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E-Cigarette Flavorings, Additives Increase Inflammation and Impair Lung Function, Study Finds

Short-term e-cigarette use causes as much or more damage as conventional cigarettes

Rockville, Md. (October 11, 2018)—Flavoring and additive ingredients in e-cigarettes may increase inflammation and impair lung function, according to new research. The study, published ahead of print in the *American Journal of Physiology—Lung Cellular and Molecular Physiology*, also found that short-term exposure to e-cigarettes was enough to cause lung inflammation similar or worse than that seen in traditional cigarette use. The research was chosen as an APS*select*article for October.

E-cigarettes, popular battery-powered devices that simulate the act of smoking a traditional cigarette, dispense a vapor derived from liquid chemicals in a refillable cartridge. The refills typically contain propylene glycol, nicotine and often flavorings. Propylene glycol—a colorless, odorless food additive—is found in numerous processed food and beverages; it is also used as a solvent in a number pharmaceuticals. E-cigarette devices and refills are not well regulated, and the long-term health effects of e-cigarette use are not widely known.

Researchers studied several groups of mice that received whole-body exposure to varying chemical combinations four times each day. Each exposure session was separated by 30-minute smoke-free intervals.

- One group was exposed to cigarette smoke ("cigarette");
- One group was exposed to e-cigarette vapor containing propylene glycol and vegetable glycerol, an odorless liquid derived from plant oils ("propylene");
- One group was exposed to e-cigarette vapor containing propylene glycol and nicotine ("propylene + nicotine") and
- One group was exposed to e-cigarette vapor containing propylene glycol, nicotine and tobacco flavoring ("flavoring").

The cigarette and e-cigarette groups were compared with a control group that was exposed to medical-grade air. Some of the animals in each group were exposed to short-term cigarette smoke or e-cigarette vapor (three days), while others were exposed for a longer term (four weeks).

The research team found an increase in markers of inflammation, mucus production and altered lung function in the propylene, propylene + nicotine and flavoring groups after three days. However, the propylene group showed fewer negative effects with long-term exposure, suggesting the additive alone elicits only a temporary irritation

that eventually subsides with continued use. In addition, two inflammation-producing proteins became elevated only in the flavoring group, suggesting that some of the many flavoring components on the market may not be safe for even short-term use.

The condition of the e-cigarette groups in comparison with the cigarette group surprised the researchers. The level of oxidative stress—stress at a cellular level—in the flavoring group was equal to or higher than that of the cigarette group. However, respiratory mechanics were adversely affected only in mice exposed to cigarette smoke and not to e-cigarette vapor after prolonged treatment. "The observed detrimental effects in the lung upon [e-cigarette] vapor exposure in animal models highlight the need for further investigation of safety and toxicity of these rapidly expanding devices worldwide," the researchers wrote.

Read the full article, "Comparison of the effects of e-cigarette vapor with cigarette smoke on lung function and inflammation in mice," published ahead of print in the *American Journal of Physiology—Lung Cellular and Molecular Physiology*. It is highlighted as one of this month's "best of the best" as part of the American Physiological Society's APS*select* program. Read all of this month's selected research articles.

NOTE TO JOURNALISTS: To schedule an interview with a member of the research team, please contact the APS Communications Office or 301-634-7314. Find more research highlights in the APS Press Room.

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Released May 26, 2015 - With the use of e-cigarettes on the rise, especially among young people, research to uncover the health effects of e-cigs is becoming increasingly important. In a new study published ahead of print in AJP-Lung, researchers find that e-cig solution and vapors—even those that are nicotine-free—damage lung health.

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1 **Comparison of the effects of e-cigarette vapor with cigarette smoke on lung**

2 **function and inflammation in mice**

3

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30 **Abstract**

31 Electronic cigarettes (e-cig) are advertised as a less harmful nicotine delivery system

32 or as a new smoking cessation tool. We aimed to assess the in vivo effects of e-

33 cigarette vapor in the lung and to compare them to those of cigarette smoke (CS). We

34 exposed C57BL/6 mice for either 3 days or 4 weeks to ambient air, CS or e-cig vapor

35 containing: i) propylene glycol/vegetable glycerol (1:1; PG:VG-Sol), ii) PG:VG with

36 nicotine (G:VG-N), or iii) PG:VG with nicotine and flavor (PG:VG- N+F) and

37 determined oxidative stress, inflammation and pulmonary mechanics. E-cig vapors,

38 especially PG:VG- N+F, increased bronchoalveolar lavage fluid (BALF) cellularity,

39 Muc5ac production, as well as BALF and lung oxidative stress markers at least

40 comparably and in many cases more than CS. BALF protein content at both time

41 points studied was only elevated in the PG:VG- N+F group. After 3 days, PG:VG-Sol

42 altered tissue elasticity, static compliance and airway resistance, while after 4 weeks,

43 CS was the only treatment adversely affecting these parameters. Airway

44 hyperresponsiveness in response to methacholine was increased similarly in the CS

45 and PGVG-N+F groups. Our findings suggest that exposure to e-cig vapor can trigger

46 inflammatory responses and adversely affect respiratory system mechanics. In many

47 cases, the added flavor in e-cigs exacerbated the detrimental effects of e-cig vapor.

48 We conclude that both e-cig vaping and conventional cigarette smoking negatively

49 impact lung biology.

50

51

52 **Keywords**: electronic cigarettes, cigarette smoking, lung inflammation, lung

53 mechanics, lung hyperresponsiveness

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2

56 **INTRODUCTION**

57 Smoking is the leading cause of morbidity and mortality in men and women and for

58 this reason smoking prevention and cessation strategies have tremendous potential

59 for improving overall public health (4). In recent years, electronic cigarettes (e-

60 cigarettes or "e-cigs") are increasingly advertised as a reduced-risk nicotine product

61 and an attractive alternative smoking-cessation tool (23, 24, 35). In fact, part of the

62 medical community believes that they can be used as a harm-reduction strategy for

63 smokers. E-cig have become rapidly popular worldwide (6, 35), although their

64 effectiveness as a smoking cessation tool has not been rigorously proven yet (29,

65 36). Currently, their effects on human health have not been adequately addressed (6,

66 11, 36). While some negative short-term health effects have already been shown (6,

67 20, 36), altogether there is still paucity of reliable data regarding long-term exposure

68 effects.

69

70 Ecig are battery–powered devices, which do not contain or burn natural tobacco.

71 They consist of a rechargeable battery, a heater, and a refillable cartridge with liquids,

72 usually consisting of propylene glycol (PG;VG), nicotine and flavourings (6, 11).

73 When the battery-powered heater is activated, it heats the solution to produce a

74 vapour containing various heat-produced ingredients of variable concentration which

75 are inhaled by the user. Quality-control of e-cig among various brands has been a

76 matter of controversy, raising concerns about their safety profile and their toxicity (12,

77 35, 46, 47). The US Food and Drug Administration (FDA) has indicated that e-cig

78 contain a number of toxins and carcinogens (i.e. nitrosamines, diethylene glycol)(8).

79 Recently, there have been attempts to regulate the market of the e-cig devices (6, 8).

80 As experiment-based scientific knowledge is still largely lacking in the field, it is crucial

81 to vigorously assess e-cig toxicity, safety and health effects.

82

3

83 The smoke of conventional cigarettes contains more than 4,000 chemicals with

84 multiple immunomodulatory and other effects on the lungs (9, 33). Compared with

85 their effects, e-cig vaping is advertised as less harmful. Based on the few published in

86 vitro or in vivo studies, e-cig vapour seems to have adverse effects on both cultured

87 cells and on experimental animals (reviewed in (6, 11). E-cig vapour induces

88 inflammation, augments the development of allergic airway inflammation in asthma

89 models, suppresses the host defence and triggers effects associated with COPD-like

90 tissue damage (26, 30, 42).   In humans, clinical manifestations such as acute

91 eosinophilic pneumonia and lipoid pneumonia (31, 43) have been related to e-cig use.

92 It has also been shown recently that acute exposure to e-cig vapor increases aortic

93 stiffness, blood pressure (16, 23) and airway resistance (45) while, conversely, it

94 decreases airway conductance (34) in healthy subjects and changes the slope of

95 phase III curve in asthmatic smokers (34). It should be stressed that all the above-

96 mentioned studies focus on the acute pathophysiological effects of the e-cig vapour

97 with regard to the respiratory and cardiovascular function.Thus, the potential longer-

98 term effects of e-cig vapour exposure in animals and humans still remain unexplored

99 and unknown.

100

101 The aim of the present study was to determine the effects of both acute (3-days) and

102 sub-chronic (4-weeks) exposure of mice to components of vaporized e-cig liquids on

103 the respiratory functional parameters and inflammatory responses, and to compare

104 them side-by side to those of air and classic cigarette smoke exposure, using a well-

105 established animal model (10).

106

107

4

108 **MATERIAL & METHODS**

109

110 **Animals**: . Sex disparity in response to chronic smoke exposure has been observed

111 in animal models (5). In the present study we used only male mice for two reasons.

