E-cigarette aerosol exposure of pulmonary surfactant impairs its surface tension reducing function.

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Abstract

E-cigarette (EC) and vaping use continue to remain popular amongst teenage and young adult populations, despite several reports of vaping associated lung injury. This popularity is due in part to the vast variety of appealing flavours and nicotine concentrations easily accessible on the market. One of the first compounds that EC aerosols comes into contact within the lungs during a deep inhalation is pulmonary surfactant. This lipid protein mixture lines the alveoli, reducing surface tension and preventing alveolar collapse. Impairment of surfactant's critical surface tension reducing activity can contribute to lung dysfunction. Currently, information on how EC aerosols impacts pulmonary surfactant remains limited. We hypothesized that exposure to EC aerosol impairs the surface tension reducing ability of surfactant. Bovine Lipid Extract Surfactant (BLES) was used as a model surfactant in a direct exposure syringe system. BLES (2ml) was placed in a syringe (30ml) attached to an EC. The generated aerosol was drawn into the syringe and then expelled, repeated 30 times. Biophysical analysis after exposure was completed using a constrained drop surfactometer (CDS). Minimum surface tensions increased after exposure to the EC aerosol. Variation in device used, addition of nicotine, or temperature of the aerosol had no additional effect. Two e-liquid flavours, menthol and red wedding, had further detrimental effects, resulting in higher surface tension than the vehicle exposed BLES. Alteration of surfactant properties through interaction with the produced aerosol was observed with a basic e-liquid vehicle, however additional compounds produced by added flavourings appeared to be able to increase inhibition. In conclusion, EC aerosols alter surfactant function through increases in minimum surface tension. This impairment may contribute to lung dysfunction and susceptibility to further injury.

53

54 # Introduction

55 Vaping and the use of e-cigarettes (ECs) has seen a marked increase over the last decade,
56 particularly amongst youth and young adults (1-3). Although originally regarded as a popular
57 smoking cessation tool, it is becoming increasingly clear that the target demographic for EC
58 marketing has shifted from smokers to teenagers and young adults (3). Most of this population
59 are not regular smokers, but instead are drawn to the attraction of efficient nicotine delivery and
60 the large variety of appealing flavours available for recreational use (1, 3). Previous studies have
61 indicated negative impacts of vaping (4). For example, in 2019 an outbreak of e-cigarette or
62 vaping associated lung injury (EVALI), resulting in over 2000 hospitalizations and 68 deaths,
63 was reported (5-7). Although studies associated the addition of vitamin E acetate with the
64 incidences of EVALI, vaping without the compound has still been linked to lung injury, and
65 there is not enough evidence to suggest vitamin E acetate to be the sole cause of injury (6, 8-10).
66 Clearly, more information regarding the impact of vaping on the lung is required.

67

68 The process of vaping involves an EC device that uses a battery powered coil (atomizer) which
69 heats an e-liquid comprised of solvents propylene glycol (PG) and vegetable glycerine (VG) to
70 produce an inhalable aerosol. Many ECs utilize sub-ohm conditions, employing low resistance
71 coils (blow 1 ohm) resulting in more rapid heating of the e-liquid and thicker more flavourful
72 clouds. Complexity in studying the effects of this habit arise from the multitude of vaping
73 options. There are presently over 16000 commercially available e-liquid flavour blends and
74 almost 500 brands of ECs currently on the market (11-13). Adding to this complexity is the fact
75 that many users make modifications to their device and/or generate their own e-liquid.

76 Furthermore, the harmful effects of vaping are not solely due to the impact of the ingredients of
77 the e-liquid since it has been demonstrated that the generation of the aerosol results in the
78 formation of a multitude of new products including formaldehyde and acetaldehyde (14, 15).
79 This effect may vary among different devices, with particulate matter reported to increase with
80 the evolution of some of the newer designs, thereby potentially increasing hazardous effects to
81 the health of vapers (4, 16). Thus, although the general concept of vaping is relatively
82 straightforward, complications in studying its effects arise from the variability and customization
83 of the vaping process.
84
85 From a respiratory perspective, one of the first substances that EC aerosol encounters in the
86 lungs is pulmonary surfactant. Surfactant is made up of 85-90% by weight phospholipids, 5-10%
87 neutral lipids, mainly cholesterol, and 10% surfactant proteins, designated SP-A, SP-B, SP-C,
88 and SP-D (17, 18) . The primary function of surfactant is to reduce surface tension in the lung.
89 To achieve this, surfactant rapidly absorbs to the surface forming the surface film, reaching a
90 surface tension of ~23 mN/m, and subsequently reduces surface tension to ~0 mN/m upon
91 compression (19, 20). This function is essential for breathing as it allows the lung to inflate and
92 deflate with minimal changes in pressure, i.e., it maintains lung compliance, and prevents
93 atelectasis (18, 21). As can be deduced from its function, absence or alterations to pulmonary
94 surfactant can contribute to lung dysfunction. Previous studies have demonstrated that some
95 ingredients of e-liquids can interfere with the function of model systems of surfactant (22-24).
96
97 Based on the above information, the objective was to investigate the impact that e-cigarette
98 aerosol has on the function of surfactant. We initially focused on the impact of the common

99 ingredients of aerosol, VG and PG, prior to testing the impact of selected additives. Using *in*
100 *vitro* methods to replicate the dynamic compression and expansion of surfactant during
101 respiration, this study focused on the biophysical changes to surfactant after direct exposure of a
102 modified natural surfactant preparation to EC aerosol. It was hypothesized that EC aerosol
103 inhibits surfactant's ability to effectively reduce surface tension.
104

105 # Methods

106 ## Materials

107 Bovine lipid extract surfactant (BLES) at 27mg/ml phospholipid concentration was obtained
108 from BLES biochemicals inc (London, ON) and was diluted to 2mg/ml in a buffer containing
109 0.9% NaCl, 1.5mM $CaCl_2$, and 2.5mM HEPES (pH 7.4). Three common vaping devices were
110 used. The Aspire Pockex with 0.6 ohm coil, and Vaporesso Gen with GT4 0.15 ohm coil, were
111 purchased from Vapour North (Pickering, ON). The Uwell Caliburn A2 with 0.9 ohm pre-
112 installed mesh coil was purchased from Fogged Up Vape Shop (London, ON). All e-liquids were
113 purchased from Vapour North.
114

115 ## Exposure to aerosol

116 As schematically shown in figure 1, to expose surfactant to the EC aerosol, 2ml of BLES was
117 placed in a 30ml syringe connected to an EC, via silicone tubing. The produced aerosol was
118 drawn into the syringe and the syringe was placed on a rocker set to rotate 15-20 times/min for a
119 30 second exposure period. The aerosol was then expelled, and this procedure was repeated 30
120 times to simulate 30 inhalations. The control group consisted of BLES (2ml at 2mg/ml) placed in

