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Biological changes in C57BL/6 mice following 3 weeks of inhalation exposure to cigarette smoke or e-vapor aerosols

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Abstract

In this study, we compared early biological changes in mice after inhalation exposure to smoke from a reference (3R4F) cigarette or aerosols from e-vapor products (MarkTen® with Carrier, Test-1, or Test-2 formulations), using standard and mechanistic endpoints. All three e-vapor products contained aerosol formers (propylene glycol, glycerol, water) and nicotine (4%); the two test products also contained flavor mixtures. C57BL/6 mice were exposed to 3R4F smoke or e-vapor aerosols by nose-only inhalation for up to 4 h/day, 5 d/wk, for 3 weeks. The 3R4F and e-vapor exposures were set to match the nicotine concentration at the noseports (~41 ug/L). Body weights were comparable across the 3R4F, e-vapor, and air control groups. The 3R4F group showed transient clinical signs of stress post-exposure, markedly reduced respiratory function during exposure, and therefore substantially lower plasma nicotine and cotinine levels compared to the e-vapor groups. Increases in terminal lung weight and in bronchoalveolar lavage findings were notable with the 3R4F group as well as microscopic changes in the respiratory tract (nose, larynx, and lung). The control and all e-vapor groups had similar minimal microscopic changes with a few exceptions, such as higher incidences in squamous metaplasia in larynx (all three e-vapor groups) and histiocytic infiltrates in the lung for Test-2 group. Overall, these findings were consistent with transcriptomics data in the lung (e.g., the 3R4F group showed the highest number of differentially expressed genes compared to the control). The Test-2 e-vapor group showed a higher number of differentially expressed genes than the other two e-vapor groups, but the magnitude of gene expression-based network perturbations in all e-vapor groups was more than 94% less than in the 3R4F group. In the lung, proteome differential regulation was detected only in the 3R4F group. In conclusion, following exposure for 3 weeks, cigarette smoke exposure induced biological responses in the respiratory tract associated with smoking-related diseases, while e-vapor exposures, even at higher nicotine intake, showed substantially reduced molecular and microscopic changes.

Methods

Female C57BL/6 mice were exposed to 3R4F smoke or aerosols generated from three e-vapor products via nose-only inhalation for up to 4 hours/day, 5 days/week, for 3 weeks. Aerosols were generated from e-vapor products using CRM81 (3-sec puffing; 55 mL/puff, 2 puffs/min) and mainstream smoke from 3R4F using CIR (2-sec puffing; 55 mL/puff, 2 puffs/min). 3R4F and e-vapor exposures were set to match the nicotine concentration at the nose ports (~41 µg/L). Following the last exposures, groups of mice were subjected to sample collection and analyses: bronchoalveolar lavage (BAL) fluids (cytology, cytokines), blood (exposure markers [plasma nicotine, cotinine], COHb) and selected organs (histopathology, transcriptomics, proteomics).

Table 1. Study Design

Group	Group Name	BAL	Exposure markers	Histopath	Omics	Total
1	Sham (Air) Control	15	6	12	8	41
2	Carrier (PG/VG/Nic)	15	6	12	8	41
3	Test-1 Mix (Carrier+flavors-1)	15	6	12	8	41
4	Test-2 Red (Carrier+flavors-2)	15	6	12	8	41
5	3R4F cigarette	15	6	12	8	41

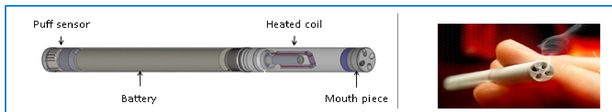

Figure 1. e-Vapor prototype product design. The cartridge contains 0.9 grams of e-Liquid. The puff-activated product generates the aerosol via a 3.5 Ω (3.9 watts) heater coil.

Results

Table 2. Exposure Characterization

Exposure Parameter (Mean ± SD)0	Sham (Air) Control (0 µg WTPM/L)	Carrier (1550 µg WTPM/L)	Test-1 Mix (1400 µg WTPM/L)	Test-2 Red (1400 µg WTPM/L)	3R4F Cigarette (550 µg WTPM/L)
Wet Total Particulate Matter, WTPM (µg/L)	0±0	1583 ±44	1410±73	1423±54	554±16
Carbon Monoxide, CO (ppm)	NM*	NM	NM	NM	676±23
Nicotine (µg/L)	ND**	43.1±3.9	43.7±2.5	43.7±3.4	40.7±2.8
Propylene Glycol, PG (µg/L)	ND	361±21	311±19	310±26	ND
Glycerol, VG (µg/L)	ND	1058±28	946±26	960±38	58.3±5.0
Particle Size Distribution (MMAD [µM] ± GSD)	ND	0.90±1.6	1.0±1.6	1.0±1.7	0.70±1.5
Acrolein (µg/L) – Wk3	BLOQ	BLOQ	BLOQ	BLOQ	3.45±0.08
Acetaldehyde (µg/L) – Wk3	0.00490±NA	0.0286±0.0024	0.0674±0.0085	0.0641±0.0183	34.9±0.2
Formaldehyde (µg/L) – Wk3	0.0216±0.0148	0.192±0.015	0.139±0.028	0.145±0.064	1.07±0.13
Propionaldehyde (µg/L) – Wk3	BFB	BFB	BFB	BFB	4.38±0.17
Crotonaldehyde (µg/L) – Wk3	BLOQ	BFB	BLOQ	BLOQ	0.787±0.019

