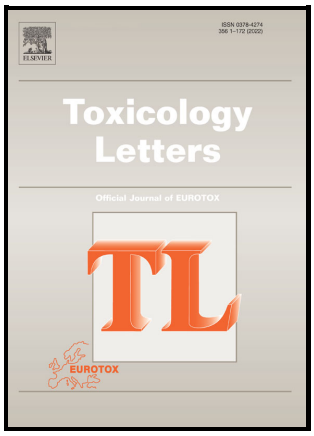

Comparative study of e-cigarette aerosol and cigarette smoke effect on *ex vivo* embryonic chick lung explants

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Comparative study of e-cigarette aerosol and cigarette smoke effect on ex vivo embryonic chick lung explants

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Running title: Electronic cigarette aerosol impairs lung morphology

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Abstract

Electronic cigarette usage has significantly expanded among young people and pregnant women in the last decade. Although there are already some data regarding

the short- and long-term consequences of e-cigarettes on human health, their effect on embryo and lung development still needs to be fully disclosed. In this sense, this study describes, for the first time, the impact of electronic cigarette aerosol on early lung development. For this purpose, ex vivo chick (Gallus gallus) embryonic lungs were cultured in vitro for 48 h in e-cigarette aerosol exposed-medium or unexposed medium. Chick lung explants were also cultured in a cigarette smoke-exposed medium for comparison purposes. Lung explants were morphologically analyzed to assess the impact on lung growth. Additionally, TNF-α levels were determined in the supernatant as a marker of pro-inflammatory response. The results suggest that electronic cigarette aerosol impairs lung growth and promotes lung inflammation. However, its impact on early lung growth seems less detrimental than conventional cigarette smoke. This work provides significant data regarding the impact of e-cig aerosol, adding to the efforts to fully understand its effect on embryo development. The validation of these effects may eventually lead to new tobacco control recommendations for pregnant women.

Key words: Smoking; Vaping; Lung Development; Branching Morphogenesis; Inflammation

1. Introduction

Combustion of conventional cigarettes (c-cig) generates smoke composed of more than 7000 substances, most of which are considered toxic or carcinogenic (Rodgman and Perfetti, 2013). In fact, smoking is responsible for more than eight million deaths per year worldwide, caused by cardiac or pulmonary diseases, namely, heart failure and chronic lung diseases or cancer (World Health Organization, 2019). Furthermore, it has been shown that maternal smoking negatively affects embryo development (Talbot and Lin, 2011). For instance, it has been reported that it affects the offspring's development, increasing the risk of developing lung diseases (Maritz and Harding, 2011; Napierala et al., 2019).

Vaping has emerged as an alternative to the c-cig and employs a device known as electronic cigarette (e-cig). Briefly, e-cigs are battery-powered devices that include a coil that reaches sufficiently high temperatures to vaporize a liquid (aka e-liquid), creating an aerosol that is subsequently inhaled (Dusautoir et al., 2021; Glantz and Bareham, 2018; Winnicka and Shenoy, 2020). The e-liquid mainly consists of vegetable glycerol and propylene glycol but may also contain nicotine (in different concentrations) and flavors (Dusautoir et al., 2021; Glantz and Bareham, 2018). Except for nicotine, e-liquid components are generally recognized as safe (GRAS) and can be found, for instance, in perfumes and food (21 CFR Part 172, 1977). However, GRAS substances are not maintained in their natural physical state in the aerosol. The high temperature of the coil, around 200 ºC to 300 ºC, leads to the thermal breakdown of e-liquid components producing noxious sub-products such as aldehyde, formaldehyde, and hemiacetals (Dusautoir et al., 2021; Kuniyoshi and Rehan, 2019; Lerner et al., 2015; Sleiman et al., 2016; Winnicka and Shenoy, 2020). Nevertheless, the amount of toxic substances detected in e-cig aerosols is lower than in c-cig smoke (Dusautoir et al., 2021; Goniewicz et al., 2014), since there is no need for combustion to deliver nicotine. Some studies point to a less harmful effect of e-cig (Farsalinos, 2021; Glantz and Bareham, 2018; McNeill et al., 2018), although the long-term effects are yet to be determined, prompting e-cig suppliers to advertise them as a safer/less harmful alternative. Therefore, e-cigs are very popular among pregnant women since they are recommended as a smoking cessation tool (Whittington et al., 2018). Nonetheless, vaping represents a risk for pregnant women since substances present in the aerosol can cross the placenta and affect the developing embryo/fetus (Jiang et al., 2018; Whittington et al., 2018). Consequently, it is crucial to determine the effect of vaping on fetal development, specifically on lung development.