121 a 30ml syringe exposed only to air, no aerosol, and placed on the rocker for the same amount of
122 time as the vaped samples. The exposed BLES was either used immediately for the analysis of
123 surface tension reduction, or frozen at -20ºC for subsequent analysis.
124
125 **Figure 1. Schematic of the *in vitro* surfactant exposure set-up.** Aerosol exposed surfactant
126 was generated using a syringe containing BLES connected directly to an EC device which
127 allowed aerosol to be drawn into the syringe and, after 30 seconds, expelled. This procedure was
128 repeated 30 times.
129

130 Biophysical analysis of surfactant

131 A constrained drop surfactometer (CDS) was used to assess the surface tension reducing
132 properties of the BLES as previously described (25, 26). Briefly, an 8.5µL drop of the 2mg/ml
133 surfactant sample was placed on the pedestal in an environmentally controlled chamber at 37 ºC.
134 A small capillary in the middle of the pedestal was attached to a computer controlled 2.5ml
135 Hamilton syringe (Hamilton Co., Reno, NV) filled with distilled water. Two minutes after
136 placing the BLES on the pedestal, the surface film of the drop was compressed and expanded via
137 the controlled movement of the water in and out of the pedestal via the syringe. A total of 20
138 compression/expansion cycles were completed at a frequency of 1 cycle/ 1.5 seconds, with an
139 area compression of approximately 20%. Aligned with the horizontal plane of the pedestal was a
140 camera which took rapid pictures of the samples (15 fps) as they underwent dynamic changes.
141 The images were then analysed by the Axisymmetric Drop Shape Analysis (ADSA) computer
142 software to obtain precise measurements of the surface tension and surface area of the samples

143 calculated based on the shape of the drop after the 2 minutes of adsorption as well as throughout
144 the repeated compression/expansion cycles (27).
145

146 Experimental design

147 Using the above procedure, a variety of exposure conditions were applied to surfactant followed
148 by biophysical analysis. Initial experiments tested the e-liquid vehicle composed of unflavoured,
149 nicotine free, e-liquid with a 50:50 VG:PG ratio using the Aspire Pockex. Subsequently, the
150 50:50 VG:PG liquid was tested in different devices, as well, other vehicle compositions (70:30
151 VG:PG and Max VG) tested with the Aspire Pockex device. The Vaporesso GEN EC was used
152 to examine the effects of temperature through adjustment of the wattage in the device settings,
153 with low (30W), medium (55W), and high (80W) temperatures. Of the three devices used,
154 resistance varied between models (0.6ohm, 0.9ohm, 0.15ohm), and due to the simplicity of the
155 Pockex and Caliburn models, atomizer temperature (through wattage) was only able to be
156 assessed with the Vaporesso device. To determine the impact of aerosolization, vehicle 50:50
157 VG:PG e-liquid was mixed directly with BLES at 10 and 20% volumes, followed by analysis on
158 the CDS.
159

160 To assess the impact of additives such as flavouring and nicotine to the vehicle, ten common e-
161 liquids were tested: Vehicle + 18mg/ml nicotine, Juicy Burst + 3 mg/ml nicotine, RY4 Double
162 (tobacco) + 18 mg/ml nicotine, RY4 Double (tobacco), Cigar Passion, Caramel Macchiato, Pink
163 Lemonade, Red Wedding (raspberry doughnut with yogurt), Cinnaswirl, Menthol, Wintergreen,
164 and Cherry. Experiments were performed in groups with multiple flavours, with each day of
165 exposures including separate unexposed and vehicle exposed BLES controls.

Statistics analysis

Data was expressed as the mean ± standard error of the mean (SEM) with a minimum of three independent replicates. Statistical analysis was performed using Graphpad Prism 9 software. Comparison of surface tensions across compression/expansion cycles for each sample were assessed using a two-way repeated measure analysis of variance (ANOVA) with a Bonferoni's post hoc test. No significant differences between cycles were seen in samples except for the red wedding flavoured aerosol. Minimum surface tensions at the twentieth compression cycle were compared between groups using a one-way ANOVA, or two tailed unpaired t-test (air vs vehicle). For red wedding and menthol aerosols, minimum surface tensions at the first, fifth, tenth, and twentieth cycles were compared to vehicle and air control using a one-way ANOVA. Each set of three bars in Fig 4 comparing different flavourings, were analysed using a one-way ANOVA with a Dunnetts post hoc test compared to air control. P values less than or equal to 0.05 were considered statistically significant.

Results

Effect of vehicle aerosol on surfactant function

Using the above protocol, BLES was exposed to vehicle (50:50 VG:PG) aerosol and analyzed on the CDS. Representative surface tension versus relative area isotherms for the fifth and tenth compression/expansion cycle are shown in Figs 1A and 1B. At the fifth compression/expansion cycle, the air-exposed BLES sample shows a reduction in surface tension by compression to values below 5mN/m (lower curve) with subsequent expansion leading to an increase in surface

188 tension with relative low amount of hysteresis (upper curve). In contrast, the vehicle exposed
189 group had higher surface tension after compression (Fig 1A). Similar results were observed at
190 the tenth cycle (Fig 1B). The results of the analysis of the minimum and maximum surface
191 tension of all compression/expansion cycles are shown in Figs 1C and 1D. The air control BLES
192 sample demonstrates low minimal surface tensions starting at the first compression that decrease
193 further over the first few cycles to values below 5mN/m. Compared to these low values observed
194 in the air control group, minimal surface tension in the vehicle exposed BLES group were
195 significantly higher, reaching minimal values of approximately 10mN/m. Maximum surface
196 tension of the control sample was approximately 37mN/m (Fig 1D), these values were not
197 significantly different than those observed in the vehicle exposed group.
198 Additional data obtained from this analysis is shown in Table 1. The surface tension after two
199 minutes of adsorption was not significantly different between the two groups. Furthermore, the
200 calculated values for the maximum area compression, as well as the area compression required to
201 reach minimum surface tension were also not significantly different between the two groups.
202
203 **Fig 2. Vehicle compression/expansion.** BLES exposed to vehicle (■) compared to air control
204 (○). **A.** Representative surface tension vs area isotherm at the fifth compression/expansion cycle.
205 **B.** Representative surface tension vs area isotherm at the tenth compression/expansion cycle. **C.**
206 Minimum surface tensions across 20 compression/expansion cycles. **D.** Maximum surface
207 tensions across 20 compression/expansion cycles. *$P<0.05$ compared to air control.
208

