for 60 s, for a total of 45 cycles, using an Applied Biosystems Veriti 96-well Thermal Cycler (Applied Biosystems, Waltham, MA). After cycling, droplets were analyzed with a QX200 droplet reader (Bio-Rad Laboratories, Hercules, CA), and the data were processed using QuantSoft version 1.7.4 software. This software identified PCR-positive and PCR-negative droplets to provide absolute quantification of the target mtDNA sequences, with the fraction of positive droplets used to calculate the target sequence concentration in copies per microliter.

2.4. Determination of plasma protein carbonyls

Plasma protein carbonyls (PCO) were measured by ELISA (Enzo Life Sciences, ALX-850-312-KI01) per kit recommendations. Carbonylated proteins from lot no. M44BZ30 (Enzo Life Sciences ALX-850-312-KI01) were used to establish the nmol carbonyls/mg protein standard. E-cig user and non-user plasma samples (60–75 mg/ml) were diluted in DNPH solution, incubated for 45 min to allow PCO derivatization, a 1:200 dilution in ELISA buffer performed, and 200 µL (1–2 µg of protein) added to each well. The plate was incubated overnight at 4 °C to facilitate protein binding. The ELISA assay was conducted according to the manufacturer's instructions, and the absorbance was read at 450 nm (TECAN Infinite® 200 PRO Plate Reader). The calibration line for all assays showed an R² value close to 0.98. The carbonyl content of the samples was calculated using the regression factors (intercept with the y-axis and line slope) obtained from the standard curve.

2.5. Determination of plasma 4-Hydroxynonenal (4-HNE) levels

Plasma 4-HNE levels were quantified via ELISA (Abcam, ab287803). Briefly, 50 µL of plasma samples and standards were loaded into 96-well plates, 50 µL of biotin-labeled 4-HNE antibody was added, and plates were incubated for 45 min at room temperature. The ELISA was carried out according to the manufacturer's protocols, and the final optical

density (OD) was measured at 450 nm. The 4-HNE levels in the samples were calculated using the regression factors (intercept with the y-axis and line slope) obtained from the standard curve.

2.6. Effects of e-cig user plasma on cell metabolism

The oxygen consumption rate (OCR) was measured using a Seahorse XF96 extracellular flux analyzer (Seahorse Bioscience, Agilent, TX, USA). To quantify metabolism, endothelial cells (EA.hy 926; 40,000 cells/well) and epithelial cells (A549; 50,000 cells/well) were plated in XF96 Cell Culture Microplates (103774-100, Agilent) and incubated overnight. Cells were treated with e-cig user and non-user plasma (1 %, 1 h). Following standard experimental procedures, EA.hy 926 and A549 cells were equilibrated in fresh XF DMEM base medium (103575-100, Agilent) containing 25 mM glucose (103577-100, Agilent), 1 mM sodium pyruvate (103578-100, Agilent), and 2 mM L-glutamine (103579-100, Agilent) for 1 h at 37 °C without additional CO₂. For the Mito Stress test, 1.0 μM oligomycin A, 1.5 μM FCCP, and 0.5 μM rotenone/antimycin for EA.hy 926 and 1.0 μM oligomycin A, two μM FCCP, and 0.5 μM rotenone/antimycin A for A549 cells were sequentially injected into each well as operated by the instrument. An energy phenotype test was used to assess the mitochondrial phenotype (103325-100, Agilent) under similar conditions, following the manufacturer's kit protocols in both cell types cells.

2.7. Effects of e-cig user plasma on cellular ROS

ROS levels induced by plasma from e-cig users in endothelial and epithelial cells were quantified using the 2',7'-dichlorofluorescein diacetate (DCFDA) Cellular ROS Detection Assay Kit (Abcam, Cambridge, UK, ab113851). Cells were cultured in standard DMEM, then collected and seeded into a 96-well microplate with a dark, clear bottom at 25,000 cells/well. After allowing the cells to adhere overnight, the media was discarded, and each well was washed with 100 μL of 1X Buffer, per kit protocol. The 1X Buffer was then replaced with 100 μL of diluted DCFDA Solution per well for cell staining. Cells were incubated with the DCFDA Solution for 1 h at 37 °C in the dark. Following incubation, the DCFDA Solution was replaced with 100 μL of 1X Buffer (1 % FBS) containing 1 % e-cig users and non-user plasma for 1 h at 37 °C in the dark. The plate was imaged and measured using a Citation5 in endpoint mode, with compounds, media, or buffer still present. ROS levels were quantified based on green fluorescence values, represented as Relative Fluorescence Units (RFU). Data extraction and image optimization were performed using Gen 5 software in Citation5.

