Online Data Supplement

Excess mucus viscosity and airway dehydration impact COPD airway clearance

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Extended Materials and Methods

Smoke exposure

Primary HBE cells were apically exposed to 2% cigarette smoke extract (CSE) [1] or DMSO vehicle (24 hr) prior to imaging. All cigarette smoke was generated from 3R4F cigarettes (University of Kentucky, Lexington, Kentucky, USA).

Standardized anesthesia and intubation protocol

Ferrets (0.6-1.8 kg in weight) were anesthetized with an IACUC-approved formulation of dexmedetomidine (0.4 mg/kg, IM) in combination with ketamine (2.8 mg/kg, IM). Ophthalmic lubricant was applied to the eyes once sedation was achieved. The animals were placed on a heated operating table until recovery. Atipamezole was given as a reversal agent at an equal volume as the dexmedetomidine.

In vivo mucociliary clearance

Sedated ferrets were placed in nose-only restraint tubes similar to the ones used for smoke exposures for administration of ^{99}Tc. Regions of interest (ROI) corresponding to the entire left and right lungs were determined as indicated by concomitant chest radiograph, and counts measured and calculated after correction for radioactive decay. Results were expressed as percentage of radioactivity present in the initial baseline image. Data are plotted as retention of radioactivity vs. time for the whole lung.

Tissue preparation

Connective tissue was removed from excised tracheal and bronchial sections before opening each along the non-cartilaginous tissue for imaging. Samples were incubated at 37°C (5% CO_2) between imaging sessions. All agents were added to the basolateral surface of the trachea (so as not to add exogenous fluid to the apical surface), and stimulants were allowed to incubate for a sufficient duration (30 min) to impart their effects. Indomethacin was used to avoid prostaglandin signaling as a mediator of pharmacologic agents [2], while low-dose carbachol helped stimulate some epithelial secretion for additional mucus transport measurements. Following these imaging treatments, each ferret trachea was then treated with a combination of higher concentration carbachol and phenylephrine, which was necessary to stimulate maximal secretion (5-30 µL collected volume) for subsequent collection and use in mucus-related assays.

Micro-optical coherence tomography (µOCT) imaging

This real-time system provides high-resolution, cross-sectional images of the airway epithelial surface, thereby allowing for simultaneous quantification of airway surface liquid (ASL) depth, periciliary layer (PCL) height, cilia beat frequency (CBF), and mucociliary transport (MCT) rate in a co-localized fashion. Multiple ROI (2-4 per transwell filter or at least 10 per trachea) were imaged to account for variability within individual samples. Cells were imaged at baseline and 24 hr post-exposure.

Particle tracking microrheology (PTM)

Fluorescent beads were imaged using TRITC (500-nm) and GFP (1- μm) channels in the same ROI, and in the same or adjacent plane. 4-5 ROI were imaged per coverslip, with 10-25 individual particles per ROI tracked using ImageJ SpotTracker (NIH); these tracks were then sorted and analyzed using custom MatLab scripts to quantify mean-squared displacement of particles over time, from which effective viscosity can be calculated [3].

Histological staining.

Formalin-fixed human and ferret airway tissues were stained with hemotoxylin and eosin (H&E) or Alcian blue/periodic acid-Schiff (AB/PAS), to assess smoke-induced changes in morphology and mucus-producing structures, respectively.

Statistical analysis.

All groups of data were analyzed using the D'Agostino-Pearson normality test to determine whether a parametric or non-parametric test should be used. Results for airway microanatomy and viscoelasticity metrics between control and smoke-exposed tissues over time were assessed by two-way ANOVA, and those between control and COPD or smoke-exposed groups were compared using unpaired Mann-Whitney or t-test. Wilcoxon was used to compare paired measures pre- and post-stimulation within each exposure group. Correlations between metrics were calculated using linear or semi-log regression methods. A univariate model was performed to assess for effects on mean MCT. A linear mixed model for repeated measures analysis was used to model MCT as a repeated measure (technical and biological replicates) using

previously published statistical methods [4, 5], and predictors included time effects, cohort effects, sex effects, smoking effects, and the potential for interaction among them. A separate linear mixed model was used to assess the relationship between MCT and mean ASL, mean PCL, and mean CBF as independent variables along with time category, smoking status, cohort, and sex. Random effects included intercept and repeated measures at baseline, or random intercept only. Unstructured random effects covariance matrix was used for each analysis. The final models omitted interaction terms between predictor variables and time or cohort, as these were not significant and did not alter conclusions. R^2 were estimated by previous methods [6].