209 **Table 1. Adsorption and compression data**

	Air Control	Vehicle Exposed
Adsorption (mN/m)	24.11 ±0.11	24.57±0.17
Maximum Area Compression (%)	20.33±0.21	20.65±0.73
Area Compression at Min Surface Tension (%)	19.27±0.31	19.41±0.90

210 Average adsorption and percent compression (±SEM) for air control and vehicle exposed groups

211 After establishing surfactant inhibition by the vehicle aerosol using a single experimental
212 condition, this observation was further explored by examining two additional vehicle
213 compositions, testing two other e-cigarette devices, examining three different wattages (i.e.,
214 temperatures) for aerosol generation, and mixing with non-aerosolized vehicle e-liquid. Fig 3A
215 displays minimum surface tension trends (mN/m ± SEM) for BLES exposed to EC aerosol with
216 each of the three different ratios of vehicle composition. Compared to air control samples, which
217 reached low minimum surface tension values, 50:50 VG:PG vehicle achieved significantly
218 higher minimum surface tensions. 70:30 VG/PG and max VG exposed samples were not
219 significantly higher compared to air control. Minimum surface tensions obtained with aerosol
220 from three different devices are shown in Fig 3B. Values for each of the devices were
221 significantly higher than the air control. Comparison among the three EC devices revealed no
222 statistically significant differences. A similar pattern was observed when comparing aerosol
223 generated at three different wattages, as seen in Fig 3C. These wattages impact the temperature
224 of the generated aerosol. The results show no difference among the varying wattages, with all
225 three exposed samples resulting in significantly higher minimum surface tension than the air
226 control. After mixing with e-liquid volumes up to 20%, no significant difference in minimum
227 surface tension was observed compared to control, shown in Fig 3D. Surface tensions after two

228 minutes of absorption, maximum area compression values, and maximum surface tensions were
229 not significantly different between groups and are shown in supplementary data (S1 and S2).
230
231 **Fig 3. Minimum surface tensions of experimental variables.** Minimum surface tensions (±
232 SEM) across 20 compression/expansion cycles for BLES exposed to three experimental
233 variables, compared to air control. **A.** Three different vehicle compositions with increasing VG
234 content. **B**. Three different EC devices. **C.** Three different wattages set on a programmable EC to
235 alter aerosol temperature. **D.** BLES mixed with three different volumes of vehicle e-liquid. N=3
236 *P<0.05 compared to air control.
237

238 Effect of commercially available e-liquid aerosols

239 Having established the effects of the vehicle on surfactant, our subsequent experiment evaluated
240 the effect of aerosols from a variety of e-liquids containing flavourings and/or nicotine. Ten
241 popular commercially available e-liquids were tested. Fig 4 summarizes the data by displaying
242 the minimum surface tension values obtained for the fifth compression cycle as compared to its
243 vehicle and air control. The values for each of the 20 compression/expansion cycles are shown in
244 the supplementary data (S2 Fig. A-I). Consistent with the above experiments, in each of the tests
245 the vehicle exposure led to higher minimum surface tension as compared to air-exposed BLES
246 (Fig 4). With the exception of two of the e-liquids, all flavours resulted in minimum surface
247 tension that were not significantly different to the unflavoured vehicle. This included samples
248 with nicotine additives. The two exceptions to this pattern were menthol and red wedding
249 flavoured aerosols, which resulted in significantly increased minimum surface tension compared
250 to both air control and the unflavoured vehicle control.

251

252 **Fig 4. Minimum surface tensions of flavoured e-liquids.** BLES exposed to aerosols
253 containing nicotine and/or flavouring additives compared to air control and unflavoured
254 vehicle at the fifth compression/expansion cycle (± SEM). Each flavour on the x-axis
255 represents BLES exposed to air, unflavoured vehicle, and vehicle + flavouring aerosols.
256 N=3-4 *P<0.05 compared to air control, #P<0.05 compared to vehicle.

257

258 A further exploration of the data obtained from menthol and red wedding e-liquids is shown in
259 Fig 5 (Menthol) and Fig 6 (Red Wedding). Isotherms for the fifth (Fig 5A) and tenth (Fig 5B)
260 cycles show menthol exposed BLES to result in minimum surface tension at ~20 mN/m, higher
261 than the vehicle exposed BLES which achieved minimum surface tension of ~10 mN/m.
262 Menthol exposure resulted in significantly higher minimum surface tension than both
263 unflavoured vehicle and air control groups at all 20 compression/expansion cycles (Fig 5C).
264 Maximum surface tension remained consistent between groups, at ~35 mN/m (Fig 5D). The
265 inhibitory effect of menthol on minimum surface tension was further enhanced with three
266 repeated exposures on consecutive days (S4). Isotherms at the fifth cycle for red wedding
267 exposed BLES, also show significant impairment, with minimum surface tension at ~20 mN/m,
268 higher than both the vehicle and air exposed groups (Fig 6A). At the tenth cycle however, the red
269 wedding flavour exposed group achieves minimum surface tension similar to that of the vehicle
270 exposed group (Fig 6B). A steep decline on a cycle-by-cycle basis can be observed for the red
271 wedding exposed group, until approx. the tenth cycle, where surface tension remains similar to
272 the vehicle exposed (Fig 6C). Both flavoured and vehicle exposed BLES showed higher
273 minimum surface tension than the air control. Red wedding further appeared to have lower

maximum surface tension during the first ten cycles, maintaining values ~30 mN/m, whereas air control groups had an average maximum surface tension of ~40 mN/m (Fig 6D).

Fig 5. Menthol compression/expansion data. BLES exposed to menthol flavoured aerosol (▲) compared to vehicle (■) and air control (○). **A.** Representative surface tension vs area isotherm at the fifth compression/expansion cycle. **B.** Representative surface tension vs area isotherm at the tenth compression/expansion cycle. **C.** Minimum surface tensions (± SEM) across 20 compression/expansion cycles. **D.** Maximum surface tensions (± SEM) across 20 compression/expansion cycles. (N=5) *P<0.05 compared to air control, #P<0.05 compared to vehicle.

Fig 6. Red wedding compression/expansion data. BLES exposed to red wedding flavoured aerosol (▲) compared to vehicle (■) and air control (○). **A.** Representative surface tension vs area isotherm at the fifth compression/expansion cycle. **B.** Representative surface tension vs area isotherm at the tenth compression/expansion cycle. **C.** Minimum surface tensions (± SEM) across 20 compression/expansion cycles. **D.** Maximum surface tensions (± SEM) across 20 compression/expansion cycles. (N=3) *P<0.05 compared to air control, #P<0.05 compared to vehicle.