2.8. Effects of e-cig user plasma on mitochondrial membrane potential ($\Delta\Psi$)

The effect of e-cig user plasma on the mitochondrial membrane potential ($\Delta\Psi$) of endothelial and epithelial cells was assessed using the Mito-ID[®] Membrane Potential Kit (CAT: ENZ-51018-K100, Enzo Life Sciences, NY, USA) according to manufacturer's instructions. Twenty-five thousand cells were seeded per well in a 96-well microplate with a dark, clear bottom and allowed to adhere overnight. Cells were washed with PBS and treated with 1 % e-cig user and non-user plasma samples. After a 1-h incubation at 37 °C, 100 μL of 2 μM MITO-ID[®] Membrane Potential Dye Loading Solution was added, followed by incubation for 30 min in the dark. The orange-to-green fluorescence ratio was measured

via RFU. Data extraction and image optimization were performed using Gen 5 software in Citation 5.

2.9. Electron microscopy analysis of mitochondrial morphology

To determine the effect of e-cig user plasma on endothelial and epithelial cells, 1×10^6 cells were plated in a 6-well plate and incubated overnight at 37 °C. Cells were then treated with 1 % e-cig user and non-user plasma for 1hr at incubator. Cells were washed with PBS and fixed with 10 % glutaraldehyde, 16 % paraformaldehyde, and 0.4M CB. Samples were then sent to the San Diego State University transmission electron microscopy (TEM) core facility for further processing and imaging.

3. Results

3.1. E-cig use associated changes in plasma exosome profiles

The NanoSight system is an advanced nanoparticle tracking analysis (NTA) instrument designed to visualize and measure particles in liquid suspensions in the nanometer range, typically from 10 nm to 1 μ m. NTA analysis demonstrated a significant decrease in the total particle profile in the plasma of e-cigarette users (Fig. 1A). Additionally, a notable change in the number of exosomes, particularly within the 20–60 nm range (Fig. 1B), was observed. These results suggest a shift in the exosome profile of e-cigarette users.

3.2. Increased oxidative damage in e-cig users

Protein carbonylation (PCO) is a form of permanent damage caused by ROS, which leads to functional impairment, and is associated with multiple diseases, including cardiovascular and chronic lung diseases. E-cig users were found to have increased levels of plasma PCO levels relative to non-users ($p < 0.003$; Fig. 1C). 4-HNE is a byproduct of oxidative damage to membrane lipids and can indicate the presence of cellular stress. E-cig users had higher levels of 4-HNE in the plasma than non-users ($p < 0.003$; Fig. 1D), suggesting that e-cig use induces cellular stress *in vivo*.

3.3. Elevated circulating mitochondrial DNA in e-cig users

Cell-free mitochondrial DNA (mtDNA) has gained recognition as a biomarker associated with cellular damage, particularly with elevated plasma levels [28]. We quantified mtDNA in plasma samples using ddPCR and an ND1 gene probe and found elevated levels of mtDNA in e-cig users relative to non-users, 70.25 and 245.06 mean copies/ μ L respectively, ($p < 0.03$; Fig. 2A).

3.4. Altered endothelial and epithelial cell metabolism by exposure to e-cig user plasma

We conducted *in vitro* assessments of metabolic effects of e-cig user plasma (1 % concentration, 1-h conditioning) via adoptive transfer on endothelial cells using the seahorse XF cell mitochondrial stress (Fig. 3A) and energy phenotype test (Fig. 3D). Relative to plasma from non-users, endothelial cells exhibited a reduction in both maximal mitochondrial respiration (%OCR, $p < 0.03$; Fig. 3B) and spare capacity ($p < 0.005$; Fig. 3C) when exposed to e-cig plasma during cell mitochondrial testing. These findings were

substantiated by plasma from e-cig users inducing a stressed mitochondrial phenotype by increasing mitochondrial metabolism ($p < 0.04$; Fig. 3E) vs. glycolysis (Fig. 3F). In response to an increase in cellular energy demand, cells are prompted to shift towards a glycolytic state. However, when e-cig plasma was present, endothelial cells could not change to a glycolytic state in basal respiration.

For A549 epithelial cells (seahorse depicted in Fig. 4A, and energy phenotype in Fig. 4D), maximal mitochondrial respiration ($p < 0.003$; Fig. 4B) and spare capacity ($p < 0.006$; Fig. 4C) significantly decreased when exposed to e-cig plasma. However, the energy phenotype test (Fig. 4D) showed that basal respiration was more glycolytic when exposed to e-cig user plasma. Conversely, mitochondrial metabolism increased ($p < 0.002$; Fig. 4E), and glycolysis decreased ($p < 0.03$; Fig. 4F) when epithelial cells were exposed to e-cig user plasma compared to non-users.