112 First, it is estimated that men smoke nearly five times as much as women

113 worldwide(21). Second, limiting our studies to male mice would be expected to

114 reduce variability allowing the use of a smaller number of animals that conforms with

115 the 3Rs principle in humaine animal research.   Due to the abundance of information

116 on C57BL6 and its susceptibility to lung injury this strain was chosen. Eight-to-twelve

117 week old male C57BL/6 wild type Pasteur Institute (Athens, Greece) or Fleming

118 Institute (Vari, Greece), weighing 16–24 g mice were exposed for 3 days or 4 weeks

119 to air (medical air grade), cigarette smoke (CS) or e-cigarette (e-cig) vapor from i)

120 propylene glycol/vegetable glycerol (1:1; PG:VG-Sol), ii) PG:VG with nicotine

121 (18mg/ml; PG:VG-N), or iii) PG:VG-N+F with flavor (tobacco blend; PG:VG-N+F). A

122 partial chemical characterization of the tobacco blend flavor that (Nobacco American

123 Tobacco) has been previously published (14). It should be mentioned that this

124 product is not a tobacco extract. Mice were maintained in standard conditions under a

125 12-hour light-dark cycle, provided a standard diet and chlorinated tap water ad

126 libitum. All procedures were in accordance to European Union Directive for care and

127 use of laboratory animals and were approved by the competent Regional Veterinary

128 Service and the ethical committee of Evangelismos Hospital.

129

130 **Cigarette smoke (CS) and e-cigarette (e-cig) vapor exposure:** The apparatus used

131 in our study is shown in Fig.1; it exposes the entire body of animals to the treatment

132 applied. The apparatus has been described before (10, 18) and has been used by us

133 in recent studies (10, 18). A pump connected with five syringes was used to create

134 positive/negative pressure cycles in order to drive flow of the smoke or vapor to a

135 chamber where the animals were kept. The chamber volume was 7,500 cm^3 and the

5

136 flow of medical air into the chamber was between 1.5 and 2L/min. The puff volume

137 was 20mL. In our chamber a smoke/air ratio of 1:6 was obtained.   All of these

138 parameters were identical between the CS and e-cig exposure. The e-cig exposure

139 was performed using the same system as for the CS exposure; the only change was

140 the use of an adaptor that held the e-cig in place due to its different diameter. Three

141 different experimental series of air, CS and e-cig vapor exposures were performed for

142 the acute study and two experimental series were performed for the sub-chronic

143 study. Control mice were exposed to medical air. For CS exposure 5 reference

144 cigarettes (3R4F University of Kentucky, USA) were used, while for vaping, 5 eRoll

145 devices (Joye Technology) were employed. The eRoll is a first generation e-cig

146 device(39) and was chosen as it was among the most widely used in Greece when

147 the study was initiated. The smoke and vapor from all 5 cigarettes or e-cig was

148 directed toward the chamber were the animals were. Mice were exposed to CS or e-

149 cig 4 times a day with 30-minute smoke-free intervals for 3 days or 4 weeks. For the

150 CS treatment 15 puffs were drawn per session which sufficed to burn the entire

151 conventional cigarette. The eRoll cartridge holds 0,4ml of liquid and contains a

152 chromium coil. 8 puffs/min for two minutes (i.e. a total of 16 puffs) were drawn during

153 each session, with 4 sessions being used per day with 30 min intervals. The animal

154 whole body exposure lasted 7 min in each session. Our protocol uses less than half

155 of the total amount of the 0.4ml in the cartridge. The cartridge was replaced after

156 every vaping session, i.e. 4 times per day. This procedure avoided overheating of the

157 chromium coil. In order to eliminate the metal decay that could be relevant to the

158 long-term treatment, we changed the chromium coil every second week. Lack of

159 overheating was empirically confirmed by regular e-cig users who vaped the eRoll

160 device at the same rate of 16 puffs over a 2 min period and reported no heating or

161 change in vapor taste.

162

6

163 **Respiratory System Mechanics:** The function of the respiratory system of mice after

164 3 days or 4 weeks exposure to cigarette smoke or e-cig vapour was estimated with

165 the use of the forced oscillation technique and by performing static pressure volume

166 curves, as previously described (19). Following 3 days or 4 weeks treatment, the

167 animals were anaesthetized with an intraperitoneal injection of ketamine (100 mg/kg)

168 and xylazine (10 mg/kg). An anterior cervical incision was performed and the animals

169 were tracheostomized below the level of banding with a tracheal cannula (20G). The

170 animals were connected to a small animal ventilator (Scireq, Montreal, Canada) and

171 ventilated with a 7 ml.kg-1 tidal volume, 150 breaths x min^{-1} and the end expiratory

172 pressure was set to 3 cmH20. Following three minutes of ventilation, an ip injection of

173 succinylcholine (8 mg/kg) was performed, to cease spontaneous breathing and after

174 one minute, three forced oscillation perturbations were performed, with one minute

175 interval, to estimate lung mechanics. A static pressure volume curves was also

176 constructed following one minute of ventilation after the last oscillation perturbation.

177 Results from repeated measures in every animal were averaged. Prior to

178 measurements (30 sec) the lung volume history was once standardized by one

179 inflation to total lung capacity, as estimated by airway opening pressure at 30 cmH20.

180 During ventilation, the heart rate was monitored to ensure adequate depth of

181 anaesthesia.

182

183 **Forced Oscillation Technique:** The forced oscillation perturbation consists of a

184 pseudorandom waveform of low frequencies (0.5-19.75 Hz) applied for 8 seconds

185 with a peak to peak volume of 3 ml/kg. Pressure and volume data are recorded and

186 the impedance of the respiratory system is calculated using the Fast Fourier

187 transformation. Impedance (Z) is then fitted to constant phase model:$Zrs(f) = Rn +$

188 $i2\pi fI + (G-iH)/(2\pi f)a$, where Rn is the Newtonian resistance of the airways, i is the

189 imaginary unit, f is the frequency, I is the inertance of the gas in the airways, G

190 represents tissue viscance (viscous dissipation of energy) and H represents tissue

7

191 elasticity and alpha can be calculated through the equation $\alpha = (2/\pi)\arctan(H/G)$.

192 Data were accepted only when the Coefficient of Determination (fit of the model) was

193 more than 0.9. Static Pressure Volume Curve: Static pressure volume curves of the

194 respiratory system were performed by gradually inflating and deflating the lungs with

195 a total volume of 40 ml/kg at seven steps each. The static compliance of the

196 respiratory system was estimated by the slope of the mid linear part in the expiratory

197 limb of the PV curve. Hysteresis (area between inspiratory and expiratory limb) was

198 automatically calculated (FlexiVent software) (44).

199

200 **Airway Hyperresponsiveness:** Twenty-four hours after the 3 days CS exposure or

201 e-cig vapour mice were anesthetized, tracheostomized, paralyzed, and ventilated with

202 Flexivent (SCIREQ Scientific Respiratory Equipment, Inc., Montreal, PQ, Canada).

203 After baseline measurements of impedance (Zrs), methacholine (2.5, 10, 20, 40, 60

204 mg/ml) or saline were delivered (Aeroneb; SCIREQ) for 10 seconds. Afterwards, a 2-

205 second forced oscillation perturbation (1–20 Hz) was performed every 10 seconds for

206 3 minutes. Before measurements and before every aerosol delivery, the volume

207 history of the lung was established with two 6-second deep inflations to a pressure

208 limit of 30 cmH$_2$O. Measurements of Zrs were fit with the constant phase model,

209 where Rn is the Newtonian resistance of the airways; G represents tissue resistance,

210 and H tissue elasticity. After each dose of methacholine, model parameters were

211 expressed as % ratio of the baseline (41, 44).

212

213 **Bronchoalveolar lavage fluid:** The animals were sacrificed by exsanguination (vena

214 cava dissection) following anaesthesia with ketamine (100mg/kg) and xylazine

215 (10mg/kg) ip 24hr after the last exposure to air, CS or e-cig vapor. After

216 exsanguination, the trachea was cannulated with a 20-gauge plastic catheter. Lungs

217 were lavaged by infusing 1 mL warm saline, three sequential times. The recovered

218 bronchoalveolar lavage fluid (BALF) was centrifuged; cells were collected and

8

219 resuspended in PBS. Differential BALF cell counts were performed on Giemsa-

220 stained cytospins and percentages of eosinophils lymphocytes, neutrophils and

221 macrophages were determined. Protein concentration was measured in the BALF

222 using the Lowry method, employing bovine serum albumin as a standard.

223

224 **Lung Histology:** Following bronchoalvolar lavage, the left lung was harvested from

225 mice and fixed using a 4% formaldehyde solution. The tissue was embedded in

226 paraffin wax, serially sectioned, and stained with hematoxylin-eosin, using standard

227 methods. Two pathologists blinded for treatment evaluated the histopathological

228 findings in the lung. A scoring system to grade the degree of lung inflammation have

229 been used based on the following histological features: (i) capillary congestion, (ii)

230 intra-alveolar haemorrhage, (iii) interstitial neutrophil infiltration, (iv) intra-alveolar

231 neutrophil infiltration, (v) focal thickening of alveolar membranes. A scale from 0 to 3

232 for each feature will be used (0: absence, 1: mild, 2: moderate, 3: most severe) (32).

233

234 **Immunohistochemistry:** Mouse lung paraffin sections 0.5 μm thick were applied to

235 positive electrical charge coated slides and left at 55^0C to remove paraffin excess.

236 Sections were deparaffinised by two consecutive treatments (5 minutes each) with

237 xylene. Rehydration was performed with graded ethanol's (90%, 80%, and 70%) for 4

238 minutes each. Antigen retrieval was subsequently performed by boiling the sections

239 with 1x Target Retrieval Solution pH 6.0 (Dako Ltd., Athens, Greece) in a steamer for

240 30 minutes. Endogenous peroxidase was blocked with 3% H_2O_2 in methanol for 15

241 minutes at room temperature. Primary antibody mouse monoclonal Mucin 5AC (clone

242 45M1, PierceThermo Scientific Inc) diluted 1:50 (v/v) in REAL™ Antibody Diluent

243 (Dako Ltd., Athens, Greece) was applied to sections and incubated for 30 minutes at

244 room temperature. Immunostaining reaction was developed using REAL™

245 EnVision™ Detection System Peroxidase/DAB+ Rabbit/Mouse (Dako Ltd., Athens,

246 Greece) incubated for 30 minutes at room temperature. Washes were performed

9

247   using Tris-Buffered Saline-Tween 20 (TBS-T) buffer for 10 minutes. Immunoreactivity

248   was detected using DAKO REAL DAB$^+$ Chromogen reagent for 5 min. Sections were

249   counterstained with Hematoxylin, dehydrated, mounted and examined. Sections from

250   which the primary antibody was omitted served as negative control. IHC slides were

251   evaluated by light microscopy and the immunosignal was scored using a

252   semiquantitative scoring system as previously described (1). An intensity score was

253   assigned representing the estimated average intensity of positive staining cells. The

254   staining intensity was classified into 4 scales scored as negative (0), weak (1+),

255   moderate (2+), and intensive (3+)(1).