Discussion

This study tested the hypothesis that EC aerosols inhibit surfactant's ability to effectively reduce surface tension. Results supported this hypothesis as exposure to EC aerosols resulted in impaired surfactant function, in particular its ability to reduce surface tension to near-zero

297 values. All exposed samples, regardless of additive, device, or vehicle composition resulted in
298 higher minimum surface tensions than air exposed samples. Furthermore, surfactant dysfunction
299 was observed in all cases of aerosol exposure with different additives, and most of the flavours
300 tested resulted in similar inhibition and minimum surface tensions to those of the vehicle.
301 Interestingly, two flavours, menthol and red wedding, resulted in significantly higher minimum
302 surface tensions compared to the vehicle. It is concluded that aerosol exposure can lead to
303 surfactant impairment which, *in vivo*, could impact lung function. This effect of EC aerosol can
304 be augmented due to certain flavour additives.
305
306 Previous studies have examined the effects of e-liquid aerosols on surfactant, with a variety of
307 experimental approaches. Distinctions in the type of surfactants used, with several studies using
308 surfactant-like lipid mixtures, as well as differences in exposure method including mixing of
309 surfactant with isolated components of the e-liquid directly, have been reported (22, 28, 29).
310 Furthermore, a variety of analyses, including structural and functional assessments have been
311 reported (24, 30). Building on these studies we used a modified natural surfactant, exposed the
312 samples to EC generated aerosols, and focused on the main function of surfactant by measuring
313 surface tension reduction. Specifically, we used BLES, a well-established commonly prescribed
314 exogenous surfactant composed of the surfactant lipids and the hydrophobic proteins B and C
315 (31). Aerosol exposure was performed in a syringe containing a surfactant suspension allowing
316 direct exposure of surfactant to the aerosol. This exposure of the surfactant before
317 compression/expansion cycling resulted in findings consistent with recent direct aerosol
318 exposure of a compressing drop (30). Analysis was done using the CDS, which was able to
319 provide extremely accurate measurements of changes in surface tension and droplet area during

320 consistent dynamic cycling with compression maintained within physiological levels (19). To
321 provide a broad assessment of the impact of EC aerosols on surfactant, we used several
322 commercially available EC devices and multiple e-liquids to accurately reflect the vaping tools
323 on the market today. Overall, we deemed that this approach allowed for effective testing of our
324 hypothesis.
325
326 One of the main goals of our study was to examine the effect of the base vehicle of e-liquid, VG
327 and PG, since these compounds are universally utilized during vaping. Although both
328 compounds are safe and frequently used humectants in a wide range of foods and consumer
329 cosmetic products (32-34), research on the effects of aerosolization and inhalation of these
330 compounds is limited. Focusing on the effect of surfactant specifically, it has been previously
331 shown that EC aerosols did not induce harmful effects on surfactant interfacial properties but
332 caused minor alterations in lateral structure arrangement after exposure (24). In this case
333 however, surfactant (Infasurf) was not directly exposed to the aerosol, but rather the aerosol was
334 bubbled into the subphase in a Langmuir-Blodgett trough before addition of the surfactant,
335 without measurement of aerosol concentrations. Alterations observed to the lateral structure are
336 nonetheless important to note, as proper arrangement of the surface film phospholipids are
337 essential to reaching near zero surface tensions upon compression (35). In two other studies,
338 impairment of surfactant function was observed. Using a lipid mixture as a model system of
339 surfactant it was observed that exposure to EC aerosol resulted in lower maximum surface
340 pressures at any given area (higher minimum surface tensions) and altered compressibility on a
341 Langmuir balance (28). Further, direct mixing of non-aerosolized PG and VG with surfactant,
342 resulted in impaired surfactant function (increased surface tension amplitude, mean surface

343 tensions, and surface elasticity), particularly in ultra-high concentrations of PG dominant
344 mixtures (29). The current study builds upon these findings, by exposing a natural surfactant to
345 aerosolized VG/PG and dynamic measurement of surfactant function. Furthermore, we showed
346 that the VG/PG induced reduction in surface activity was consistent among several different sub-
347 ohm devices with very low resistance coils and different wattages for aerosol generation.
348 Although the sub-ohm devices are popular amongst users, large variation in resistance and the
349 amount of current passed through the coil exist beyond those tested in the current study. Overall,
350 the findings support a modification in dynamic surface-active properties of surfactant due to
351 vehicle aerosol exposure.
352
353 Having established a general, albeit mild, inhibition of surfactant by EC aerosol in general, our
354 second goal was to examine the effect of different flavored e-liquids. Rationally selecting
355 specific e-liquids among the approximately 16000 flavours currently available is difficult,
356 however, we picked popular e-liquids in different categories (tobacco alternatives, drinks, candy,
357 and dessert) (13). Interestingly, whereas most e-liquids showed similar results to vehicle,
358 menthol and red wedding resulted in further inhibition of surfactant function. The mechanism of
359 inhibition of surfactant by menthol has been recently studied and indicates a direct interaction of
360 this flavouring with surfactant phospholipids and proteins thereby impacting the surface tension
361 reducing surface film (30). The specific process by which red wedding flavouring impacts
362 surfactant is not clear, and studies of the lateral organization of the surfactant film, for example
363 by atomic force microscopy could explore this further. Nevertheless, the pattern of inhibition in
364 which high surface tensions are only observed in the early compression/expansion cycles would
365 suggest that chemicals are inserted into the surface film but are removed, or squeezed out, during

366 repeated compression and expansion (19, 36). In contrast, it appears as if surfactant remains
367 inhibited by menthol exposure across compression/expansion cycles, suggesting a more
368 permanent alteration of the surface film. A recent study, showing similar effects to menthol as
369 the current study, did indeed show such alteration of the surface film using atomic force
370 microscopy (30). Regardless of the specific mechanism, the data clearly indicate that variability
371 exist between the impact of aerosols from different e-liquids. Unlike in food and beverages, the
372 chemicals in e-liquids are not highly regulated, and not all the specific ingredients are disclosed.
373 For example, a study examining the chemical concentrations in JUUL pod-based e-liquids
374 identified 59 chemicals, with menthol, vanillin, and ethyl maltol having the highest
375 concentrations, with the remaining 56 chemicals having concentrations of <1.0mg/mL (37).
376 Considering our data demonstrated enhanced surfactant inhibition with 2 out of 12 e-liquid
377 samples selected, we speculate that a substantial fraction of the commercially available e-liquid
378 flavours available have a negative impact on surfactant function.
379
380 Further complicating the study of EC aerosol interactions with the surfactant system is the
381 generation of various compounds during the aerosolization process. For example, flavour
382 aldehydes have been shown to react causing the formation of PG acetals (38). Benzaldehyde
383 (cherry flavouring), ethylvanillin, vanillin, cinnamaldehyde, and citral were examined to
384 determine reaction products after vaping and were shown to produce PG acetals that remained
385 stable for hours (38). It has also been reported that aerosolization, and subsequent degradation of
386 VG/PG can lead to the generation of harmful chemicals, including carbonyl compounds such as
387 formaldehyde, acetaldehyde, and acrolein, as well as volatile organic compounds, tobacco-
388 specific nitrosamines, metals, free radicals, and reactive oxygen species in EC aerosols (15, 39-