3.5. Altered mitochondrial membrane potentials and increased ROS in cells exposed to e-cig user plasma

Mitochondrial membrane potential increases in endothelial and epithelial cells exposed to plasma from e-cig users ($p < 0.009$ and $p < 0.008$; Fig. 5A & C) suggest a loss of mitochondrial membrane integrity, leading to electron leakage from the mitochondria and increased free electrons within the cell. Consequently, this resulted in elevated levels of intracellular ROS in endothelial and epithelial cells ($p < 0.003$ and $p < 0.001$; Fig. 5B & D), which may be the drivers for the increased protein and lipid oxidative damage detected in e-cig users (Fig. 1).

3.6. The effects of e-cig user plasma on cell mitochondrial morphology

Intracellular mitochondrial morphology was assessed using TEM in e-cig user and non-user plasma environments. In the presence of control plasma, mitochondria in endothelial and epithelial cells displayed a dynamic tubular shape, with well-defined cristae and densely packed matrices, reflecting high metabolic activity (Fig. 6A-B). However, exposure to e-cig user plasma resulted in mitochondria that were elongated with an irregular tubular configuration, accompanied by a significant reduction in matrix density, disordered parallel cristae, and disrupted double membrane structure, indicating compromised structural integrity (Fig. 6C-D). Moreover, significant alterations in mitochondrial size, quantity, and cristae quality were observed in cells exposed to e-cig user plasma (Fig. 6C-D). These findings visually confirm mitochondrial dysfunction induced by factors within e-cig user plasma.

4. Discussion

The existing literature on e-cig-induced oxidative stress remains inconclusive [8,13], with studies yielding conflicting findings on potential harm. The long-term health implications of e-cig use, especially regarding the ability to induce oxidative modifications in plasma biomolecules and the subsequent downstream cellular and metabolic effects, need further elucidation. Our study was designed to address the knowledge gaps in the field by investigating the impact of e-cig use on plasma constituents and the effects of plasma

from e-cig users on cell models of metabolism. Our analysis of plasma from e-cig users revealed significant alterations in exosome profiles and increased levels of protein carbonyls, lipid peroxidation, and mitochondrial DNA as evidence of cellular stress. Our study showed that the components present in the plasma of e-cig users reduced maximal mitochondrial respiration rates in human endothelial and epithelial cells via adoptive transfer *in vitro*. Furthermore, we observed that e-cig plasma markedly elevated metabolic stress, mitochondrial membrane potential, and reactive oxygen species (ROS) levels in endothelial and epithelial cells, and also produce morphological changes in mitochondria observed through TEM analysis. Our work involved two primary aspects: the *in vivo* evaluation of e-cig aerosol-induced stress on subjects, particularly within their plasma, and the *in vitro* analysis of cellular responses to exposing endothelial and epithelial cells to plasma from e-cig users. Our approach provided a broad assessment of the impact of e-cig exposure on the cardiovascular system, encompassing both systemic and cellular effects.

Plasma exosomes are essential mediators of intercellular communication and play a vital role in maintaining homeostasis [22]. Plasma contains multiple extracellular vesicles of different sizes ranging from 50 to 120 nm, typically called exosomes. The exosomes of various sizes and quantities can significantly affect disease diagnosis and progression [22]. Our results revealed a significant decrease in exosomes in the 20–60 nm size range, suggesting vesicles in this size range may be necessary for maintaining cellular homeostasis. Additionally, the total vesicle number in e-cig user plasma showed a significant decrease. Singh et al. [29] also reported a reduction in the exosome count in the plasma of e-cig users and changes in the exosome non-coding RNA profile in e-cig users. Kaur et al. [24] reported a distinct set of microRNAs among subjects exposed to e-cig vapor. Our results align with other findings suggesting that exosomes and their cargo may contribute to cellular stress and altered physiology in e-cig users.

Stress-induced cell dysfunction and death are vital consequences of mtDNA leakage. Consequently, quantifying mtDNA in biological fluids can be a valuable biomarker for tracking disease progression. We employed the ddPCR method to quantify the mt DNA in plasma. ddPCR is a precision-oriented and susceptible molecular biology method that provides the means for the absolute quantification of nucleic acids, notably cell free mtDNA [27,30]. Recent studies have associated e-cig exposure with mtDNA damage and Toll-like receptor 9 (TLR9) activation-mediated atherosclerosis [31,32]. Li et al. [31] also observed elevated levels of damaged mtDNA in the blood of mice exposed to e-cig vapor [31,32]. E-cig aerosols have been shown to induce mitochondrial stress and promote DNA fragmentation in lung fibroblasts, directly impacting mitochondrial function [33]. Our results are in line with prior studies showed elevated levels of mt DNA in human e-cig user plasma. Additionally, conventional and electronic cigarettes disrupt the expression of iron transporters and detoxifying enzymes, leading to mitochondrial depolarisation in brain vascular endothelial cells [34]. Moreover, mtDNA can activate innate immune responses, which may contribute to chronic inflammation [8,35]. E-cig aerosols contain substantial concentrations of highly reactive aldehydes [36,37] which interact with plasma proteins and lipids to form protein carbonyl adducts in the presence of oxygen. This is closely correlated with increased oxidation of LDL proteins observed in e-cig users. 4-HNE is a lipid peroxidation product implicated in oxidative stress and related pathologies [38].