In humans, lung development begins around the fourth week of gestation, and branching morphogenesis commences around the fifth week, setting the structural basis of the respiratory tree (Schittny, 2017). Lung development is usually studied

using mammalian models (Nogueira-Silva et al., 2013; Wongtrakool et al., 2007); however, the avian model has recently emerged as an attractive alternative to study branching morphogenesis due to its similarities with mammalian lung development at early stages. For instance, secondary bronchi emerge laterally in the chicken lung, mimicking one of the subroutines of mammalian lung development (Maina, 2006; Metzger et al., 2008). In both cases, lung tissue is constituted by a tube of epithelial cells endoderm-derived surrounded by a layer of mesenchyme mesoderm-derived. The epithelial-mesenchymal interactions are crucial for proper organogenesis, and the molecular mechanisms underlying the interactions are conserved between the two models (Fernandes-Silva et al., 2017; Moura et al., 2016, 2015, 2014, 2011). Nonetheless, the avian embryonic lung can only be used as a surrogate to study early branching morphogenesis because, at some point, lungs begin to differ morphologically and start to reflect some species-specific characteristics (for example, parabronchi vs. alveoli).

Considering the lack of data regarding the effect of e-cig in embryonic development, we assessed the impact of electronic cigarette aerosol on early lung development and uncovered its effect in the initial stages of branching morphogenesis.

2. Materials and methods

2.1 Ethical statement

This work was carried out in the chicken (Gallus gallus) embryo that does not require ethical approval from a review board or ethical commission, which is in accordance with the Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes. The Portuguese Directive 113/2013 of 7 August 2013 does not limit the use of non-mammalian embryos.

2.2 E-cig aerosol and c-cig smoke acquisition

The e-cig aerosol/c-cig smoke acquisition process was adapted from a previous report (Romagna et al., 2013). 20 mL of 199 medium (Sigma, USA) were put in a Büchner flask. To obtain aerosols from the electronic cigarette (power supply: 20 W), the side-arm of a Büchner flask was connected to a vacuum pump (N035.1.2 AN.18, KNF Lab, Germany) by a hose; this connecting tube had a small hole on the pump´s side to decrease the vacuum pressure thus creating suction comparable to human breathing. E-cig was filled with e-liquid (composition: 16 mg/mL nicotine, < 40 % vegetable glycerin, < 60 % propylene glycol and menthol flavor; ρ = 1.13 g/mL) and weighed. Then, it was connected to the neck of the flask via a tube. By pressing the e-cig knob for 3 s and activating the pump for the same period (pressure < 200 hPa), a puff was made and allowed to be in contact with the medium for 1 min. After this period, the aerosol was released, and the e-cig was weighed. This procedure was repeated until 200 mg of e-liquid were consumed (on average 17 to 18 puffs), as described in (Romagna et al., 2013).

To obtain smoke from the conventional cigarette (commercial cigarette not conditioned; composition: 0.8 mg nicotine, 10 mg Tar; 10 mg carbon monoxide /cigarette), the side-arm of the flask was connected to a 50 mL syringe. The c-cig was connected to the neck of the flask via a tube. The c-cig was lit, and, with the syringe, a 35 mL puff was made and allowed to be in contact with the medium for one minute. The authors followed the ISO 3308:2012 guideline for generating smoke from c-cig regarding puff volume, duration, and frequency. This process was repeated until the cigarette was fully consumed (on average, 7 to 8 puffs), and only after the last puff was the smoke released from the flask (ISO/TC 126, 2012). The acquisition was performed in both cases in the chemical hood and with gentle rocking (100 min^{-1}); moreover, both procedures were performed at room temperatures (22 °C ± 2 °C) and relative humidity of (60 ± 5) %.

2.3. Gas Chromatography-Mass Spectrometry analysis

Nicotine content was analyzed by Gas Chromatography-Mass Spectrometry (GC-MS) after extracting the aerosol/smoke-exposed medium, as previously described (Coelho et al., 2020). Briefly, 100 µL of 4-nonanol (35.4 mg/L; 818773, Merck, USA) and 400 µL dichloromethane (SupraSolv, Merck) were added to 8 mL of diluted (5x) exposed medium. Samples were stirred vigorously with a magnet for 15 min at room temperature. The samples were then left at -20 ºC for 10 min and subsequently centrifuged (860 g, 30 min). The nicotine-enriched dichloromethane phase was recovered using a Pasteur pipette. This organic phase was then dried with anhydrous sodium sulfate (≥ 99 %, Fluka, USA) to remove aqueous contamination. The obtained solution was analyzed by GC-MS (GC: Varian CP-3800, MS: Varian Saturn 2000, USA). The GC-MS analysis was performed according to (Coelho et al., 2020). A calibration curve was prepared using pure nicotine solutions (N3876, Sigma) following the above-mentioned dichloromethane extraction procedure.