389 41). In a non-targeted study, over 115 volatile organic compounds were found in a single puff of
390 EC aerosol (42). Further, compared to the composition of the non-aerosolized liquid,
391 aerosolization produced more than 80 additional compounds beyond the 60 identified within the
392 liquid itself (42). It has also been reported that higher power EC devices increase reactive oxygen
393 species production (43). However, it should be noted that in the current study, no differences
394 were observed between three devices, as well as no significant differences between power levels
395 (wattage) on our highest power device (Vaporesso), suggesting that either the lowest wattage
396 was able to maximally effect the surface tension of the surfactant, or that reactive oxygen species
397 production was not a major contributor to the observed surfactant inhibition. Furthermore, no
398 significant differences were seen when mixing the non-aerosolized e-liquid directly with BLES,
399 indicating the importance of aerosolization for inhibition. Overall, the published data suggest that
400 the inhibition of surfactant by EC aerosols may not only be the results of the incorporation of e-
401 liquid components but also due to additional components generated during aerosolization. This
402 includes those generated from flavour additives, which can either insert into the surfactant and/or
403 chemically react with surfactant lipids and proteins. As such, it is important that future
404 regulations regarding vaping and e-liquid composition should base recommendations on studies
405 that have evaluated the health impact after aerosol generation.
406
407 Although the current study examined the effects of EC aerosols on surfactant *in vitro*, the
408 rationale for our experiments was the interaction of aerosols with surfactant in the lung, thus it is
409 important to extrapolate our findings to the *in vivo* situation. Although we observed a slight
410 decrease in surface tension reducing ability after exposure, such minor surfactant dysfunction
411 may not have a major impact *in vivo*. Nevertheless, a clinical study by Dr. Vardavas and

412 colleagues demonstrated that using an EC device for only five minutes led to an immediate,
413 subtle, decrease in lung mechanics in a group of volunteers (44). Considering the rapid nature of
414 this effect on lung mechanics, it would be consistent with a rapid direct interaction of the aerosol
415 with surfactant, rather than, for example, a cellular mechanism. A second potential consequence
416 of altered surfactant function may be a susceptibility to lung injury. Thus, although vaping
417 induced alterations to surfactant may not be sufficient to cause lung dysfunction, in scenarios
418 where the surfactant is further challenged it may become more impaired and ultimately affect
419 lung function. For example, it has been shown that surfactant that contains lyso-phospholipids
420 was more susceptible to inhibition by serum proteins as compared to control (45). It should also
421 be noted that *in vivo* vaping may impact other aspect of surfactant, such as the expression of the
422 surfactant associated proteins. Two previous studies show different results in this regard with one
423 study observing dysregulation of certain genes, but no significant alterations to surfactant
424 proteins A, B, C, or D (46), but another reporting decreased expression of surfactant proteins
425 (23). Difference in experimental set up, specific vaping conditions and exposure times may have
426 contributed to these differences. Future studies examining the acute impact of EC aerosols on
427 surfactant *in vivo*, as well as susceptibility of aerosol exposed surfactant to further inhibition, are
428 warranted.
429
430 Our study has several limitations. First the current study uses an exogenous surfactant, BLES, to
431 test the impact of EC aerosol. All studies on the effect of vaping have utilized either exogenous
432 surfactant or an even more limited, lipid model system. The rationale for using exogenous
433 surfactant is that it contains all surfactant lipids and the hydrophobic proteins, SP-B and C, and
434 has consistent functional properties. Nevertheless, future studies with natural surfactants from

435 animal and/or humans would provide additional information of the impact of the hydrophilic
436 surfactant protein A, as well as other components that co-isolate with surfactant obtained from
437 lung lavage. A second limitation is that nicotine was only tested individually as well as with two
438 flavour combinations. As no additional affect was observed, we neglected to test nicotine further,
439 however it is possible that nicotine could have an interaction with specific flavours to potentially
440 affect the impact on surfactant. Lastly, only the effects of acute exposure on surfactant were
441 tested. People that vape will do so in a prolonged fashion which may have additional
442 consequences. For example, previous *in vivo* work on mice exposed daily to EC aerosols for
443 several weeks showed altered lipid homeostasis in alveolar macrophages, as well as
444 accumulation of phospholipids in bronchoalveolar lavage fluid (23). The impact of EC aerosols
445 on surfactant *in vivo* may thus be affected by acute direct effect as observed in the current
446 experiments, as well as prolonged effects related to the impact on surfactant synthesis and
447 metabolism.
448

449 # Conclusion

450 This study demonstrated that the aerosol produced from the e-liquid vehicle (VG/PG) was able to
451 alter surfactant function independent of nicotine or device style, with some flavourings providing
452 further inhibition. From these results we conclude that acute EC aerosol exposure leads to
453 impairment of the pulmonary surfactant system through increases in minimum surface tension.
454 We speculate that this has potential detrimental effects in the *in vivo* scenario by contributing to
455 lung dysfunction and making the lung more susceptible to further injury.
456

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589 # Supporting information

590 **S1 Fig. A-D. Maximum surface tensions of experimental variables.** Maximum surface
591 tensions (± SEM) across 20 compression/expansion cycles for BLES exposed to three
592 experimental variables, compared to air control. **A.** Three different vehicle compositions with
593 increasing VG content. **B**. Three different EC devices. **C.** Three different wattages set on a
594 programmable EC to alter aerosol temperature. **D.** BLES mixed with three different volumes of
595 vehicle e-liquid. N=3 *P<0.05 compared to air control.

596 **S2. Table. Adsorption and compression data.** Average adsorption and percent compression
597 (±SEM) for control and exposed groups under four experimental conditions: changing vehicle
598 composition, using different EC devices, varying temperature through increasing wattage, and
599 mixing of non-aerosolized e-liquid directly with BLES.

