SUPPLEMENTAL MATERIALS

Impairment of Endothelial Function by Cigarette Smoke is not Caused by a Specific Smoke Constituent, but by Vagal Input from the Airway

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Detailed supplemental methods for acrolein and acetaldehyde gas generation and exposure conditions:

To generate acrolein and acetaldehyde gases, we heated the respective gas permeation tubes using an EcoFlex gas generator (KIN-TEK Analytical, La Marque, TX). The generator was run for 1 hour to stabilize and ensure a steady flow rate at 40°C. A photoionization detector (ppbRae 3000) was used to measure the flow of aldehyde gases and to assess the steady flow. Once the flow rate was stabilized, the tubes were placed in the inner glass chamber of the generator and the generator was run at high flow rate of 5000 sccm, followed by lowering the flow rate to 786 sccm for acrolein tubes and 225 sccm for acetaldehyde tubes. These flow rates produced steady gas flow at 3.2 ppm for acrolein and 11.8 ppm for acetaldehyde.

The inhalation LC50 for acrolein in rats is 8 ppm over 4 hours according to the Material Safety Data Sheet from Matheson. To ensure that our acrolein concentration would be tolerable from an animal welfare standpoint, the following calculation was performed: Sidestream smoke from 1 cigarette contains ~437 µg of acrolein.[16] Our sidestream smoke exposure experiments use smoke from ~1/3 of a single cigarette, or ~145 µg acrolein, initially diluted in a ~0.02 m^3 chamber, which equals 7250 µg/m^3 or 7.25 mg/m^3 acrolein; this equals a starting concentration of ~3.16 ppm[50] before venting excess smoke to reach the highest tested sidestream particle concentrations in our previous exposure experiments. Comparable calculations for acetaldehyde (LC50 of 14,000 ppm/4 hours in rats (MSDS) and 1,600 µg/cigarette[16]) result in a concentration of ~14.8 ppm in our exposure chamber before venting excess smoke, so we used that as a maximum exposure concentration that is well under toxicity levels.

Table S1. Exposure conditions and FMD results for each rat in the order of exposure in the experiment shown in Figure 1.

Rat #	*Exposure Condition*	*Smoke level (µg/m³ RSP)*	*Pre-FMD*	*Post-FMD*
201	Air	N/A	3.92	3.77
202	Conventional nicotine plus menthol	600	10.87	6.12
203	Reduced nicotine plus menthol	600	20.51	12.50
204	Reduced nicotine plus menthol	600	16.67	15.91
205	Reduced nicotine	600	17.07	11.63
206	Air	N/A	10.71	7.02
207	Conventional nicotine	600	6.90	3.77
208	Reduced nicotine	600	15.22	8.89
209	Conventional nicotine plus menthol	600	12.07	6.45
210	Conventional nicotine plus menthol	600	12.20	9.30
211	Conventional nicotine	600	17.07	12.20
212	Reduced nicotine plus menthol	600	7.55	5.77
213	Reduced nicotine	600	13.64	14.58
214	Conventional nicotine	600	13.46	3.39
215	Reduced nicotine plus menthol	600	11.54	8.93
216	Air	N/A	8.70	6.82
217	Conventional nicotine	600	8.33	1.96
218	Reduced nicotine plus menthol	600	12.50	10.20
219	Conventional nicotine	600	11.76	11.54
220	Conventional nicotine plus menthol	600	11.32	9.80
221	Reduced nicotine	600	18.18	2.00
222	Air	N/A	12.73	7.27
223	Reduced nicotine	600	7.55	3.57
224	Reduced nicotine plus menthol	600	12.96	12.50
225	Conventional nicotine plus menthol	600	14.89	10.64
226	Reduced nicotine	600	7.69	4.17
227	Conventional nicotine	600	17.02	9.80