2.4 In vitro lung explants

Lung explant culture was performed as previously described (Moura, 2019). Briefly, fertilized eggs were incubated for 4.5 d to 5 d, and b2 stage lungs (two secondary buds per bronchus) were dissected using a stereomicroscope (Olympus SZX16, Japan). Then, the lungs were transferred to a floating nucleopore polycarbonate membrane with 8 µm pore size (Whatman, USA) in a 24-well plate, previously soaked in 400 µL of 199 medium for 1 h. Afterwards, lungs were randomly assigned one of 3 experimental conditions (n ≥ 16 biological replicas/condition, obtained in four independent experiments): control (unexposed medium), e-cig/aerosol, or c-cig/smoke-exposed undiluted medium. Before use, the control and exposed medium were filtered through a 0.22 µm filter (Sarstedt, Germany) and properly supplemented (Moura, 2019). Lungs were kept in culture at 37 °C and 5 % CO_2 for 48 h; the medium was renewed every 24 h, and the post culture medium was collected and stored at -80 ºC. In parallel, a different set of lung explants were cultured in the

presence of 100 μM or 1 mM nicotine [(−)-nicotine hydrogen tartrate salt; SML1236, Sigma] following the same protocol.

2.5 Morphometric analysis

Lung growth was monitored by photographing the explants at D0 (0 h) and D2 (48 h) with a camera (Olympus U-LH100HG) coupled to a stereomicroscope (Olympus SZX16). The morphometric parameters were assessed using the AxioVision Rel. 4.9.1 software (Carl Zeiss, Germany) to outline the inner and outer compartments of the lung, as formerly described (Moura, 2019). Total and epithelium area and perimeter were determined at D0 and D2. Results were expressed as a D2/D0 ratio.

2.6 Enzyme-linked immunosorbent assay

TNF-α levels were measured in the culture media, collected at D1 (24 h) and D2 (48 h), by ELISA (E-EL-Ch0215, Elabscience, USA) according to the manufacturer's recommendations. D1 and D2 TNF-α amounts (from the same explant) were added up to determine the total TNF-α. Six biological replicas per condition were used, and each sample was run in duplicate.

2.7 Statistical analysis

Statistical analysis was performed with SPSS 25.0 (IBM, USA) software.

2.7.1 Nicotine quantification

The results were analyzed by the non-parametric Mann-Whitney test because the normality test failed. Data are presented as mean ± SD; the statistical difference was set at $p < 0.05$.

2.7.2 Quantitative Morphometric and TNF-α data

Since the normality test did not fail, the results were analyzed through a one-way ANOVA, and, for multiple comparisons, the post-hoc Fisher's Least Significant Difference (LSD) test was used. Data are presented as mean ± SD; the statistical difference was set at $p < 0.05$.

3. Results

3.1. Nicotine is present in higher amounts in the e-cig aerosol-exposed medium

A schematic diagram of the acquisition system used to obtain e-cig aerosol, and c-cig smoke-exposed medium is displayed in Figure 1. To certify the efficiency of this acquisition system, it was necessary to confirm that diffusion to the culture medium was occurring appropriately. Nicotine is present in both the c-cig and the e-liquid, so we choose to quantify it in the exposed medium. Nicotine was detected in both extracts by GC-MS (Figure 2A), thus validating the acquisition process. Nicotine levels in the e-cig aerosol-exposed medium were approximately (12.63 ± 1.71) mg/L, whereas in the smoke-exposed medium was roughly (7.15 ± 1.02) mg/L (Figure 2B).