256

257   **Determination of oxidative stress in the lung and the BALF:** Biomarkers of

258   oxidative stress were determined by measuring malondialdehyde and protein

259   carbonylation in the lung tissue. *MDA measurement***:** Lung tissue samples were

260   pulverized and then minced in a small volume of ice-cold 20 mm Tris-HCl buffer, pH

261   7.4, in a 1:10 w/v ratio, and homogenized. After centrifugation at 3000 g for 10 min

262   at 4 °C, the clear homogenate supernatant was used for biochemical assay. For the

263   determination of MDA, 0.65 mL of 10.3 mmol/L N-methyl-2-phenyl-indole in

264   acetonitrile was added to 0.2 mL of tissue sample. After vortexing for 3-4 s, 0.15 mL

265   of 15.4 mol/L methanesulfonic acid was added and samples were mixed well, closed

266   with a tight stopper, and incubated at 45 °C for 40 min. The samples were then

267   cooled on ice, centrifuged, and the absorbance was measured

268   spectrophotometrically at 586 nm. A calibration curve, made with standard MDA

269   solutions (from 2 to 20 nmol/mL), was also run for quantitation. Measurements were

270   performed in triplicate. MDA levels were expressed as µmol/mg protein (3). *Protein*

271   *carbonylation***:** A modification of the technique of Levine et al., (27) based on

272   spectrophotometric measurement of 2,4-dinitrophenylhydrazine (DNPH) derivatives

273   of PCs, was used to quantify protein carbonyl content in the lung or the BAL fluid of

274   the mice. Briefly, 100 µL of the homogenized lung tissue or of the BAL fluid was

10

275   incubated either with 500 mL DNPH or 2 mol/L HCl for 1 h at room temperature. The

276   samples were then reprecipated with 600 µL 20% trichloroacetic acid, incubated for

277   5 min on ice, and subsequently extracted with ethanol:ethyl acetate (1:1, v:v), 3

278   times at 11,000 g for 10 min at 4°C. The pellets were carefully drained and

279   dissolved in 6 mol/ L-guanidine solution in HO. The difference between the spectra

280   of the DNPH-treated sample and the HCl control was determined at 360 nm, and the

281   results are expressed as nmol PC/mg protein, using a molar extinction co-efficient of

282   22000 mol/L. Protein concentration was determined using the Lowrey assay.

283

284   **Determination of Cytokine in the BALF:** Cytokines levels (TNF-α, IL-1β, IL-6) in

285   the BALF were measured in 100µl BALF using the manufacturer's protocol (HS

286   Quantikine; R&D Systems, Minneapolis, MN). To determine the tissue cytokine levels,

287   lungs were homogenized as for the MDA measurements, and TNF-α, IL-1β and IL-6

288   were measured in the supernatant corresponding to similar amounts of protein (2mg

289   protein).

290

291   **Statistical Analysis.** Results are presented as means± SD of the number of

292   indicated observations. Statistical analysis was performed with Sigma Stat software

293   (SPSS 11.5, Chicago, IL, USA) using nonparametric tests for continuous variables

294   (Kruskall-Wallis, Mann-Whitney U). Differences were considered significant when

295   p<0.05.

296

11

297 **RESULTS**

298 **Bronchoalveolar lavage fluid (BALF) cellularity and protein content.** Total cell

299 counts in BALF of mice exposed to e-cig vapor for 3 days were increased in all

300 groups compared to air-breathing mice, mainly due to macrophage influx. BALF

301 cellularity in CS-exposed mice was also increased compared to air-breathing mice,

302 due to macrophage influx (90% of total cell count) and to a lesser extent to

303 neutrophils (7.1% of total cell count) (Figure 2A and 2B). BALF cell-free protein

304 content was increased only in the PG:VG-N+F group (p=0.001), compared to air-

305 breathing mice (Figure 2C).

306 The results were different after 4 weeks of exposure to cigarette smoke or e-cig vapor

307 (Figure 2D-F). Total BALF cell count was elevated only in the CS (p=0.0001) and PG:

308 VG-N+F (p=0.0001) groups, again mainly due to macrophage influx.These results

309 indicate that neither the PG:VG vehicle or nicotine addition to e-cig affected these

310 parameters at 4 weeks of vaping, however the addition of flavor to nicotine-containing

311 e-cig is capable of significantly inducing macrophage influx into the BALF. BALF

312 cellularity of CS mice also increased due to macrophage (87.5% of total cell count)

313 and neutrophil (8.2% of total cell count) influx (Figure 2D and 2E). BALF protein

314 content was elevated in the PG:VG-N (p=0.014) and PG:VG-N+F (p=0.003) groups

315 only, compared to air-breathing mice (Figure 2F), suggesting, that exposure to

316 nicotine through inhalation of e-cig vapor, but not through CS exposure, can increase

317 BALF protein content at this time point; in this case, addition of flavor to the e-cig had

318 no further effect.

319

320 **Markers of oxidative stress in the bronchoalveolar lavage fluid (BALF).** A 3 day

321 exposure to CS or e-cig vapor resulted in i) increased levels of malondialdehyde in

322 the PG:VG (p=0.016) and PG:VG-N+F (p=0.03), and ii) increased protein carbonyls in

323 all cigarette/e-cig-exposed groups compared to air-breathing mice (Figures 3A and

12

324   3C) [CS mice (p=0.013), PG:VG-Sol (p=0.03), PG:VG-N (p=0.022) and PG:VG-N+F

325   (p=0.004)].

326   Four weeks' exposure to CS or e-cig vapor resulted in i) increased levels of

327   malondialdehyde in CS (p=0.011), PG:VG-N (p=0.002) and PG:VG-N+F (p=0.003)

328   groups, ii) increased levels of protein carbonyls in BALF in CS (p=0.009), PG:VG-N

329   (p=0.019) and PG:VG-N+F (p=0.004) groups compared to air-breathing mice (Figure

330   3E and 3G). The only group that did not show an increase in BALF protein oxidation

331   markers at 4 weeks relative to air was the one exposed to PG:VG.

332

333   **Markers of oxidative stress in lung tissue.** Three days' exposure to CS or e-cig

334   vapor resulted I in) increased malondialdehyde levels were observed in the CS

335   (p=0.006), PG:VG-Sol (p=0.004) and PG:VG-N+F (p=0.011) groups, while ii)

336   increased lung protein carbonyls in the CS (p=0.004) and PG:VG-N+F (p= 0.004)

337   compared to air-breathing mice in lung tissue (Figure 3B and 3D). From these results,

338   one can conclude that the nicotine in the e-cig vapor, seems to mitigate the effect of

339   PG:VG-Sol, whereas the additional inclusion of flavor reverses this effect.   This

340   pattern is broadly reminiscent of the pattern in oxidation markers seen in BALF

341   (Figure 3A and 3C).

342

343   Four weeks' exposure to CS or e-cig vapor resulted in i) increased malondialdehyde

344   levels only in the CS and PGVG-N+F groups, and ii) similarly, increased protein

345   carbonyls levels in CS (p= 0.037) and in PGVG-N+F (p= 0.005) groups (Figure 3F

346   and 3H). Again, as seen at 3 days of exposure, the addition of flavor to the nicotine-

347   containing e-cigarette seemed to elevate the oxidation markers in the lung tissue to

348   levels similar to CS.

349

350   **Levels of pro-inflammatory cytokines in lung homogenates.** Exposure to CS or e-

351   cig vapor for 3 days resulted in increased levels of interleukin-1β and IL-6 in the

13

352 PG:VG-N+F group only (IL-1β, p= 0.047 and IL-6, p= 0.047, Figure 4B, 4C) without

353 affecting TNF-α levels (Figure 4A). Following 4 weeks of exposure to either CS or to

354 e-cig vapor, in agreement with the 3 day exposure, TNF-α levels remained

355 unchanged (Figure 4D). However, in contrast to the shorter exposure, where IL-1β

356 and IL-6 were significantly increased only in the PG:VG-N+F group, in this longer

357 exposure, levels of these two cytokines were significantly elevated (IL-1β, IL-6,

358 p=0.047) only in the CS-exposed mice (Figure 4E and 4F).

359

360 **Lung histopathology and Muc5a immunohistochemistry.** Exposure of mice for 3

361 days to CS in comparison to air or e-cig vapor resulted in a pronounced focal

362 thickening and interstitial inflammation. E-cig exposure failed to produce statistically

363 significant changes in the combined score (Figure 5A). The effects of inhaling CS was

364 equally pronounced after 4 weeks of exposure (Figure 5B). Moreover, it seems that

365 the increase in the score is more prominent at 3 days vs 4 weeks. Muc5ac protein

366 was homogenously detected in the apical surface of bronchial epithelial cells. CS,

367 PG:VG-Sol and PG:VG-N+F groups exhibited a pronounced Muc-5ac production in

368 the airways of mice upon 3-day exposurecompared to air-exposed mice (Figure 6A

369 and 6B).

370

371 **Measurements of respiratory system mechanics following CS or e-cig vapor**

372 **inhalation.** In addition, we assessed the functional consequences of CS in mice, so

373 we determined airway resistance, tissue elasticity and static compliance in the various

374 groups. Surprisingly, after 3 days of CS or e-cig vapor exposure, all three parameters

375 were significantly changed only in the PG:VG-Sol group. This group presented

376 increased airway resistance (p=0.004), tissue elasticity (p=0.001) and decreased

377 static compliance (p=0.001), compared to either the air-exposed mice or to the CS-

378 inhaling group (p=0.005 vs CS for airway resistance; p=0.001 for tissue elasticity and

379 p=0.001 for decreased compliance) (Figure 7A-C).After a four-week exposure to

14

380 cigarette smoke, the observed pattern was different; only the CS-exposed mice

381 presented significantly altered functional parameters.