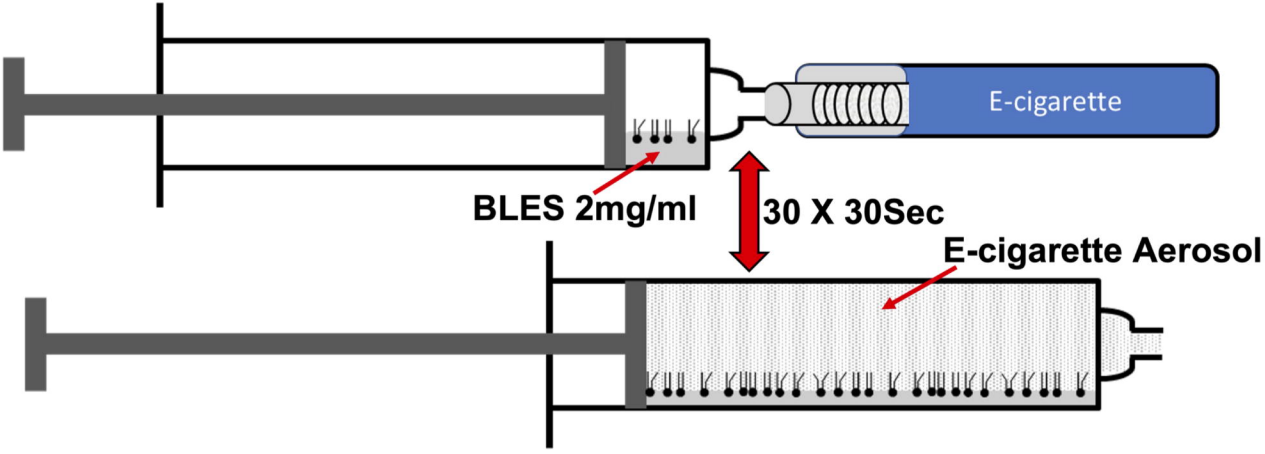
600

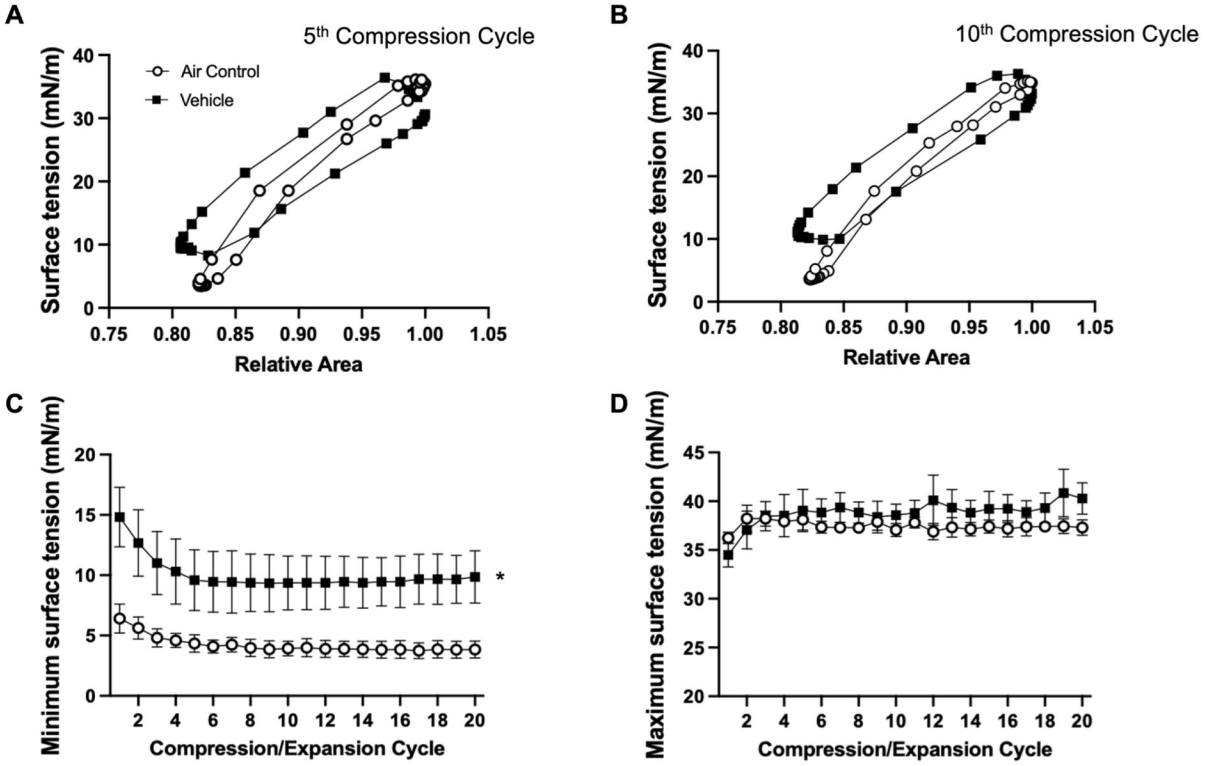
	Adsorption (mN/m)	**Maximum Area Compression (%)**	**Area Compression at Minimum Surface Tension (%)** *
Vehicle Composition			
Air Control	24.61±0.22	26.49±0.56	23.32±0.21
50VG	24.78±0.92	26.68±0.24	24.29±0.70
70VG	26.62±0.31	27.58±0.62	25.08±0.56
MaxVG	24.64±0.34	26.06±0.14	23.73±0.13
Devices			
Air Control	24.52±0.19	23.97±0.54	22.54±0.28
Caliburn	24.96±0.27	26.16±0.85	24.76±0.78
Pockex	24.70±0.23	25.61±0.67	24.36±0.59
Vaporesso	24.96±0.18	26.37±0.57	24.82±0.27
Temperature			
Air Control	23.65±0.18	20.13±0.30	18.29±0.36