*NM = Not Measured; **ND = Not Detected; NA = Not applicable (in the case of SD, only two replicates were analyzed so no SD value generated)
BFB = Below field blank concentration; BLOQ = Below lower limit of quantification

Table 3. Biological Endpoints

Exposure Parameter (Mean ± SD, as applicable)	Sham (Air) Control (0 µg WTPM/L)	Carrier (1550 µg WTPM/L)	Test-1 Mix (1400 µg WTPM/L)	Test-2 Red (1400 µg WTPM/L)	3R4F Cigarette (550 µg WTPM/L)
Biomarkers of Exposures (Day 17)					
Carboxyhemoglobin, COHb (%)	0.9±0.5	1.3±0.3[b]	1.1±0.1[b]	1.5±0.2[b,c]	46.9±1.9[a]
Plasma Nicotine (ng/mL)	<BLOQ(1.93)	1112±1019[b]	417±57[b]	506±168[b]	119±11
Plasma Cotinine (ng/mL)	<BLOQ(8.12)	3210±1556[b]	1940±534[b]	1730±320[b]	413±36
Respiratory Physiology (Wk 2)					
Respiratory Rate (breaths/min)	231.7±17.7	174.6±16.6[a,b]	171.5±9.9[a,b]	193.2±20.4[a,b,c]	96.6±19.1[a]
Tidal Volume (mL/breath)	0.19±0.02	0.22±0.05	0.19±0.03	0.21±0.05	0.18±0.05
Minute Volume (mL/min)	43.0±4.3	39.1±8.7[b]	33.7±6.0[b]	39.7±9.2[c]	19.3±6.6[a]
Total Inhaled Mass – 1 hr (mg)[#]	NA	3.67±0.81[a,b]	2.94±0.53[a,b]	3.51±0.81[a,b]	0.64±0.22[a]
Body and Organ Weights					
Body Weight (g) – Day 1	20.593 ±1.044	20.468±1.049	20.890±1.222	20.656±1.227	20.921±1.125
Body Weight (g) – Day 19	20.580±0.846	20.726±1.474	21.095±1.298	19.925±1.740[c]	20.505±1.213
Lung (g) – Wk 3	0.155±0.0.018	0.154±0.010[b]	0.160±0.017[b]	0.153±0.012[b]	0.194±0.022[a]
Thymus (g) – Wk 3	0.035±0.009	0.028±0.004[b]	0.032±0.008[b]	0.022±0.008[a,b,c]	0.044±0.007[a]
Liver (g) – Wk 3	0.987±0.088	1.010±0.072[b]	1.007±0.093[b]	0.993±0.138[b]	0.874±0.059 [a]
Histopathology – Wk 3: Incidence (Severity)*					
Nose> Respiratory epithelium, Squamous Metaplasia	0/12	0/11	0/12	3/12 (1.0)	12/12 (1.3)
Larynx – Epiglottis> Respiratory epithelium, Squamous Metaplasia	1/11 (1.0)	8/11 (1.0)	6/12 (1.0)	5/12 (1.2)	11/11 (1.9)
Lung> Infiltrate, Cellular, Histiocytic	2/12 (1.0)	0/11	0/12	7 /12 (1.0)	12/12 (1.7)
Lung> Infiltrate, Cellular Mixed, Multifocal	0/12	0/11	1/12(1.0)	1/12 (1.0)	12/12 (1.1)

a: $p \leq 0.05$ compared to Sham (Air) Control; b: $p \leq 0.05$ compared to 3R4F cigarette; c: $p \leq 0.05$ compared to Test-1 Mix
* Average severity (calculated by the sum of the severity scores divided by the incidence)
NA = Not applicable

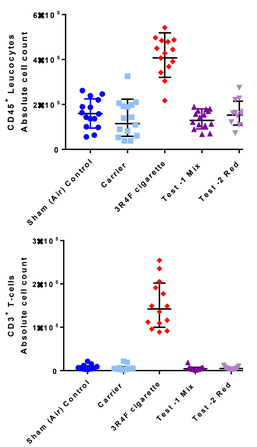

Fig 2. BAL Fluid Immunophenotyping by Flow Cytometry
The 3R4F group showed significant increases in BAL leucokytes (Top) and T-cells (Bottom), while all the e-vapor groups had comparable levels to the Sham control.