382

383 **Determination of airway hyperresponsiveness.** To test for changes in pulmonary

384 reactivity to a standard experimental airway stimulus, we challenged mice that had

385 previously undergone a 3 day exposure to the same five inhaling regimes, to various

386 doses of methacholine. Only two groups, the CS group and the PG:VG-N+F vapor-

387 exposed group,exhibited increased airway hyperresponsiveness to increasing doses

388 of methacholine, compared to air-exposed mice (Figure 8A and 8B).

389

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392 **DISCUSSION**

393 The recent advent of e-cig and their rapidly increasing use among smokers puts

394 pressure on the scientific community to generate well-controlled preclinical and

395 clinical studies of pulmonary function, in order to compare side-by-side the effects of

396 "classical" CS to the effects of vapors from e-cig. Up to now, there is only a limited

397 number of in vitro studies on human and murine pulmonary epithelial cell lines,

398 fibroblasts and stem cells ((2, 15, 37); studies reviewed in(6)) that have addressed

399 the acute toxicological profile of refill liquid contents of e-cig. In a recent study of

400 Scheffler et al (38), direct exposure of primary bronchial epithelial cells to e-cig vapor

401 containing glycerol/propylene glycol, induced oxidative stress that was less

402 pronounced, compared to the stress induced by conventional CS. In addition, the

403 effect of chemicals found in the e-cig flavors has been shown to also be responsible

404 for toxic effects on pulmonary fibroblasts (2, 7). Recent studies have addressed the

405 pulmonary effects of e-cig in animals models in vivo (17, 22, 26, 42) and while they

406 are very informative, they are characterized by either use of a single time of

407 exposure, by focus on inflammatory and immune responses alone in the absence of

408 addressing functional lung mechanics and lastly, only occasionally do they address

409 the effect of specific e-cig components, such as the widely used vehicle, PG:VG, or

410 added flavors. To complete this gap, in our in vivo study in mice, we a) varied the

411 length of exposure to either CS and e-cig vapor, and b) we examined the effects of

412 nicotine and flavorings added to the main "vehicle" in e-cig (PG:VG), on inflammatory

413 markers and functional parameters of the exposed lungs.

414 Pulmonary irritation and other effects of propylene glycol (a major constituent of e-cig

415 fluid "vehicle") have been noted in humans(6, 20). In agreement with these

416 observations, our study showed that the propylene glycol containing vehicle (PG:VG-

417 Sol) was able to increase BALF cellularity, induce oxidative modifications, raise

418 epithelial Muc5A production and negatively impact lung mechanics at 3 days of

16

419 exposure.   These effects were mostly absent following exposure to PG:VG for 4

420 weeks, suggesting a transient respiratory irritation by the vehicle that subsides upon

421 further exposure.

422

423 As far as nicotine content in e-cig liquids and its biological effects are concerned,

424 most previous studies have generally reported a "nicotine effect", usually by

425 comparison to "air", without being able to distinguish whether the observed changes

426 are due to the e-cig liquid vehicle, to the nicotine or to the flavours, if present. In our

427 experiments, nicotine addition was found to exert variable effects either aggravating,

428 causing no change or ameliorating the detrimental effects of e-cig vapour depending

429 on the parameter and time point studied.   E-cig toxicity when tested in vitro with

430 embryonic and adult cells was not attributed to nicotine, but correlated with the

431 number and concentration of chemicals used to flavour fluids(2).   Only one in vivo

432 study, that of Garcia-Across et al, has shown clear nicotine-dependent effects on

433 mouse airway hyperactivity, mucin production, distal airspace enlargement, cytokine

434 and protease expression and impaired ciliary beat frequency (17).

435

436 Hundreds of flavours have been proven safe as food additives but there are no data

437 about their impact to the respiratory system, and it is well-known that the route of

438 exposure plays a significant role in the response to xenobiotics. In vitro, as well as

439 human studies have revealed potential hazards of e-cig vapor due to their flavoring

440 components (17, 20, 26, 40). In our study tobacco flavored, nicotine-containing e-cig

441 vapor (PG:VG-N+F) showed significant effects in BALF cellularity and protein content

442 after both 3 days and 4 weeks of exposure. Notably, the determination of the two

443 oxidative markers (MDA, carbonyls) showed that the flavor contained in e-cig vapor

444 allowed for a marked effect in both the BALF and the lung; in the latter case, the

445 effect was not shown by nicotine-alone e-cig exposure (PG:VG-N). Along the same

446 lines, at the 3 day exposure, again, among the various e-cig exposure regimes, only

17

447   the PG:VG-N+F–exposed mice showed increases in IL-6 and IL-1β. Last, PG:VG-

448   N+F exposure alone among e-cig-inhalation regimes was able to exacerbate the

449   methacholine response, as did CS. Thus, in many of the parameters studied herein,

450   the hazardous effects noted after exposure to e-cig vapors were exacerbated or

451   persisted longer when flavoring was added to the refill liquid. Given the thousands of

452   flavors available world-wide, meticulously examination of these liquids and their

453   components to proof which ones are safe would be a daunting task.

454

455   Comparison of the effects of CS vs e-cig vapor yielded interesting results. Both short

456   term (3 days) and long term (4 weeks) exposure to e-cig vapor resulted in an increase

457   in BALF cellularity, BALF protein and oxidative stress that were more pronounced in

458   the e-cig group or equal between e-cig and CS. In line with our observations, Lerner

459   et al (26) reported that exposure of mice to e-cig diminished lung glutathione levels.

460   Levels of inflammatory cytokines exhibited a different pattern compared to BALF

461   cellularity and oxidative stress. After 3 days of exposure only PG:VG-N+F increased

462   the levels of IL-1β and IL-6 in the lung, whereas after 4 weeks the effect of PG:VG-

463   N+F was absent and only CS elevated IL-1β and IL-6 in the lung. Our observations

464   are in accordance with the study of Lerner et al (26) where acute (3 days) exposure to

465   e-cig containing both nicotine (16 mg) and classic tobacco flavor elicited an increase

466   in the levels of pro-inflammatory mediators, such as IL-6, in the BALF. In a different

467   study where mice were exposed for 2 weeks to e-cig vapor (42), although

468   macrophage influx in the BALF was seen, IL-6 levels in the BALF were lower than in

469   mice that were exposed to air. This discrepancy may be related to the different time of

470   exposure between the two studies and to the different compartment analysed (BALF

471   in their work vs lung homogenate herein).   It should be noted that in the work of

472   Sussan et al (42) it is not entirely clear whether the exposure of these mice was to

473   flavored or unflavored e-cig.

18

474

475 To further examine the potential harmful effect on the airways and compare CS and

476 e-cig effects, we measured mucin production. After 3 day exposure CS, PG:VG-Sol

477 and PG:VG-N+F exposure showed higher levels of Muc-5ac in the airways compared

478 to air-exposed mice. Of note, among mucins, Muc5ac is a predominant gel-forming

479 mucin induced in allergic murine lungs (13), suggesting a fundamental effector role in

480 airway hyperreactivity. Our finding regarding Muc5ac in mice exposed to CS and

481 PG:VG-N+F correlates with the observation that these groups of mice also exhibited

482 increased airway reactivity after methacholine challenge. In line with this, Lim et al

483 (28) reported that e-cig vapour exacerbated the allergy-induced asthma symptoms in

484 mice, although in this latter model the e-cig liquid was not vaporized, but was instilled

485 intra-tracheally. Complementing our findings, Garcia-Arcos et al, also reported that

486 long term exposure to nicotine-containing e-cig vapor increased airway

487 hyperreactivity to methacholine (17).

488

489 Our results provide evidence for impairment of functional lung parameters in mice

490 after short term exposure to e-cig vapors. Interestingly, resistance, elastance and

491 compliance were only affected in the PG:VG-Sol group. Larcombe et al. have also

492 reported decrements in parenchymal lung function at both functional residual capacity

493 and high transrespiratory pressures after exposure to e-cig vapors(25). The above-

494 mentioned preclinical data is in agreement with human studies; Vardavas et al. (45)

495 have shown that short term exposure to e-cig vapour can increase the impedance

496 and peripheral airway flow resistance, suggesting that airway constriction due to e-cig

497 use is a result of the irritant effects of propylene glycol. After more prolonged

498 exposure to e-cig vapors, the effect on pulmonary mechanics disappeared. At this

499 time point, pulmonary mechanics were adversely affected in CS exposed mice.

500

19

501   To study the effects of CS or e-cig vapors on lung structure we used histology and

502   determined inflammatory cell intra-alveolar and interstitial recruitment, congestion and

503   induced intra-alveolar and interstitial oedema. Interestingly, we found a high lung

504   injury score only in CS exposed mice both after 3 days or 4 weeks of exposure,

505   compared to e-cig vapour- or air-exposed mice. Our data are aligned with the

506   evidence of the less toxic effect of e-cig vapor compared to tobacco smoke (37),

507   especially regarding the loss of lung integrity in mice (22), albeit e-cig vapor exposure

508   clearly promotes pulmonary inflammatory effects (6).

509

510   In summary, we have shown that all ingredients of e-cig refills, including the vehicle

511   propylene glycol/vegetable glycerine, can cause changes in lung injury and

512   respiratory mechanics. These effects were exacerbated by the addition of flavouring

513   to the e-cig. The observed detrimental effects in the lung upon e-cig vapour exposure

514   in animal models highlight the need for further investigation of safety and toxicity of

515   these rapidly expanding devices worldwide.

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518 **REFERENCES**

519 1.   Allred D, Harvey J, Berardo M, and Clark G. Prognostic and predictive

520 factors in breast cancer by immunohistochemical analysis. Mod Pathol 11: 155-168,

521 1998.