228	Reduced nicotine	600	12.50	11.54
229	Conventional nicotine	600	10.00	8.51
230	Reduced nicotine plus menthol	600	13.73	7.84
231	Reduced nicotine plus menthol	600	20.00	15.00
232	Conventional nicotine	600	16.00	7.14
233	Reduced nicotine	600	19.15	10.00
234	Conventional nicotine plus menthol	600	15.56	15.91
235	Conventional nicotine plus menthol	600	13.04	10.64
236	Conventional nicotine plus menthol	600	17.78	6.00
237	Air	N/A	9.30	9.30
239	Air	N/A	9.62	11.32
240	Reduced nicotine plus menthol	200	9.52	8.51
241	Conventional nicotine plus menthol	200	12.24	4.17
242	Conventional nicotine	200	9.80	9.43
243	Reduced nicotine	200	14.00	7.69
244	Conventional nicotine plus menthol	200	12.50	8.51
245	Reduced nicotine	200	9.80	6.90
246	Conventional nicotine	200	10.42	5.56
247	Reduced nicotine plus menthol	200	10.20	7.69
248	Conventional nicotine	200	7.69	0.00
249	Reduced nicotine	200	13.95	15.56
250	Conventional nicotine plus menthol	200	19.15	13.33
251	Conventional nicotine	200	14.58	10.64
252	Conventional nicotine plus menthol	200	17.65	13.46
253	Reduced nicotine	200	11.54	12.00
254	Reduced nicotine plus menthol	200	6.67	10.64
255	Conventional nicotine	200	9.43	1.79
256	Air	N/A	11.76	13.04
257	Reduced nicotine	200	10.20	7.27
258	Reduced nicotine plus menthol	200	3.45	12.96
259	Reduced nicotine plus menthol	200	5.71	4.17
260	Conventional nicotine plus menthol	200	8.62	6.06

261	Conventional nicotine	200	8.33	3.51
262	Reduced nicotine	200	6.56	1.64
263	Conventional nicotine	200	9.23	4.41
265	Reduced nicotine	200	7.81	7.69
266	Conventional nicotine plus menthol	200	6.90	3.39
267	Reduced nicotine plus menthol	200	6.67	3.39
269	Conventional nicotine	200	9.09	3.45
270	Conventional nicotine plus menthol	200	9.26	3.51
271	Conventional nicotine	200	11.90	4.44
272	Reduced nicotine plus menthol	200	19.61	9.26
273	Conventional nicotine plus menthol	200	11.54	12.77
274	Reduced nicotine	200	8.77	3.13
275	Reduced nicotine plus menthol	200	5.36	3.70
276	Reduced nicotine	200	10.71	8.62
278	Air	N/A	12.00	11.54
279	Air	N/A	9.09	5.17
280	Reduced nicotine plus menthol	200	5.08	3.64
281	Reduced nicotine plus menthol	200	9.62	5.88
282	Reduced nicotine	600	6.90	5.00

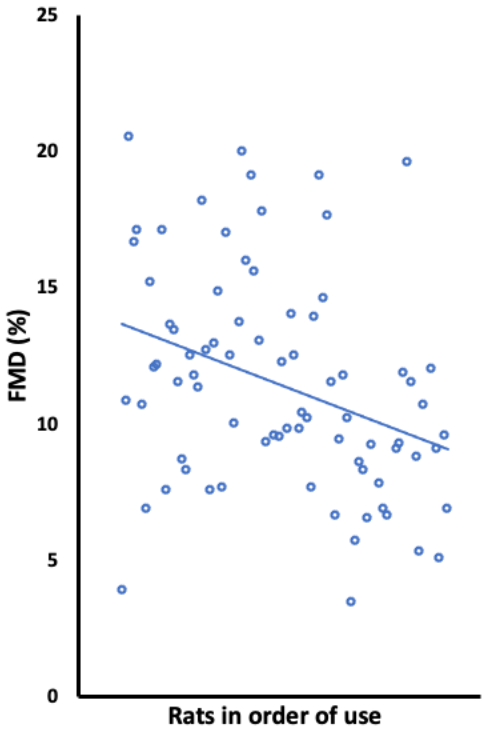

Figure S1. Pre- and Post- exposure FMD trend over time in rats in the experiment shown in Figure 1.