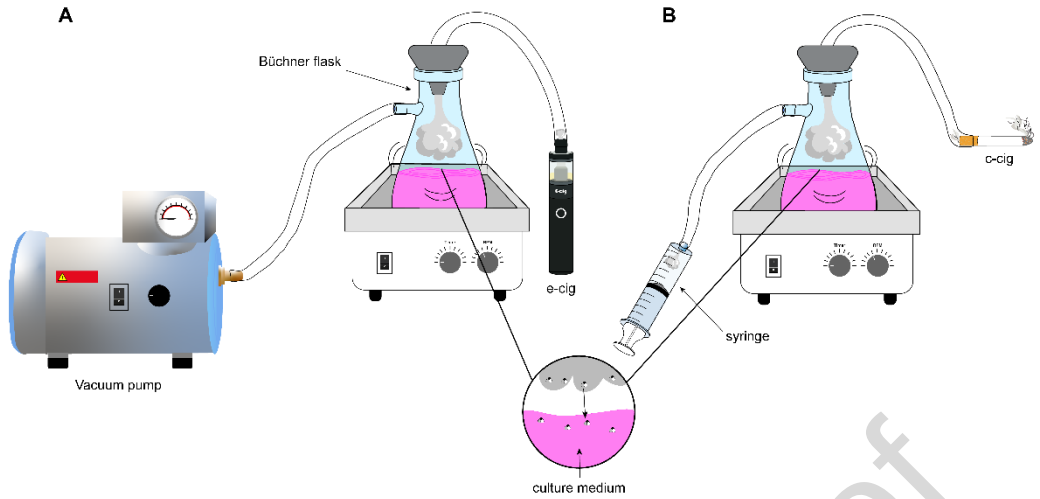

Figure 1. E-cig aerosol/cigarette smoke acquisition system. Experimental setting to obtain (A) e-cig aerosol-exposed medium: the vacuum pump was connected to the sidearm of a 500 mL Büchner flask, whereas the device was connected by a tube through the neck; (B) c-cig smoke-exposed medium: the syringe was connected to the sidearm of a 500 mL Büchner flask while a hose connected the cigarette through the neck of the flask. In both cases, 20 mL of culture medium was poured into the flask.

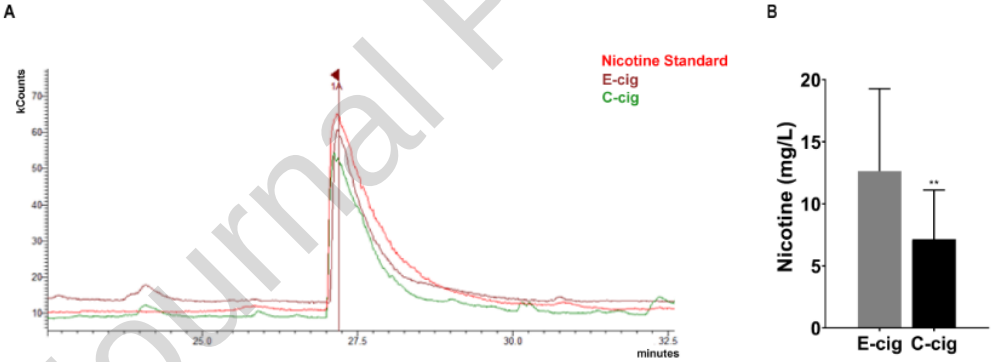

Figure 2. Nicotine quantification by GC-MS. (A) Representative example of pure nicotine standard (red), e-cig (dark red) and c-cig (green) chromatogram: elution time (*t*) *vs*. signal (kCounts). Peaks: nicotine, detected by MS (± 27 min). (B) Nicotine quantification in the e-cig and c-cig exposed media. Results are expressed in mg/L (n = 14). Data are represented as mean ± *SD*. **p < 0.01 *vs*. E-cig by Mann Whitney test.

3.2. E-cig aerosol causes a mild impairment in lung growth comparing to c-cig smoke

To determine the potential effects of e-cig aerosol on lung development, stage b2 lungs were cultured in vitro, for 48 h, with unexposed (control), aerosol (e-cig), or

smoke (c-cig) exposed medium (Figure 3). E-cig aerosol-exposed explants (Figure 3D) look smaller than controls and present thicker mesenchyme distally (Figure 3B). Tobacco smoke is known to be toxic; for this reason, a batch of explants was treated with a smoke-exposed medium to ascertain the relative grade of toxicity in the e-cig aerosol-exposed explants. Smoke-exposed explants (Figure 3F) were noticeably