Our results indicate increased levels of 4-HNE in the plasma of e-cig users. Studies have demonstrated e-cig vapor exposure induces the formation of 4-HNE in bronchial epithelial cells, underscoring the role of 4-HNE in the pathophysiology of vaping-related lung injury [39]. E-cig aerosol exposure has been shown to exacerbate cardiovascular oxidative stress, as evidenced by higher levels of 4-HNE-induced protein adducts in ALDH22 cardiomyocytes compared to wild-type ALDH2 cardiomyocytes [37].

The current literature on e-cig-induced oxidative stress has conflicting findings, leaving the potential harm of e-cig use unresolved [15,31]. The long-term health effects of e-cig use, particularly the capacity to induce oxidative stress, need further study [40,41]. Given that circulating plasma metabolites play a crucial role in regulating overall metabolic processes [40,41], examining how e-cig use might induce chronic stress by altering metabolic profiles is essential. To address this knowledge gap, we employed an adaptive transfer model to investigate the effects of e-cig plasma mediators on endothelial and epithelial cells. Epithelial cells in the lungs are the initial point of contact for aerosols, whereas endothelial cells form the first line of defense exposed to plasma mediators in the circulatory system. Our approach focused on the potential pathologic effects of e-cig use in cellular models. Many reports indicate that the plasma from e-cig users contains various metabolites [42], toxic chemicals [1,43] and extracellular vesicles [24,24]. The impact of these plasma components on cells remains poorly understood and is a relatively underexplored area within the e-cig research [43]. Therefore, we explored the effects of plasma on mitochondrial metabolism and function by adding e-cig plasma to endothelial and epithelial cells.

Plasma metabolites can influence cellular physiology [40,41]. E-cig vapors contain various chemical constituents that can dysregulate plasma metabolism [42], potentially affecting mitochondrial function and overall metabolism [42,44]. We investigated the connection between e-cig plasma components and mitochondrial respiration. Using the seahorse mito stress test to quantify mitochondrial metabolism, we observed a significant decrease in maximal respiration, indicated by reduced oxygen consumption in endothelial and epithelial cells. Previous reports suggest that the plasma components inhibit mitochondrial metabolism, which aligns with findings from other studies using similar aerosols [45]. Specifically, cinnamaldehyde in flavoured e-cig liquids can disrupt mitochondrial respiration and glycolytic function in bronchial epithelial cells, reducing intracellular ATP levels and impairing cilia motility [20]. Additionally, JUUL menthol e-cig pods have been shown to disrupt mitochondrial respiration by causing proton leakage and decreasing complex levels [21,42]. Furthermore, e-cig use can induce stem cell toxicity, resulting in mitochondrial changes and impaired autophagy [17]. These cellular alterations can lead to damaged cells, accelerated aging, and mitochondrial disorders, raising concerns about the long-term health implications for e-cig users. We assessed the mitochondrial phenotype using the Energy phenotype test, which evaluates mitochondrial status under high-energy stress status demand conditions. The baseline mitochondrial activity remained unchanged in endothelial cells supplemented with e-cig plasma. However, the mitochondria transitioned from a quiescent state to a stress phenotype under stress. The baseline was initiated with a glycolytic state in epithelial cells with e-cig plasma exposure. Upon stress induction, mitochondria in cells exposed to plasma from e-cig users shifted to a stress mitochondrial phenotype. The mitochondrial phenotypic shift may be due to mitochondrial gene expression and

network reprogramming [46,47]. Our results align with reports on similar mechanisms observed in other studies, which identified a distinct plasma signature associated with human mitochondrial disease by analyzing mitochondrial function in muscle cells [48]. E-cigs induced dysregulation of mitochondrial gene expression and caused alterations in the mitochondrial networks [2,38]. The mitochondrial phenotype was further confirmed using TEM analysis of cells treated with e-cig plasma. In endothelial and epithelial cells treated with control plasma, mitochondrial structure, and cristae organization were well-maintained. However, the endothelial and epithelial cells treated with e-cig plasma showed significant morphological disruptions, including loss of cristae and inner mitochondrial membrane integrity. These structural changes may underlie the observed decrease in maximal respiration and the shift to a stress phenotype in the metabolic assays. To our knowledge, no previous studies have detailed the effects of e-cig plasma on mitochondrial morphology to this extent.