Supplemental Tables

Table S1. Univariate linear regression analysis for mean MCT in steady-state conditions. By ferret (N=54).

Predictor	β	P-value	R^2
Smoke	-3.09	**< 0.028**	0.089
CBF	1.34	**< 0.007**	0.129
PCL	0.530	0.608	0.005
ASL	-0.40	0.114	0.047
Female	-0.22	0.879	0.000

Table S2. Tissue donor demographics.

	Non-Smoker (n = 3)	**Healthy Smoker** (n = 6)	**COPD** (n = 3)
Gender	Female (1) Male (2)	Male (6)	Female (2) Male (1)
Age (years)	44 ± 19.8	44.5 ± 9.8	51.3 ± 9.1
Race	White (2) Black (1)	White (6)	White (1) Black (2)
Donors with emphysema	0	0	1
Smoking history (Pack-years)	N/A	34.3 ± 17.5	36.7 ± 29.3

Supplemental Figure Legends

Figure S1. Deposition of Tc^{99}-DTPA in ferret tissues. (A) Representative uncorrected images of Tc^{99}-DTPA deposition in the lungs acquired at baseline (Time 0). **(B)** Total deposition of radiolabel at Time 0 was quantified before assessment of clearance. **(C)** Systemic absorption of Tc^{99}-DTPA as monitored by uptake into forearm muscles.

Figure S2. Schematic of experimental protocol for ferret trachea. Upon euthanization, the trachea was removed from each animal and immediately dissected for μOCT imaging, followed by cholinergic stimulation of mucus secretion for assessment of mucus properties such as microrheology and percent solids by weight. Thick borders indicate endpoint collection throughout the protocol. Capitalized words indicate key timepoints for endpoints. Abbreviations: μOCT = micro-optical coherence tomography, PTM = particle tracking microrheology.

Figure S3. Distribution of μOCT-quantified airway epithelial function by region of interest (ROI). μOCT was used to measure **(A)** ASL depth, **(B)** PCL height, **(C)** CBF, and **(D)** MCT rate at multiple ROI per ferret trachea, to account for the local microenvironment. Data is presented as box-and-whisker (Tukey method). n = 1078 videos.

Figure S4. Cigarette smoke exposure reduces MCT in primary HBE cells. (A) Representative μOCT stills of primary HBE cells after 24-hour apical treatment with 2% cigarette smoke extract (CSE) or DMSO control. Red line = ASL. Yellow line = PCL. **B-**

C: Quantification of *in vitro* ASL **(B)** and MCT **(C)** following exposure. *n* = 10 ROI, **p < 0.01, ***p < 0.001 compared to vehicle control, as determined by unpaired Mann-Whitney (ASL) and t-test (MCT).

Video S1. Representative μOCT video of mucus transport in a trachea excised from an air control ferret.

Video S2. Representative μOCT video of mucus transport in trachea from a smoke-exposed ferret.

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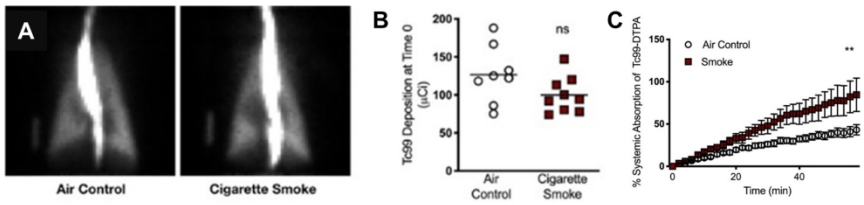