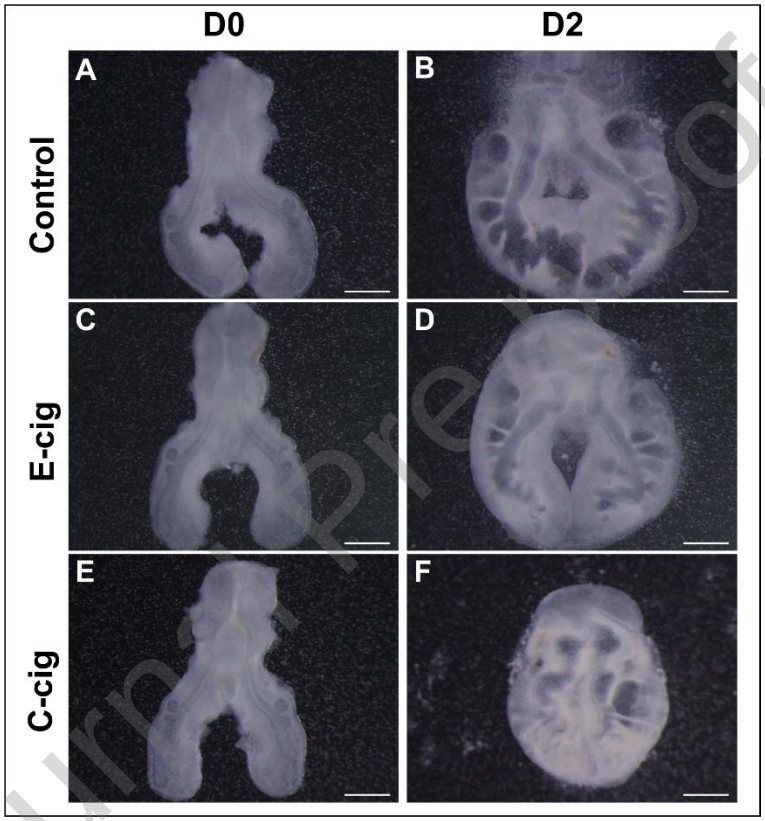

Figure 3. *In vitro* chick lung explants. Representative examples of stage b2 lung explant culture at D0 (A, C, E) and D2 (B, D, F). Experimental conditions: control (A-B), e-cig/aerosol (C-D), and c-cig/smoke (E-F) exposed medium ($n \geq 16$). Scale bar:

smaller than control and e-cig aerosol-exposed explants (Figure 3B and D, respectively).

To accurately assess the effect of each treatment on the whole lung and the epithelium and better reflect the impact on lung growth, morphometric analysis was performed (Figure 4). A schematic representation of how the morphometric data were obtained is illustrated in Figure 4A. This method is widely used to examine the impact of different culture media on lung morphogenesis; it is used to quantify lung growth

(Moura, 2019) and discriminate between the epithelial compartment and the whole lung. The e-cig aerosol-exposed explant parameters were, in general, similar to controls (Figure 4B); however, a 10 % statistically significant decrease in the total area and perimeter was detected. Smoke-exposed explants showed a statistically significant decrease in all morphometric parameters compared to controls [15 % to 30 %] and e-cig aerosol-exposed explants [12 % to 34 %]. It is worth mentioning that this greater impact of c-cig was observed with a considerably lower amount of puffs (7 to 8) compared to e-cig puffs (17 to 18).