The structural loss of mitochondria we observed with exposure to e-cig plasma may be due to a decrease in mitochondrial membrane potential and an increase in reactive oxygen species in the e-cig plasma-treated state. Mitochondrial membrane potential (MMP) is critical for preserving the electrochemical gradient necessary for ATP production, serving as a key regulator of cellular metabolism and energy production [49]. Maintaining optimal MMP levels is essential to prevent excessive reactive oxygen species (ROS) production within mitochondria [38]. Elevated MMP can lead to increased ROS generation, resulting in cellular damage and contributing to various metabolic disorders [49]. Our findings on ROS and MMP changes in response to exposure to e-cig plasma are consistent with the TEM analysis of mitochondrial morphology. Our results suggest e-cig aerosols interact with cells, inducing stress and releasing byproducts and metabolites. These byproducts and metabolites in the plasma may circulate throughout the body and contribute to oxidative stress-mediated mitochondrial dysfunction.

Our work has some limitations. There was a limited subset of subjects in this study that restricts the absolute sample size. This limitation constrains definitive statistical analyses and points to the need for future research with larger cohorts, specific effects of e-cigarette flavors, and devices on oxidative stress and mitochondrial function. Additionally, factors such as diet, physical activity, and environmental exposures may significantly influence oxidative stress and mitochondrial health in the context of e-cigarette use and should be considered in subsequent studies. There was insufficient plasma volume to isolate exosomes from the plasma of e-cig users and perform plasma metabolomics analysis. In the future, conducting exosome cargo analysis related to e-cig-mediated oxidative stress and mitochondrial physiology would shed additional light on the impacts of e-cig use on metabolism. Further research is warranted to investigate the impact of e-cig aerosols on specific exosome components as they relate to cell metabolism and mitochondrial dynamics.

In conclusion, we showed a shift in the exosome profile in the plasma of e-cig users, novel evidence of mitochondrial stress in the plasma of e-cig users, and evidence for mitochondrial stress in endothelial and epithelial cells exposed to plasma from e-cig users. Our findings indicate that e-cig plasma induces mitochondrial stress and alters mitochondrial dynamics in a manner not previously documented in the literature. These results suggest that e-cig use

exerts deleterious effects on cells that are critical to the cardiovascular system. Our study reinforces growing concerns about the health risks associated with e-cig use and highlights the potentially substantial public health implications of e-cigs.

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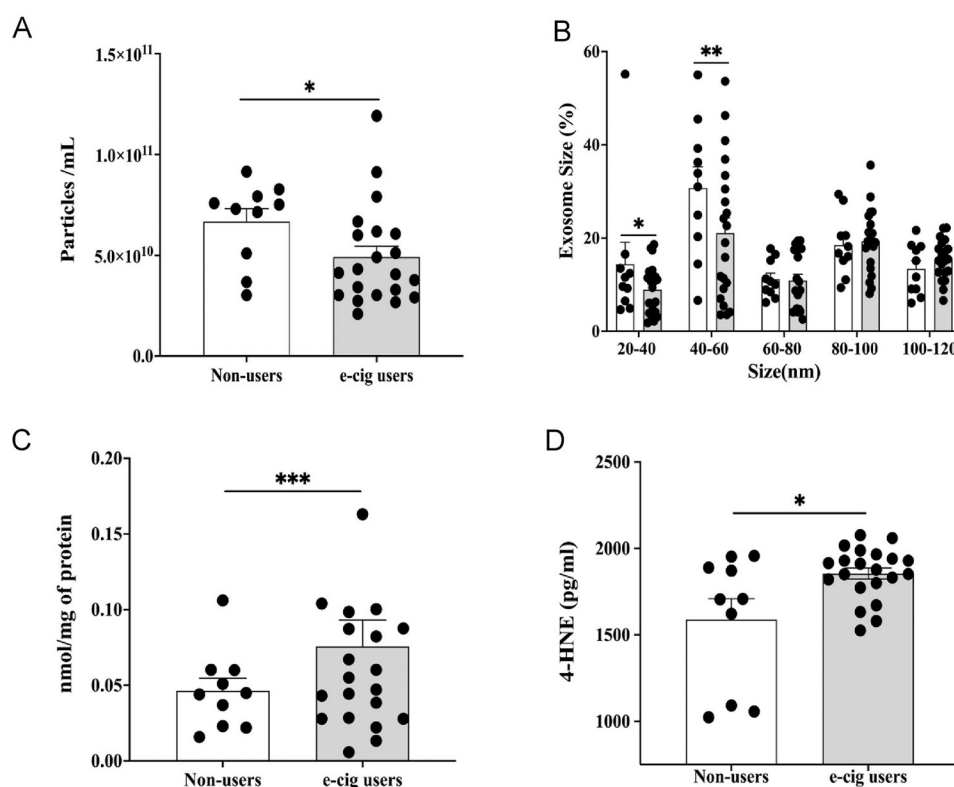


Fig. 1. E-cig use alters nanoparticles, proteins and lipids within the plasma of vapers.