522 2.   Bahl V, Lin S, Xu N, Davis B, Wang Y, and Talbot P. Comparison of

523 electronic cigarette refill fluid cytotoxicity using embryonic and adult models. Reprod

524 Toxicol 34: 529-537, 2012.

525 3.   Bibli S-I, Andreadou I, Glynos C, Chatzianastasiou A, Toumpanakis D,

526 Zakynthinos S, Vasilakopoulos T, Iliodromitis EK, and Papapetropoulos A. Exposure

527 to cigarette smoke abrogates the beneficial effect of ischemic postconditioning. Am J

528 Physiol Heart Circ Physiol 311: H1321-H1332, 2016.

529 4.   Camenga DR and Klein JD. Tobacco Use Disorders. Child Adolesc Psychiatr

530 Clin N Am 25: 445-460, 2016.

531 5.   Carey MA, Card JW, Voltz JW, Germolec DR, Korach KS, and Zeldin DC.

532 The impact of sex and sex hormones on lung physiology and disease: lessons from

533 animal studies. Am J Physiol Lung Cell Mol Biol 293: L272-L278, 2007.

534 6.   Chun LF, Moazed F, Calfee CS, Matthay MA, and Gotts JE. Pulmonary

535 Toxicity of E-cigarettes. Am J Physiol Lung Cell Mol Physiol: L193-L206, 2017.

536 7.   Clapp PW, Pawlak EA, Lackey JT, Keating JE, Reeber SL, Glish GL, and

537 Jaspers I. Flavored E-cigarette Liquids and Cinnamaldehyde Impair Respiratory

538 Innate Immune Cell Function. Am J Physiol Lung Cell Mol Physiol, 2017.

539 8.   Cobb NK and Abrams DB. E-Cigarette or Drug-Delivery Device? Regulating

540 Novel Nicotine Products. N Engl J Med 365: 193-195, 2011.

541 9.   Crotty Alexander L, Shin S, and Hwang J. Inflammatory Diseases of the

542 Lung Induced by Conventional Cigarette Smoke: A Review. Chest 148: 1307-1322,

543 2015.

21

544   10.   D'hulst AI, Vermaelen KY, Brusselle GG, Joos GF, and Pauwels RA. Time

545   course of cigarette smoke-induced pulmonary inflammation in mice. Eur Resp J 26:

546   204-213, 2005.

547   11.   Dinakar C and O'Connor GT. The Health Effects of Electronic Cigarettes. N

548   Engl J Med 375: 1372-1381, 2016.

549   12.   Etter J-F and Bullen C. Electronic cigarette: users profile, utilization,

550   satisfaction and perceived efficacy. Addiction 106: 2017-2028, 2011.

551   13.   Evans CM, Raclawska DS, Ttofali F, Liptzin DR, Fletcher AA, Harper DN,

552   McGing MA, McElwee MM, Williams OW, Sanchez E, Roy MG, Kindrachuk KN, Wynn

553   TA, Eltzschig HK, Blackburn MR, Tuvim MJ, Janssen WJ, Schwartz DA, and Dickey

554   BF. The polymeric mucin Muc5ac is required for allergic airway hyperreactivity. Nat

555   Commun 6: 6281-6281, 2015.

556   14.   Farsalinos K, Gillman I, Melvin M, Paolantonio A, Gardow W, Humphries K,

557   Brown S, Poulas K, and Voudris V. Nicotine Levels and Presence of Selected

558   Tobacco-Derived Toxins in Tobacco Flavoured Electronic Cigarette Refill Liquids. Int

559   J Environ Res Public Health 12: 3439-3452, 2015.

560   15.   Farsalinos KE, Romagna G, Allifranchini E, Ripamonti E, Bocchietto E,

561   Todeschi S, Tsiapras D, Kyrzopoulos S, and Voudris V. Comparison of the Cytotoxic

562   Potential of Cigarette Smoke and Electronic Cigarette Vapour Extract on Cultured

563   Myocardial Cells. Int J Environ Res Public Health 10: 5146-5162, 2013.

564   16.   Fogt DL, Levi MA, Rickards CA, Stelly SP, and Cooke WH. Effects of Acute

565   Vaporized Nicotine in Non-Tobacco Users at Rest and during Exercise. Int J Exerc

566   Sci 9: 607-615, 2016.

567   17.   Garcia-Arcos I, Geraghty P, Baumlin N, Campos M, Dabo AJ, Jundi B,

568   Cummins N, Eden E, Grosche A, Salathe M, and Foronjy R. Chronic electronic

569   cigarette exposure in mice induces features of COPD in a nicotine-dependent

570   manner. Thorax 71: 1119-1129, 2016.

22

571 18. Glynos C, Dupont L, Vassilakopoulos T, Papapetropoulos A, Brouckaert P,

572 Giannis A, Joos G, KR B, and Brusselle G. The Role of Soluble Guanylyl Cyclase in

573 Chronic Obstructive Pulmonary Disease. Am J Respir Crit Care Med 188: 789-799,

574 2013.

575 19. Glynos C, Toumpanakis D, Loverdos K, Karavana V, Zhou Z, Magkou C,

576 Dettoraki M, Perlikos F, Pavlidou A, Kotsikoris V, Topouzis S, Theocharis S,

577 Brouckaert P, Giannis A, Papapetropoulos A, and Vassilakopoulos T. Guanylyl

578 Cyclase Activation Reverses Resistive Breathing–Induced Lung Injury and

579 Inflammation. Am J Respir Cell Mol Biol 52: 762-771, 2015.

580 20. Grana R, Benowitz N, and Glantz SA. E-Cigarettes: A Scientific Review.

581 Circulation 129: 1972-1986, 2014.

582 21. Guindon G and Boisclair D. Past, current and future trends in tobacco use:

583 HNP discussion paper. . Washington: The World Bank; 2003 Available from:

584 http://escholarship.org/uc/item/4q57d5vp, 2003.

585 22. Husari A, Shihadeh A, Talih S, Hashem Y, El Sabban M, and Zaatari G.

586 Acute Exposure to Electronic and Combustible Cigarette Aerosols: Effects in an

587 Animal Model and in Human Alveolar Cells. Nicotine Tob Res 18: 613-619, 2016.

588 23. Ioakeimidis N, Vlachopoulos C, and Tousoulis D. Efficacy and Safety of

589 Electronic Cigarettes for Smoking Cessation: A Critical Approach. Hel J Cardiol 57: 1-

590 6, 2016.

591 24. Kalkhoran S and Glantz SA. E-cigarettes and smoking cessation in real-

592 world and clinical settings: a systematic review and meta-analysis. Lancer Resp Med

593 4: 116-128, 2016.

594 25. Larcombe N, Janka M, Mullins B, Berry L, Bredin A, and Franklin P. The

595 effects of electronic cigarette aerosol exposure on inflammation and lung function in

596 mice. Amer J Physiol Lung Cell Mol Physiol 313: L67-L79, 2017.

597 26. Lerner CA, Sundar IK, Yao H, Gerloff J, Ossip DJ, McIntosh S, Robinson R,

598 and Rahman I. Vapors Produced by Electronic Cigarettes and E-Juices with

23

599 Flavorings Induce Toxicity, Oxidative Stress, and Inflammatory Response in Lung

600 Epithelial Cells and in Mouse Lung. PLoS ONE 10: e0116732, 2015.

601 27.　　Levine R, Garland D, Oliver C, Amici A, Climent I, Lenz A, Ahn B, Shaltiel S,

602 and Stadtman E. Determination of Carbonyl Content in Oxidatively Modified Proteins.

603 Methods Enzymol 186: 464-478, 1990.

604 28.　　Lim HB and Kim SH. Inhallation of e-Cigarette Cartridge Solution Aggravates

605 Allergen-induced Airway Inflammation and Hyper-responsiveness in Mice. Toxicol

606 Res 30: 13-18, 2014.

607 29.　　Lundbäck B, Katsaounou P, and Lötvall J. The up-rise in e-cigarette use –

608 friend or foe? Respir Res 17: 52, 2016.

609 30.　　Martin EM, Clapp PW, Rebuli ME, Pawlak EA, Glista-Baker E, Benowitz NL,

610 Fry RC, and Jaspers I. E-cigarette use results in suppression of immune and

611 inflammatory-response genes in nasal epithelial cells similar to cigarette smoke. Amer

612 J Physiol Lung Cell Mol Physiol 311: L135-L144, 2016.

613 31.　　McCauley L, Markin C, and Hosmer D. An unexpected consequence of

614 electronic cigarette use. Chest 141: 1110-1113, 2012.

615 32.　　Murao Y, Loomis W, Wolf P, Hoyt D, and Junger W. Effect of Dose of

616 Hypertonic Saline on Its Potential to Prevent Lung Tissue Damage in a Mouse Model

617 of Hemorrhagic Shock. Shock 20: 29-34, 2003.

618 33.　　Öberg M, Jaakkola MS, Woodward A, Peruga A, and Prüss-Ustün A.

619 Worldwide burden of disease from exposure to second-hand smoke: a retrospective

620 analysis of data from 192 countries. Lancet 377: 139-146, 2011.

621 34.　　Palamidas A, Tsikrika S, Katsaounou PA, Vakali S, Gennimata S-A,

622 kaltsakas G, Gratziou C, and Koulouris N. Acute effects of short term use of e-

623 cigarettes on airways physiology and respiratory symptoms in smokers with and

624 without airways obstructive diseases and in healthy non smokers. Tob Prev Cessat 3,

625 2017.

24

626 35.   Pepper JK and Brewer NT. Electronic nicotine delivery system (electronic

627 cigarette) awareness, use, reactions and beliefs: a systematic review. Tob Control 23:

628 375-384, 2014.

629 36.   Pisinger C and Døssing M. A systematic review of health effects of electronic

630 cigarettes. Prev Med 69: 248-260, 2014.

631 37.   Romagna G, Allifranchini E, Bocchietto E, Todeschi S, Esposito M, and

632 Farsalinos KE. Cytotoxicity evaluation of electronic cigarette vapor extract on cultured

633 mammalian fibroblasts (ClearStream-LIFE): comparison with tobacco cigarette smoke

634 extract. Inhal Toxicol 25: 354-361, 2013.