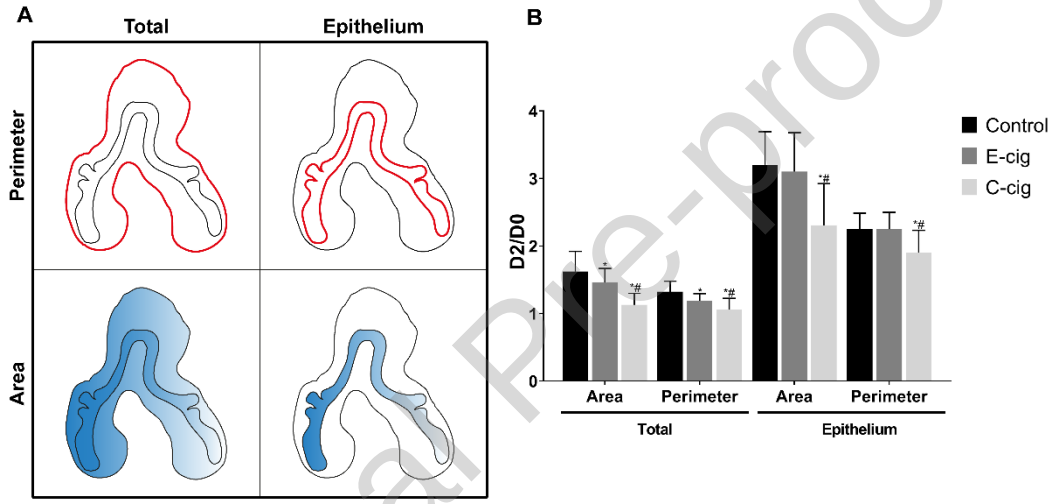

Figure 4. Morphometric analysis of chick lung explants. (A) Schematic representation of the morphometric analysis. The whole lung and the epithelial compartment were delineated (red line) in D0 and in the corresponding D2 explant image to obtain total and epithelial perimeter, respectively (upper panel). The area delimited by the red line, highlighted in blue, corresponds to the total and epithelial area, respectively (lower panel). (B) Control, e-cig/aerosol, and c-cig/smoke-exposed explants were evaluated at D0 and D2 for total and epithelial area/perimeter; results are expressed as D2/D0 ratio ($n \geq 16$). Data are represented as mean ± *SD*. * $p < 0.05$ *vs*. control; # $p < 0.05$ *vs*. E-cig by one-way ANOVA.

To discard whether the impact on lung morphology was due to nicotine, lung explants were exposed to two concentrations of nicotine, 100 µM (16.2 mg/L of nicotine; similar to e-cig exposed medium) or 1 mM (162.2 mg/L of nicotine; 10 times e-cig exposed medium). Explants exposed to both concentrations are similar to controls (Supplementary Figure S1A-F), sustaining the hypothesis that constituents of the e-cig

other than nicotine may be responsible for the observed effects. Nevertheless, the morphometric analysis revealed a statistically significant decrease in the epithelial perimeter of 1 mM nicotine-treated explants compared to controls (Figure S1G).

3.3. E-cig aerosol-exposed medium promotes pro-inflammatory cytokine release

Several authors have shown that exposure to tobacco smoke causes inflammation in vitro and in vivo (Czekala et al., 2019; Devi and Moharana, 2020; He et al., 2015). Additionally, it has been described that e-cig aerosol can also promote inflammation in vitro (Scott et al., 2018). TNF-α is a pleiotropic cytokine and an endogenous mediator of acute and chronic pro-inflammatory responses. For this reason, the levels of Tumor necrosis factor α (TNF-α) released into the medium after 48 hours in culture were evaluated.

Post-incubation culture medium, collected at 24 h and 48 h, was used to quantify the TNF-α levels released during culture. After quantification, the TNF-α data from the same explant was summed up to provide information on the overall culture process (Figure 5). A statistically significant increase in TNF-α levels was detected in the e-cig aerosol and smoke-exposed explants compared to controls (9x and 7x larger, respectively). Also, TNF-α levels are higher (around 1.3x) in e-cig aerosol-exposed explants when compared to smoke-exposed explants, but without statistical

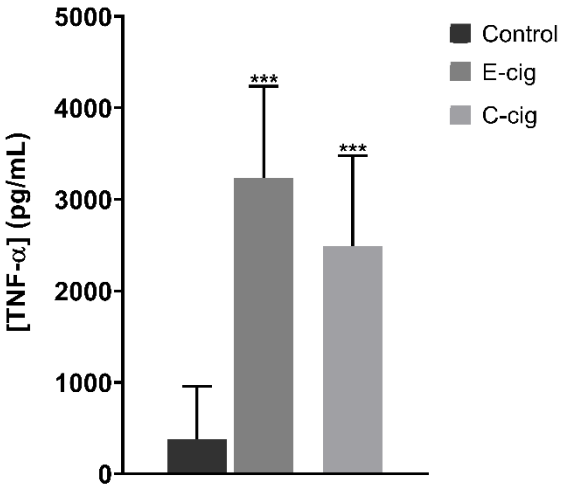

Figure 5. Determination of TNF-α levels in the culture medium. Control, e-cig/aerosol, and c-cig/smoke-exposed media were assessed for TNF-α levels at D1 and D2. Total TNF-α was calculated by adding D1 and D2 amounts (n = 6). Data are represented as mean ± *SD*. ***p < 0.001 vs. control by one-way ANOVA.

significance (p = 0.33).