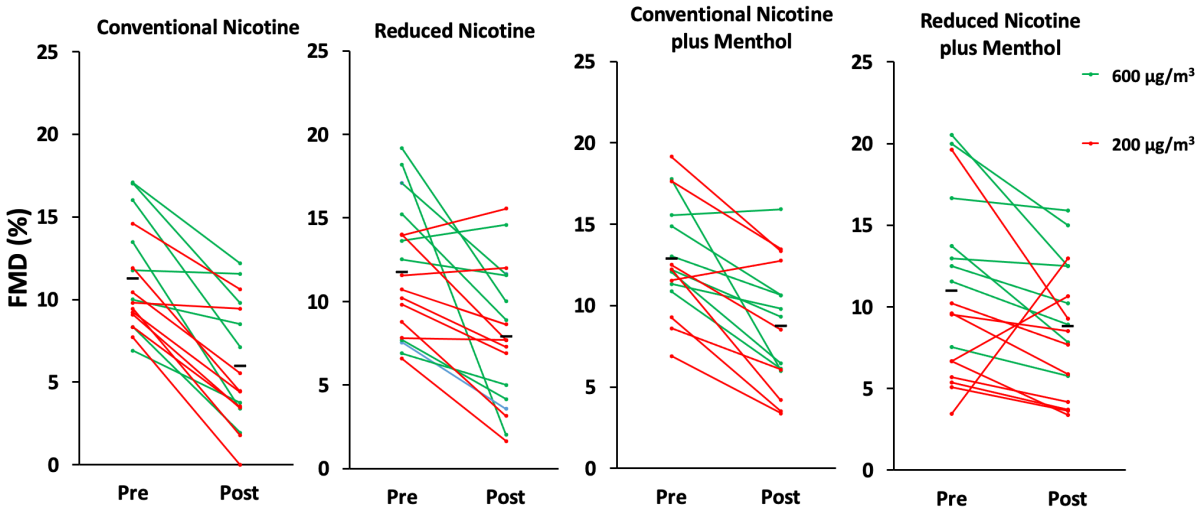

Figure S2. FMD in rats exposed to secondhand smoke from conventional and reduced nicotine with and without menthol addition. Green and red lines indicate rats exposed to smoke levels of ≈600 and ≈200 µg/m^3 RSP respectively.

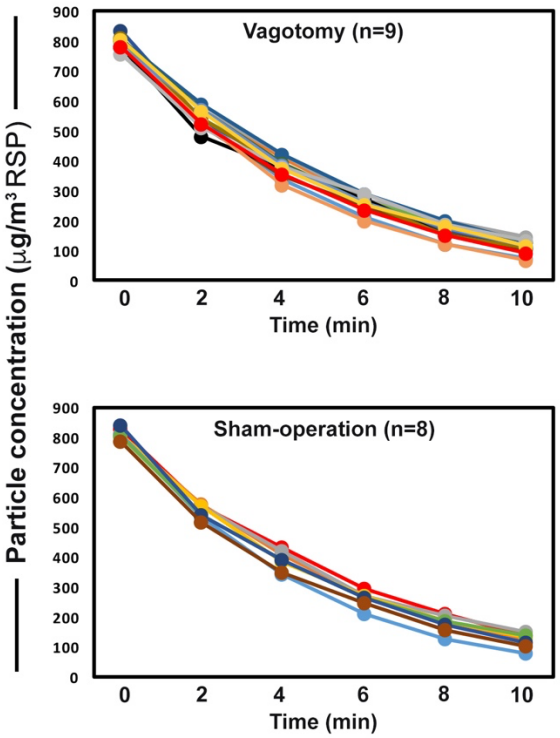

Figure S3. Decreasing smoke levels over time for vagotomy experiment shown in Figure 4 A-C. Levels of respirable suspended particles from tobacco sidestream smoke over 10 minutes for each rat. Mean starting concentration (mean±SD): 790±18 µg/m^3 and 809±16 µg/m^3; mean concentration over time: 62±4.2 µg/m^3 and 6±4.0 µg/m^3; and total exposure derived from the area under the curve: 2240±119 µg/m^3 x min and 2305±126 µg/m3 x min, respectively for vagotomy and sham-operation groups. RSP, respirable suspended particles.

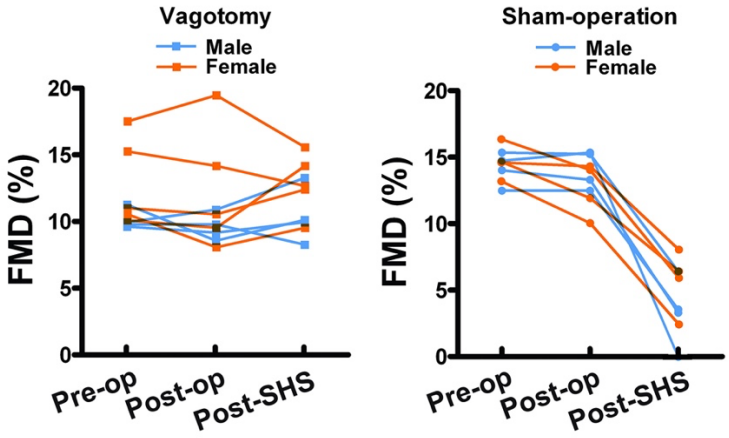

Figure S4. Distribution of FMD by gender in vagotomy and sham-operation groups.