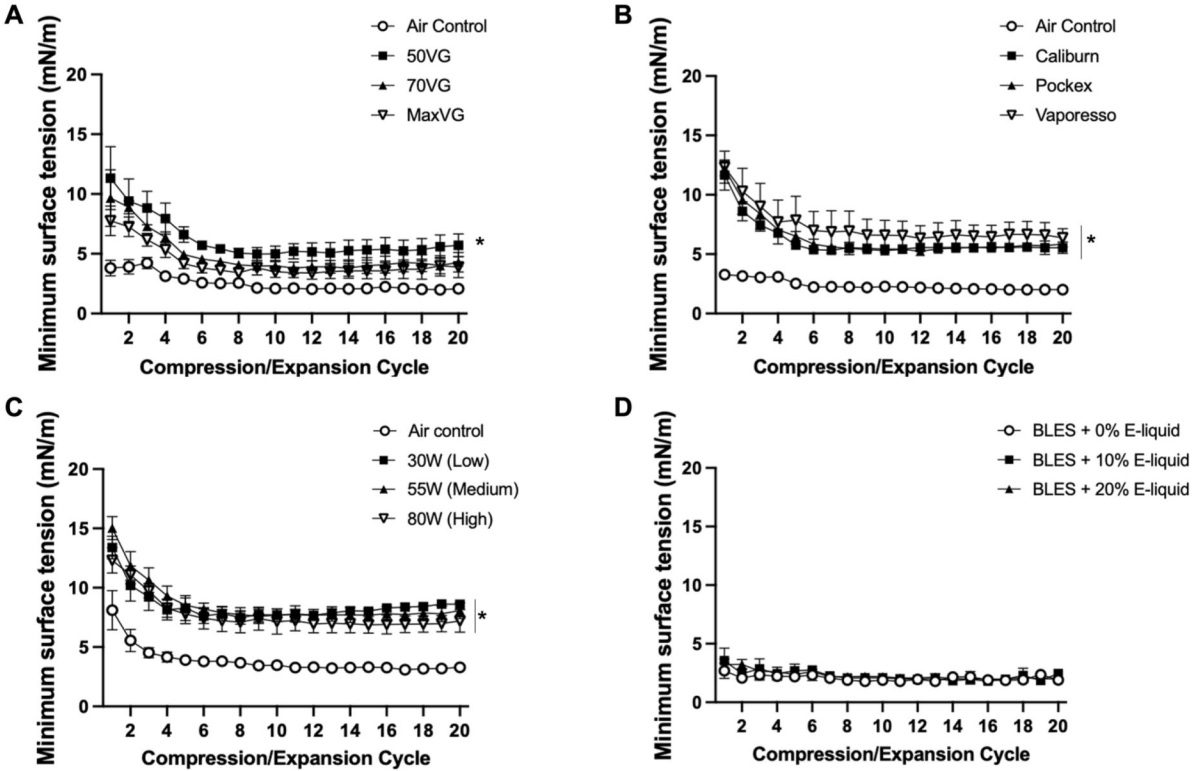
30W (Low)	23.68±0.07	21.42±0.61	18.88±0.64
55W (Medium)	23.62±0.10	21.41±0.65	18.79±0.65
80W (High)	23.53±0.15	21.29±0.48	18.64±0.44
Direct Mixing E-liquid			
BLES+0% E-liquid	24.24±0.33	24.20±0.39	21.15±0.42
BLES+ 10% E-liquid	24.28±0.39	24.02±0.28	21.06±0.36
BLES+20% E-liquid	24.18±0.23	23.78±0.37	21.02±0.41

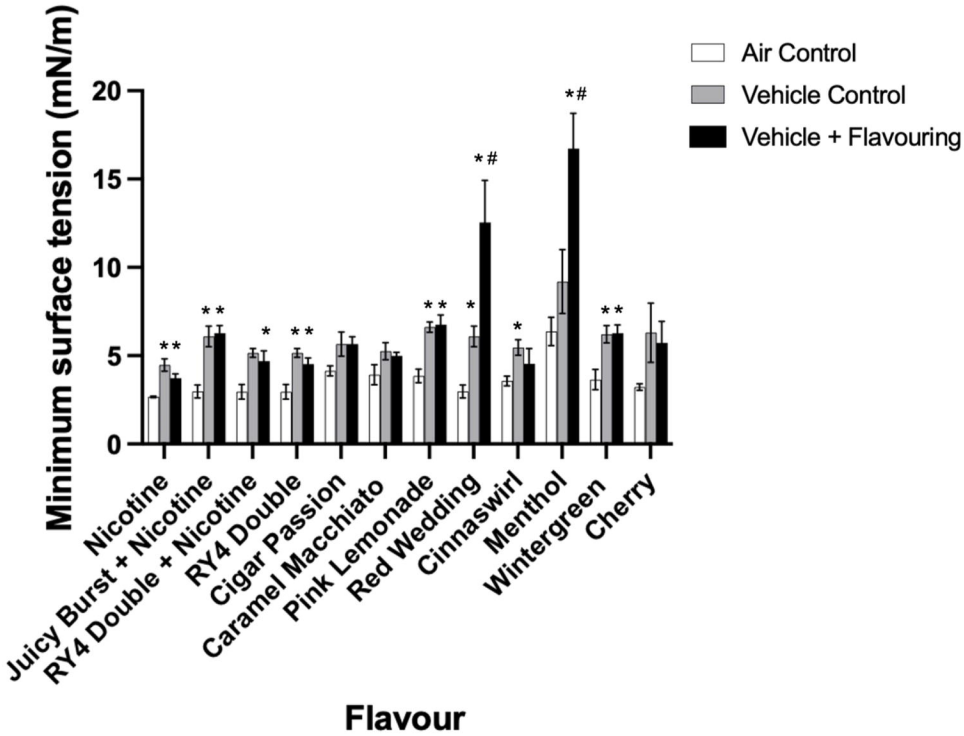
S3 Fig. A-I. Minimum surface tensions (± SEM) across 20 compression/expansion cycles for BLES exposed to nine flavourings/additives compared to unflavoured vehicle control. N=3.

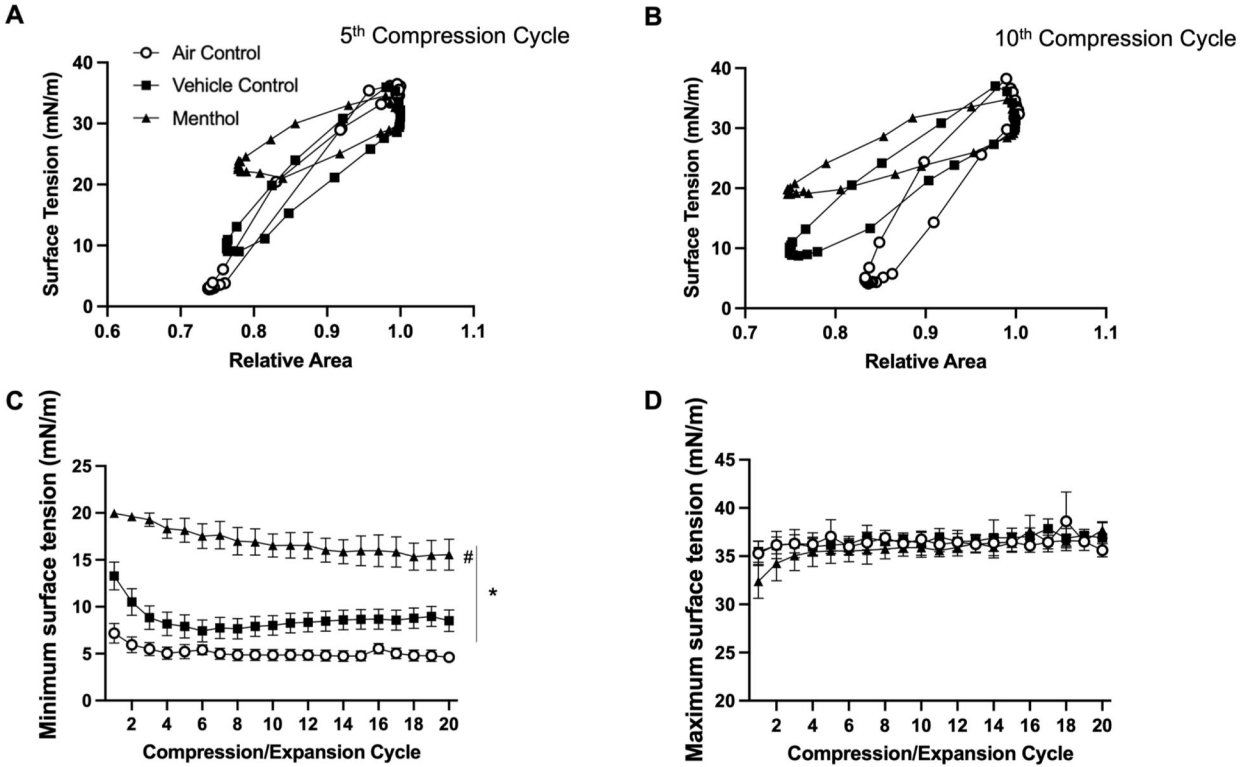
S4 Fig. Minimum surface tensions (±SEM) across 20 compression/expansion cycles for BLES exposed to menthol and vehicle aerosols as well as air control after one day and three days of consecutive exposure. N=3 *P<0.05 compared to day one.