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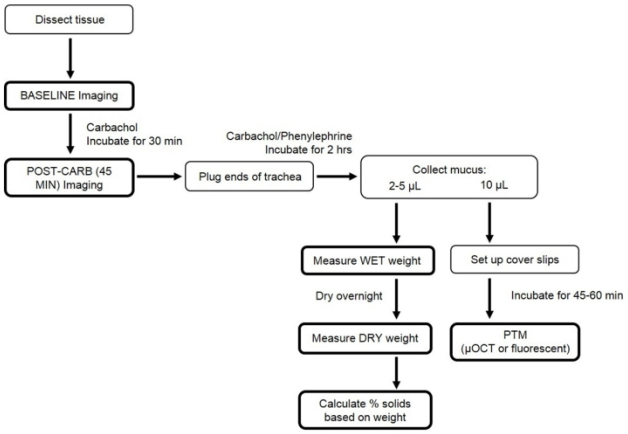

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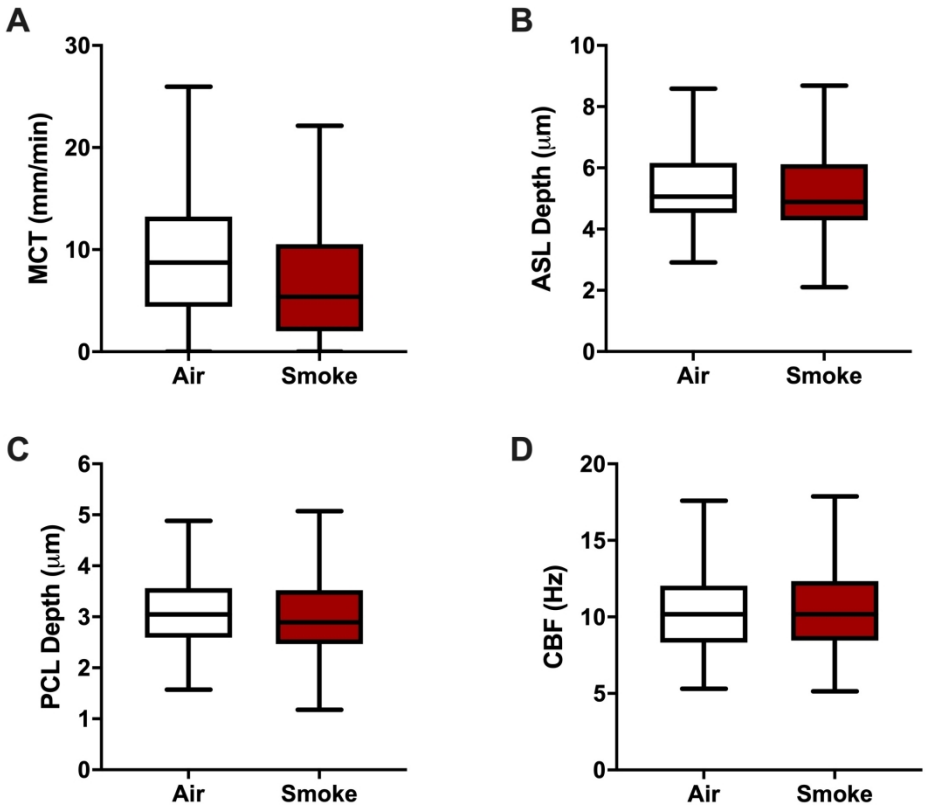

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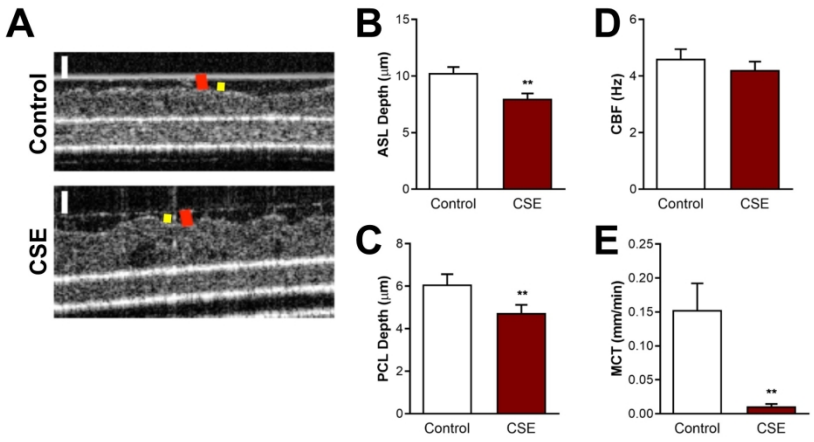

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