Major Resources Table

In order to allow validation and replication of experiments, all essential research materials listed in the Methods should be included in the Major Resources Table below. Authors are encouraged to use public repositories for protocols, data, code, and other materials and provide persistent identifiers and/or links to repositories when available. Authors may add or delete rows as needed.

Animals (in vivo studies)

Species	Vendor or Source	Background Strain	Sex	Persistent ID / URL
Rat	Simonsen Laboratory, CA Charles River, MI	Sprague-Dawley	Male and female	

Antibodies

None

Other

Description	Source / Repository	Persistent ID / URL

ARRIVE GUIDELINES

The ARRIVE guidelines (https://arriveguidelines.org/) are a checklist of recommendations to improve the reporting of research involving animals. Key elements of the study design should be included below to better enable readers to scrutinize the research adequately, evaluate its methodological rigor, and reproduce the methods or findings.

Study Design

Experiment 1: *Assessment of FMD impairment with reduction of nicotine level and addition of menthol*

Groups	Sex	Age	Number (prior to experiment)	Number (after termination)	Littermates (Yes/No)	Other description
Group 1 Air (Control)	Male	8–10 weeks	9	0	No	
Group 2 Conventional Nicotine (high dose)	Male	8–10 weeks	8	0	No	
Group 3 Conventional Nicotine plus Menthol (high dose)	Male	8–10 weeks	8	0	No	
Group 4 Reduced Nicotine (high dose)	Male	8–10 weeks	8	0	No	

Group 5 Reduced Nicotine plus Menthol (high dose)	Male	8–10 weeks	8	0	No	
Group 6 Conventional Nicotine (low dose)	Male	8–10 weeks	9	0	No	
Group 7 Conventional Nicotine plus Menthol (low dose)	Male	8–10 weeks	8	0	No	
Group 8 Reduced Nicotine (low dose)	Male	8–10 weeks	9	0	No	
Group 9 Reduced Nicotine plus Menthol (low dose)	Male	8–10 weeks	10	0	No	

Experiment 2: *Assessment of FMD impairment with inhalation of unsaturated aldehydes acrolein and acetaldehyde*

Groups	**Sex**	**Age**	**Number (prior to experiment)**	**Number (after termination)**	**Littermates (Yes/No)**	**Other description**
Group 1 Air (Control)	Male/Female	8–10 weeks	8	0	No	
Group 2 Acrolein	Male/Female	8–10 weeks	8	0	No	
Group 3 Acetaldehyde	Male/Female	8–10 weeks	8	0	No	
Group 4 Cigarette	Male/Female	8–10 weeks	8	0	No	

Experiment 3: *Assessment of FMD impairment with inhalation of inert carbon nanoparticles*

Groups	**Sex**	**Age**	**Number (prior to experiment)**	**Number (after termination)**	**Littermates (Yes/No)**	**Other description**
Group 1 Air (Control)	Male	8–10 weeks	8	0	No	
Group 2 Particles	Male	8–10 weeks	8	0	No	
Group 3 Cigarette	Male	8–10 weeks	8	0	No	

Experiment 4: *Assessment of FMD impairment recovery after inhalation of inert carbon nanoparticles*

Groups	Sex	Age	Number (prior to experiment)	Number (after termination)	Littermates (Yes/No)	Other description
Group 1 Air (Control)	Male	8–10 weeks	8	0	No	
Group 2 Particles	Male	8–10 weeks	8	0	No	

Experiment 5: *Assessment of vagus nerve role in FMD impairment caused by sidestream smoke*

Groups	Sex	Age	Number (prior to experiment)	Number (after termination)	Littermates (Yes/No)	Other description
Group 1 Sham (Control)	Male/Female	8–10 weeks	8	0	No	
Group 2 Vagotomy	Male/Female	8–10 weeks	9	0	No	

Sample Size:

For FMD data, power calculation was based on standard deviations from within-group comparisons in several previous repeated-measures FMD experiments. It was determined that N=8 per group was sufficient to achieve 0.8 power to detect FMD change of 3.5 percentage points with a significance level of 0.05.

Inclusion Criteria

Male and female Sprague-Dawley rats (Simonsen Laboratory, CA and Charles River, MI) at 8–10 weeks of age with body weights of 200–250 g.

Exclusion Criteria

None.

Randomization

Rats were randomly assigned to different exposure conditions based on the experiment.

Blinding

The investigator was blinded to exposure conditions during FMD procedure, analysis of ultrasound images, and subsequent calculations.