635 38.   Scheffler S, Dieken H, Krischenowski O, Förster C, Branscheid D, and

636 Aufderheide M. Evaluation of E-Cigarette Liquid Vapor and Mainstream Cigarette

637 Smoke after Direct Exposure of Primary Human Bronchial Epithelial Cells. Int J

638 Environ Res Public Health 12: 3915, 2015.

639 39.   Schick SF, Blount BC, 3rd PJ, Saliba NA, Bernert JT, Hellani AE, Jatlow P,

640 Pappas RS, Wang L, Foulds J, Ghosh A, Hecht SS, Gomez JC, Martin JR, Mesaros

641 C, Srivastava S, Helen GS, Tarran R, Lorkiewicz PK, Blair IA, Kimmel HL, Doerschuk

642 CM, Benowitz NL, and Bhatnagar A. Biomarkers of exposure to new and emerging

643 tobacco delivery products. Amer J Physiol Lung Cell Mol Physiol 313: L425-L452,

644 2017.

645 40.   Sherwood CL and Boitano S. Airway epithelial cell exposure to distinct e-

646 cigarette liquid flavorings reveals toxicity thresholds and activation of CFTR by the

647 chocolate flavoring 2,5-dimethypyrazine. Respir Res 17: 57, 2016.

648 41.   Simoes D, Vassilakopoulos T, Toumpanakis D, Petrochilou K, Roussos C,

649 and Papapetropoulos A. Angiopoietin-1 Protects against Airway Inflammation and

650 Hyperreactivity in Asthma. Am J Respir Crit Care Med 177: 1314-1321, 2008.

651 42.   Sussan TE, Gajghate S, Thimmulappa RK, Ma J, Kim J-H, Sudini K,

652 Consolini N, Cormier SA, Lomnicki S, Hasan F, Pekosz A, and Biswal S. Exposure to

25

653   Electronic Cigarettes Impairs Pulmonary Anti-Bacterial and Anti-Viral Defenses in a

654   Mouse Model. PLoS ONE 10: e0116861, 2015.

655   43.   Thota D and Latham E. Case report of electronic cigarettes possibly

656   associated with eosinophilic pneumonitis in a previously healthy active-duty sailor. J

657   Emerg Med 47: 15-17, 2014.

658   44.   Toumpanakis D, Kastis G, Zacharatos P, Sigala I, Michailidou T, Kouvela M,

659   Glynos C, Divangahi M, Roussos C, Theocharis S, and Vassilakopoulos T. Inspiratory

660   Resistive Breathing Induces Acute Lung Injury. Am J Respir Crit Care Med 182: 1129-

661   1136, 2010.

662   45.   Vardavas C, Anagnostopoulos N, Kougias M, Evangelopoulou V, Connolly

663   G, and Behrakis P. Short-term pulmonary effects of using an electronic cigarette:

664   impact on respiratory flow resistance, impedance, and exhaled nitric oxide. Chest

665   141: 1400-1406, 2012.

666   46.   Wollscheid KA and Kremzner ME. Electronic cigarettes: Safety concerns and

667   regulatory issues. Am J Health Syst Pharm 66: 1740-1742, 2009.

668   47.   Zhu S-H, Gamst A, Lee M, Cummins S, Yin L, and Zoref L. The Use and

669   Perception of Electronic Cigarettes and Snus among the U.S. Population. PLoS ONE

670   8: e79332, 2013.

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672

673 **Figure Legends**

674

675 **Figure 1. Equipment and treatment protocol.** (A) The proprietary modified chamber

676 used for both CS and e-cigarette vapor exposure of mice. (B) Schematic

677 representation of the protocol of exposure to CS and E-cigarette vapour

678

679 **Figure 2**: **Effects on BALF cellularity and protein content.** Following an acute (3

680 day, A-C) and a sub-chronic 4 week (D-F) exposure to CS and e-cig vapor, BALF was

681 obtained and following centrifugation, total cellularity was determined (A and D).

682 Differential counts, to assess cell-type-specific contribution (B, D) were performed on

683 Giemsa-stained cytospins. Cell-free protein was determined in the supernatant (C, F).

684 Values are expressed as mean±SD; n=5-10 mice * $p<0.05$ Vs Air, # $p<0.05$ Vs CS.

685 BALF: bronchoalveolar lung fluid, CS: cigarette smoking, PG:VG-Sol propylene

686 glycol/vegetable glycerol, PG:VG-N: propylene glycol/vegetable glycerol with nicotine,

687 PG:VG-N+F: propylene glycol/vegetable glycerol with nicotine and tobacco blend

688 flavor.

689 **Figure 3: Determination of markers of oxidative stress following smoking and**

690 **vaping.** Following an acute (3 days, A-D) and a sub-chronic 4 weeks (E-H) exposure

691 to CS and e-cigarette vapour MDA (A, B, E F) and protein carbonyl (C, D, G, H)

692 oxidative markers were determined in the BALF (A, C, E, G) and lung homogenate (B,

693 D, F, H).Values are expressed as mean±SD; n=5-8 mice * $p<0.05$ Vs Air, # $p<0.05$ Vs

694 CS. BALF: bronchoalveolar lung fluid, MDA: malondialdehyde CS: cigarette smoking,

695 PG:VG-Sol propylene glycol/vegetable glycerol, PG:VG-N: propylene glycol/vegetable

696 glycerol with nicotine, PG:VG-N+F: propylene glycol/vegetable glycerol with nicotine

697 and tobacco blend flavor.

27

698 **Figure 4: Determination of markers of inflammation following smoking and**

699 **vaping.** Following an acute (3 days, A-C) and a sub-chronic 4 weeks (D-F) exposure

700 to CS and e-cig vapor, cytokines were measured using commercial kits in lung

701 homogenates. A, D: TNF-α; B, E, IL-1β; C, E: IL-6. Values are expressed as

702 mean±SD n=5 mice * $p < 0.05$ Vs Air. TNF-α: Tumor Necrosis Factor-α, IL: Interleukin,

703 CS: cigarette smoking, PG:VG-Sol propylene glycol/vegetable glycerol, PG:VG-N:

704 propylene glycol/vegetable glycerol with nicotine, PG:VG-N+F: propylene

705 glycol/vegetable glycerol with nicotine and tobacco blend flavor.

706

707 **Figure 5: Lung histological changes are evident after smoking, but not after**

708 **vaping.** Following an acute (3-days, A) and a sub-chronic 4-week (B) exposure to CS

709 and e-cig vapor, the left lung was obtained and histopathology was performed on

710 hematoxylin-eosin-fixed sections according to Murao et al, 2003 (26).Values are

711 expressed as mean±SD; n=5-8 mice * $p < 0.05$ Vs Air, # $p < 0.05$ Vs CS. CS: cigarette

712 smoking, PG:VG-Sol propylene glycol/vegetable glycerol, PG:VG-N: propylene

713 glycol/vegetable glycerol with nicotine, PG:VG-N+F: propylene glycol/vegetable

714 glycerol with nicotine and tobacco blend flavor.

715

716 **Figure 6: Changes in lung Muc-5ac following smoking and vaping.** After 3 days

717 of exposure to CS or e-cig vapor, mouse lung paraffin sections were stained using an

718 anti-Mucin 5ac antibody (A). IHC slides were evaluated by light microscopy and the

719 immune signal was scored (B) using a semi quantitative scoring system as previously

720 described (1). Values are expressed as mean ± SD; n=5-6 mice * $p < 0.05$ Vs Air, #

721 $p < 0.05$ Vs CS. CS: cigarette smoking, PG:VG-Sol propylene glycol/vegetable

722 glycerol, PG:VG-N: propylene glycol/vegetable glycerol with nicotine, PG:VG-N+F:

723 propylene glycol/vegetable glycerol with nicotine and tobacco blend flavor.

28

724 **Figure 7: Changes in respiratory system mechanics following smoking and**

725 **vaping.** Mouse lung function was measured using a forced oscillation before the

726 onset of cigarette exposure (baseline) and following exposure of mice for either 3

727 days (A-C) or 4 weeks (D-F) to CS or e-cig vapor. Parameters determined include

728 airway resistance (R, figures A, D), static compliance (C, figures B, F) and tissue

729 elasticity (H, figures C, E). Values are expressed as mean ± SD; n=5-12 mice *

730 $p<0.05$ Vs Air, # $p<0.05$ Vs CS. CS: cigarette smoking, PG:VG-Sol propylene

731 glycol/vegetable glycerol , PG:VG-N: propylene glycol/vegetable glycerol with

732 nicotine, PG:VG-N+F: propylene glycol/vegetable glycerol with nicotine and tobacco

733 blend flavor.