A. Nanoparticle tracking analysis quantification of exosomes and macroparticles in the plasma of e-cig users and non-users, found no difference in total particle counts (Mann-Whitney test, $P < 0.031$). **B.** E-cig users had alterations in exosome size distribution, with fewer 20–40 nm and 40–60 nm particles, relative to non-users (Mann-Whitney test, $P < 0.01$). **C.** Plasma from e-cig users had elevated levels of protein carbonyls (T test, $P < 0.003$), and **D.** elevated levels of 4-HNE (T test $P < 0.003$). Sample sizes: non-users ($n = 10$) and e-cig users ($n = 21$).

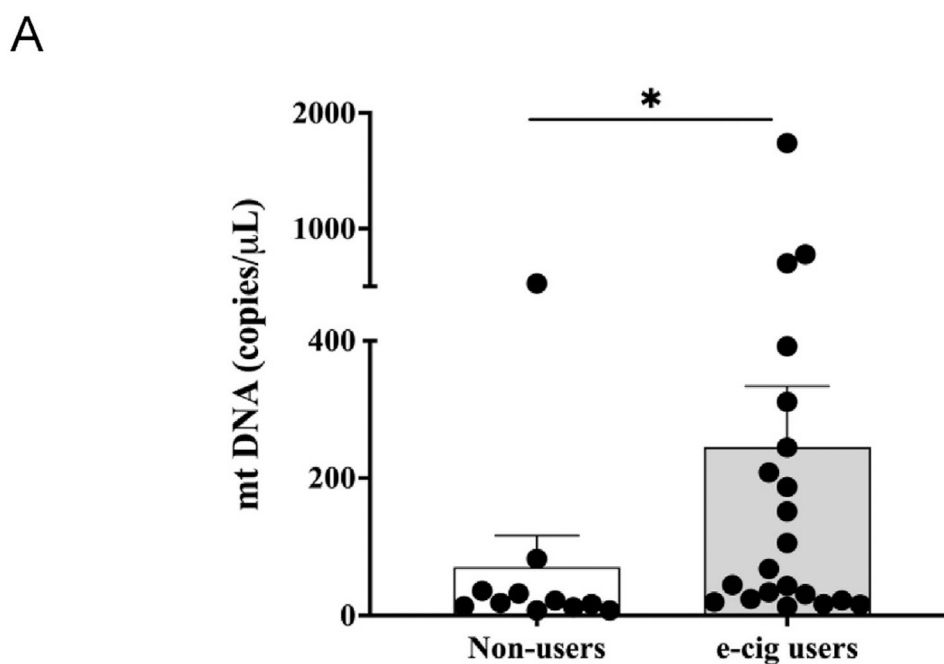


Fig. 2. Circulating mtDNA is elevated in the plasma of e-cig users.

A. Mitochondrial (mt) DNA levels in the plasma of e-cig users and non-users, expressed in copy numbers (Mann-Whitney test, $P < 0.03$). Sample sizes: non-users ($n = 10$) and e-cig users ($n = 21$).

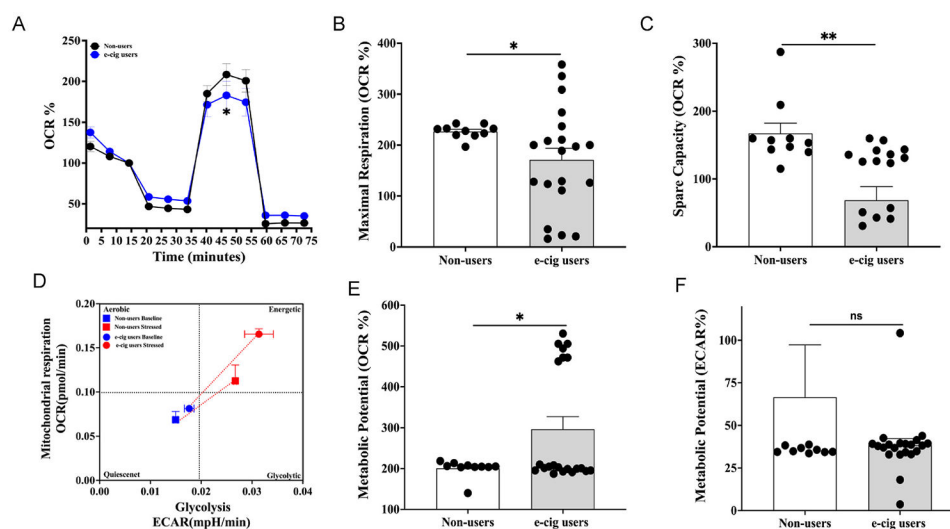


Fig. 3. Exposure to mediators within e-cig plasma alters endothelial cell metabolism.