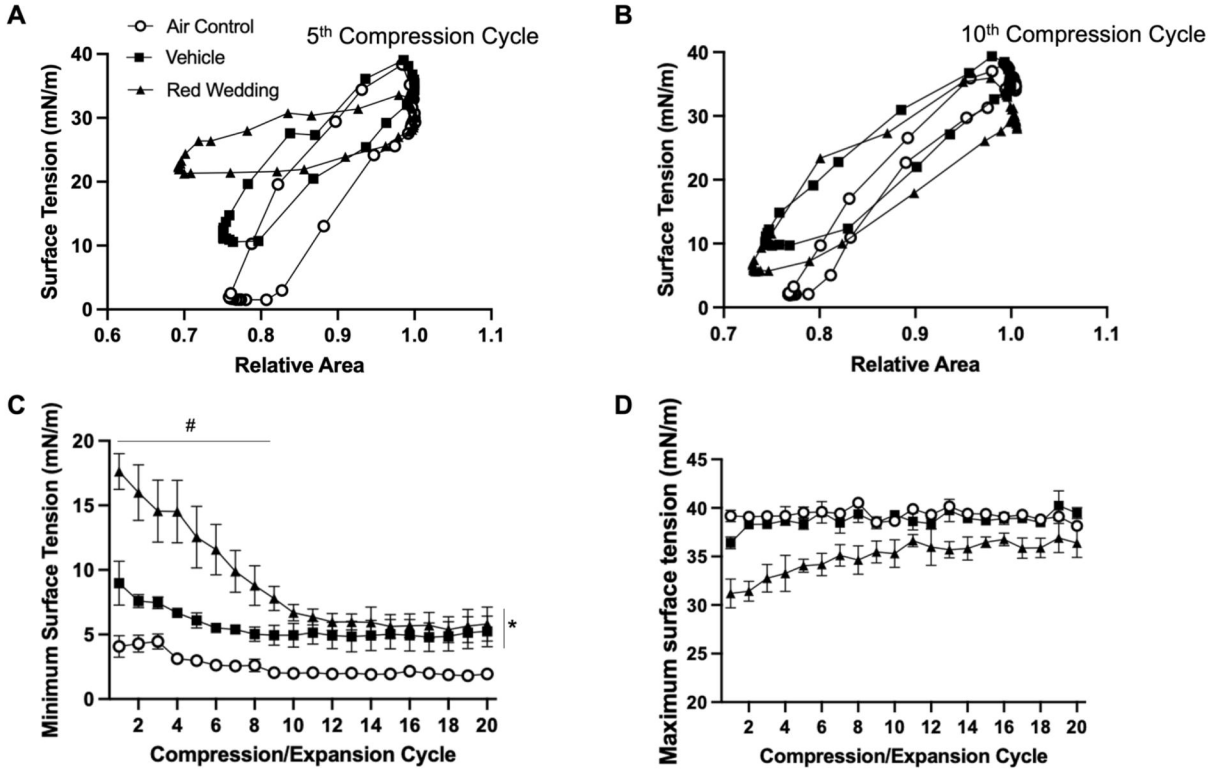

E-cigarette
BLES 2mg/ml
30 X 30Sec
E-cigarette Aerosol


A
5th Compression Cycle
Air Control
Vehicle
Surface tension (mN/m)
Relative Area
B
10th Compression Cycle
Surface tension (mN/m)
Relative Area
C
Minimum surface tension (mN/m)
Compression/Expansion Cycle
*
D
Maximum surface tension (mN/m)
Compression/Expansion Cycle


A
Minimum surface tension (mN/m)
Compression/Expansion Cycle
Air Control
50VG
70VG
MaxVG
*
B
Minimum surface tension (mN/m)
Compression/Expansion Cycle
Air Control
Caliburn
Pockex
Vaporesso
*
C
Minimum surface tension (mN/m)
Compression/Expansion Cycle
Air control
30W (Low)
55W (Medium)
80W (High)
*
D
Minimum surface tension (mN/m)
Compression/Expansion Cycle
BLES + 0% E-liquid
BLES + 10% E-liquid
BLES + 20% E-liquid


Minimum surface tension (mN/m)
20
15
10
5
0
Air Control
Vehicle Control
Vehicle + Flavouring
*#
*#
* *
* *
*
* *
* *
*
*
* *
Nicotine
Juicy Burst + Nicotine
RY4 Double + Nicotine
RY4 Double
Cigar Passion
Caramel Macchiato
Pink Lemonade
Red Wedding
Cinnaswirl
Menthol
Wintergreen
Cherry
Flavour


A
5th Compression Cycle
Air Control
Vehicle Control
Menthol
Surface Tension (mN/m)
Relative Area
B
10th Compression Cycle
Surface Tension (mN/m)
Relative Area
C
Minimum surface tension (mN/m)
Compression/Expansion Cycle
#
*
D
Maximum surface tension (mN/m)
Compression/Expansion Cycle


A
Air Control
Vehicle
Red Wedding
5th Compression Cycle
Surface Tension (mN/m)
Relative Area
B
10th Compression Cycle
Surface Tension (mN/m)
Relative Area
C
#
*
Minimum Surface Tension (mN/m)
Compression/Expansion Cycle
D
Maximum surface tension (mN/m)
Compression/Expansion Cycle