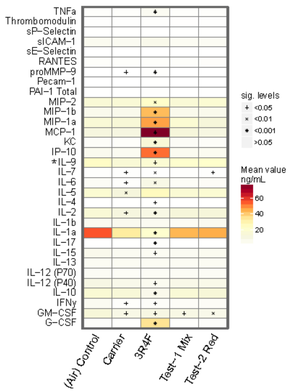

Fig 3. Cytokine Profiling in the BAL Fluid
The color scale reflects the magnitude of the estimated differences between groups. *P*-values represented significant differences between the treatment groups compared with the air-control.
The most notable changes were observed in the 3R4F group, showing up-regulation of cytokines (e.g., MCP-1, IP-10, MIP-1, G-CSF). In contrast, there are only minimal changes in all e-vapor groups (an increase in GM-CSF) and few mediators in the carrier group compared with the sham-control. *IL-9 concentration used in this heatmap is 10 times lower than the actual mean.

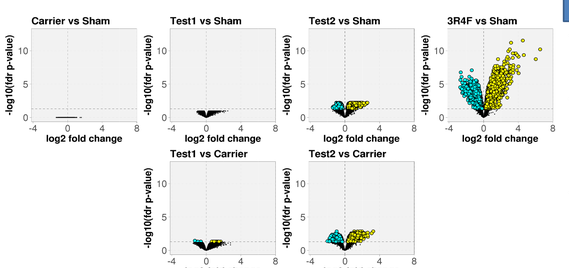

Fig 4. Volcano Plots of the mRNA Response Profiles in Lung
For each gene, the expression change is plotted on the x-axis, and the statistical significance (fdr<0.05) is plotted on the y-axis. Yellow and cyan dots highlight genes that are statistically, significantly up- and down-regulated, respectively. There was no difference in differentially expressed genes (DEG) between Carrier, Test-1, and the sham control. There were 1750 and 1032 genes significantly up- and down-regulated in Test-2, compared to sham-control. In contrast, 4028 and 4601 genes were significantly up- and down-regulated in the 3R4F compared to sham-control.

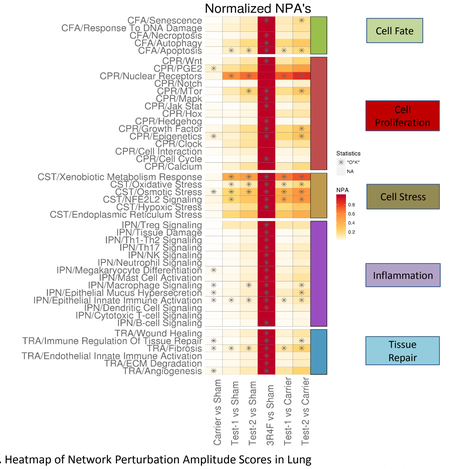

Fig 5. Heatmap of Network Perturbation Amplitude Scores in Lung
All tested networks showed the highest perturbation following 3R4F exposure compared to the sham control. Some networks were perturbed following e-vapor exposures (e.g., cell proliferation, cell stress); however, the amplitude was lower than that observed following the 3R4F exposure.
A network is perturbed if the two companion statistics (O and K), derived to inform on the specificity of the NPA score with respect to the described biology in the network, are significant ($p < 0.05$).

Summary

- An inhalation exposure system successfully generated and delivered e-vapor aerosols of a respirable size to a nose-only mouse exposure system. The exposure regimen used for 3R4F as well as for e-vapor exposures (with matching aerosol nicotine levels) was tolerated by mice.
- The 3R4F group showed signs of respiratory function depression, increased lung weight, and biochemical and microscopic changes in respiratory tracts. The e-vapor groups had significantly higher plasma nicotine and cotinine levels than the 3R4F, yet displayed minimal microscopic changes that were similar to the sham control: Few differences compared with the sham control were squamous metaplasia in larynx (all three e-vapor groups) and histiocytic infiltrates in the lung (Test-2 group).
- Similar to the histopathological findings, the 3R4F group showed the highest number of DEGs compared with the sham-control. Among e-vapor groups, the Test-2 group showed a relatively higher number of DEGs. However, the magnitude of gene expression-based network perturbations in all e-vapor groups was substantially lower (>94%) than in the 3R4F group. Additionally, proteome differential regulation in the lung was increased only in the 3R4F group compared with the sham-control.

In conclusion, following three weeks of exposure, cigarette smoke induced biological responses in the respiratory tract associated with smoking-related diseases, while e-vapor exposure, even with higher nicotine intake, showed substantially reduced molecular and microscopic changes.

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