A. Seahorse XF96 mitochondrial stress test profile demonstrates diminished %OCR in endothelial cells exposed to plasma from e-cig users (%OCR, $P < 0.01$). **B.** Maximal respiration was reduced in endothelial cells exposed to e-cig user plasma (%OCR, $P < 0.03$). **C.** E-cig plasma exposure reduced spare capacity in endothelial cells (%OCR, $P < 0.005$). **D.** Mitochondrial energy phenotype map of non-users (squares) and e-cig users (circles), at baseline (blue) and under stress (red). **E.** E-cig plasma exposure increased the metabolic potential (%OCR, $P < 0.045$). **F.** Lactic acid production during glycolysis was unchanged. Statistical analysis with Mann-Whitney test; Sample sizes: non-users ($n = 10$) and e-cig users ($n = 21$).

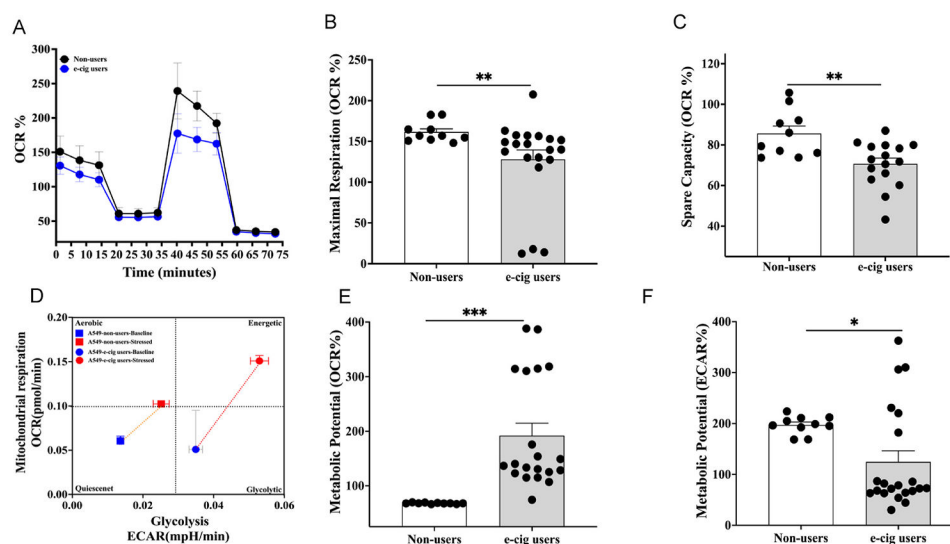


Fig. 4. Exposure to mediators within e-cig plasma alters epithelial cell metabolism.

A. Seahorse XF96 mitochondrial stress test profile demonstrates similar % OCR in epithelial cells exposed to plasma from e-cig users and non-users. **B.** Maximal respiration was reduced in epithelial cells exposed to e-cig user plasma (% OCR, $P < 0.003$). **C.** E-cig plasma exposure reduced spare capacity in epithelial cells (% OCR, $P < 0.006$). **D.** Mitochondrial energy phenotype map of non-users (squares) and e-cig users (circles), at baseline (blue) and under stress (red). **E.** E-cig user plasma exposure increased the metabolic potential of epithelial cells (% OCR, $P < 0.01$). **F.** Lactic acid production during glycolysis was reduced in epithelial cells exposed to e-cig user plasma (% OCR, $P = 0.03$). Mann-Whitney test; Sample sizes: non-users ($n = 10$) and e-cig users ($n = 21$).