734

735 **Figure 8: Airway hyperresponsiveness in animals exposed to cigarette smoke**

736 **or e-cig vapor.** Airway responsiveness mice was assessed before and after

737 methacholine challenge (2.5, 10, 20, 40, 60 mg/ml), 24 four hours following a 3 day

738 exposure to room air, CS or e-cigarette vapour. **(A)** Airway resistance (R) as percent

739 increase of baseline in response to methacholine. **(B)** Area under curve (AUC) for

740 airway resistance. Values are expressed as mean±SD; n=5 mice * $p<0.05$ Vs Air, #

741 $p<0.05$ Vs CS. CS: cigarette smoking, PG:VG propylene glycol/vegetable glycerol,

742 PG:VG-N: propylene glycol/vegetable glycerol with nicotine, PG:VG-N+F: propylene

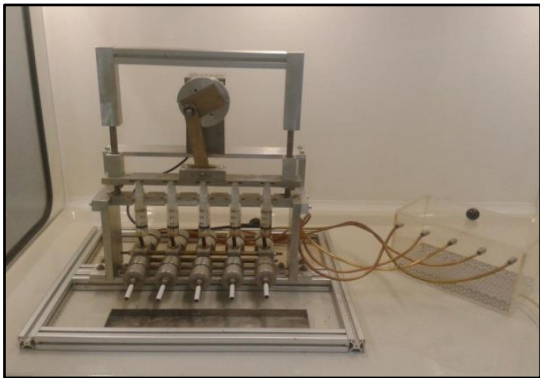743 glycol/vegetable glycerol with nicotine and tobacco blend flavor.
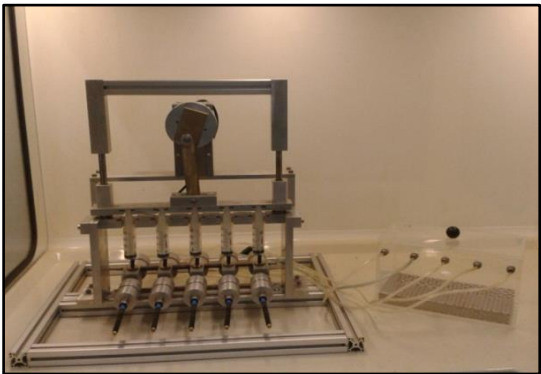
744

745

746

29

Figure 1

A

Modified cigarette smoking chamber

cigarette smoking

B

Modified e-cigarette smoking chamber

e-cigarette vaping

C

3R4F cigarette

D

Battery Atomizer head Atomizer cone Cartridge

e-cigarette

E

3 days → X ——— cigarette smoking ——— 4 weeks >

——— E-cig. vaping ——— 4 weeks >

3 days → X

Lung Tissue
BAL fluid
• *Cellularity*
• *Protein content*
• *Oxidaive stress*
• *Cytokines*
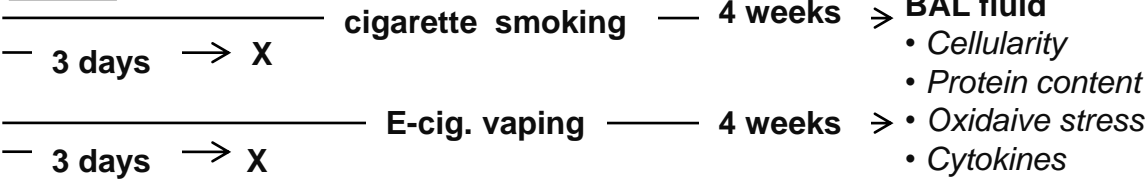
Groups:

1. **Air**

2. **Cigarette smoking (**3R4F University of Kentucky, USA) **(CS)**

3. **Propylene glycol/ vegetable glycerol 1:1 (PV:VG)**

4. **Propylene glycol/ vegetable glycerol with nicotine 18mg/mL (PV:VG-N)**

5. **Propylene glycol/vegetable glycerol with nicotine 18mg/mL plus tobacco blend flavour 4% (PV:VG-N+F)**
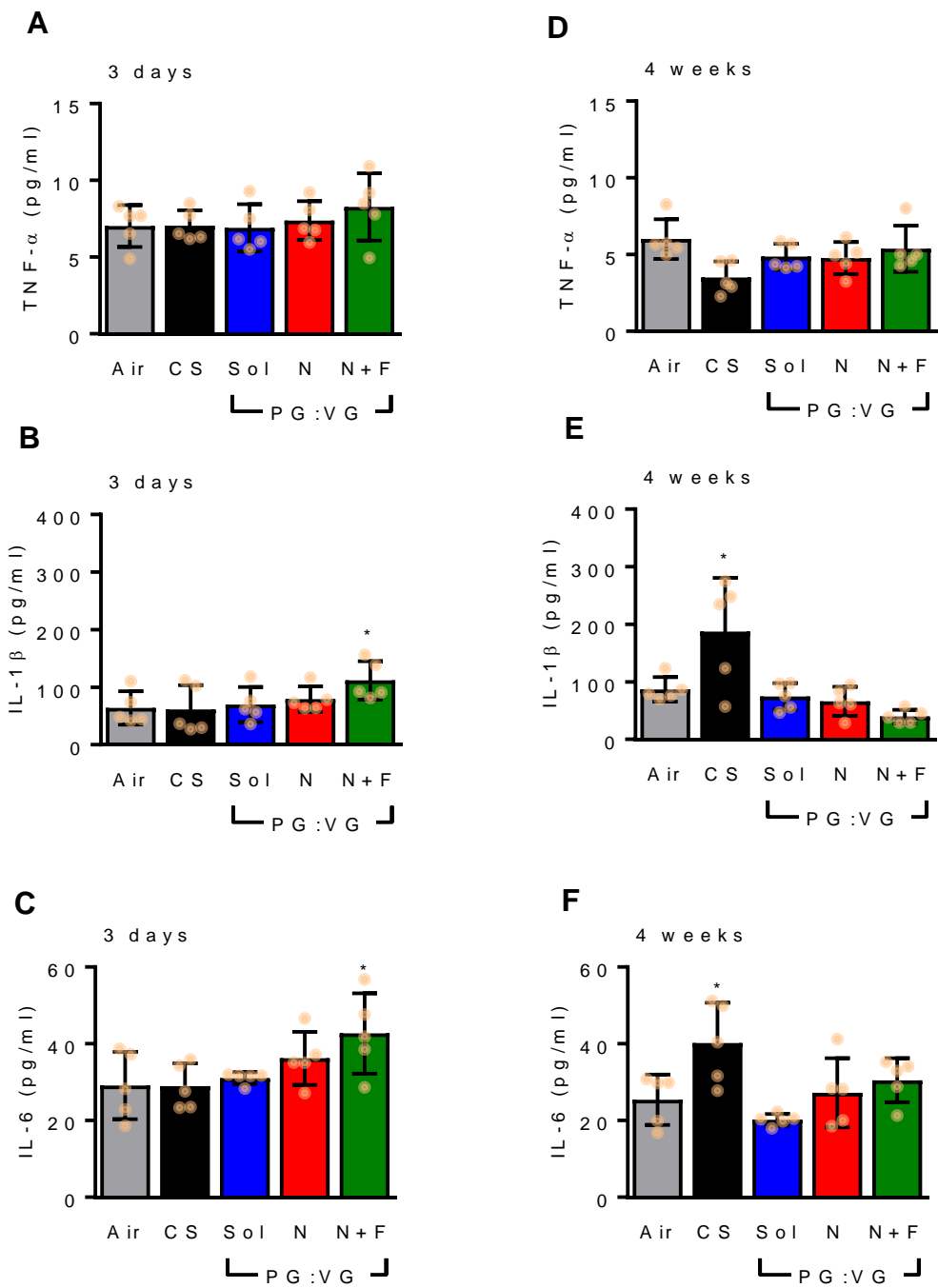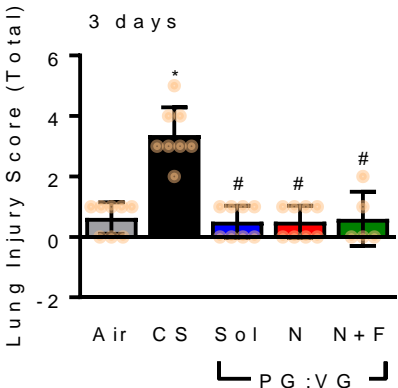
Figure 2
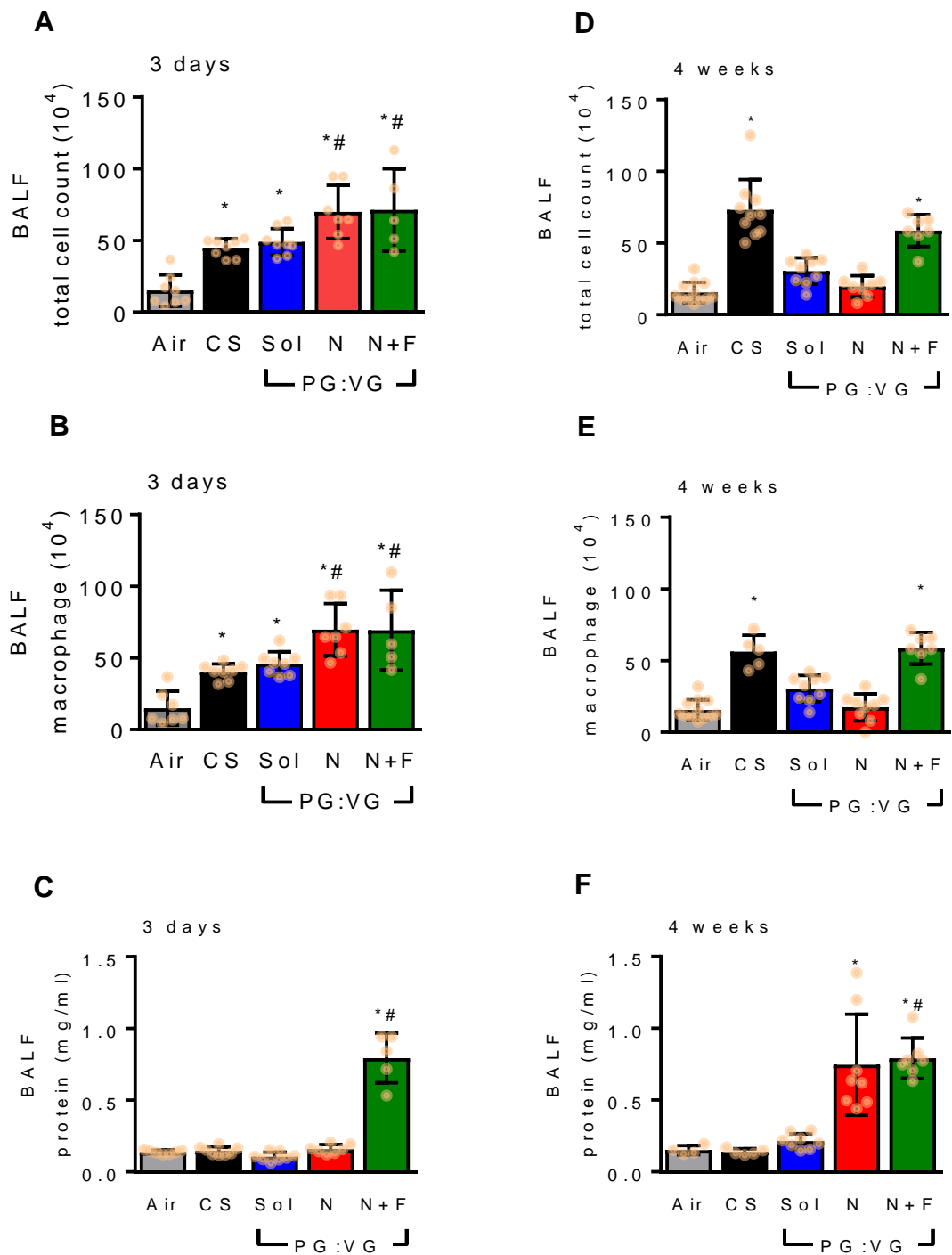
Figure 3