4. Discussion

Nicotine delivery systems, such as electronic cigarettes, have been extensively publicized as less noxious than conventional cigarettes. Even though the e-cig aerosol has fewer toxic components than smoke, the potentially damaging effects of inhaling aerosol cannot be discarded (Goniewicz et al., 2014). The toxic effect of smoke on lung development has already been documented (Gibbs et al., 2016; McEvoy and Spindel, 2017); however, the impact of e-cig aerosol is not entirely understood. Therefore, this work aimed to assess the impact of e-cig aerosol on embryonic lung explants, using the chicken embryo as a model. Moreover, explants were cultured in a smoke-exposed medium for comparison purposes. To the best of our knowledge, this is the first study analyzing the effect of aerosol/smoke on a whole organ in vitro. Several studies have evaluated the impact of aerosol exposure in vitro in cell lines (Cervellati et al., 2014; Scott et al., 2018), 3D models (Czekala et al., 2019), and even adult human lung explants (Rankin et al., 2019); however, none of them used a whole embryonic organ.

After a thorough optimization of the system required to obtain an enriched e-cig aerosol/c-cig smoke culture medium, the culture medium was analyzed by GC-MS to detect nicotine, the common substance in both cigarettes. With this approach, we were able to quantify nicotine in an originally nicotine-free medium, proving that the acquisition system was properly settled and that aerosol/smoke compounds diffused into the culture medium. In our experimental conditions, nicotine levels in the e-cig aerosol-exposed medium were significantly higher (43 %) than in the smoke-exposed medium (Figure 2B). To a certain extent, this result was expected since the 200 mg of e-liquid used to prepare the e-cig aerosol-exposed medium has more nicotine (approximately 2.83 mg, considering $\rho = 1.13$ g/mL) than the c-cig used to prepare smoke-exposed medium (0.8 mg). We found a wide range of nicotine levels in different experimental settings in the literature (Dusautoir et al., 2021; Etter et al., 2013; Farsalinos et al., 2015; Goniewicz et al., 2013; Trehy et al., 2011). Different acquisition

methods, electronic cigarette devices, and e-liquids make it virtually impossible to compare different studies (Dusautoir et al., 2021). For example, voltage values directly impact the presence of noxious compounds in the aerosol. Dusautoir et al. (2021) reported that the nicotine level per puff varies with e-cig power supply, and, specifically, more powerful devices release more nicotine per puff than c-cig (Dusautoir et al., 2021).

Plasma nicotine concentrations reported in real-life scenarios are far less than those detected in our experimental setting. Yan et al. 2015 conducted a randomized, partially single-blinded, six-period crossover study and evaluated plasma nicotine levels in smokers and vapers under controlled conditions (Yan and D'Ruiz, 2015). In this study, vapers consumed between 119 to 257 mg of e-liquid (similar to the one used in this study), and smokers consumed 1 to 3 cigarettes, depending on the group. The maximum plasma nicotine levels were detected after 90 min: 0.017 mg/L for e-cig and 0.029 mg/L for c-cig. In the whole organism, plasma nicotine derived from cigarette smoking has a half-life of 2 hours because it is rapidly metabolized in the adult liver to cotinine which has an elimination half-life of ~16 hours (Benowitz, 2009; Benowitz et al., 2009). This fact may explain the differences. Still, nicotine easily crosses the placenta without being metabolized (Pastrakuljic et al., 1998). In fact, it has been shown that nicotine levels are 15% and 88% higher in fetal circulation and amniotic fluid, respectively, than maternal blood levels (Luck et al., 1985; Wickström, 2007). Moreover, nicotine metabolism is slower in the fetal liver, which means that the nicotine half-life in the fetus is longer (Kuniyoshi and Rehan, 2019; Lambers and Clark, 1996). Consequently, fetal cells, including the developing lung, might be exposed to higher nicotine concentrations for extended periods. In vitro studies such as the present one are crucial to determine e-cig aerosol, cigarette smoke, and nicotine's effect on lung development to better understand the impact on fetal development.

At the morphological level, e-cig aerosol-exposed embryonic lung explants appeared similar to controls; however, the morphometric analysis revealed a decrease

in total area and perimeter, suggesting that aerosol is impairing lung growth. The presence of toxic substances in different e-cig aerosols has already been documented, even though they are present in significantly lower amounts than smoke (Dusautoir et al., 2021; Goniewicz et al., 2014; Rankin et al., 2019). Nonetheless, their presence may account for the slight decrease in lung growth. Conversely, smoke negatively influences overall lung growth, which is in agreement with the literature (Cheng et al., 2016). Compared to smoke-treated explants, e-cig aerosol-exposed explants exhibited a better outcome. To a certain extent, this result was expected, considering that it is well established that smoke contains more deleterious substances than aerosols (Carmines, 2002). Worth to highlight that the effects observed were achieved using a conditioned media obtained from more than double number of puffs in the e-cig vs. the c-cig conditioned media. In our study we obtained different nicotine concentrations in the conditioned media (14.32 mg/L vs. 8.17 mg/L). To overcome this difference, we made an assay in which e-cig-conditioned media was diluted (1:2, 1:3, 1:4) to match the nicotine concentration values obtained in smoke-conditioned media. Lung explants treated with e-cig diluted media were similar to controls and distinct from matched concentration smoke-exposed lungs (data not shown).