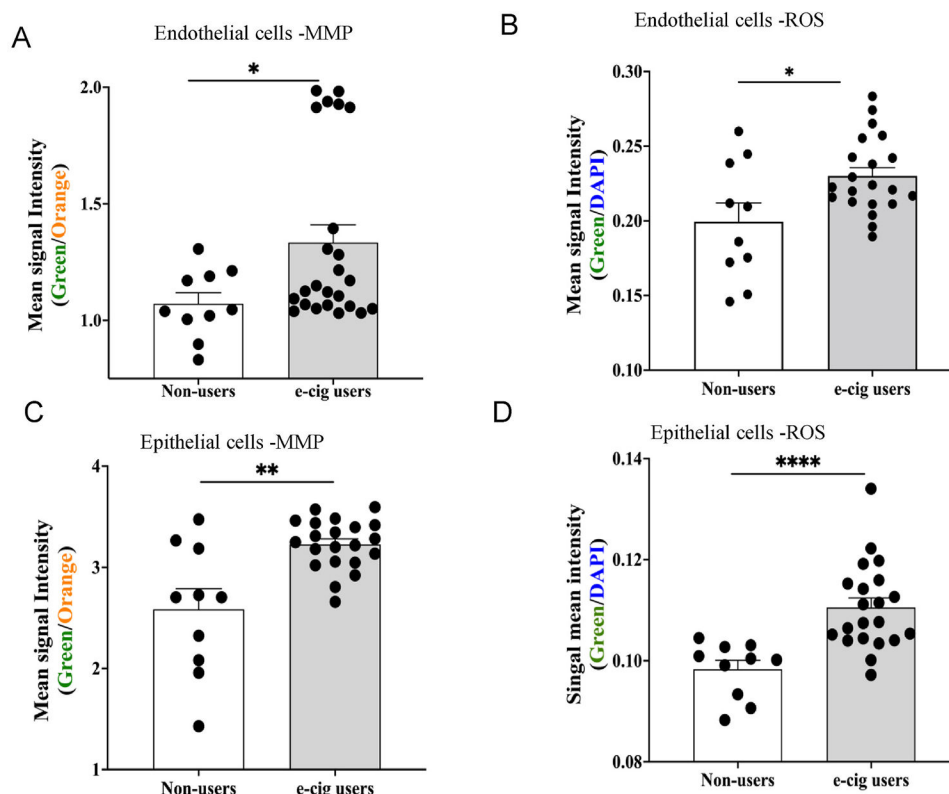


Fig. 5. Increased mitochondrial membrane potential and ROS levels of endothelial and epithelial cells treated with e-cig plasma.

Mitochondrial membrane potential (MMP) was determined using MITO-ID cationic dye, which changes fluorescence from green to yellow. Intracellular ROS was determined using Cell ROX Green, quantified with the Cytation-5. **A.** Membrane potential of EA.hy 926 endothelial cells ($p < 0.01$). **B.** Intracellular ROS levels were increased in EA.hy 926 endothelial cells by exposure to plasma from e-cig users versus non-users ($p < 0.01$). **C.** Membrane potential of A549 epithelial cells ($p < 0.01$). **D.** Intracellular ROS levels were increased in A549 cells exposed to e-cig user plasma ($p < 0.001$). Mann-Whitney test; Sample sizes: e-cig users ($n = 21$) and non-users ($n = 10$).

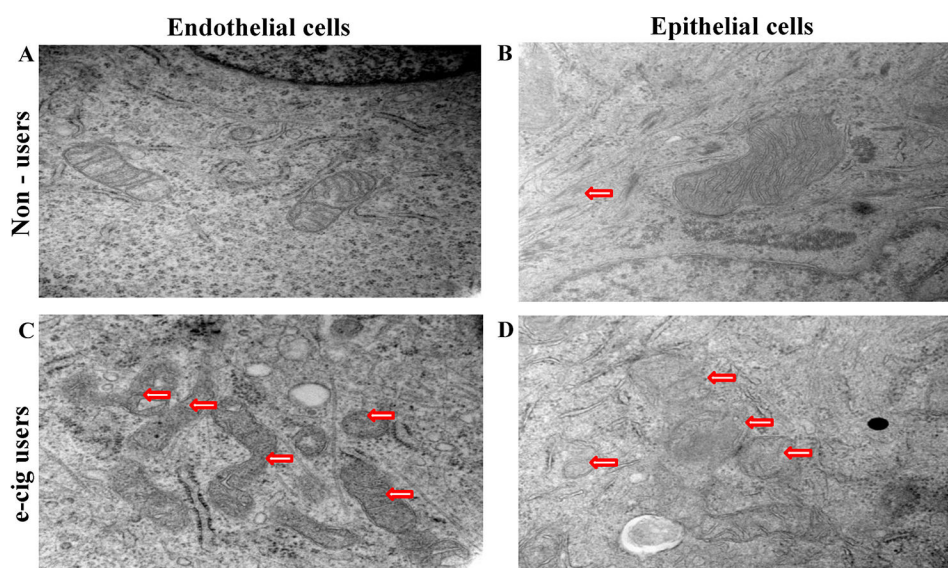


Fig. 6. E-cig user plasma induces alterations in mitochondrial morphology in endothelial and epithelial cells.

A. Endothelial and **B.** epithelial cells exposed to non-user plasma demonstrate normal mitochondrial morphology. Incubation of **C.** endothelial and **D.** epithelial cells with e-cig user plasma led to changes in size and quantity of mitochondria, as well as elongation, irregular tubular configuration, decreased matrix density, disordered parallel cristae, and disrupted double membrane structure. Magnification of 4200 $\times$, with a scale of 500 nm.