Figure 4

Figure 5

A

Air CS
PV:VG PV:VG-N
PV:VG-F
50 μm

3 days

Lung Injury Score (Total)

Air CS Sol N N+F

PG:VG

B

Air CS
PV:VG PV:VG-N
PV:VG-F
50 μm

4 weeks

Lung Injury Score (Total)

Air CS Sol N N+F

PG:VG
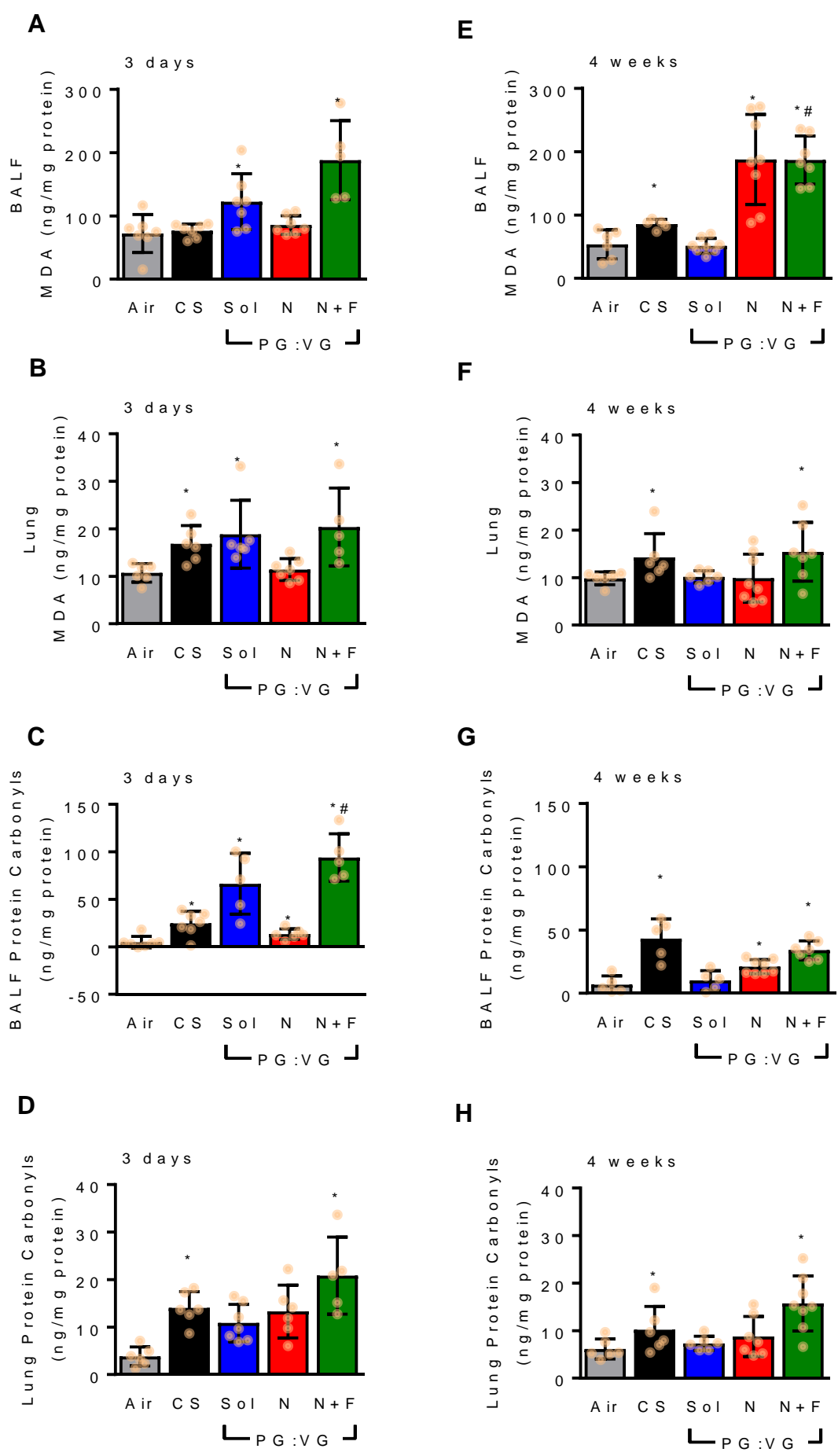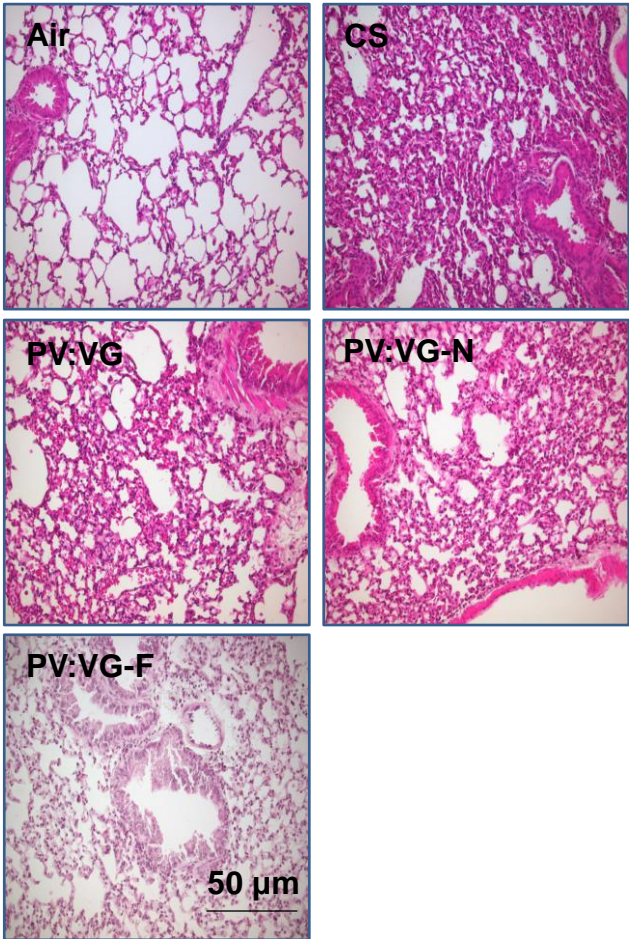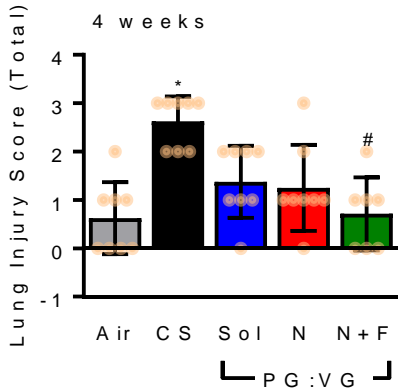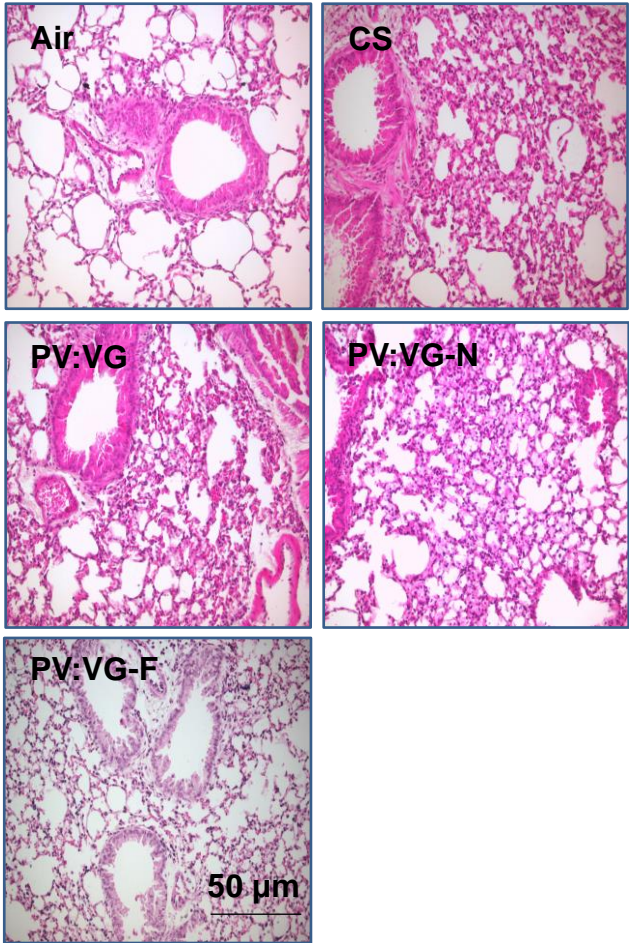
Figure 6

Figure 7

Figure 8

A

R (% of baseline)

- Air
- CS
- PG:VG
- PG:VG-N
- PG:VG-N+F

1.5×10^3
1×10^3
5×10^2
0

0 2,5   10   20   40   60

methacholine (mg/ml)

B

AUC

800
600
400
200
0

*
*#

Air CS Sol N N+F

└ PG:VG ┘