It has been reported that the negative effect of smoking, and potentially vaping, on lung development is mediated by nicotine (Gibbs et al., 2016; Spindel and McEvoy, 2016; Wongtrakool et al., 2012). Bearing this in mind, we assessed the impact of nicotine in chick lung explants. Our results showed a significant decrease in epithelial perimeter only in 1 mM nicotine-exposed explants (Figure S1); this concentration is 10 times greater than the nicotine levels in the e-cig aerosol-exposed medium. Previous studies have shown that mouse lung explants treated with 1 µM nicotine display an increase in lung branching compared to controls (Wongtrakool et al., 2007; Wuenschell et al., 1998). We did not test 1 µM concentration, but we did not find differences between control and 100 µM- treated chick lung explants. These results show that the effect of e-cig aerosol-exposed medium on lung development is not triggered by

nicotine but most likely by other aerosol-derived compounds present in the medium, such as carbonyl and polycyclic aromatic hydrocarbon compounds (Dusautoir et al., 2021).

A recent study demonstrated that e-cig aerosol exposure does not impair tissue histology and cell viability on a 3D tissue model (EpiAirway^{TM}) compared to the air-exposed tissue (Czekala et al., 2019). Our findings are in agreement with this study. Conversely, they also disclosed no differences between smoke, aerosol, and control when the 3D tissue model was exposed to 9 smoke puffs. In our experimental setting, the consumption of a conventional cigarette took, on average, 7 to 8 puffs, and we found significant differences between groups. This divergence may be explained if we consider that the acquisition system (smoking machine) and the experimental model differ considerably from our experimental design. Nonetheless, the authors report an alteration of tissue architecture and decreased viability after exposure to 27 puffs of conventional cigarette smoke.

One of the main effects of aerosol/smoke exposure in vitro, using different cell lines, is the inflammatory response mediated by cytokine release (Lerner et al., 2015; Pareek et al., 2019). In this sense, we investigated whether there was also cytokine release in the whole organ culture. TNF-α mediates pro-inflammatory responses, and it is known to be involved in apoptosis, cell survival, proliferation, and cell differentiation (Faustman and Davis, 2010). Data shows that, compared to controls, both aerosol and smoke promote TNF-α release similarly. However, when we normalize the TNF-α levels to the nicotine levels ([TNF-α]/[nicotine]), this ratio is ~1.4x times higher for c-cig conditioned media compared to e-cig media but still without statistically significant differences (data not shown). This value contemplates nicotine contribution and it seems aligned with the differences observed between the e-cig and c-cig at the morphological level. It has been shown that ammonia exposure leads to an increase in the mRNA expression of tumor necrosis factor α in the adult chicken lung (Bai et al., 2021). Ammonia is as a chemical, not biological insult, that can be compared to the

chemical insult lung explants are exposed in the conditioned media. Moreover, pro-inflammatory cytokine release has been detected in the fluid surrounding dental implants of vapers compared to nonsmokers (Al-Aali et al., 2018). Additionally, e-cig aerosol with nicotine promotes an increase in TNF-α levels compared to nicotine-free aerosol exposure and untreated controls in alveolar macrophages (Scott et al., 2018). The nicotine levels detected in that report were approximately ten times higher than the nicotine concentration obtained in the current study.

5. Conclusion

Our data suggest that the e-cig aerosol impairs lung growth and promotes pro-inflammatory cytokine release, in our ex vivo approach. Nevertheless, the effects are, in fact, less adverse than smoke. Still, e-cig aerosol alters the normal pattern of airways and parenchymal development, potentially impacting pulmonary function and leading to lifelong postnatal diseases. Deciphering these questions is crucial to strengthen the recommendations regarding the use of electronic cigarettes during pregnancy. We recognize that this is a descriptive study and that further studies are needed to pinpoint the effect of each of the aerosol components in the developing lung.

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Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Highlights

- E-cig aerosol impairs lung growth but to a lesser extent than c-cig smoke;
- TNF-α induction was similar upon e-cig aerosol or c-cig smoke exposure;
- The impact on lung growth was not due to the difference in nicotine content.